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Volvoxrhodopsin, a Light-Regulated Sensory Photoreceptor of the Spheroidal Green Alga *Volvox carteri*

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Somatic cells of the multicellular alga *Volvox carteri* contain a visual rhodopsin that controls the organism's phototactic behavior via two independent photoreceptor currents. Here, we report the identification of an opsinlike gene, designated as volvoxopsin (*vop*). The encoded protein exhibits homologies to the opsin of the unicellular alga *Chlamydomonas reinhardtii* (chlamyopsin) and to the entire animal opsin family, thus providing new perspectives on opsin evolution. Volvoxopsin accumulates within the eyes of somatic cells. However, the *vop* transcript is detectable only in the reproductive eyeless gonidia and embryos. *vop* mRNA levels increase 400-fold during embryogenesis, when embryos develop in darkness, whereas the *vop* transcript does not accumulate when embryos develop in the light. An antisense transformant, T3, was generated. This transformant produces 10 times less volvoxopsin than does the wild type. In T3, the *vop* transcript is virtually absent, whereas the antisense transcript is predominant and light regulated. It follows that *vop* expression is under light-dependent transcriptional control but that volvoxopsin itself is not the regulatory photoreceptor. Transformant T3 is phototactic, but its phototactic sensitivity is reduced 10-fold relative to the parental wild-type strain HK10. Thus, we offer definitive genetic evidence that a rhodopsin serves as the photoreceptor for phototaxis in a green alga.

INTRODUCTION

Volvox carteri is a simple spheroidal multicellular alga with many features that recommend it as a model for studying the process of cytodifferentiation (Kirk and Harper, 1986) and the development of photoreception in lower eukaryotes. Individuals of this species contain only two distinct cell types—16 large reproductive cells (gonidia) and 2000 to 4000 somatic cells that cannot divide. The somatic cells are arranged in a single layer at the surface of the transparent sphere, whereas the 16 gonidia are located below the surface, where they have no direct contact with the external medium (Kirk et al., 1991). All somatic cells are biflagellate and possess eyespots, which, together with the photoreceptor and the downstream signaling machinery, form the functional eye. The eyes are responsible for guiding the organism to places of optimal light conditions. The orientation of the individual somatic cells within the spheroid combined with the three-dimensional pattern in which their flagella beat cause the spheroid to rotate in a counterclockwise direction as it moves (Hoops, 1993). The two flagella of each cell beat synchronously and in an almost precisely parallel fashion, but they beat toward the posterior of the spheroid and slightly to the right, causing the spheroid to rotate to the left as it moves forward (Hoops, 1993, 1997). Anterior cells possess larger and more sensitive eyes than do posterior ones (Sakaguchi and Iwasa, 1979; Hoops, 1997).

In *V. carteri*, the photophobic response involves a cessation of flagellar movement (on–off response) and not a switch to a different beating mode of the sort that causes slow backward movement in most of its unicellular relatives (flagellar reprogramming; Tamm, 1994). When a *V. carteri* spheroid is illuminated from one side, its rotation causes the cells to pass repeatedly between the shaded and the lit sides, with the consequence that their flagella slow down or accelerate beating, turning the colony either toward or away from the light (Foster and Smyth, 1980). All light-induced behavioral responses show action spectra with maxima between 490 and 520 nm (Schletz, 1976; Sakaguchi and Iwasa, 1979), suggesting that in *V. carteri*, a rhodopsin similar to the chlamyopsin of its unicellular relative *Chlamydomonas reinhardtii* serves as the functional photoreceptor.

Rhodopsin-mediated photoreceptor currents were recently analyzed in a *dissolver* mutant of *V. carteri*. In this mutant, flash-induced photocurrents were restricted to the eyespot region of somatic cells and were detectable from whole cells and excised eyes. Flash-induced photocurrents are a composite of a fast and transient Ca^{2+}-carried P_F current and a slowly activated H^+-carried P_S current. P_F is a high-intensity response, whereas P_S is more dominant at low flash energies (Braun and Hegemann, 1999).

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Here, we present evidence identifying an opsin gene and its apoprotein in *V. carteri*. We created an antisense transformant, which enabled us to study volvoxopsin expression through the life cycle under different light conditions. Finally, the antisense transformant provides a genetic tool to test whether the identified rhodopsin is the photoreceptor for phototaxis.

RESULTS

Volvoxopsin Primary Sequence

A λ ZAPII *V. carteri* cDNA library was screened with fragments of the chlamyopsin cDNA (Deininger et al., 1995). Only one clone, vop10, was positive in the second round. It contained a 1549-bp-long cDNA insert coding for a protein of 244 amino acids and a molecular mass of 26,760 D (Figure 1). The deduced protein sequence is 61% identical to chlamyopsin if aligned without gaps (Figure 2), and it definitely belongs to the same algal opsin family. Chlamyopsin is the only detectable retinal binding protein in the unicellular flagellate alga *C. reinhardtii* (Kröger and Hegemann, 1994) and the most abundant protein in eyespot preparations of this species (Deininger et al., 1995). Chlamyopsin and the encoded opsinlike *V. carteri* protein are 89% similar between residues 69 and 205, whereas they differ significantly at their N termini and considerably more at their C termini. The high overall similarity suggests that volvoxopsin is the apoprotein of the photoreceptor that controls photomovement responses. In contrast to all known animal and bacterial opsins, volvoxopsin, like chlamyopsin, contains many charged residues (Figure 2), including 42K, 16E, and 9D. Consequently, membrane-spanning segments are not as evident in hydropathy plots of the deduced amino acid sequences of the algal proteins as they are in most other membrane receptors (data not shown).

Relationship between Volvoxopsin and Members of Other Opsin Families

The conclusion that the *vop* gene encodes an opsin sequence that belongs to the algal opsin family and to the animal opsin superfamily is based on the finding that the protein sequence is 61% identical to that of chlamyopsin. Both algal sequences contain a typical retinal binding site at the C-terminal end of the sequence (Figure 3). Moreover, the organization of the two algal opsin genes is also similar in regard to the location of the introns and the length of the exons.

Both algal opsins show significant similarity to the entire animal opsin family. The degree of similarity to the invertebrate subgroup is higher than to the vertebrate subgroup. Volvoxopsin is most closely related to the Drosophila opsin Rh6 (Huber et al., 1997), including 49 identical amino acid

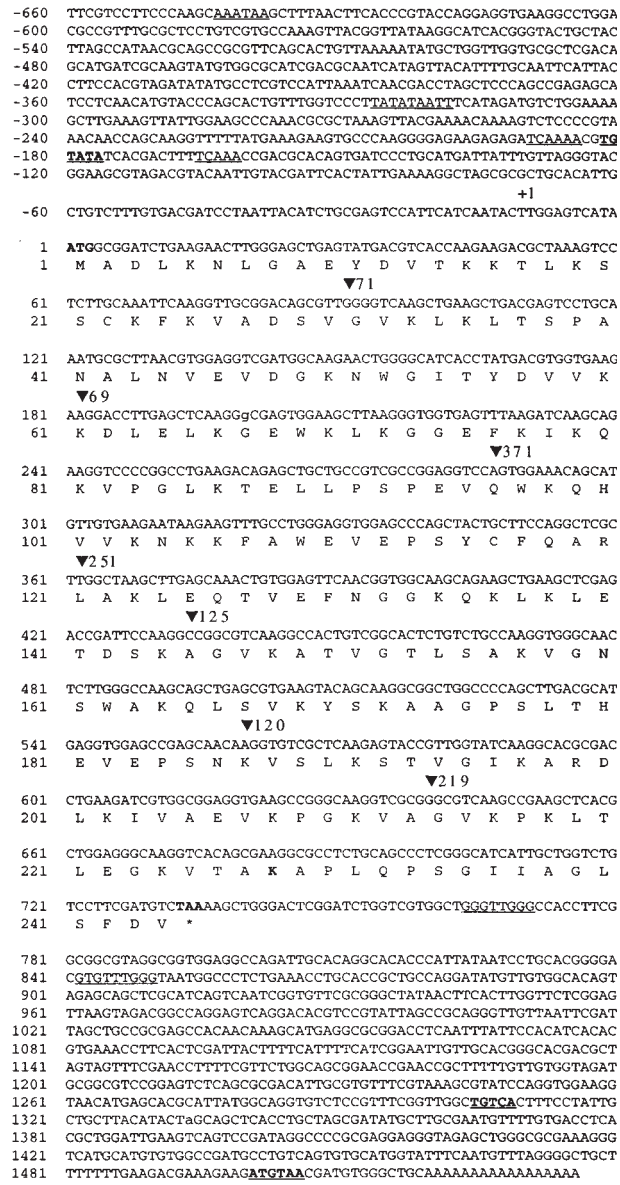

Figure 1. Nucleotide Sequence of the cDNA and the Deduced Amino Acid Sequence of Volvoxopsin.

