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CHARACTERIZATION OF THE \textit{EYE2} GENE REQUIRED FOR EYESPOT ASSEMBLY IN \textit{CHLAMYDOMONAS REINHARDTII}

by

DOUGLAS GORDON WILLIAMS ROBERTS

A Dissertation Submitted to the Faculty of the

DEPARTMENT OF BIOCHEMISTRY

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

1999
As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Douglas Gordon Williams Roberts entitled Characterization of the EYE2 gene required for eyespot assembly in Chlamydomonas reinhardtii and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

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<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlamydomonas strains and media</td>
<td>66</td>
</tr>
<tr>
<td>Chlamydomonas genomic DNA isolation</td>
<td>66</td>
</tr>
<tr>
<td>Chlamydomonas transformation</td>
<td>66</td>
</tr>
<tr>
<td>Light microscopy</td>
<td>68</td>
</tr>
<tr>
<td>Flanking sequence plasmid rescue from tagged eyespot mutant strains</td>
<td>68</td>
</tr>
<tr>
<td>RNA isolation from Chlamydomonas</td>
<td>69</td>
</tr>
<tr>
<td>Total protein isolation from Chlamydomonas</td>
<td>69</td>
</tr>
<tr>
<td>Western analysis</td>
<td>70</td>
</tr>
<tr>
<td>DNA sequencing</td>
<td>70</td>
</tr>
<tr>
<td>Southern analysis</td>
<td>70</td>
</tr>
<tr>
<td>PCR amplifications</td>
<td>71</td>
</tr>
<tr>
<td>Oligonucleotide synthesis</td>
<td>71</td>
</tr>
<tr>
<td>Site-directed mutagenesis</td>
<td>71</td>
</tr>
<tr>
<td>Plasmid construction</td>
<td>76</td>
</tr>
<tr>
<td>Population-based phototaxis assays</td>
<td>78</td>
</tr>
<tr>
<td>Camera flash photoshock assays</td>
<td>79</td>
</tr>
<tr>
<td>Electrophysiology</td>
<td>79</td>
</tr>
<tr>
<td>Phototaxis assays (motion analysis)</td>
<td>80</td>
</tr>
<tr>
<td>Photoshock assays (motion analysis)</td>
<td>81</td>
</tr>
</tbody>
</table>
# TABLE OF CONTENTS - Continued

**Chapter 3: Photobehavior and ion influx assays of eyespot assembly mutants**

- **Abstract**
- **Introduction**
- **Results**
  - Photoshock behavior of the eyespot mutants
  - Phototactic behavior of the eyespot mutants
  - Signal transduction analysis of the eyespot mutants
- **Discussion**

**Chapter 4: Cloning of the EYE2 gene from *Chlamydomonas reinhardtii***

- **Abstract**
- **Introduction**
- **Results**
  - Plasmid rescue of flanking DNA from eye2-2 and *min1-5*
  - Tests of recovered *min1-5* flanking DNA as probes for genomic DNA
  - Tests of recovered flanking eye2-2 DNA as probes for genomic DNA
  - Genomic library screen for EYE2
  - Complementation of the eye2-1 defect with isolated cosmids
  - Subcloning of cosmid inserts into pARG7.8
  - Phenotype rescue by cosmid subclones
# TABLE OF CONTENTS - Continued

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sequencing the 4 kb insert of C/B-10</td>
<td>153</td>
</tr>
<tr>
<td>Gene prediction using GCG Codonpreference and Genemark</td>
<td>154</td>
</tr>
<tr>
<td>RT-PCR of wildtype <em>Chlamydomonas</em> mRNA</td>
<td>164</td>
</tr>
<tr>
<td>Extension of the <em>EYE2</em> cDNA sequence</td>
<td>174</td>
</tr>
<tr>
<td>Sequence of the partial <em>EYE2</em> cDNA</td>
<td>178</td>
</tr>
<tr>
<td>Discussion</td>
<td>194</td>
</tr>
</tbody>
</table>

**Chapter 5: Functional analysis of the *EYE2* gene and protein**

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>199</td>
</tr>
<tr>
<td>Introduction</td>
<td>200</td>
</tr>
<tr>
<td>Results</td>
<td>201</td>
</tr>
</tbody>
</table>

Demonstration of the mutation in the eye2-I strain: 201

Demonstration that the 503 aa ORF is the likely coding region for EYE2: 202

Sequence comparison of the EYE2 protein to the TRX family: 203

Site-directed mutagenesis of the EYE2 protein: 209

Epitope tagging of the EYE2 protein: 211

Discussion                                                             | 217  |

**Chapter 6: General discussion and prospective future studies**

The expression pattern of the *EYE2* gene and protein: 224

The location of the EYE2 protein in *Chlamydomonas*: 226
<table>
<thead>
<tr>
<th>Topic</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Identification of other proteins which interact with the EYE2 protein</td>
<td>226</td>
</tr>
<tr>
<td>Screening for suppressors of an eye2 mutation</td>
<td>227</td>
</tr>
<tr>
<td>The role of the thioredoxin active site motif in the EYE2 protein</td>
<td>228</td>
</tr>
<tr>
<td>The role of the RCRxxPQR repeat motif in the EYE2 protein</td>
<td>230</td>
</tr>
<tr>
<td>The role of the uORF in control of EYE2 expression</td>
<td>231</td>
</tr>
<tr>
<td>References</td>
<td>234</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Figure 1. Organization of the photoreceptive apparatus in *Chlamydomonas*....................19

Figure 2. The architecture of a *Chlamydomonas* cell.................................................23

Figure 3. Reflection and absorption functions of the eyespot that lead to directional
sensitivity.............................................................................................................28

Figure 4. Ultrastructure of the eyespot apparatus of *Chlamydomonas reinhardtii*, and
organization of the eyespot as an interference reflector........................................32

Figure 5. Angle of incident light and its effects on eyespot reflection.........................36

Figure 6. Overview of events during *Chlamydomonas* photobehavior..........................40

Figure 7. Enlargement of the eyespot region..............................................................45

Figure 8. Phototactic behaviors of *Chlamydomonas*...................................................52

Figure 9. Photoshock (photophobic) response of *Chlamydomonas*.........................56

Figure 10. Behavioral responses to light in a population of *Chlamydomonas*..............59

Figure 11. Photoshock behavior of wildtype and *minl-2* strains grown in minimal
media......................................................................................................................90

Figure 12. Representation of cell swimming behavior in the phototaxis assay............94

Figure 13. Phototactic orientation of wildtype and *eye2-l* at various light intensities...97

Figure 14. Phototactic orientation of various mutant strains at selected light
intensities..............................................................................................................105
LIST OF FIGURES - Continued

Figure 15. Electrophysiology of selected mutant strains in response to a high intensity light flash .................................................................................................................................110

Figure 16. Insertional mutagenesis and recovery of flanking DNA in *Chlamydomonas* .............................................................................................................................130

Figure 17. Restriction maps of the recovered flanking sequence plasmids ..........................................................................................................................133

Figure 18. *min1-5* flanking sequence probe fails to identify a unique sequence in the *Chlamydomonas* genome ........................................................................136

Figure 19. *eye2-2* flanking sequence probe identifies a unique band in wildtype genomic DNA ...................................................................................................................139

