



# Switch in rod opsin gene expression in the European eel, *Anguilla anguilla* (L.)

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The rod photoreceptors of the European eel, *Anguilla anguilla* (L.), alter their wavelength of maximum sensitivity ( $\lambda_{\max}$ ) from *ca.* 523 nm to *ca.* 482 nm at maturation, a switch involving the synthesis of a new visual pigment protein (opsin) that is inserted into the outer segments of existing rods. We artificially induced the switch in rod opsin production by the administration of hormones, and monitored the switch at the level of mRNA accumulation using radiolabelled oligonucleotides that hybridized differently to the two forms of eel rod opsin. The production of the deep-sea form of rod opsin was detected 6 h after the first hormone injection, and the switch in rod opsin expression was complete within four weeks, at which time only the mRNA for the deep-sea opsin was detectable in the retinal cells. It is suggested that this system could be used as a tractable model for studying the regulatory control of opsin gene expression.

**Keywords:** European eel; *Anguilla anguilla*; opsin gene expression; visual pigment

## 1. INTRODUCTION

Animal photoreceptors contain visual pigments, the function of which is to absorb light as the first step in the process of vision. Visual pigments comprise a protein (opsin) and a chromophore which, in vertebrates, is either the aldehyde of retinol (vitamin A1), or that of 3,4-dehydroretinol (vitamin A2). It is both the nature of the chromophore and the electrostatic interactions that occur between the opsin and chromophore, determined by the amino acid sequence of the opsin, that determine the absorption spectrum and wavelength of maximum sensitivity ( $\lambda_{\max}$ ) of the visual pigment (Hubbard & Sperling 1973; Yokoyama 1995).

Opsins belong to the large family of G-protein-linked cell membrane receptors which includes such diverse molecules as  $\beta$ -adrenergic receptors, serotonin receptors, and muscarinic acetylcholine receptors. Despite the diversity in function there is a high degree of structural similarity within the family (Baldwin 1993), indicative of an origin from a common ancestral gene. The study of one member of the super family is thus likely to reveal information relevant to the understanding of other G-protein-linked receptors, including those involved in a range of diseases. A main advantage of studying opsins, when compared with other members of the G-protein-linked receptor super family, is that they are synthesized within retinal tissue at exceptionally high concentrations, providing an excellent source of opsin and associated components. The genomes of many species of vertebrates contain more than one opsin gene, encoding different opsins, but individual photoreceptors generally express only one opsin. Nevertheless, more than one opsin has been detected both in the photoreceptors of some fish (see, for example, Archer &

Lythgoe 1990; Wood & Partridge 1993) and mammals (Rölich *et al.* 1994; Szel *et al.* 1995; Desjardin *et al.* 1995). Further understanding of the regulation of genes encoding opsins, and, by implication, those encoding other G-protein-linked receptors, may well depend on the development of an animal model in which opsin expression can be artificially manipulated. Such a model system is presented by the Atlantic eel.

Atlantic eels, *Anguilla anguilla* (L.) and *A. rostrata*, are catadromous teleosts which spawn in the Sargasso Sea (52–62° W, 24–28° N). Having hatched, leptocephalus larvae drift in the surface waters of the Gulf Stream to the European, North African and North American coastlines and enter the river and lake systems as glass eelers. They remain in fresh water as yellow eels for several years, after which they migrate downstream and back to the Atlantic as sexually mature silver eels (Tesch 1977). During the downstream migration, and subsequent entry into the deep sea, the  $\lambda_{\max}$  of the rod visual pigments shifts from *ca.* 523 nm (referred to as a P523<sub>2</sub> pigment, i.e. a pigment with a wavelength of maximum sensitivity at 523 nm and using the 3,4-dehydroretinal chromophore) to *ca.* 482 nm (referred to as a P482<sub>1</sub> pigment), by both chromophore exchange (from 3,4-dehydroretinal to retinal) and the synthesis of a new rod opsin protein (Carlisle & Denton 1959; Beatty 1975).