Underlined sequences at the 5′ end indicate AT-rich segments with a potential regulatory function. The potential RNA start site is in boldface and underlined. Underlined sequences at the 3′ end are motifs that are identical in chlamyopsin cDNA. The two polyadenylation signals are also in boldface and underlined. The sequence data have EMBL accession numbers Z69301 (the cDNA) and Y11204 (the genomic sequence). Intron positions are indicated by triangles. Asterisk indicates stop codon; +1 indicates assumed RNA start.

positions (21%), whereas chlamyopsin is most related to Calliphora opsin (Huber et al., 1990), covering 54 identical residues (23%). Interestingly, most vertebrate opsin genes contain four introns, of which introns I1 and I2 are at the same positions as the algal introns I2 and I4 (Nathans and Hogness, 1983). At the position of the algal intron I7, an insertion is found in all animal opsin genes (Zucker et al., 1985). It follows that all eukaryotic opsins derived from a common ancestral gene and the algal introns I2, I4, and I7 predate the algal–animal divergence.

Compared with animal rhodopsins, the algal sequences are truncated at both ends and do not possess hydrophilic C-terminal tails. The putative retinal binding site of the volvoxopsin, which is coded by exon 8 and is close to the C terminus, is less similar to chlamyopsin than is the rest of the protein (Figure 3). However, this region of volvoxopsin is of especially high similarity to the invertebrate homologs. Exon 8 is the most striking feature, indicating an evolutionary relationship between algal and animal light receptors. In contrast, the retinal binding motifs of archeaen opsins show, like the whole proteins, very little relationship to the corresponding sites of eucaryotic opsins.

Volvoxopsin possesses two lysine residues within this C-terminal hydrophobic and potentially transmembrane domain, but because the $AKA_{227–229}$ motif is identical to retinal binding motifs found in several cephalopod opsins (Figure 3), Drosophila Rh6, and melanopsin, K-228 is likely to be preferred for retinal attachment in volvoxopsin. In contrast, the binding site KGAA in chlamyopsin shows a closer resemblance to the retinal binding sites of Calliphora opsin, Drosophila opsins 1 to 4, and opsins from many other insects. Outside the retinal binding region, short animal opsin-related motifs are interspersed along the whole volvoxopsin sequence. C-22 and C-116 could form a disulfide bridge that is important for chromophore formation in animal light receptors.

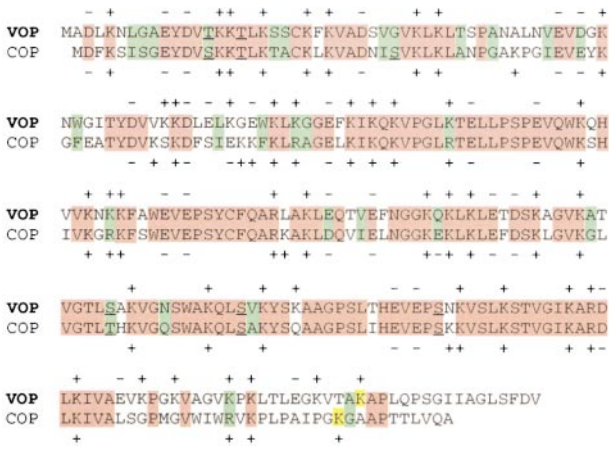

Figure 2. Amino Acid Sequence Comparison between Volvoxopsin (VOP) and Chlamyopsin (COP).

Identical amino acids are indicated by red boxes; conservative substitutions are indicated in green. The latter are defined as pairs of residues belonging to one of the following groups: STPAG, MILV, HRK, FYW, and NDQ (Dayhoff et al., 1978). Charged amino acids are labeled with (+) or (−). The putative retinal binding lysines are shown in yellow. Protein kinase C consensus sites are underlined.

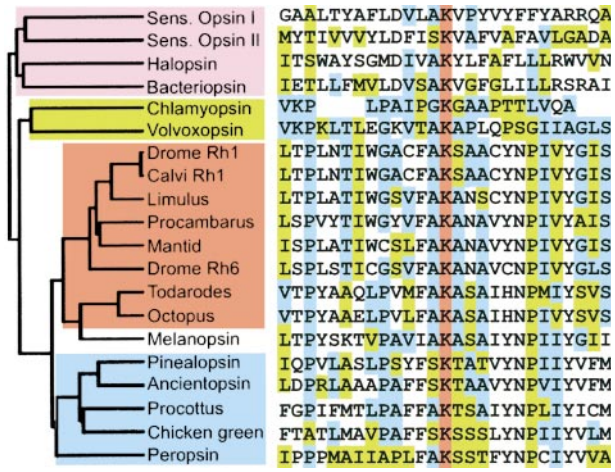

Figure 3. Phylogenetic Comparison of Retinal Binding Sites.

Putative retinal binding regions are shown from volvoxopsin (exon 8 minus the last three amino acids), chlamyopsin, opsins of *Halobacterium salinarium*, and several animal opsins. Amino acids identical in one algal opsin and one other opsin are shaded in blue; conservative exchanges are shown in green, and the putative retinal binding lysines are shown in red. Note that chlamyopsin may be aligned only by introducing a gap. Sens. Opsin, sensory opsin; Drome, *Drosophila melanogaster*; Calvi, *Caliphera vicina*.

A functionally important feature for rhodopsins is their ability to activate G proteins. As concluded from electrical measurements, volvoxopsin activates two eye-specific conductances. One activation pathway involves a second messenger (Braun and Hegemann, 1999). In eyespot preparations of *C. reinhardtii* and *Spermatozopsis similis*, G proteins have already been identified (Hegemann and Harz, 1993; Calenberg et al., 1998). The triplet DRY or ERY, present in all G protein–activating receptors, does not exist in the algal sequences, but the cytoplasmic 5 to 6 loop, which is the second important segment for G protein activation, is similar in algae. After analyzing many 5 to 6 loops of invertebrate opsins, we propose that the sequence HEExxKKVSLK/RS/T (where x indicates any amino acid) is the ancestral sequence of this region, to which more amino acids were added later.

In contrast to animal opsins, algal opsins exhibit characteristics that are shared with several ion channels. The lysine-rich sequences, interspersed with hydrophobic amino acids (residues 62 to 79), are reminiscent of S4 stretches of voltage-gated or cGMP-gated channels. The sequence

VSLKSTVGI (residues 188 to 196) is identical in both algal opsins and is reminiscent of the pore loop region of voltage-gated potassium channels. Because flash-induced photoreceptor currents begin at high flash energies with a delay of <50 msec, we had concluded that one type of eyespot channels is activated by the rhodopsin through a direct contact or that the rhodopsin itself exhibits a low light–regulated conductance (Harz et al., 1992; Holland et al., 1996; Braun and Hegemann, 1999). If algal rhodopsins fulfill such a dual role, this would elegantly explain the two different eye-specific photocurrents (Braun and Hegemann, 1999).