Figure 20. Identification of the wildtype genomic region in cosmid pools .................................................................................................................................143

Figure 21. Identification of single cosmids containing the wildtype genomic region ....146

Figure 22. Sequence of the bottom strand of the 4 kb insert from plasmid C/B-10 ....156

Figure 23. Predictions of coding potential by the programs GCG Codonpreference and GeneMark for the 4 kb genomic sequence of C/B-10 ..................................................................161

Figure 24. Identification of *EYE2* PCR products amplified from a wildtype *Chlamydomonas* reverse transcribed mRNA sample ........................................166

Figure 25. Identification of *EYE2* cDNA PCR products from a wildtype *Chlamydomonas* cDNA library .........................................................................................169
LIST OF FIGURES - Continued

Figure 26. Summary of PCR on wildtype RT-mRNA and Pazour cDNA library...........171

Figure 27. Extension of 5' and 3' borders of EYE2 cDNA by PCR.................................176

Figure 28. Sequence of the partial EYE2 cDNA and predicted EYE2 protein
sequence........................................................................................................................................180

Figure 29. Organization of the EYE2 gene in the C/B-10 genomic sequence.............185

Figure 30. Representation of the layout of the EYE2 gene in the
four kb genomic clone................................................................................................................190

Figure 31. Multiple sequence alignment of the TRX active site motif of the EYE2
protein with Thioredoxin superfamily members.................................................................207

Figure 32. EYE2-3xMYC fusion protein is undetectable in whole cell extracts........216
LIST OF TABLES

Table 1. *Chlamydomonas* strains used in this study ........................................67

Table 2. Oligonucleotides used in this study. *EYE2* sequencing primers ............72

Table 3. Oligonucleotides used in this study. *EYE2* cDNA PCR primers ............73

Table 4. Oligonucleotides used in this study. *EYE2* mutagenesis/cloning ..........75

Table 5. Plasmids used in this study ....................................................................77

Table 6. Photoshock responses of eyespot mutant strains ...............................87

Table 7. Photoreceptor currents of eyespot mutant strains ..............................112

Table 8. Mutant strains used for discussion of photobehavior ..........................115

Table 9. Results of transformation of cosmid clones into *eye2-l arg7-2* ............149

Table 10. Results of transformation of cosmid subclones into *eye2-l arg7-2* .........152

Table 11. Predicted and experimentally verified non-coding sequence information
for the *EYE2* gene ...............................................................................................192
**ABSTRACT**

*Chlamydomonas reinhardtii* is a unicellular biflagellate green alga which has the ability to perceive external light and alter its swimming behavior in response. A specialized light sensing structure, the eyespot, is responsible for this sensory ability. The eyespot is composed of photoreceptors and signal transduction components thought to be localized to the plasma membrane of the cell, and an organized arrangement of carotenoid-filled lipid granules in the chloroplast underlying the plasma membrane. To identify the components involved in eyespot assembly, a screen for eyespot assembly mutants (Lamb, et al., 1999) identified four new loci deficient in eyespot assembly. Herein, we demonstrate the eyespot assembly mutants are generally capable photobehaviors, though the phenotype of the mutant strain affects the nature and manner in which the photobehaviors are affected. Additionally, we report on the isolation of a tagged allele of *EYE2*. Using DNA adjacent to the tag site, the *EYE2* gene was isolated. Attempts to localize the protein *in vivo* were unsuccessful. The *EYE2* gene encodes a protein which contains a putative thioredoxin active site motif. Transformants harboring a mutant *EYE2* gene with the more C-terminal cysteine changed to serine possess eyespots and are phototactic. This substitution has only been observed to be tolerated in protein disulfide isomerasas; we hypothesize that the function of *EYE2* in eyespot assembly may be through the rearrangement of disulfide bonds in substrate proteins that function more directly in eyespot assembly.
This dissertation deals with a sensory organ, the eyespot, in the flagellated unicellular green alga *Chlamydomonas reinhardtii*. Mutants at the *EYE2* locus result in the loss of pigmented eyespot granules and a reduced ability to carry out positive phototaxis. The major focus of this work is the physiological characterization of eyespot assembly mutants, and isolation and characterization of the *EYE2* gene, that is required for eyespot assembly *Chlamydomonas reinhardtii*.

Chapter One describes the organization of the *Chlamydomonas* cell, and the asymmetric placement of the eyespot, relative to cytoskeletal components. Also discussed are the structure and function of the eyespot, the organization of the components of the eyespot, the function of other components required for visual responses and the previous studies of eyespot assembly mutants of *Chlamydomonas*. Chapter Two describes the materials and methods used in this study.

Chapters Three, Four, and Five describe the experiments designed to further our understanding of eyespot assembly in *Chlamydomonas*. Chapter Three details the capacity of various eyespot assembly mutants to carry out basic visual processes. Chapter Four recounts the isolation of the *EYE2* gene and a partial cDNA clone. Chapter Five relates the characterization of the *EYE2* gene and its mechanism of action in eyespot assembly. Chapter Six is a delineation of the logical sequence of future experiments designed to elucidate the mechanism of *EYE2* function in eyespot assembly.
Chapter 1: Introduction

Green algae have the ability to perceive external light and adjust their behavior in response. Unlike photoreceptive bacteria that are only capable of detecting light intensity, green algae can sense both the intensity and direction of a light source. Known photobehaviors of green algae are positive phototaxis (swimming toward a light source), negative phototaxis (swimming away from a light source), photoshock, also known as the photophobic response (a cessation of motion followed by backward swimming in response to high intensity light) and photokinesis (adjusting swimming speed as a function of light intensity) [for reviews, see Witman, 1993; Kreimer, 1994; Hegemann, 1997; Sineshchekov and Govournova, 1999; Kreimer, 1999]. These behaviors are thought to be critical for the survival of algae, because photosensory responses allow cells to move into a region of light intensity optimal for photosynthesis (photoaccumulation), while avoiding light that is intense enough to cause photoinhibition and damage to the photosynthetic apparatus.

*Chlamydomonas reinhardtii* is a unicellular green alga that is known to exhibit all of the above photobehaviors. Chlamydomonads, as well as other green algae, have unique cellular features that allow the cells to exhibit these behaviors. A primary adaptation that allows for algal photoreception is a specialized structure known as the eyespot (or stigma). This structure is thought to be required for efficient phototactic behavior, but is not thought to be required for the photoshock response (Witman, 1993), and its requirement for photokinesis is unknown.
*Chlamydomonas* is an excellent organism for the study of eyespot assembly. *Chlamydomonas reinhardtii* is amenable to both classical and molecular genetic approaches to complex problems. The aim of this study is to elucidate how the components of the eyespot in *Chlamydomonas* are assembled into a competent photoreceptive unit.

**Section 1.0**

**Components of the photoreceptive system**

Four basic components are involved in photobehavioral responses in *Chlamydomonas*: a signal receiver (the photoreceptor), a signal enhancer (the pigment granule layers), a response generator (the signal transduction pathway), and a response effector (the two flagella). Figure 1A shows a whole-cell view of the components involved in *Chlamydomonas* photoreception. The pigment granule layers can be observed by light microscopy as a bright reddish-orange spot located approximately equatorially in the cell, if the end of the cell containing the two flagella is viewed as being one pole. The line marked B in figure 1A would denote the equatorial plane, dividing the cell into an anterior half, containing the flagella, and the posterior half. In wildtype cells, only one eyespot is seen, near the surface of the cell. The presence of the eyespot in the cell automatically defines an asymmetry for the cell (Holmes and Dutcher,
Figure 1.