The shift in rod  $\lambda_{\max}$  accompanying natural maturation in *A. anguilla* can be experimentally induced by gonadotropin administration. Using this approach, Wood & Partridge (1993) measured visual pigments within rod photoreceptors using microspectrophotometry (MSP) and monitored the shift in visual pigment  $\lambda_{\max}$  in single cells. They demonstrated that the new 'deep-sea' opsin was expressed within existing rod photoreceptors and formed a shortwave sensitive visual pigment ( $\lambda_{\max}$  482 nm) that could be detected by MSP at the base (vitreal end) of existing rod outer segments after one

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week of hormone treatment. At this stage the old 'fresh-water' opsin was detectable, forming a relatively longwave-sensitive visual pigment at the distal (scleral) end of the same rod outer segments.

In this study, we extend the observations of the speed of the hormone-induced switch in opsin gene expression using molecular biological techniques. The changes in retinal mRNA were followed using oligonucleotide probes specific to the freshwater and deep-sea forms of eel rod opsin. The switch in opsin gene expression was found to be fast, new gene product being detectable within 6 h of a single hormone injection.

## 2. MATERIAL AND METHODS

### (a) *Animals*

Two-year-old male eels (mean length  $\pm$  s.d.,  $372 \pm 26$  mm; mean weight  $\pm$  s.d.,  $97.0 \pm 22.1$  g;  $n=37$ ) were obtained from a commercial eel farm. Animals were kept in artificial seawater (salinity 35 p.p.t.) at  $15^\circ\text{C}$  under a controlled light regime (12L:12D) provided by 0.6 m long, fluorescent tubes (Thorn EMI Ltd, UK, 75/85 W White 3500). A group of eels ( $n=28$ ) were each subcutaneously injected, at weekly intervals, with 250 I.U. of human chorionic gonadotropin (HCG) and 1 mg of salmon pituitary extract (SPE), both being delivered in 0.1 ml of Hanks' balanced saline. A further seven eels were used as control groups, and were injected with 0.2 ml of Hanks' balanced saline at the beginning and end of the experimental period (i.e. when the first and last hormone-injected eels were sacrificed). Hormone-treated eels were subsampled for RNA extraction (see next paragraph) and collection of blood at 6, 12, 24, 36 and 48 h, and at one, two and four weeks after the first hormone injection. Before all injections, eels were anaesthetized by immersion in 0.3% w/v 3-aminobenzoic acid ethyl ester (MS222). No morbidity and no mortality occurred over the course of the experiment.

### (b) *Production of cDNA standards from immature and mature eels*

An additional four eels were used to produce single-stranded complementary DNA (cDNA) from immature and mature eels. This cDNA was used to test the specificity of molecular probes designed to anneal to the deep-sea and freshwater opsins. A total of two eels were removed at the time of the first hormone treatment, held in the dark for 1 h, sacrificed and enucleated. The two remaining eels were artificially matured by weekly injections with hormones for four weeks, after which they too were sacrificed and enucleated. One eye from each animal was used for microspectrophotometric measurement and the other eye was used for RNA extraction and cDNA production.

### (c) *Microspectrophotometry*

To confirm the nature of the opsin being expressed by immature and hormone-treated eels, and the specificity of the opsin-specific probes, microspectrophotometric measurements of the visual pigments in individual retinal rods were made from the eels that were used to produce the cDNA standards. The microspectrophotometer (MSP) is a computer-controlled, wavelength-scanning instrument that has been fully described elsewhere (Partridge 1986, 1989; Partridge & De Grip 1991). Retinae were placed in Dulbecco 'A' phosphate buffered saline (PBS; pH 7.3) diluted to  $368 \text{ mOsm kg}^{-1}$ . A sample of retina, ca.  $1 \text{ mm}^2$ , was placed on a coverslip with a drop of PBS containing both 10%