Opsin Gene Structure

The volvoxopsin-encoding gene, *vop*, comprises seven introns of sizes varying between 69 and 370 bp. Introns I1 and I2 are remarkably small (71 and 69 bp), as in several other *V. carteri* genes (Fabry et al., 1993). The transcription start has not been determined experimentally; however, the 5′ region contains several AT-rich segments of a sort that is assumed to serve as a promotor element in other *V. carteri* genes. TGTATA at position −178 is the most likely transcription start site. It resembles the only AT-rich element, TGTAAA, in the 5′ region of the chlamyopsin gene (Fuhrmann, 1996). The start codon is part of a start consensus sequence, with a G residue at position +4 and an A residue at position −3 (Kozak, 1981). The codon usage of the *vop* gene is biased toward G and C at the third position, but this preference is less stringent for *vop* than it is in highly expressed algal genes.

vop Expression during the *V. carteri* Life Cycle

vop was expressed in *Schizosaccharomyces pombe* and affinity purified by means of an added histidine tag (see Methods). Anti-volvoxopsin antibodies were raised in rabbits. The antibodies identified a single protein with an apparent molecular mass of 30 kD in *V. carteri* total membrane fractions. This molecular mass is larger than is the one deduced from the sequence, probably due to post-translational modification or a positive net charge at neutral pH. The modification is not glycosylation, because treatment with anhydrous hydrofluoric acid did not alter volvoxopsin migration during PAGE. During all stages of the life cycle, which requires 48 hr and two light and dark periods for completion under our conditions (Figure 4), volvoxopsin was detectable by protein gel blotting.

To assign the volvoxopsin content to certain cell types, somatic cells were separated from gonidia or embryos (Figure 5). In somatic cells, volvoxopsin was present during all stages of the 48-hr asexual life cycle. After mild disruption of somatic cells, eyespot membranes were enriched by sucrose density gradient centrifugation, according to Deininger et al. (1995). Unfortunately, *V. carteri* eyespot membranes did not fractionate as a sharp band, because eyespot size and pigmentation differ between anterior and posterior cells. The lipid fraction, S1, from the top of the gradient and the green membrane fraction, S3, only contained small amounts of volvoxopsin. As expected for a visual receptor, the somatic volvoxopsin was enriched in the orange eyespot fraction, S2, suggesting that volvoxopsin is localized within the eyespot area, as chlamyopsin is in the unicellular relative *C. reinhardtii* (Deininger et al., 1995). However, as a surprising result, the volvoxopsin concentration was found to be higher in the total membrane fraction of gonidia, which lack visible eyespots, than in the total membrane fraction of somatic cells. Considering that the volume of a single gonidium at the end of the life cycle is >500 times larger than that of a somatic cell, the total volvoxopsin amount in a gonidium is dramatically higher than in a somatic cell. During the process of embryogenesis, the volvoxopsin concentration further increased two- to threefold (Figure 5).

RNA of whole spheroids was probed for the *vop* transcript on RNA gel blots by using a *vop* cDNA. Among the RNA from unsynchronized spheroids, only one faint band of 1.7 kb was identified, which is in agreement with the size of the sequenced *vop* cDNA. To track *vop* expression during the

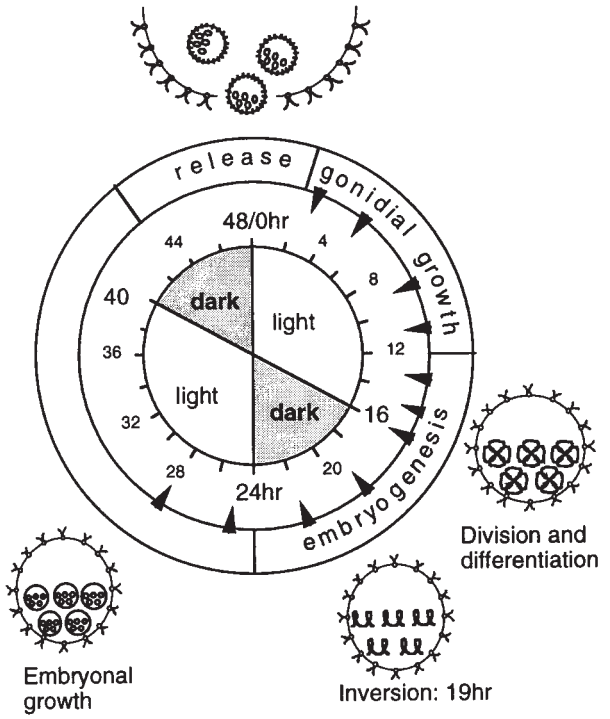

Figure 4. Asexual Life Cycle of Strain HK10 under a 16-Hr-Light/8-Hr-Dark Cycle.

Colonies were collected at the times indicated by arrowheads. Gonidia or embryos were separated from somatic cells and further analyzed as described in the legends to Figures 5 and 6.

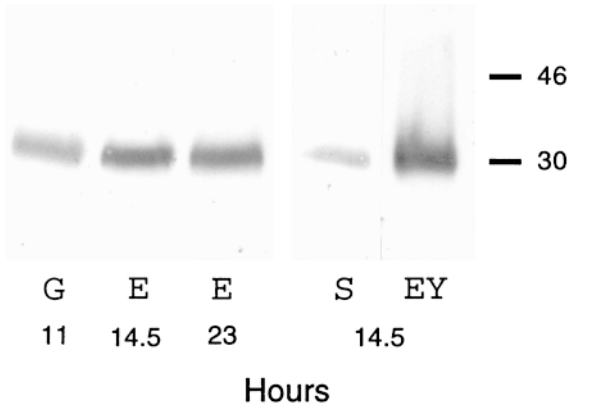

Figure 5. Volvoxopsin Concentration in Selected Membrane Preparations as Tested by Protein Gel Blotting.

The numbers below the lanes indicate the hours at which spheroids were harvested. Numbers at right indicate molecular mass markers in kilodaltons. E, embryos; EY, enriched eyespot membranes; G, gonidia; S, somatic cells.

cell cycle and to distinguish between *vop* transcripts in different cell types, RNA was extracted separately from somatic cells and from gonidia or embryos, prepared from spheroids of a synchronized culture. The *vop* RNA level was quantified by competitive reverse transcription–polymerase chain reaction (RT-PCR). Initially, we anticipated that volvoxopsin would be synthesized in juvenile somatic cells during the light period after embryogenesis (Figure 4) to provide the young spheroid with maximal phototactic sensitivity at a time when it is released from the mother sphere. Surprisingly, however, the *vop* transcript was detectable in neither maternal somatic cells nor somatic cells of the juveniles (certainly $<$300 transcripts per microgram of RNA).

Next, isolated gonidia and embryos were tested for the presence of the *vop* transcript. It was detectable in gonidia during the entire light period between spheroid release (time 0 hr) and the beginning of embryogenesis (Figure 6A). Shortly after release, the RNA level was low, 2000 to 3000 transcripts per microgram total RNA, corresponding to only 10 to 50 molecules per gonidium. It increased 30-fold during the next 10 hr. During embryogenesis, the transcript accumulated again $>$400-fold, with a brief stop during the inversion process. The maximum was reached shortly after the light came back on, and then it rapidly declined to a level of $<$2000 transcripts per juvenile spheroid or just a few molecules per cell. Strictly speaking, the competitive RT-PCR experiments could only show the presence of the short 200-bp fragment from the 5′ end that the primers were designed to amplify. However, RNA gel blotting confirmed the low *vop* RNA level in young embryos and demonstrated a low turnover based on the absence of degradation products (Figure 6B). At the end of the dark period at 22 hr, degradation started, and the transcript disappeared almost completely during the next 6 hr. These results show that accumulation of the *vop* transcript and its degradation are precisely regulated during the developmental cycle. The PCR results suggest that in addition to the developmental stage, darkness is an important factor that promotes accumulation of the *vop* transcript, whereas light triggers its degradation.