Organization of the photoreceptive apparatus in Chlamydomonas.

A. Whole cell view.

The eyespot is located equatorially if the cell is regarded with the flagellar (anterior) end as being polar. The placement of the eyespot breaks the symmetry of the cell and thus the two flagella are no longer equivalently located with respect to the eyespot. This asymmetry allows for the designation of the flagellum proximal to the eyespot as the *cis*-flagellum, and the distal flagellum is designated the *trans*-flagellum. If one examined the cell from the anterior end, it would be evident that the eyespot is displaced approximately 45° from the plane of the flagellar beat. The photoreceptor is located adjacent to the eyespot, and is presumed to be localized to the plasma membrane in the region overlying the eyespot pigment granules.

B. Enlargement of the eyespot region.

The pigment granules of the eyespot are located underlying the chloroplast envelope, and are subtended by a specialized region of thylakoid membrane (not shown). The photoreceptor molecules are thought to lie in the plasma membrane overlying the pigment granules.
A

10 nm

Eyespot - pigment granules and photoreceptors

B

Chlamyopsin (?)

Chloroplast Envelope

Eyespot pigment granules
1989). If the cell is imagined to be divided by a plane denoted by the line marked A in Figure 1A, then the cell is divided longitudinally into two halves, one that contains the eyespot and one that does not. The half of the cell containing the eyespot contains one flagellum, denoted the cis-flagellum. The other half of the cell has no eyespot, and the flagellum contained in this half is denoted the trans-flagellum.

The cell can also be divided into two halves by another plane, in this case the plane of the page. The two flagella beat in the same plane, and as drawn, the plane of the flagellar beat would lie in the page. The eyespot is offset 45° from the plane of the flagellar beat; this feature of the photoreceptive apparatus is significant for the precise tracking of light direction. The cell rotates as it swims, tracing a helical path if unperturbed. The offset of the eyespot from the flagellar beating plane is such that, during the time period when signaling from the photoreceptor to the flagella takes place, (for a more detailed discussion, see sections 3.0 and following) the cell rotates along the axis defined by the plane marked A in figure 1A and the flagellar beat plane lies in the same plane as the direction of incident light. The result of this delayed response is that the cell steers in the appropriate direction.

Figure 1B shows the arrangement of the pigment granules and the photoreceptor in more detail. The photoreceptor molecules are presumed to be localized in a patch in the plasma membrane, in a region just overlying the eyespot pigment granule layers in the chloroplast. Both of these components, as well as the other elements of the visual system will be discussed in more detail in later sections.
Section 2.0

Directional sensitivity due to asymmetric placement of visual apparatus

The eyespot is located asymmetrically within the cell, and this is a requirement for a directionally sensitive photoreceptive system (Foster and Smyth, 1980). Proper positioning cues for the eyespot granules, photoreceptors and signaling machinery are thought to be provided by the association of these components with specific cytoskeletal elements in the cell. *Chlamydomonas* has two flagella, each flagellum is associated with a basal body, and both flagella and basal bodies are located at the anterior of the cell. Each basal body is associated with two microtubule rootlets, a two-membered rootlet and a four-membered rootlet which extend down from the anterior end of the cell and terminate at approximately the equator of the cell. In *Chlamydomonas*, every cell contains a basal body which was formed in the prior cell division (the daughter basal body), and one that is at least one cell cycle older (the parental basal body). The eyespot in a wildtype cell is always observed located near the four-membered microtubule rootlet emanating from the daughter basal body (Figure 2).

The plane of cell division lies directly over the eyespot, just adjacent to the four-membered microtubule rootlets. To summarize what happens to the eyespot during cell division, the first event is that eyespots at the cell surface disappear. A loss of reflectivity by the eyespot has been observed prior to cell division (Morel-Laurens and
**Figure 2.**

The architecture of a Chlamydomonas cell.

A. A polar view looking down from the anterior end of the cell. The flagella are represented by the thick black lines. The daughter basal body (grey circle with the black outline) has two rootlets attached, a four-membered microtubule rootlet (four lines), and a two-membered microtubule rootlet (two lines). The parental basal body (black circle) has similar rootlets attached. Each rootlet is located 90° away from its neighboring rootlets. The eyespot (grey ellipse) is always associated with the four-membered rootlet emanating from the daughter basal body.

B. A longitudinal section view of the Chlamydomonas cell. The daughter basal body is the grey circle with the black outline, and the parental basal body is the black circle. Each has its associated microtubule rootlets as described in part A. The one flagellum extends out from each basal body, the *cis*-flagellum is associated with the daughter basal body, and the *trans*-flagellum is associated with the parental basal body. The eyespot (grey ellipse) is associated with the four-membered microtubule rootlet originating from the daughter basal body. In this representation, both flagella lie in the plane of the page. The orientation of the rootlets relative to the flagella is such that two rootlets would actually project out from the surface of the page (black rootlets), while the other two rootlets would project into the page (dashed rootlets).
Bird, 1984; Holmes and Dutcher, 1989). This is presumably due to the fact that the pigment granule layers become more disorganized (Holmes and Dutcher, 1989; Gaffal et al., 1993; Yang and Tsuboi, 1999), or that they are internalized deep within the chloroplast (Morel-Laurens and Bird, 1984). After cell division, new eyespots appear, located next to the four membered microtubule rootlet associated with the daughter basal body (Holmes and Dutcher, 1989, Gaffal et al., 1993; Yang and Tsuboi, 1999). Currently unknown is what happens to the pigment granules while cell division occurs. Two possibilities are, first that the eyespot pigment granules are completely lost and then resynthesized (Holmes and Dutcher, 1989; Yang and Tsuboi, 1999), and second, that the granules might become more disorganized, distributed to each daughter cell and then reorganized adding new components to produce the organized multi-layered granule stack (Morel-Laurens and Bird, 1984; Yang and Tsuboi, 1999).

The exact order of assembly of eyespot components is unknown, but it is clear that the microtubule rootlets are assembled prior to the placement of the eyespot pigment granules (Holmes and Dutcher, 1989). It seems probable that co-localization of the eyespot with the four-membered microtubule rootlet emanating from the daughter basal body is mediated by some difference in this rootlet; the four-membered rootlet might serve to nucleate both the positioning of the pigment granules in the chloroplast as well as the photoreceptors in the plasma membrane. Alternatively, the rootlet could position one of the visual components, for instance the photoreceptors, and the photoreceptors could signal for proper positioning of the eyespot pigment granules. It is possible that either the
four membered microtubule rootlet emanating from the daughter basal body is structurally
distinct from the others in its polypeptide composition, or the proteins of the four
membered microtubule rootlet emanating from the daughter basal body are modified in a
manner that is different from the other rootlets. However, nothing definitive is known for
the positioning signals except for the fact that when the cell is finished with division, the
visual apparatus is found in association with the four-membered rootlet attached to the
daughter basal body.