dextran (250 kRMM) to stop cell movement, and 50 mM hydroxylamine to remove photoproduct absorbance to wavelengths shorter than 400 nm (Knowles & Dartnall 1977). All procedures were done under dim red light (Kodak safelight No. 1A). Approximately 60 absorption spectra were measured from the rod outer segments of each animal. Each scan was subjected to an acceptance procedure (for justification, see Levine & MacNichol 1985; Partridge & De Grip 1991) and only absorbance spectra with high signal-to-noise ratios and insignificant absorbance at wavelengths greater than 650 nm were used in further analysis.

Accepted absorbance spectra were averaged and analysed by the template fitting technique employed by Wood & Partridge (1993). The theoretical absorbance spectra of different mixtures of all four rod visual pigments produced by the eel (P482; P501; P493; P523; Carlisle & Denton 1959; Beatty 1975; Wood *et al.* 1992) were calculated by using visual pigment templates (Bridges 1967; Partridge & De Grip 1991) and were best-fitted to the normalized averaged MSP absorbance spectra. Best-fitting was achieved by altering the ratio of A1:A2 chromophore and the ratio of freshwater to deep-sea opsins, and identifying the visual pigment mixture that fitted the MSP data with the lowest root-mean-square (RMS) deviation. The calculation of RMS deviation was restricted to the range 0.8 maximum absorbance on the shortwave limb of the spectral absorbance data to 0.2 maximum absorbance on the longwave limb, this being the most artefact-free part of MSP absorbance spectra (Levine & MacNichol 1985; Partridge & DeGrip 1991).

### (d) *Molecular biology*

Preparations of total retinal RNA were made from the eyes of all animals and corresponding cDNAs made by reverse transcription and PCR amplification. After sacrifice, the eyes from each animal were frozen in liquid nitrogen and stored at  $-70^\circ\text{C}$  for subsequent extraction of nucleic acids. At the end of the hormone injection programme all eyes were thawed and processed simultaneously. Each eye was homogenized individually in 600  $\mu\text{l}$  of lysis buffer (containing 10 mM EDTA, 100 mM Tris HCl (pH 8.0), 35 mM NaCl, 70 mM SDS and 2 M Urea) and incubated at  $37^\circ\text{C}$  for 10 min. The nucleic acids were extracted using a standard phenol-chloroform-isoamyl alcohol (25:24:1) technique (Sambrook *et al.* 1989), after which they were resuspended in double-distilled water, with DNAase added to digest the DNA. The resulting RNA was precipitated with ethanol and redissolved in 100  $\mu\text{l}$  of distilled water, giving an estimated total RNA concentration of  $1\text{--}2 \mu\text{g } \mu\text{l}^{-1}$ . From this, cDNA was synthesized using a first-strand cDNA synthesis kit (Pharmacia Biotech Inc.). A sample of cDNA from each eel was used in a PCR reaction to amplify a specific region of the rod opsin encoding sequences using the primers 5'-ACTTCCG(A/C)TTTGGTGAGA-3' and 5'-GGAGGTCACCCGCATGGT-3'. The PCR reaction conditions were 30 s at  $94^\circ\text{C}$ , 30 s at  $55^\circ\text{C}$ , 60 s at  $72^\circ\text{C}$ , with the reaction being cycled 35 times and terminated with a final 10 min at  $72^\circ\text{C}$ .