Antisense Transformant

To further explore the influence of light on volvoxopsin regulation, a transformant with reduced *vop* expression was created. Hallmann et al. (1997) were able to achieve gene

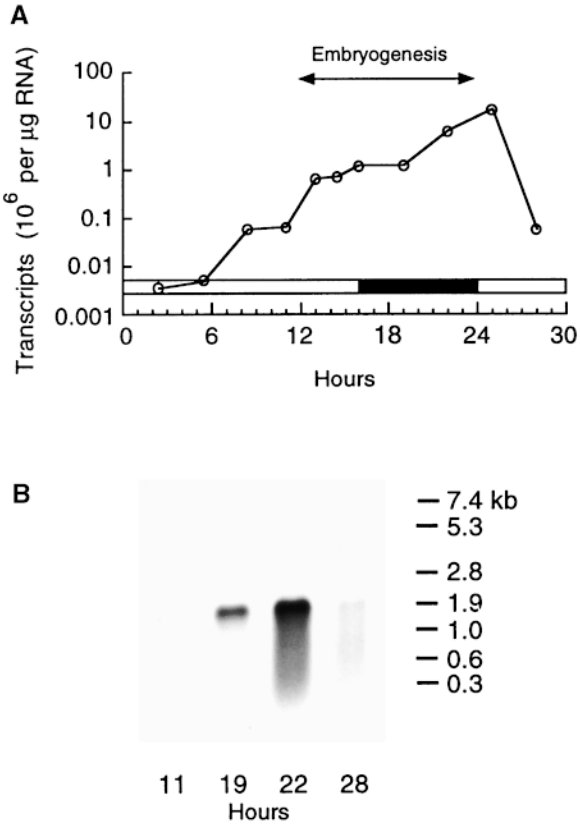

Figure 6. Accumulation of the *vop* Transcript.

(A) Number of *vop* transcripts in gonidia and developing embryos, as determined by competitive RT-PCR (log scale). At 0 hr, the spheroids were released from the mother spheroid. The concentration was determined by competitive RT-PCR.

(B) RNA gel blot analysis of gonidial and embryonal mRNA. The RNA gel blot was hybridized with the digoxigenin-labeled coding region of the *vop* cDNA. Numbers at right indicate molecular length markers in kilobases.

replacement in *V. carteri* under very special circumstances. However, our attempts to create a *vop* knock-out mutant were unsuccessful. To at least partially overcome this problem, an antisense construct was made basically as reported by Kobl (1997). Because, in green algae, introns near the 5′ end interact with the promotor region and enhance transcription (Lumbreras et al., 1998), we inverted only the 3′ region of the *vop* gene, namely, the region corresponding to exons 6 to 8 (nucleotide positions +1341 to +1920; Figure 7A). *V. carteri* was transformed with this construct plus a selectable marker (*nitA*) plasmid. Nit$^+$ transformants appeared after 1 week. Spheroids were collected at hour 14 of the life cycle (Figure 4), total membrane fractions were purified from whole spheroids (somatic cells plus gonidia), and they were probed for volvoxopsin concentration by protein gel blotting. Strains T3 and T4 contained reduced volvoxopsin concentrations (Figure 7B). The reduction was more pronounced in T4. In the wild type, the *vop* gene is a single-copy gene (Figure 7C) and the only gene of this receptor family. Based on scanning the DNA gel blot, the construct inserted 10 times in T3 and 13 times in T4 (Figure 7C). Initially, both transformants, T3 and T4, were considered as suitable control strains for further studies of volvoxopsin-triggered events. However, both strains were difficult to synchronize. T3 cells were only slightly unsynchronous, with the juvenile release being distributed over a time of 8 hr versus 3 to 4 hr in the wild type. Synchronous growth of T4 was not achieved at all. Spheroids grew and divided approximately two times more slowly than did HK10 spheroids and in a totally uncoordinated manner. This observation lends support to the earlier suggestion that a rhodopsin is involved in *V. carteri* synchronization (Kirk, 1998). Due to this complication, only T3 was used in all further experiments.

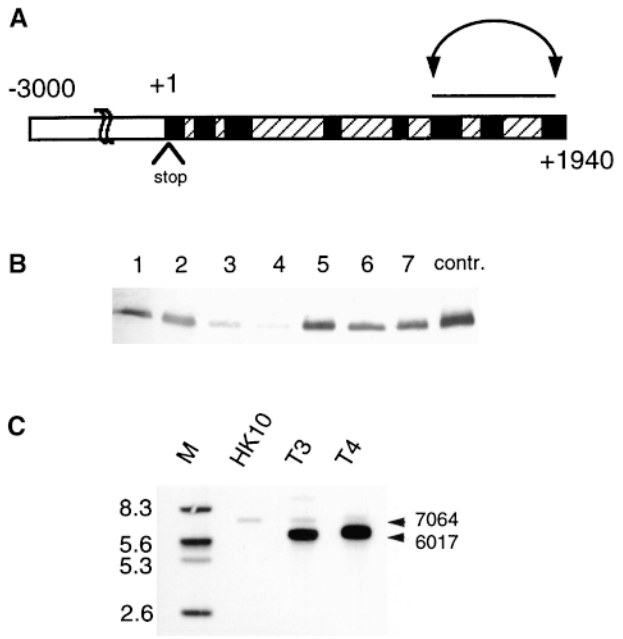

Figure 7. Antisense Transformants.

(A) The *vop* antisense construct, which covers the genomic region from positions −3000 to +1940, with an inverted 3′ region, including exons 6 to 8 and introns 6 and 7. Black boxes, exons; hatched boxes, introns; open boxes, untranslated upstream region.

(B) Volvoxopsin concentrations in total membrane fractions of the Nit$^+$ transformants 1 to 7 and the control strain HK10 (contr.) were analyzed by protein gel blotting.

(C) DNA gel blot analysis. Genomic DNA from the wild-type strain HK10 and the antisense transformants T3 and T4 were digested with ScaI. The DNA gel blot was hybridized with the digoxigenin-labeled coding region of the *vop* cDNA. For the *vop*-containing fragment in the wild type, a length of 7064 bp was predicted from the sequenced DNA fragment. For the transformants, a length of 6017 bp was calculated from the ScaI site within the antisense construct and the one in the pBluescript KS−. Note that T3 contains one *vop* insertion with an altered restriction pattern. Lane M, length markers given in kilobases.

Light Regulation of *vop* Transcription and Translation

vop transcription was further evaluated by two sets of experiments. First, the *vop* RNA was quantified in embryos and juvenile spheroids that had developed in continuous light and not under an 8-hr dark period. During the time period after inversion, the amount of *vop* message in embryos was reduced by >10-fold relative to that in embryos that had developed in darkness (Figure 8A); the protein level was reduced three- to fourfold (Figure 8, inset). The overall time course, however, was unchanged. The maximal RNA level was achieved at the 25-hr time point, which is 1 hr after the beginning of the light cycle. Thereafter, *vop* RNA rapidly declined within the next 3 hr. This result confirms that light plays an important role in regulating the accumulation of the *vop* transcript.

Apparently, light downregulates the *vop* transcript by a mechanism that up to this point has remained unclear. To determine whether volvoxopsin itself downregulates its own transcript, the *vop* message was quantified in the antisense transformant T3 (Figure 8B). The level of *vop* message (sense plus antisense) was twofold lower in the T3 transformant than it was in the wild type, although the antisense construct had inserted into the genome 10 times. The time course profile, that is, the induction and disappearance of the *vop* transcript over time, was left unchanged. However, the concentration of the sense RNA, when tested independently of the antisense transcript, was reduced >10 times (Figure 8B). Thus, for spheroids that develop in darkness, the sense RNA level correlates with the amount of the product. However, because the time course of the antisense transcript exactly follows that of the sense transcript in the wild type, we conclude that volvoxopsin is responsible for neither *vop* mRNA accumulation nor its rise or decay.