Section 2.1

Arrangement of the eyespot pigment granules

The pigment granules in green algae are an ordered array of carotenoid-filled lipid
granules. In *Chlamydomonas*, as well as other green algae, the pigment granules are
located within the chloroplast near the chloroplast envelope. In *Chlamydomonas*, the area
of the carotenoid granules is about 1.3 μm², or about 1% of the surface area of the cell
(Kreimer, 1999). The pigment granules of *Chlamydomonas reinhardtii* are arranged as
multiple layers (usually two, three or four) of granules. The first layer of granules is
tightly packed between the chloroplast envelope and a layer of thylakoid membrane, while
the subsequent layers are sandwiched between single layers of thylakoid membrane. This
arrangement of alternating layers of granules and membranes is crucial for the function of
the eyespot (Foster and Smyth, 1980) as the regularity of the spacing between the layers
Section 2.2

Function of the pigment granule layers: Shading and reflecting

The visual apparatus of *Chlamydomonas* is sensitive to the direction of light as well as the absolute intensity of the external light source. The pigment granules are a critical component in allowing the cell to sense light direction. Figure 3 shows how the pigment granule layers function in general terms to allow for directional sensitivity in phototaxis. Part A considers a situation where the light source is on the same side of the cell as the eyespot. Light will enter the cell with the potential to activate the photoreceptors directly. If this does not occur, the light will strike the eyespot and be reflected back onto the area containing the photoreceptors with the potential to activate them again. Reflection by the eyespot in this manner has been demonstrated using confocal microscopy (Kreimer and Melkonian, 1990; Kreimer, 1994; and Schaller and Uhl, 1997). Part B of Fig. 3 shows a situation in which the light is entering the cell from the side opposite the eyespot. In this case the light must pass through the entire cell body before it could activate the photoreceptors. As this situation requires that the light pass through the chloroplast, it is probable that some of the light signal will be attenuated by absorption by the photosynthetic machinery.

Experimental evidence for light attenuation by the carotenoid granules of the
Figure 3.

Reflection and absorption functions of the eyespot that lead to directional sensitivity.

A. Light entering the cell from the side with the eyespot will either be absorbed by the photoreceptor directly, or will be reflected back onto the photoreceptor by the eyespot.

B. Light entering the cell body must pass through the chloroplast where it can be absorbed by the photosynthetic apparatus. Light that passes through the cell body without being absorbed can be absorbed by the eyespot pigment granules. Light not absorbed by either the chloroplast or the eyespot pigment granules will be reflected back into the chloroplast.
A Light reflected onto photoreceptor

B Light absorbed
eyespot comes from Crescitelli et al. (1992) and Kreimer et al. (1992). The influence of the cell body and chloroplast on shading of the photoreceptor has been demonstrated by examination of the attenuation of light from different directions in eyespot deficient mutants. Measurements of the front-to-back contrast of wildtype cells (Sineshchekov, 1991; Harz et al., 1992) indicates an eight-fold difference in light reaching the photoreceptor location in cells illuminated from the side with the eyespot versus cells illuminated through the cell body. The same measurements performed in an eyeless (eye1-3) mutant of *Chlamydomonas* that conditionally lacks pigmented granules. Under conditions where eye1-3 does not contain pigmented granules, there was still a twofold front-to-back contrast in cells without an eyespot, indicating that some shading could still occur due to absorption of light by the cell body (Morel-Laurens and Feinleib, 1983; Sineshchekov et al., 1989). Other experiments with the eye1-3 mutant indicated that this eyeless strain could still respond to external light, but that phototactic orientation in these strains was inefficient (Morel-Laurens and Feinleib, 1983). Thus, the absence of an eyespot decreases the directional sensitivity of the visual apparatus, and without the pigmented granules, phototactic steering is imprecise.

Analysis of the composition of the eyespot granules in both *Chlamydomonas* and *Spermatozopsis similis* demonstrated that the carotenoid pigments contained in the granules are primarily β,β-carotene, β,Ψ-carotene and lycopene (Ohad et al., 1969; Grung et al., 1994). This specific carotenoid composition in the eyespot lipid granules is the primary determinant for the absorption properties of the eyespot. Eyespots reflect light
maximally if the wavelength is about 530-550 nm, while purified granules absorb
maximally at 550 nm. This is in good agreement with the action spectrum for
photobehavior, which shows a maximal sensitivity at about 503 nm and essentially no
response at wavelengths above 550 nm. However, the composition of the eyespot lipid
granules alone is insufficient to explain the directional sensitivity of the eyespot apparatus
and the high degree of sensitivity shown by the photoreceptive apparatus.

Section 2.3

Ultrastructure of the eyespot apparatus

Electron microscopy of *Chlamydomonas* cells shows a highly organized structure
for the eyespot region (Walne and Arnott, 1967; Melkonian and Robenek, 1984). Cross-
sections of whole cells that contain the eyespot region show a close packing of the
components of the visual apparatus. **Figure 4** shows a representation of an electron
micrograph of a cross-section through the eyespot region. The prominent feature of the
eyespot is the carotenoid-filled lipid granules contained within the chloroplast (Nakamura
et al., 1973). These are seen as tightly packed granules which are hexagonal in shape
(denoted eyespot pigment granules, represented as circles). In *Chlamydomonas
reinhardtii*, the eyespot contains multiple layers of granules. The first layer lies just under
the chloroplast envelope (OCM and ICM), and just below the first granule layer is a
thylakoid membrane (T). Adjacent to the outer chloroplast membrane is a region of
Figure 4. Ultrastructure of the eyespot apparatus of *Chlamydomonas reinhardtii*, and organization of the eyespot as an interference reflector.

A cross sectional view of the eyespot shows the organization of the components of the visual apparatus. The eyespot pigment granules are located within the chloroplast, with the first layer of pigmented granules located just underneath the outer and inner chloroplast membranes, labeled OCM and ICM respectively. Just underneath the first layer of pigmented granules (Eyespot pigment granules) is a layer of thylakoid membrane (Thylakoid layers). The next layer of granules lies a fixed distance from the first thylakoid layer and is itself subtended by another thylakoid layer. This arrangement is then repeated for all subsequent layers of the eyespot: fixed space, granule layer and thylakoid. Adjacent to the outer chloroplast membrane is a region of closely appressed plasma membrane (PM), which is thought to be the location of the photoreceptor molecules (PR patch).
Eyespot pigment granule layers
Thylakoid layers

ICM
OCM
PM
PR patch
plasma membrane (PM) which is in close proximity to the outer chloroplast membrane, and this is thought to be the location of the photoreceptors (PR patch).

The membranes found in the eyespot region are highly specialized (Melkonian and Robenek, 1980; Melkonian and Robenek, 1984; Robenek and Melkonian, 1981). As shown by freeze-fracture electron microscopy, both the plasma membrane and the outer chloroplast membrane in the eyespot region are densely packed with intramembrane particles, while the thylakoid layers have an atypically low density of intramembrane particles. Most of the particles in the plasma membrane range in diameter from about 8 to 12 nm, while those of the outer chloroplast membrane are smaller, with most in the range of 2 to 6 nm in diameter. It is generally believed that these particles are proteins involved in sensing the light and in signaling once light input is detected (Melkonian and Robenek, 1980; Melkonian and Robenek, 1984; Nultsch, 1983).