Replicate samples of the PCR products were separated on two 1.0% agarose gels and blotted onto Zeta probe hybridization membranes (Bio-Rad) using 0.4 M NaOH. One membrane was probed with the freshwater opsin-specific probe (FWPRO; 5'-GGTGAAGTGGCAGATA-3'), and the other membrane was probed with the deep-sea opsin-specific probe (DSPRO; 5'-GGAGAAGTGAACATG-3'). Both probes were radiolabelled with  $^{32}\text{P}$  using T4 polynucleotide kinase (Pharmacia Biotech) and hybridized at  $45^\circ\text{C}$  for 16 h in phosphate buffer (50 mM

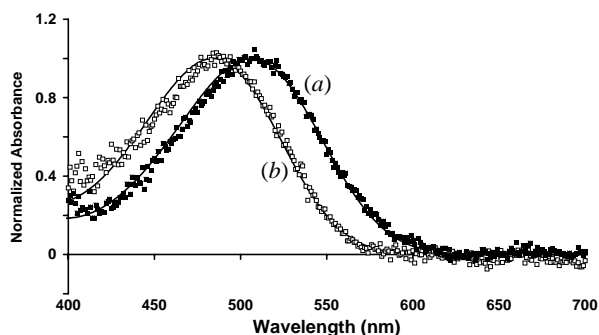


Figure 1. Normalized average absorbance spectra of visual pigments measured from rods of non-injected and hormone-treated eels. Averaged data (symbols) are shown with the best-fitting visual pigment template mixture (continuous line). (a) Average absorbance spectrum obtained from retinal rods of non-injected eels.  $\lambda_{\max}$  506.2 nm fitted with a visual pigment template based on a freshwater-to-deep-sea opsin ratio of 100:0, and retinal-to-3,4-dehydroretinal ratio of 7:3. (b) Average absorbance spectrum obtained from the retinal rods of eels treated with hormones for four weeks.  $\lambda_{\max}$  484.4 nm fitted with a visual pigment template based on a freshwater opsin-to-deep-sea opsin ratio of 0:100, and retinal-to-3,4-dehydroretinal ratio of 4:1.

$\text{PO}_4^-$ , pH 7.2, 1 mM EDTA, 7% SDS). The hybridization of the probes to the PCR fragments was visualized by autoradiography of the membranes (Hyperfilm, Amersham).

#### (e) Serum steroid assays

Immediately after sacrifice, ca. 2 ml of blood was collected from each eel. Blood samples were left to clot at room temperature for 3 h, after which the serum was removed and centrifuged at 2500  $g$  for 15 min before being frozen at  $-20^\circ\text{C}$  until it was assayed for steroids by radioimmunoassay. The assay used antiserum raised in rabbit,  $^{125}\text{I}$ -testosterone, and anti-rabbit antiserum raised in donkey to determine the change in testosterone production during the course of the experiment. Full details of the assay are described by Parkinson & Follett (1995).

### 3. RESULTS

#### (a) Visual pigment microspectrophotometry

Eels killed at the start of the experiment had rod visual pigments with  $\lambda_{\max}$  values greater than 501 nm (mean  $\pm$  s.d.,  $506.2 \pm 1.2$  nm;  $n=62$ ; see figure 1), whereas those killed after four weeks of hormone injections had retinal rods with  $\lambda_{\max}$  values less than 501 nm (mean  $\pm$  s.d.,  $484.0 \pm 1.4$  nm;  $n=61$ ; see figure 1). The visual pigment mixture that best fitted the absorbance spectra recorded from the rods of eels before hormone injections was based on a retinal:3,4-dehydroretinal chromophore ratio of 7:3 and a freshwater opsin content of greater than 95%. In contrast, the template fitting method suggested that the visual pigment found in the rods of the eels that had received four injections of hormones was greater than 95% deep-sea opsin and that the retinal:3,4-dehydroretinal chromophore ratio was 4:1.

#### (b) Molecular biology

A preliminary Northern blotting experiment demonstrated that insufficient mRNA was obtained from

individual eel eyes and, in consequence, PCR amplification and Southern blots were used. The primers used in the PCR amplified a region between base positions 434 and 761 of the opsin coding sequences of *A. anguilla* (numbering as in fig. 1 of Archer *et al.* 1995). This region was chosen because it included sequences that could be probed by using opsin-specific oligonucleotides (FWPRO and DSPRO) that could differentiate between the eel freshwater and deep-sea rod opsin encoding sequences. The sequences of these opsin-specific probes were designed by comparing the eel freshwater and deep-sea opsin sequences determined by Archer *et al.* (1995) and identifying the most dissimilar region. The PCR products were separated on agarose gels and, in all cases, showed a single band corresponding to the expected 300 bp fragment of the opsin-encoding region of cDNA, fragment sizes being confirmed by comparison with a set of  $\phi\text{X}174$  markers (see figure 2).