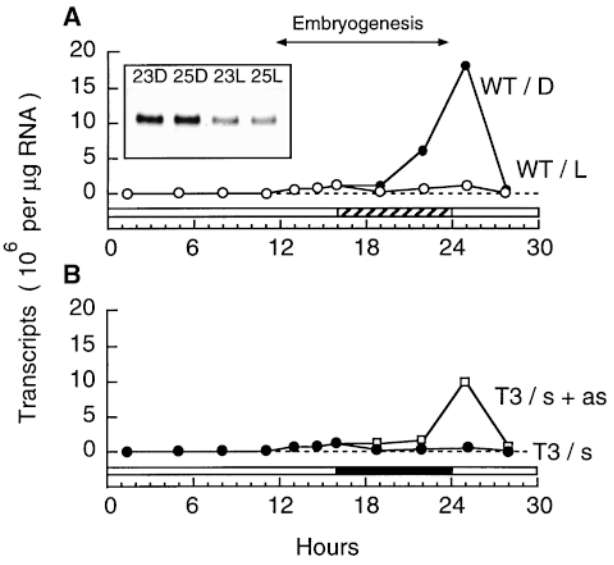

Figure 8. Comparison of *vop* Transcript Accumulation in HK10 and T3.

(A) Accumulation of the *vop* transcript in embryos of HK10 in light and darkness. Between 16 and 24 hr, spheroids were kept either in darkness (D) or light (L). The inset shows the corresponding volvoxopsin concentrations in the young spheroids under conditions both before (23 hr) and after (25 hr) the light had come back on. WT, wild type.

(B) Accumulation of the sense (s) and antisense (as) transcript in the antisense transformant T3.

Phototaxis

Phototaxis was measured in a population assay. Spheroids were placed into a small Petri dish and illuminated from one side. Due to the low number of spheroids per milliliter, a sharp migration front, as seen in the phototaxis assay developed for *C. reinhardtii* (Hegemann et al., 1988), could not be seen with *V. carteri*. We measured the accumulation of spheroids in a defined area at the dark side of the dish (Figure 9A). As already mentioned by Luntz (1931), phototaxis in Volvox is extremely sensitive to environmental factors ("Die Lichtreaktionen sind überaus launenhaft" or "Light reactions are extremely capricious"). For achieving reproducible migration, the cells were aerated in growth medium until just before the measurements were started. Juveniles, just released from the mother spheroid, showed the strongest phototactic reaction, which is consistent with the observation that Luntz (1931) made with *V. minor*. However, in contrast to *V. minor*, the age of a *V. carteri* culture is of great importance. As long as the culture was in the logarithmic growth phase, spheroids from any developmental stage showed either no or weak phototactic responses. When the culture approached the stationary phase, all spheroids stopped their development shortly after release or at a very early phase of embryogenesis, and the spheroids became phototactic. The spheroids remained relatively small, and they contained fewer somatic cells, indicating that fewer cell divisions had occurred during the previous embryogenesis (Figures 9B and 9C). The gonidia did not divide any more or passed only through a few cell division cycles. Because synchrony is difficult to achieve in a late-logarithmic culture, we used a 48-hr-light/dark regime, which facilitates synchronization. Spheroids were only phototactic during the light phase, even when growth and development were already completely disabled. It was conceivable that the low phototactic activity of *V. carteri* during the dark period was caused by the reduced energetic state. This possibility, however, could be excluded, because maintaining *V. carteri* in darkness at any other time of the life cycle for several hours did not reduce the phototactic rate.

At the growth temperature of 28°C, we observed simultaneously positive and negative phototaxis over a wide intensity range of actinic light, which precluded quantitative measurements of phototaxis. At 20°C, however, the response was consistently negative and reproducible as long as the other conditions, such as the light regime during growth and the age of the culture, were kept constant. Interestingly, we were not able to see any positive phototaxis at this temperature, no matter how low the intensity of the actinic light was.

For establishing stimulus–response curves, fresh spheroids were used for every data point. The time course of three representative recordings is shown in Figure 9D. The maximal slope of such a recording was plotted versus the photon irradiance (Figure 9E). The dynamic range of the stimulus–response curve covered only 1.5 orders of magnitude of the actinic light. Above 10^{17} photons m^{-2} sec^{-1}, the phototactic rate approached the swimming speed (Hegemann et al., 1988) and did not further increase at higher light intensities. The threshold, defined as the lowest photon irradiance at which phototaxis occurs, was determined by extrapolation of the log–linear part of the curve to zero. This is a measure of the absolute light sensitivity of the strain. As repeatedly emphasized by Foster and colleagues, the threshold in green alga, as in animal vision, is proportional to the number of photoreceptor molecules per cell, provided the efficiency of the signaling system remains unchanged (Foster and Smyth, 1980; Hegemann et al., 1988; Foster et al., 1994).

The major result of the phototaxis experiments is that the sensitivity of the transformant T3 was shifted by a factor of >10 to higher light intensities. The fact that the phototactic rate of T3 at high intensities reached the wild-type level indicates that T3 spheroids are able to migrate with the same speed toward the light, provided the light intensity is high enough. This observation makes it unlikely that the multiple integration of the antisense construct into the genome might have destroyed other genes responsible for phototaxis or motility. Thus, the T3 transformant provides genetic evidence that volvoxopsin serves as the photoreceptor for phototaxis. The lower slope of the T3 stimulus–response curve

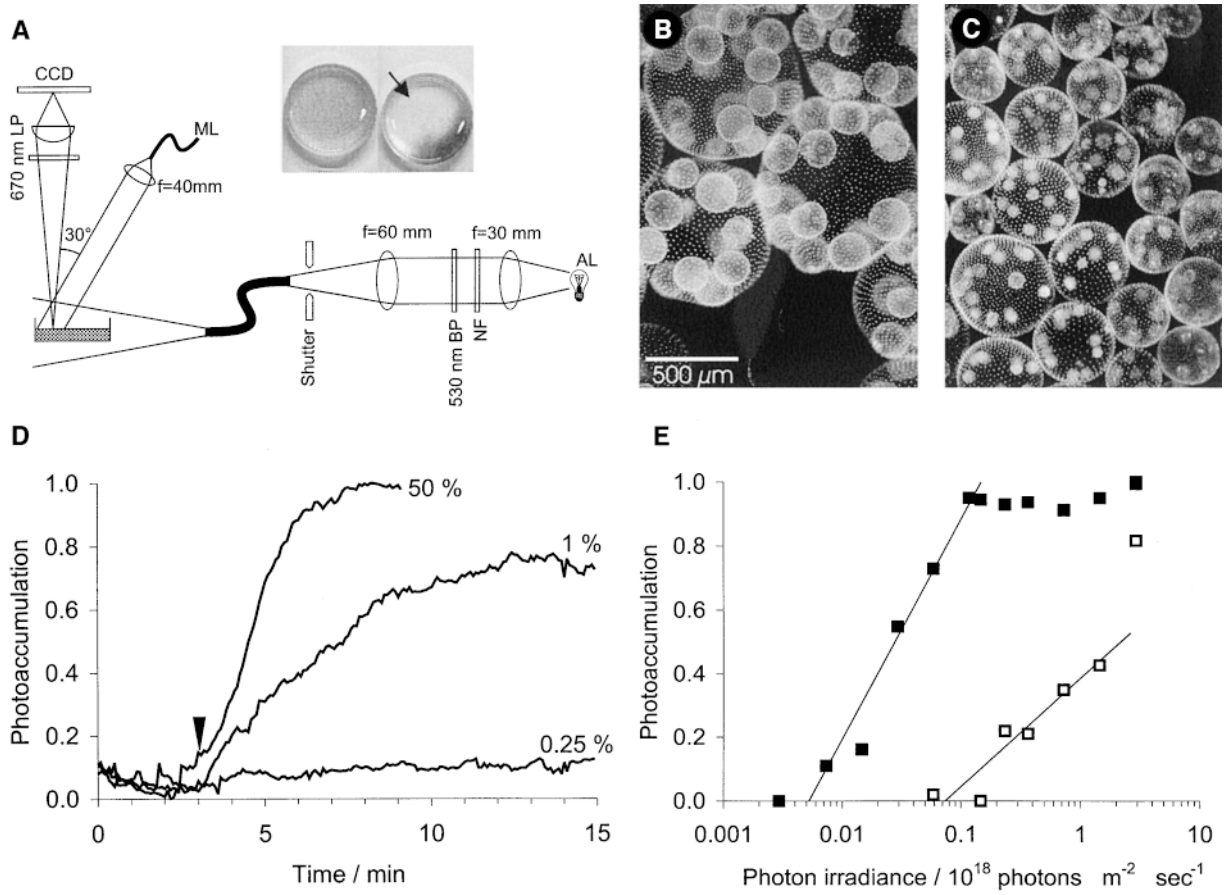

Figure 9. Phototaxis of *V. carteri* Spheroids.