For each subsequent layer of granules, there is another layer of thylakoid membrane associated with each layer of granules. All layers of thylakoid membrane found in eyespots are observed to be associated more closely with the granule layer above it, with a regular spacing between that thylakoid layer and the granule layer below it. The regular spacing of the layers of membrane and pigmented granules is thought to be critical for the function of the eyespot.

Section 2.4
Function of the pigment granule layers: The optics of eyespot reflection

Because the pigment granules are known to aid the cell in both detecting light direction and improving the absolute sensitivity of the photoreceptive apparatus, an important question is how the structure and function of the eyespot allow this to take place. Theoretical considerations provided a convincing hypothesis for the basis for eyespot reflectivity. The eyespot was proposed to function as a quarter-wave interference reflector (Foster and Smyth, 1980). In general terms, an interference reflector is a regular alternating arrangement of materials of two different refractive indices. Such an arrangement is represented in Figure 4.

In addition to the reflective properties the eyespot has with respect to the wavelength of light, the reflection maxima produced by the eyespot will change depending on the angle of incidence of the external light. Theoretical considerations by Foster and Smyth (1980) led to the prediction that the maximal reflectivity of the eyespot would be achieved by light striking the eyespot at an angle perpendicular to the plane of the eyespot granules (i.e. 90°) (Fig. 5A). If the angle of incidence was gradually decreased from 90° toward either 0° (Fig. 5B) or 180° (Fig. 5C), the intensity of the reflected light would decrease as well. This prediction means that the amount of light striking the photoreceptor when the angle of incidence is parallel to the eyespot is minimal. As the angle is then increased, the reflection intensity will increase until the highest reflection intensity is achieved when the light is exactly perpendicular to the eyespot. Reflection
Figure 5.

Angle of incident light and its effects on eyespot reflection.

A. Angle of incident light is 90°. In the case where the angle of incident light is perpendicular to the plane of the eyespot, the reflections are maximal at the location of the plasma membrane overlying the eyespot when the wavelength of the incident light is that which produces maximal phototactic sensitivity.

B. Angle of incident light is 0°. When the angle of incidence is parallel to the plane of the eyespot, no eyespot reflection occurs.

C. Angle of incident light is 180°. When the angle of incidence is parallel to the plane of the eyespot, no eyespot reflection occurs.
intensity by the eyespot at various angles has been measured (Kreimer and Melkonian, 1990, Kreimer et al., 1992) and the predictions of Foster and Smyth (1980) were supported. Reflection intensities at angles parallel to the eyespot were not detected, and were unmeasurable until an angle of 20° (or 160°) was achieved. The intensity of the observed reflections was found to increase as predicted (by Foster and Smyth, 1980) when the angle of incidence was gradually increased from parallel to normal to the eyespot.

The dependence of the eyespot reflection intensity on the angle of incident light is another feature of the eyespot which is critical for efficient phototactic orientation. Because the reflection intensity is highest when the light direction is perpendicular to the eyespot, the probability of phototactic response is greatest when the cells are oriented with their eyespot perpendicular to the light beam. Because the reflection intensity is minimal when the angle is parallel to the eyespot, behavioral responses are non-existent when cells are swimming directly into or directly away from the light. Thus maximal orientation is achieved when a cell is swimming directly toward or away from the light. In such a case, the cell has minimized dI/dθ (the change in light intensity as a function of angle of rotation) that is sensed by the photoreceptors while the cell rotates. In theory, a cell which is swimming exactly toward or away from the light cannot distinguish between these two possibilities, as they both satisfy the condition where dI/dθ detected by the photoreceptors is constant (near zero). Schaller et al., (1997) provided an explanation for how *Chlamydomonas* can sense whether it has attained proper orientation when dI/dθ is
zero by proposing that positioning of the eyespot either on the inside or the outside of the helical path during the rotation of the forward swimming cell would allow for determination of proper orientation, either by having a "forward raked" or "backward raked" eyespot. The placement of the eyespot relative to the helical path would be determined by which flagellum was dominant (beating more strongly), either cis or trans. These observations provide a convincing picture of how the eyespot provides the cell with the capability to sense light direction efficiently.

Section 3.0

A general model for photobehaviors in *Chlamydomonas*

To review the basic events involved in photobehavior, as shown in Figure 6, the initial event is the activation of the rhodopsin photoreceptor(s) (R) presumed to be located in the plasma membrane overlying the eyespot. Following activation of the photoreceptor(s) (R*), a signal is generated due to the influx of calcium localized to the region of the eyespot (PRC). The speed with which this influx is generated is thought to reflect a tight coupling between the photoreceptors and calcium channels in the plasma membrane in the eyespot region. It is thought that the photoreceptors themselves may actually pump calcium in a light-dependent manner (Deininger et al., 1995) or that the photoreceptors may form a complex with the calcium channels. The eyespot calcium current, termed the PRC, is then thought to propagate by electrical spreading over the
Figure 6. Overview of events during Chlamydomonas photobehavior.


The primary event during photoreception is the absorption of a photon by the rhodopsin photoreceptor (R). This causes its activation to the signaling form $R^*$, which subsequently triggers the calcium influx in the plasma membrane overlying the eyespot giving rise to the photoreceptor current (PRC). The PRC then propagates throughout the plasma membrane, and apparently triggers voltage-gated calcium channels in the plasma membrane, generating the flagellar current (FC). Depending on the intensity of the original light stimulus, one of three behavioral paths is chosen.

Positive phototaxis is performed when the original light intensity is low; the intraflagellar calcium concentration $[Ca^{2+}]_f$ is thought to increase slightly, leading to an increase in the beat amplitude of the trans-flagellum and to the reduction in the beat amplitude of the cis-flagellum, causing positively phototactic steering.

Negative phototaxis occurs at higher light intensities; increasing photon absorption leads to an increased PRC, and a subsequent increase in the intraflagellar calcium concentration. This increase causes the flagellar response to be the opposite of the one observed in positive phototaxis, and the cells turn away from the light.

During the photoshock response, very high light intensity leads to the activation of many photoreceptors, and this activation ultimately results in a large increase in concentration of intraflagellar calcium. This calcium influx causes the flagella to switch from the normal beat pattern to an undulatory one, with resultant backward swimming.
Changes in flagellar protein phosphorylation

- Flagellar steering
- Positive phototaxis

- Flagellar steering
- Negative phototaxis

- Flagellar undulation
- Photoshock
plasma membrane to the region of the flagellar membrane. This event is thought to trigger a voltage-gated calcium influx in the flagellar membrane, called the flagellar current (FC). At low light intensities, the flagellar calcium influx is undetectable, but is still thought to be present.