Autoradiographs were obtained from gels probed with opsin-specific probes and the intensity of the different hybridization bands gave some indication of the amounts of amplified freshwater or deep-sea opsin sequence present (figure 2). Confirming MSP measurements, the radiolabelled freshwater probe hybridized to the cDNA fragments amplified from uninjected eels, and the deep-sea probe hybridized to the PCR fragments produced from the hormone-injected eels that were examined by both MSP and molecular biological methods (figure 2; lanes FW and DS). The deep-sea probe also hybridized, albeit to a much lesser degree, to the PCR fragments obtained from the uninjected eels, possibly owing to the transcription of a small amount of deep-sea opsin in the untreated animals, or as a result of some probe cross-hybridization. When probed with the radiolabelled freshwater probe, hybridization bands were observed with cDNA amplified from the freshwater opsin standard, the control animals, and 22 of the 28 hormone-injected animals (i.e. those injected with hormones for up to two weeks). In contrast, PCR products from the remaining four animals (i.e. those killed up to four weeks from the first hormone injection) did not hybridize to the freshwater-specific probe. Hybridization bands, produced when the PCR fragments were probed with the radiolabelled deep-sea opsin-specific probe, are present in the lanes containing the deep-sea opsin standard and PCR products from all hormone-injected animals except one which was killed at 24 h. (Note: although the density of DS hybridization bands is very variable, this is likely to be a PCR artefact rather than a reflection of different levels of DS mRNA production in different individuals.) In contrast, only very faint bands can be seen when the PCR products from the saline-injected animals and freshwater standard were probed with the deep-sea opsin-specific probe.

#### (c) Steroid assay

Blood samples were taken from all eels killed during the experiment and the concentration of testosterone measured by radioimmunoassay. Testosterone levels in the control eels remained at undetectable levels (i.e. below  $0.06 \mu\text{g ml}^{-1}$ ) throughout the whole experiment. In contrast, testosterone levels in the hormone-injected eels increased rapidly over the four weeks of gonadotropin