(A) Design for phototaxis experiments. The Petri dish was illuminated by actinic light (AL) of 530 nm from one side in such a way that the light cone illuminated the whole dish. The actinic light was selected by a 530-nm band path filter (BP) and attenuated by exchangeable neutral density filters (NF) (100% equals 2.9×10^{18} photons m^{-2} sec^{-1}). The concentration of the spheroids was recorded by their chlorophyll fluorescence from a 100 mm^2 spot at the rear side of the dish by using 400-nm chopped measuring light (ML; 4.2×10^{18} photons m^{-2} sec^{-1}). The signal was recorded by a CCD camera equipped with a 670-nm-long path filter (LP).

(B) and **(C)** Spheroids of a log-phase culture shortly before release (~1 hr **[B]**) and of a stationary culture at the same time point **(C)**.

(D) Photoaccumulation. The photoaccumulation signal is plotted versus time (100% light intensity equals 2.9×10^{18} photons m^{-2} sec^{-1}). The arrowhead indicates the time when the actinic light was switched on.

(E) Phototactic sensitivity. The maximal slope of the accumulation signal was calculated and plotted versus the photon irradiance. The straight lines mark the extrapolated threshold irradiances. Compared with that of HK10 (filled squares), the threshold determined for T3 (open squares) is shifted by a factor of 15 to higher photon irradiance.

compared with that of the wild type is explained by the moderate unsynchrony of the T3 culture.

DISCUSSION

Rhodopsin Is the Photoreceptor for Phototaxis

That rhodopsins are the photoreceptors for phototaxis and phobic responses in green algae has been shown before by behavioral studies with Chlamydomonas mutants that are unable to synthesize retinal. In these "blind" mutants, *all-trans*-retinal and modified analogs reconstituted the phototaxis and phobic responses. The action spectrum was shifted according to the size of the conjugated polyene system of the analog (reviewed in Spudich et al., 1995). Chlamyopsin from the unicellular alga *C. reinhardtii* was the first algal opsin to be purified and sequenced. Chlamyopsin binds retinal, shows a rhodopsin-like absorption spectrum, and is localized within the eyespot overlaying membrane where, together with the optical apparatus, it may form a

functional eye (Beckmann and Hegemann, 1991; Deininger et al., 1995; Fuhrmann et al., 1999). Strictly speaking, however, it has not been definitely proven that chlamyopsin is the apoprotein of the photoreceptor that triggers phototaxis. Based on sequence similarity, gene structure, and its enrichment in eyespot fractions, volvoxopsin is the second member of the algal opsin family. In Volvox, the missing link between the opsin and phototaxis has been closed by showing that the reduction in the number of opsin molecules per cell correlates with reductions in phototactic sensitivity.

However, to measure phototaxis in *V. carteri* was unexpectedly difficult. The fact that only stationary cultures showed a strong phototactic response and only at low temperature indicates that phototaxis in multicellular algae is a poorly understood phenomenon. The poor phototaxis is inconsistent with photocurrent measurements conducted before (Braun and Hegemann, 1999) and with the recording of flagellar beating changes in response to light (Sakaguchi and Iwasa, 1979). Flash-induced photoreceptor currents are robust and may be recorded over a wide temperature range of between 10 and 30°C. Likewise, flagellar beating in response to light pulses is transiently decelerated at high temperatures and accelerated at low temperatures but is observable over a wide temperature range. Unfortunately, the highly reproducible photocurrents can be recorded only from a dissolver mutant, which releases individual cells during the process of inversion. However, this mutant could not be transformed. In contrast, flagellar beating is difficult to quantify. In addition, high-intensity red light ($>$600 nm) is needed for the recording, which already partially desensitizes the rhodopsin system.

Although the localization has not been directly shown for *V. carteri*, there is good evidence that in somatic cells, volvoxopsin is localized within the eyespot region. First, volvoxopsin is more abundant in eyespot-enriched membranes than in total membrane fractions. Second, its *C. reinhardtii* homolog is definitively localized within the eyespot overlaying membranes and is the major protein of purified eyespot membranes (Deininger et al., 1995). And third, a photoreceptor that mediates phototaxis must generate its directivity by a closed coupling to the optical system, which in green algae is the interference reflector constituted by the eyespot pigment granules.

vop Expression

During the time of maximal volvoxopsin synthesis, all cells of the young spheroid are still linked by a network of cytoplasmic bridges (Green and Kirk, 1981). These bridges are the major reason why neither RNA nor protein can be studied separately in the presumptive somatic and reproductive cells at these times. Still, it may be concluded that the somatic cells acquire their final volvoxopsin levels before the light comes back on after embryogenesis. The volvoxopsin level is probably higher in cells at the anterior side of the spheroids compared with those at the posterior end because the anterior cells have larger eyes. Because all cells grow during the light period and dramatic protein synthesis is stimulated in light, the relative volvoxopsin concentration declines within the next 16 hr.

The accumulation of the *vop* transcript and the synthesis of volvoxopsin early during embryogenesis in darkness, almost one day before it was used as a phototaxis receptor in somatic cells, initially suggested that volvoxopsin is involved at an earlier stage in processes other than movement responses. Such an alternative function could be the stimulation of protein translation in the light, which in both embryonic cell types is a requirement for final cellular differentiation. Kirk and Kirk (1985) showed that this process has an opsinlike action spectrum, which is only 30 nm red-shifted relative to the action spectra for phototaxis and photoreceptor currents (Schletz, 1976; Braun and Hegemann, 1999). It is still unclear whether the volvoxopsin presented here is the apoprotein of the receptor that regulates this process. However, considering that presumptive somatic cells as well as presumptive gonidia possess short flagella already during the time between spheroid inversion through the end of the dark period (Hoops, 1993), obviously the machinery of the visual system including the effector system is set up early during embryogenesis. This very much resembles the situation in animals, in which the eye is displayed also at a very early stage of development (Gehring, 1998).

METHODS

All biochemical studies were performed with wild-type *Volvox carteri* f *nagariensis* HK10 (female). The *V. carteri nitA*-defective strain 153-48, originally found as a nitrogen-requiring derivative of HK10 (Adams et al., 1990), was kindly provided by D. Kirk (St. Louis, MO). The λ ZAPII *V. carteri* cDNA library was originally prepared by B. Cresnar and given to us by R. Schmitt (University of Regensburg). The *V. carteri* genomic EMBL3 library was provided by K. Godl (University of Regensburg).

All strains were grown synchronously under a 16-hr-light/8-hr-dark regime (14 W m^{-2}) in standard medium (Provasoli and Pinter, 1959) at 28°C.

Identification of the *vop* cDNA and Gene

The *vop* cDNA was identified by screening a λ ZAPII *V. carteri* cDNA library. Plaques from 20 plates (8000 plaques per plate) were transferred to nylon membranes, denatured, and UV cross-linked. The filters were hybridized with a digoxigenin-labeled chlamyopsin cDNA fragment in 40% formamide and 5 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate) at 42°C and finally washed with 0.1 × SSC and 0.1% SDS at 60°C. Hybridization was detected with an anti–digoxigenin alkaline phosphatase conjugate, according to the instructions of the supplier (Boehringer Mannheim). Clone 10/1 was

excised, recloned into XL1 Bluescript (Stratagene, La Jolla, CA), and sequenced in both directions using T7 Sequenase (Amersham).