*In vitro* data are suggestive that small magnitude changes in calcium concentration are important in phototactic steering, as small changes in calcium concentration cause differential flagellar responses in cells with demembranated flagella (Kamiya and Witman, 1984). The calcium influx into the flagella is thought to result in a change in the phosphorylation state of several flagellar proteins, resulting in a change in flagellar beat pattern. The differential responses of the two flagella are known to be involved in control of phototactic steering from *in vivo* experiments. At low light intensity, the trans-flagellum has an increased beat amplitude, while the cis-flagellum has a reduced beat amplitude, resulting in turning toward the light source. At somewhat higher intensity, the flagellar response is the opposite, and cells turn away from the light source. At very high light intensities, the flagellar calcium influx is detectable, and the change in flagellar calcium concentration is of much higher magnitude. This large calcium influx into the flagella is thought to result in a change in the phosphorylation state of several flagellar proteins, resulting in a drastic change in flagellar beat pattern, with the flagella switching from the normal breaststroke beat pattern to a mode of undulatory beating. The result of this undulatory beat pattern is that the cell switches from normal forward swimming to swimming directly backward. This is the response known as photoshock.
Section 3.1

The photoreceptor

The action spectrum for phototactic and photophobic responses in *Chlamydomonas* is suggestive of a retinal pigment as the prosthetic group for the photoreceptor protein for both photoshock and phototactic responses (Foster, et al., 1984; Kröger and Hegemann, 1994). Wildtype strains of *Chlamydomonas* show the highest sensitivity when stimulated with light of 503 nm, and the action spectra for photobehaviors in *Chlamydomonas* resemble the action spectrum observed for the vertebrate retinal-based photoreceptor, rhodopsin (Beckmann and Hegemann, 1991). Experimental evidence supports this hypothesis, as addition of exogenous retinal and its analogs can restore phototactic behavior to two carotenoid-deficient mutants, *FN 68* mutant (*carJ*) (Foster et al., 1984; Foster and Saranak, 1988; Hegemann et al., 1988), and *CC2359* (*itsJ-30*) (Hegemann et al., 1991; Lawson et al., 1991; Takahashi et al., 1991).

Incubation of cells with tritiated retinal resulted in the labeling of a single 32 kDa band on an SDS gel (Beckmann and Hegemann, 1991). This molecular weight is within the expected range of other known opsins (30-40 kDa). This latter observation led Deininger et al. (1995) to purify the $^3$H-retinal labeled band from purified eyespot fractions, and obtain the cDNA for the putative photoreceptor, which they named chlamyopsin. In contrast to all other known opsins, which are members of the seven-
transmembrane helix family, the chlamyopsin sequence was predicted to contain only four transmembrane helices. An unusual structural prediction for chlamyopsin was not altogether unexpected. Previous speculation held that the opsin may be a highly specialized protein. Chlamyopsin was thought to comprise a part of the calcium channel at the plasma membrane, a component known to be involved in photobehavior, or itself be a light-induced ion channel (specific for calcium), similar to the bacterial chloride ion pump halorhodopsin. To date, neither of these hypotheses has been tested. It should be noted, however, that these results do not conclusively demonstrate that the protein called chlamyopsin is the in vivo photoreceptor in Chlamydomonas. It is possible that the protein Deininger et al. (1995) have isolated is a retinal binding protein that has a function other than photoreception.

The location of the photoreceptor also remains unknown. Theoretical considerations led Foster and Smyth (1980) to hypothesize that the photoreceptors must be located asymmetrically in the cell; the photoreceptors were hypothesized to be located either in the plasma membrane or the outer chloroplast membrane in the region of the pigment granule layers. Freeze fracture electron microscopy (Melkonian and Robenek, 1980) demonstrated a high density of intramembrane particles in the plasma membrane overlying the eyespot, and it was proposed that these may be the photoreceptor molecules or the calcium channel responsible for the generation of the PRC. Figure 7 shows the arrangement of the pigment granules and the photoreceptor, while the chlamyopsin has been represented in the plasma membrane, this localization remains unsubstantiated.
Figure 7.

Enlargement of the eyespot region.

The pigment granules of the eyespot are located underlying the chloroplast envelope, and are subtended by a specialized region of thylakoid membrane (not shown). The photoreceptor molecules are thought to lie in the plasma membrane overlying the pigment granules.
Chlamyopsin (?)

Eyespot pigment granules

Chloroplast Envelope
Using polyclonal antisera generated against the purified chlamyopsin, Deininger et al. (1995) were able to visualize the protein in vivo by immunofluorescence. They observed a small fluorescent spot at the cell surface in wildtype cells, as well as in the carotenoid-deficient strain CC2359 (itsI-30) which was previously demonstrated (Lawson and Satir, 1994) to lack carotenoid-filled granules. In the wildtype strain, they were unable to demonstrate co-localization of the photoreceptor and the pigment granule layers; nevertheless, these observations led them to speculate that the photoreceptor was localized in a patch overlying the pigment granule layers, and that the presence of the eyespot pigment granule layers was not required for appropriate placement of the photoreceptor molecules.

Section 3.2

**Signal transduction: calcium channels**

Following activation of the photoreceptors, the cell must possess a way to transmit the visual information received by the photoreceptor to the cellular machinery which will ultimately cause the behavioral response by the flagella. Both phototactic responses and the photophobic response are strongly dependent on the presence of extracellular calcium. Measurements of ion currents made on both single cells (Harz and Hegemann, 1991; Holland et al., 1996; Nonnengäßer et al., 1996; Holland et al., 1997) and populations of
cells (Sineshchekov et al., 1992, Pazour et al., 1995; Matsuda et al., 1998) subjected to high-intensity light flashes revealed two ion influxes into the cells. The first influx is quite fast, peaking 0.5 ms after the light pulse, while the second influx is slower, occurring 10 to 30 ms after the light flash. The timing of the second influx is dependent on the intensity of the applied light flash. Flashes of higher intensity cause the second ion influx to occur more rapidly after the illumination. For both sets of inward currents, the action spectrum for the ion influxes matched the action spectrum for the behavioral responses, indicating that the currents are probably involved directly in the signal transduction pathway for photoreception. Suction pipette recording at different locations on single cells illuminated with high-intensity light was indicative that the initial ion influx was localized to the eyespot region, while the second influx was localized to the flagella (Harz and Hegemann, 1991). Thus, the first current was named the photoreceptor current (PRC) and the second was named the flagellar current (FC). At light intensities sufficient to cause the photoshock response, the amplitude of the flagellar current appears to be independent of the intensity of the light used to trigger the response. Thus it has been concluded that the flagellar current is a voltage-gated event that is initiated only when the photoreceptor current amplitude is sufficient to activate it (Harz et al., 1992). However, at lower (phototactic) light intensities, the FC is undetectable. It is generally believed that at low light, the PRC still triggers the FC, but the amplitude of the FC is below the threshold of detection. Biochemical evidence indicates that sub-micromolar changes in the flagellar calcium concentration are sufficient to alter the flagellar beat pattern required for
phototaxis (Kamiya and Witman, 1984); therefore the undetectable FC at low light intensity is not surprising.

Section 3.3

Signal transduction: other processes

The short interval between the applied light flash and the onset of ion influxes has led to the suggestion that the kinetics of this response are too rapid to allow for signal amplification by G-proteins as is commonly seen in vertebrate rhodopsin signaling (Harz et al., 1992), although this hypothesis remains controversial (Sineshchekov et al., 1991; Calenberg, et al., 1998). More recent work with measurements of Ca\(^{2+}\) influx at low light intensities indicate that at very low (positive phototaxis-inducing) intensities, the kinetics of the Ca\(^{2+}\) signaling are more complex (Braun and Hegemann, 1999), leaving open the possibility of a G-protein signal amplification pathway involved in the visual response (Schlicher et al., 1995; Linden and Kreimer, 1995; Calenberg et al., 1998; Braun and Hegemann, 1999).