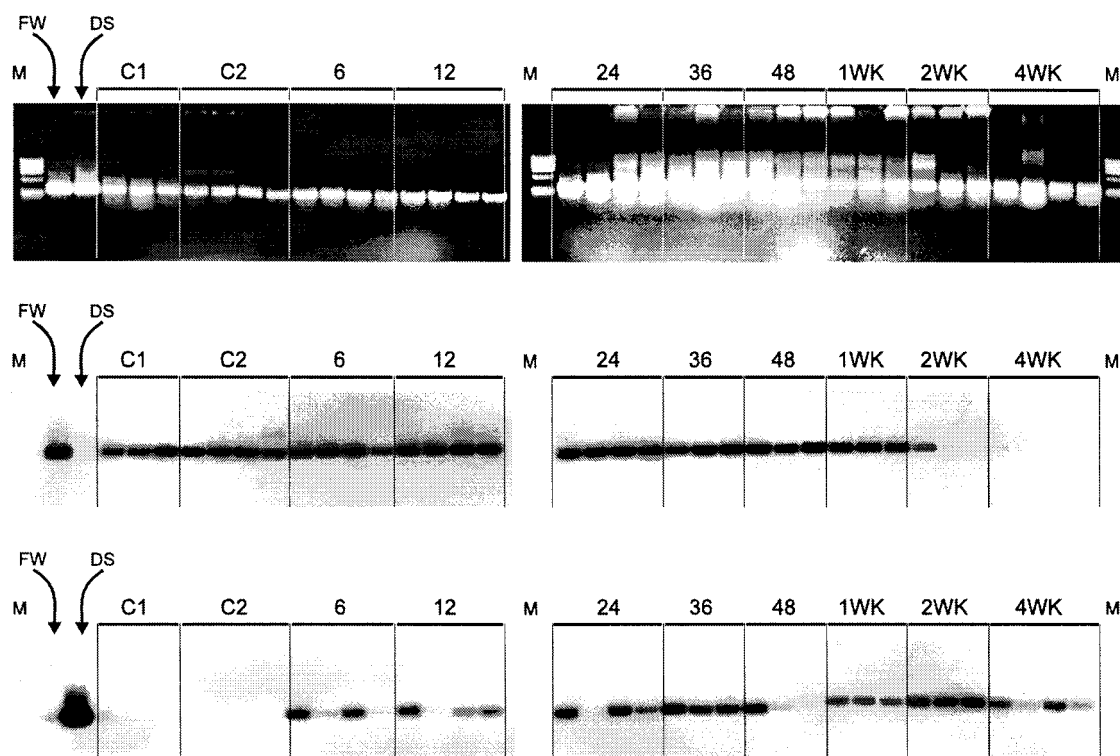


Figure 2. Opsin-specific cDNA amplified from the mRNA extracts from the uninjected, saline-injected and hormone-injected eels. The upper panel shows the size of bands produced in the PCR amplification compared with the  $\phi$  X174/HaeIII markers (loaded into the lanes labelled M). The size of the markers, from the largest to the smallest, are as follows (in base pairs): 1358, 1078, 872, 603, 310, 281, 271, 234, 194, 118 (the last six bands are not resolved in this gel system). The gel, in which ethidium bromide-stained DNA was visualized using an ultraviolet transilluminator, shows that the size of the band produced during the PCR of the cDNA is *ca.* 300 bp in every sample. The numbers above the horizontal bars indicate the animals from which the PCR bands were produced: FW and DS mark the lanes containing cDNA from the freshwater and deep-sea standards, respectively; C1, control eels killed before the course of hormone injections; C2, control eels killed a week after the fourth hormone injection was given to the experimental animals; 6, 12, 24, 36 and 48 indicate the time, in hours after the first hormone injection, at which the animals were killed; 1WK, 2WK and 4WK, indicate the number of weeks after the first hormone injection at which the animals were killed. The middle panel shows the autoradiograph obtained from the hybridization between the PCR fragments and the radiolabelled freshwater opsin-specific probe. The lower panel shows the autoradiograph obtained from the hybridization between the PCR bands and the radiolabelled deep-sea opsin-specific probe. In the middle and lower panels the lane labelling is identical to that used in the top panel.

administration from a concentration ( $\pm$ s.d.) of  $0.09 \pm 0.01$  mg ml<sup>-1</sup> 6 h after the first treatment with hormones, to a concentration ( $\pm$ s.d.) of  $0.50 \pm 0.14$   $\mu$ g ml<sup>-1</sup> after four weeks of hormone injections.

#### 4. DISCUSSION

The administration of HCG and SPE causes a switch from the expression of freshwater opsin to the deep-sea form in the retinal rods of immature European eels (Wood & Partridge 1993). This switch involves a change in the rates of expression of the two intronless rod opsin genes known to occur in this species (Archer *et al.* 1995; Fitzgibbon *et al.* 1995). Nevertheless, it is extremely unlikely that gonadotropins act directly on the retina; all somatic effects of these hormones being mediated indirectly via steroids produced by the gonads (Hoar 1983). Levels of blood testosterone increased throughout the experiment in response to injected gonadotropin, and it is likely that steroids act, directly or indirectly, on retinal rods to control the switch in rod opsin production.