The gene and flanking regions were sequenced from two λ EMBL3 clones, LH1 and L8. Eighty thousand plaques of a genomic EMBL3 library were screened using the digoxigenin-labeled volvoxopsin cDNA coding region as probe under stringent conditions. DNA was isolated from the two positive clones. Clone LH1 was digested with PstI, clone 8/1 was digested with BamHI, and each fragment was ligated into pBluescript KS− (Stratagene). The volvoxopsin cDNA (clone 10/1) has EMBL accession number Z69301, and the genomic sequence (LH1/L8) has EMBL accession number Y11204.

vop Expression in *Schizosaccharomyces pombe* and Antibody Generation

The volvoxopsin cDNA was cloned into the expression vector *pNEU* (Hildebrandt and Oesterhelt, 1996), a derivative of pREP1 (Maundrell, 1993), and designed for high-yield expression of membrane-spanning receptors. The yeast strain *S. pombe leu1-32* was transformed with this vector by electroporation, according to standard protocols. Transformants were grown in EMM medium (Dianova, Hamburg, Germany) in the presence of 2 mM thiamine. Finally, cells were transferred to thiamine-free medium, which induced *vop* expression via the *nmt1* promotor. Eighteen hours after induction, cells were harvested, and membranes were prepared as described by Ficca et al. (1995). Volvoxopsin was expressed to a level of ~10% of the yeast total membrane protein. The added histidine tag allowed volvoxrhodopsin purification by Ni–affinity chromatography. Antibodies were raised in rabbits.

Membrane Preparation and Immunodetection

Approximately 10,000 spheroids were collected from a young culture and were disrupted in an L-Douce-homogenizer. The suspension was sonicated and centrifuged at 100,000*g* at 4°C for 1 hr. The membrane pellet was resuspended and subjected to Tris–Tricine SDS-PAGE. Antibody reactivity was tested by protein gel blot analysis, using the anti-volvoxopsin antiserum at a 1:5000 dilution and an alkaline phosphatase–coupled goat anti–rabbit second antibody (1:2000; Boehringer Mannheim). At these concentrations, the preimmune serum did not recognize any *V. carteri* proteins.

For separate analysis of gonidia or embryos, spheroids were collected at the end of the light phase during which embryogenesis begins. The spheroids were disrupted by homogenization, and the suspension was passed through a 100-mm sieve. The flowthrough contained gonidia and embryos and some individual somatic cells, whereas matrix-imbedded somatic cells were retained. The flowthrough was centrifuged repetitively at 1000*g* for 2 min. The gonidia and embryo pellet and the supernatant containing sheets with somatic cells were each sonicated and centrifuged at 10,000*g* for 10 min and 100,000*g* for 40 min. The final membrane pellets were free of intracellular and extracellular matrix proteins and did not contain cytoskeleton components (Wenzl and Sumper, 1982). The membrane pellets were further processed as described above.

Eyespot membranes were prepared according to Deininger et al. (1995), with slight modification. Briefly, spheroid sheets still containing the somatic cells from a 20-liter culture were disrupted at 1700 psi in a Parr disruption bomb. The lysate was centrifuged at 200,000*g* for 1 hr. The dark-green membrane pellet was resuspended and homogenized, and the membranes were fractionated by sucrose density gradient centrifugation (10 to 60% [w/w]). The gradient contained three fractions: S1, a lipid fraction at the top; S2, a single orange membrane fraction between 20 and 35% sucrose; and S3, a green fraction reaching from 50% to the bottom. S2 was washed free of sucrose and was subjected to SDS-PAGE.

DNA and RNA Preparation

For DNA preparation, young spheroids were isolated from mother spheroids 2 hr after the beginning of the light cycle by homogenization with an L-Dounce-homogenizer. The young colonies were repeatedly washed by centrifugation. DNA was prepared according to standard protocols. For RNA preparation, either gonidia or embryos were released from spheroids of a synchronized culture by homogenization. RNA was prepared according to Chomczynski and Sacci (1987) and further purified by using oligo(dT)–cellulose. One microgram of mRNA was loaded on a 0.9% formamide gel and transferred to a Hybond-N^+ (Amersham) nylon membrane. *vop* RNA was detected at high stringency using the digoxigenin-labeled coding sequence of the *vop* cDNA as the probe.

For quantification of *vop* transcript levels, the 5′ terminal part was reverse transcribed by using the TITAN-One tube reverse transcription–polymerase chain reaction (RT-PCR) system (Boehringer Mannheim), 250 ng RNA, and the reverse primer Vo4rev (5′-CACGTCATAGGTGATGC-3′). The cDNA was amplified after the addition of the forward primer Vo5 (5′-GATAATGGCGGATCTGAAG-3′; 35 cycles at 55°C annealing temperature).

Generation of an Antisense Transformant

The genomic region between +1341 and +1920 was cut out of the clone LH1 with PstI, purified, and religated into the same vector in the reverse orientation (LH1/pBluescript KS−). (The PstI cleavage site at −3560 was removed beforehand.) A stop codon was introduced at position +31 by inserting an extra A residue using the Quick Change site-directed mutagenesis kit (Stratagene). The antisense construct was identified among the transformants by using PCR. The plasmid was purified and cotransformed into *153-48 Nit-A* with pVcNR15 as a selection plasmid by bombardment of the cells with DNA-coated microprojectiles (Schiedlmeier et al., 1994). After 7 days, *V. carteri* transformants appeared. Total membrane fractions, DNA, and RNA were prepared as described above. The antisense message was amplified by RT-PCR using the two forward primers Vo21 (5′-CGAGAGCATCCTCAACATG-3′) and Vo34 (5′-CGTCCCACGGCGTTCATTCAC-3′).

Phototaxis

For phototaxis experiments, HK10 was grown under a 32-hr-light/16-hr-dark cycle. Spheroids (4000 per mL) at the early stationary phase were concentrated twofold and immediately transferred to a 30-mm-diameter Petri dish. An area of 100 mm^2 at the rear end of the dish was illuminated with 400 nm of light (15 nm half bandwidth [hbw]) every 5 sec for a duration of 30 msec, and the *V. carteri* spheroids within this area were monitored by their chlorophyll fluorescence via a CCD camera (Figure 9). Actinic light of 530 nm (15 nm hbw) was applied horizontally from the other side. The spheroids were recorded for 3 min in darkness followed by 8 to 20 min under actinic il-

lumination. The signal was fed into a personal computer and analyzed using the Till Photonics software package Till Vision (T.i.l.l. Photonics, Gräfelfing, Germany).

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REFERENCES

Adams, L.R., Stamer, K.A., Miller, J.K., McNally, J.G., Kirk, M.M., and Kirk, D.L. (1990). Patterns of organellar nuclear inheritance among progeny of two geographically isolated strains of *Volvox carteri*. Curr. Genet. **18,** 141–153.

Beckmann, M., and Hegemann, P. (1991). *In vitro* identification of rhodopsin in the green alga *Chlamydomonas*. Biochemistry **30,** 3692–3697.

Braun, F.J., and Hegemann, P. (1999). Two light-activated conductances in the eye of the green alga *Volvox carteri*. Biophys J. **76,** 1668–1678.

Calenberg, M., Brohsonn, U., Zedlacher, M., and Kreimer, G. (1998). Light- and Ca^{2+}-modulated heterotrimeric GTPases in the eyespot apparatus of a flagellate green algae. Plant Cell **10,** 91–103.

Chomczynski, P., and Sacci, N. (1987). Single-step method of RNA isolation by acid-guanidium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162,** 156–159.

Dayhoff, M.O., Schwarz, R.F., and Orcutt, B.C. (1978). In Atlas of Protein Sequences and Structure, Vol. 5, Suppl. 3, M.O. Dayhoff, ed (Silver Spring, MD: National Biomedical Research Foundation), pp. 345–352.