G-proteins have been detected in eyespot preparations from *Chlamydomonas* (Korolkov, et al. 1989), as well as a related green alga *Spermatozopsis similis* (Schlicher et al., 1995). An *in vitro* study by Linden and Kreimer (1995) observed that addition of calcium to isolated eyespot preparations would cause an alteration of the phosphorylation
state of several proteins in the eyespot preparation. Since a calcium influx in the eyespot region is a component of the visual process, they concluded that the phosphorylation/dephosphorylation events might also be important in vision. Additionally, Schlicher et al. (1995) observed several bands in eyespot preparations that cross-reacted with antibodies against soybean Ca^{2+}-dependent protein kinases.

Recently, however, more compelling evidence for the involvement of G-proteins in algal vision was obtained. In isolated preparations of eyespots from Spermatozopsis, light was shown to modulate GTPase activity in the extracts, with maximal inhibition of GTPase activity observed when the preparations were illuminated with 502 nm light. Increasing intensity of light at this wavelength further reduced GTPase activity, while increasing intensities of light at other wavelengths did not. This result was the first which demonstrated a link between light of wavelengths which causes phototactic behavior and the activity of putative eyespot G-proteins. Interestingly, when antisera prepared against the putative *Chlamydomonas* photoreceptor chlamyopsin was incubated with the eyespot preparations, a loss of light-dependent GTPase inhibition was observed (Calenberg et al., 1998). It was also observed that Spermatozopsis eyespots contained a band of about 32 kDa which cross-reacted with the antisera against chlamyopsin and could also be labeled with ^3^H-retinal, indicating that this alga contains a protein immunologically related to the putative *Chlamydomonas* photoreceptor. In spite of the increasing evidence for G-protein involvement in signal transduction during photoreception, the only process clearly involved in visual signaling is due to changes in calcium concentration.
The calcium influx in the eyespot region following illumination is thought to cause a membrane depolarization that then spreads over the plasma membrane of the cell. Voltage-gated calcium channels in the flagellar membrane are thought to open, and the opening of the flagellar channels is observed as the flagellar current. The calcium influx in the flagella causes the two flagella to respond to the change in calcium concentration by altering their beat pattern. The molecular mechanism of calcium control of flagellar beat pattern changes remains unknown.

Section 3.4

Flagellar responses in photobehavior

The flagellar responses that control phototactic steering are thought to be due to biochemical differences in the composition of the two flagella (Kamiya and Witman, 1984), which result in a differential sensitivity to calcium concentration, and subsequently differential flagellar beat amplitudes. Figure 8 shows the flagellar responses involved in phototactic steering. A cell undergoing positive phototaxis will respond to a low light intensity by making the cis-flagellum beat weakly, while the trans-flagellum beats more strongly. The net result is that the cell turns toward the light. Conversely, a cell undergoing negative phototaxis will have the opposite flagellar response. Light of higher intensity causes the cis-flagellum to beat more strongly, while the trans-flagellum beats
Figure 8. Phototactic behaviors of Chlamydomonas.

Adapted from Rüffer and Nultsch, (1997)

A. A cell in the dark (labeled dark) beats with essentially identical beat strokes for both flagella, represented by a time course of the positions of each flagellum during the beat cycle. Position 1 is the starting position for the flagella, which extend upward to position 2, and then downward to positions 3 and 4 to generate the propulsive force for swimming. Detection of low light (light bulb and arrow) by the eyespot causes the cis-flagellum to beat weakly and the trans-flagellum to beat strongly for two or three beat strokes, and this is shown by the decreased beat amplitude of the cis-flagellum shown in positions 1 through 4 for the cell. Conversely, the trans-flagellum has an increased beat amplitude for positions 1 through 4. The net result of the flagellar responses is that the cell turns in the direction of the light (positive phototaxis).

B. A cell in the dark (labeled dark) beats with essentially identical beat strokes for both flagella, represented as in part A. Detection of more intense light (light bulb and arrows) by the eyespot causes the cis-flagellum to beat strongly and the trans-flagellum to beat weakly for two or three beat strokes, and this is shown by the increased beat amplitude of the cis-flagellum shown in positions 1 through 4 for the cell. Conversely, the trans-flagellum has a decreased beat amplitude for positions 1 through 4. The net result of the flagellar responses is that the cell turns away from the light (negative phototaxis).
A. Positive phototaxis

2. Cell turns towards light.

B. Negative phototaxis

2. Cell turns away from light.
more weakly. This flagellar response causes the cell to turn away from the direction of the incident light.

Evidence in support of the phototactic steering mechanism shown in Fig. 8 was provided by analysis of the flagellar beat pattern of illuminated cells held on micropipettes. In cells which were known to be positively phototactic, a small increase in light intensity caused the \emph{trans}-flagellum to beat more strongly. Conversely, a small decrease in light intensity resulted in the \emph{cis}-flagellum beating more strongly (Rüffer and Nultsch, 1991; Rüffer and Nultsch, 1997). The same experiment was done using immobilized cells from a negatively phototactic population. An increase in the light intensity resulted in the \emph{cis}-flagellum beating more strongly, while a decrease in light intensity resulted in the \emph{trans}-flagellum beating more strongly (Rüffer and Nultsch, 1991; Rüffer and Nultsch, 1997).

The rotation of the cell during swimming causes the visual apparatus to experience periodic changes in light intensity, (mimicked by the step-up and step-down stimuli used in the experiments of Rüffer and Nultsch, 1991; Rüffer and Nultsch, 1997); the differential responses seen in the two flagella in response to the step-up and step-down stimuli ensure that as the cell rotates the proper phototactic orientation is maintained.

The effects of calcium on the flagella that result in phototactic steering are thought to be due to differences in the biochemical properties of the two flagella. The normal calcium concentration in the flagella is thought to be around $10^{-8}$ M (Witman, 1993). From experiments using cells with demembranated flagella (Kamiya and Witman, 1984), it was demonstrated that changes in calcium concentration caused changes in flagellar beat
pattern similar to what is seen in photobehavior. The normal calcium concentration of the flagella is thought to be about $10^{-8}$ M. Kamiya and Witman (1984) observed that changes of an order of magnitude in calcium concentration would induce the changes in flagellar beat pattern. It has been proposed that the effect of calcium on the flagella is to cause a calcium-dependent change in the phosphorylation state of proteins in the flagella (Horst and Witman, 1993; Witman, 1993; King and Dutcher, 1997).