One method by which the opsin shift could be effected is by steroid control of the transcription of the two forms of rod opsin gene, with the rate of deep-sea rod opsin gene transcription being promoted by elevated levels of testosterone. Such steroid-mediated control of gene transcription has been previously described in rat hepatoma cells (O'Farrell 1975; Ivarie & O'Farrell 1978) and in liver cells (Schutz *et al.* 1975; for a review, see Yamamoto 1985). In such systems the enhancement of gene expression is dependent on the binding of steroid hormone to an enhancer element that is linked to the promoter sequence of the target gene. Such enhancer elements can be several kilobases away from the start site of transcription, and may be either upstream or downstream of the coding sequence (Yamamoto 1985).

Alternatively, steroid hormones may act on rod opsin gene expression by altering the stability of transcribed mRNA, previous studies having shown that the post-translation half-life of mRNAs can be substantially increased by the presence of steroid hormones (Brock & Shapiro 1983; Raghov 1987). In this study, and that of Wood & Partridge (1993), low levels of expression of the

deep-sea rod opsin were observed in control animals untreated with gonadotropins. Possibly, therefore, the deep-sea opsin gene is transcribed at a rate similar to the freshwater opsin gene, but differences in the stability of the deep-sea and the freshwater opsin mRNAs result in different steady-state concentrations of gene product. If this is the case, the deep-sea opsin mRNA must be less stable than the freshwater opsin mRNA in the absence of testosterone. The similarities of the coding sequences of the two mRNAs (81.4% identical over the whole coding nucleotide sequence; Archer *et al.* 1995) do not exclude the possibility that differences in mRNA structure cause differential mRNA stabilities. Studies have found that it is the nature of the 5' and/or the 3' untranslated regions of the mRNA transcripts that are responsible for the differential stabilities of various mRNAs (Furuichi *et al.* 1977; Caput *et al.* 1986; Brawerman 1987). Archer *et al.* (1995) did not sequence the full-length opsin cDNAs of the eel, thus precluding a full comparison of the untranslated domains of the two eel opsin mRNAs with consensus sequences determining mRNA stabilities (Furuichi *et al.* 1977; Caput *et al.* 1986; Raghov 1987).

We have shown in this study that the induction of the expression of the deep-sea opsin by hormone treatment is fast, the corresponding mRNA being measurable 6 h after the first hormone treatment (see figure 2). We can therefore confidently assert that the induction of deep-sea opsin expression occurs in less than this time, and may occur only minutes after the first hormone injection. In contrast, we can be much less certain of the rate at which transcription of the freshwater opsin gene is terminated by hormone treatment. In hormone-injected eels, both deep-sea opsin and freshwater opsin mRNAs co-existed for several weeks. The continuing production of the freshwater opsin may not be owing to the continued transcription of the freshwater opsin gene, but may, instead, be owing to the translation of previously transcribed mRNA persisting in the photoreceptors. Eukaryotic mRNAs have extremely variable half-lives, ranging from a few minutes to several days (Raghov 1987). Thus, if the freshwater opsin mRNA is slow to break down, the switch in opsin gene transcription could be considerably faster than is suggested by microspectrophotometry (Wood & Partridge 1993) or by measurements of freshwater opsin encoding mRNA (this study). Unfortunately PCR amplification is inherently non-quantitative and, even though great efforts were made to standardize all experimental protocols, and all PCR amplifications were run simultaneously on all samples, we cannot be certain about the concentrations of freshwater opsin mRNA at different points in the experiment. Nevertheless, the switch of the rod opsin expression was complete within four weeks from the time of the first hormone injection, at which time only the mRNA for the deep-sea opsin was present in the retinal cells. Because of the similarities with other receptors, it is not unreasonable to suggest that further understanding of the regulatory control of opsin gene expression could have implications for understanding the regulatory mechanism employed by other members of this super family of G-protein-linked receptors. The artificially inducible shift in opsin expression observed in the eel may prove a useful and tractable model for such studies.

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