Deininger, W., Kröger, P., Hegemann, U., Lottspeich, F., and Hegemann, P. (1995). Chlamyrhodopsin represents a new type of sensory photoreceptor. EMBO J. **14,** 5849–5858.

Fabry, S., Jacobsen, A., Huber, H., Palme, K., and Schmitt, R. (1993). Structure expression and phylogenetic relationships of a family of *yptK* genes encoding small G-proteins in the green alga *Volvox carteri*. Curr. Genet. **24,** 229–240.

Ficca, A.G., Testa, L., and Valentini, G.P.T. (1995). The human β2-adrenergic receptor expressed in *Schizosaccharomyces pombe* retains its pharmacological properties. FEBS Lett. **377,** 140–144.

Foster, K.W., and Smyth, R.D. (1980). Light antennas in phototactic algae. Microbiol. Rev. **44,** 572–630.

Foster, K.W., Saranak, J., Patel, N., Zarilli, G., Okabe, M., Kline, T., and Nakanishi, K. (1984). A rhodopsin is the functional photoreceptor for phototaxis in the unicellular eucaryote *Chlamydomonas*. Nature **311,** 756–759.

Fuhrmann, M. (1996). Aufbau und Sequenz des Chlamyopsin Gens. Diplomarbeit (University of Regensburg: Regensburg, Germany).

Fuhrmann, M., Oertel, W., and Hegemann, P. (1999). A synthetic geen coding for the green fluorescent protein (GFP) is a versatile reporter in *Chlamydomonas reinhardtii.* Plant J.**19,** 1–10.

Gehring, W. (1998). Master Control Genes in Development and Evolution. (New Haven, CT: Yale University Press).

Green, K.J., and Kirk, D.L. (1981). Cleavage pattern, cell lineages and development of a cytoplasmic bridge system in *Volvox* embryos. J. Cell Biol. **91,** 441–452.

Hallmann, A., Rappel, A., and Sumper, M. (1997). Gene replacement by homologous recombination in the multicellular green alga *Volvox carteri*. Proc. Natl. Acad. Sci. USA **94,** 7469–7474.

Harz, H., Nonnengässer, C., and Hegemann, P. (1992). The photoreceptor current of the green alga Chlamydomonas. Philos. Trans. R. Soc. Lond. Ser. B **338,** 39–52.

Hegemann, P., and Harz, H. (1993). Photoreception in *Chlamydomonas*. In Signal Transduction: Prokaryotic and Simple Eukaryotic Systems, J. Kurjan and B.J. Taylor, eds (New York: Academic Press), pp. 279–307.

Hegemann, P., Hegemann, U., and Foster, K.W. (1988). Reversible bleaching of *Chlamydomonas reinhardtii* rhodopsin *in vivo.* Photochem. Photobiol. **48,** 123–128.

Hildebrandt, V., and Oesterhelt, D. (1997). Overexpression of proteins with seven transmembrane spanning segments. In Foreign Gene Expression in the Fission Yeast *Schizosacchromyces pombe*, Y. Giga-Hama and H. Kumagai, eds. (New York: Springer-Verlag), pp. 79–90.

Holland, E.-M., Braun, F.-J., Nonnengässer, C., Harz, H., and Hegemann, P. (1996). The nature of rhodopsin triggered photocurrents in *Chlamydomonas*. I. Kinetics and influence of divalent ions. Biophys. J. **70,** 924–931.

Hoops, J.H. (1993). Flagellar, cellular and organismal polarity in *Volvox carteri*. J. Cell Sci. **104,** 105–117.

Hoops, J.H. (1997). Motility in the colonial and multicellular Volvocales: Structure, function and evolution. Protoplasma **199,** 99–112.

Huber, A., Smith, D.P., Zuker, C.S., and Paulsen, R. (1990). Opsin of calliphora peripheral photoreceptors R1-6. J. Biol. Chem. **265,** 17906–17910.

Huber, A., Schultz, S., Bentrup, J., Groell, C., Wolfram, U., and Paulsen, R. (1997). Molecular cloning of *Drosophila* Rh6 rhodopsin: The visual pigment of a subset of R8 photoreceptor cells. FEBS Lett. **406,** 6–10.

Kirk, D.L. (1998). *Volvox*: Molecular Genetic Origins of Multicellularity and Cellular Differentiation. (New York: Cambridge University Press).

Kirk, D.L., and Harper, J.F. (1986). Genetic, biochemical and molecular approaches to *Volvox* development and evolution. Int. Rev. Cytol. **99,** 217–293.

Kirk, D.L., Kaufman, M.R., Keeling, R.M., and Stamer, K.A. (1991). Genetic and cytological control of the asymmetric divisions that pattern the *Volvox* embryo. Development **1** (suppl.)**,** 67–82.

Kirk, M.M., and Kirk, D.L. (1985). Translational regulation of protein synthesis, in response to light, at a critical stage of *Volvox* development. Cell **41,** 419–428.

Kobl, I. (1997). Einsatz optimierter Reportergene, antisens und kompetitive PCR-Verfahren für die molekulargenetische Analyse von *V. carteri.* PhD. Dissertation (Regensburg, Germany: University of Regensburg).

Kozak, M. (1981). Possible role of flanking nucleotides in recognition of the AUG initiator codon by eucaryotic ribosomes. Nucleic Acids Res. **9,** 81–123.

Kröger, P., and Hegemann, P. (1994). Photophobic responses and phototaxis in *Chlamydomonas* are triggered by a single rhodopsin photoreceptor. FEBS Lett. **341,** 5–9.

Lumbreras, V., Stevens, D.R., and Purton, S. (1998). Efficient foreign gene expression in *Chlamydomonas reinhardtii* mediated by an endogenous intron. Plant J. **14,** 441–447.

Luntz, A. (1931). Untersuchungen über die Phototaxis. I. Mitteilung: Die absoluten Schwellenwerte und die relative Wirksamkeit von Spektralfarben bei grünen und farblosen Einzellern. Z. Vergl. Physiol. **14,** 68–92.

Maundrell, K. (1993). Thiamine-repressible expression vectors pREP and pRIP for fission yeast. Gene **123,** 127–130.

Nathans, J., and Hogness, D.S. (1983). Isolation, sequence analysis and intron–exon arrangement of the gene encoding bovine rhodopsin. Cell **34,** 807–814.

Provasoli, L., and Pinter, I.J. (1959). Artificial media for freshwater algae: Problems and suggestions. In The Ecology of Algae, Special Publication No: 2: Pymatuning Laboratory of Field Biology, C.A. Tyron and R.T. Hartman, eds (Pittsburgh, PA: University of Pittsburgh), pp. 84–96.

Sakaguchi, H., and Iwasa, K. (1979). Two photophobic responses in *Volvox carteri*. Plant Cell Physiol. **20,** 909–916.

Schiedlmeier, B., Schmitt, R., Müller, W., Kirk, M.M., Gruber, H., Mages, W., and Kirk, D.L. (1994). Nuclear transformation of *Volvox carteri.* Proc. Natl. Acad. Sci. USA **91,** 5080–5084.

Schletz, K. (1976). Phototaxis bei *Volvox*. Pigmentsysteme der Lichtrichtungsperzeption. Z. Pflanzenphysiol. **77,** 189–211.

Spudich, J.L., Zacks, D.N., and Bogomolni, R.A. (1995). Microbial sensory rhodopsins: Photochemistry and function. Israel J. Chem. **35,** 495–513.

Tamm, S. (1994). Ca^{2+} channels and signaling in cilia and flagella. Trends Cell Biol. **4,** 305–310.

Wenzl, S., and Sumper, M. (1982). The occurrence of differently sulfated cell surface glycoproteins correlates with defined developmental events in *Volvox*. FEBS Lett. **143,** 311–315.

Zucker, C.S., Cowman, A.F., and Rubin, G.M. (1985). Isolation and structure of a rhodopsin gene from D. melanogaster. Cell **40,** 851–858.