The response of the flagella during the photophobic response is much simpler. Cells illuminated with light of high intensity display a behavior in which they transiently stop swimming, swim backward for a short time and then resume swimming randomly until a subsequent stimulus is received. The flagellar response during this behavior is completely different (Fig. 9). After the high-intensity illumination, both flagella straighten out, resulting in the cessation of motion. Following this, the flagellar beat pattern changes from the normal breaststroke pattern seen in phototactic cells to one in which the flagella undulate (Rüffer and Nultsch, 1995). This undulation results in the backward swimming behavior of the photoshock response. Calcium is also involved in the control of the flagellar photoshock response, isolated axonemes from wildtype cells switch from the normal breaststroke beat pattern to the undulatory beat pattern at external calcium concentrations above $10^{-6}$ M (Hyams and Borisy, 1975, Hyams and Borisy, 1978). Following this calcium response, it is generally believed that calcium pumps within the flagella are activated, thus lowering the concentration of calcium within the flagella and allowing the return to normal flagellar beat patterns.
The cell receiving a stimulus of high light intensity responds by stopping all motion and straightening out its flagella. The two flagella then begin to undulate, causing the cell to swim directly backward. After a brief period, the cell then resumes swimming normally.
Photoshock

Cell swims backward
Section 3.5

General considerations on the photobehaviors of *Chlamydomonas*

While the absolute intensity at which an individual cell undergoes a given photobehavior depends on a variety of parameters (including prior light exposure history and phase of the cell cycle), the intensity-response curve for photobehaviors is always similar. At lower intensities, positive phototaxis is observed (Fig. 10, dotted line). As the intensity increases, the proportion of cells in the population that are undergoing positive phototaxis increases, resulting in accumulation of the population of cells at the point of illumination (photoaccumulation). At higher intensity, the cells then change behavior, switching from positive phototaxis to negative phototaxis (Fig. 10, dashed line). This switch is referred to as the reversal of the sign of phototaxis (Morel-Laurens, 1987). At light of these higher intensities, the population of cells will start to swim away from the light. This response would allow the cells to avoid light intensities that would damage the photosynthetic apparatus, and generate radical oxygen species that could damage the chloroplast. If the light intensity is increased further, the cells undergo the photoshock response (Fig. 10, solid line). Individual cells undergoing the photoshock response transiently cease all motion, swim directly backward for a brief period and subsequently return to normal swimming. A common sensory system is thought to be responsible for all three types of photobehavior (Foster et al., 1984; Uhl and Hegemann, 1990; Kröger and
Figure 10.

Behavioral responses to light in a population of Chlamydomonas.

At low light intensities (below the threshold of the photoreceptor apparatus), there is no response. As light intensity increases, the cells in the population will start to respond by swimming toward the light source, a behavior known as positive phototaxis (dotted line). As the intensity increases, there is a higher probability that more cells will respond, and the population will eventually accumulate in the light.

Upon achieving some threshold intensity, the positively orienting cells will then start to reverse the sign of phototaxis, swimming away from the light, which is called negative phototaxis (dashed line). At very high light intensities, the cells abruptly stop swimming and transiently swim backward. This behavior is known as the photoshock (or photophobic) response, and is represented by the solid line. It should be noted that this representation is idealized, and assumes a lack of any adaptational processes during the course of observation. In a realistic setting, a cell undergoing the photoshock response in the presence of continued high light stimulus would quickly become partially desensitized, and switch from the photoshock response to a negatively phototactic response. This would enable the cell to migrate away from the potentially damaging high light environment.
Positive phototaxis
Negative phototaxis
Photoshock

Cell response

Light Intensity, arbitrary units
Section 4.0

Mutations affecting eyespot assembly

*Chlamydomonas* is amenable to genetic studies, owing to its haploid genome, and the ease of mutagenesis. *Chlamydomonas* can easily be mated, and vegetative diploids can also be isolated. Biochemical purification of eyespots in *Chlamydomonas reinhardtii* has been done (Deininger et al., 1995), but faces technical difficulties owing to the presence of a cell wall, and contamination of eyespot preparations by the large chloroplast. Thus the study of eyespot assembly in *Chlamydomonas* is more easily done by genetic methods.

Four loci have been described which have defects in the formation of eyespots, *EYE1, LTS1, CAR1* and *PTX4*. Mutants that describe these loci are phototaxis deficient and have abnormalities in their eyespots. However, the *car1* and *lts1* mutants are not regarded as eyespot assembly mutants because the defects in both of these strains are in the biosynthesis of carotenoid pigments. Therefore, since the carotenoids which would be packaged into the pigment granules are absent, the strains do not possess pigmented granules.

The *eye1-1* mutant was isolated as a UV-induced mutant (Hartshorne, 1952), and displayed a lack of carotenoid granules. The mutant was originally thought to be
completely phototaxis deficient, but later experiments using \textit{eye\textsubscript{1-3}} (formerly known as \textit{ey627} and later found to be an allele of \textit{EYE1}, Lamb et al., 1999) demonstrated that the strain was still competent for phototactic behavior, but that phototactic orientation was imprecise (Morel-Laurens and Feinleib, 1983). Electron and light microscopy of the \textit{eye\textsubscript{1-3}} mutant demonstrated that the structure of the eyespot was conditionally organized; rapidly dividing cultures were observed to possess a disorganized eyespot (Morel-Laurens and Bird, 1984), which had lower reflection intensities than wildtype (Kreimer et al., 1992). This disorganization in rapidly dividing cultures is responsible for the poor photoorientation in this strain. When the eyespots of \textit{eye\textsubscript{1-3}} are disorganized, the strain has only about one quarter the wildtype amplitude photoreceptor current (Kreimer et al., 1992). However, in cultures which divide more slowly, the \textit{eye\textsubscript{1-3}} strain is observed to possess organized eyespots, essentially indistinguishable from wildtype eyespots in their reflective properties and organization at the subcellular level. Thus, it has been proposed (Morel-Laurens and Bird, 1984; Kreimer et al., 1992) that the defect in \textit{eye\textsubscript{1-3}} is not an all-or-none defect in granule assembly, but a defect in which the strain assembles its granules more slowly, such that the phenotype is only observed in rapidly dividing cultures.

Pazour et al., (1995) described an insertional mutant which displayed a different eyespot phenotype. The \textit{ptx4-I} mutant was isolated as a non-phototactic mutant, but the cells each possessed more than one eyespot. This strain was capable of the photoshock response, indicating that all the necessary components for photobehavior were present.
The strain was also wildtype for motility and calcium dependent flagellar responses. However, the light induced calcium influxes in ptx4-l were defective, the strain has a greatly reduced photoreceptor current and an altered flagellar current when stimulated with high intensity light flashes. The PTX4 gene has not yet been isolated.

More recently, Lamb et al (1999), have described many UV-induced mutants which affect eyespot assembly in Chlamydomonas. Because this screen failed to find mutants in previously known loci, the screen was not saturating. These mutations fall into four complementation groups, and map to four different loci. All four classes of mutants are deficient in phototaxis at a defined light intensity (Lamb et al., 1999), although the ability of individual cells of these strains to undergo photoshock or phototaxis at other light intensities is unknown. Additionally, all are capable of growing photoautotrophically, indicating that they are not defective in carotenoid biosynthesis. eye2 and eye3 mutants are eyeless, and appear to lack pigmented granules. However, eye3 mutants appear to be similar to eye l mutants in that these strains are capable of recovering wildtype phototactic behavior in stationary phase. The eye2-l mutant has never been observed to recover wildtype phototactic behavior under any conditions. When growing exponentially, both eye2 and eye3 strains are found to contain an occasional lipid-containing granule as shown by electron microscopy, although it is unknown if these are eyespot pigment granules or another kind of lipophilic granule in the chloroplast (Kreimer, 1994).

mlt l mutants are similar to ptx4-l in that they have multiple eyespots within the same cell. Multi-eyed cells of mlt l strains were observed to have variations in the
placement of the supernumerary eyespot. One eyespot was generally located in the wildtype position, while the secondary eyespots were most frequently located near the base of the flagella (Anderson and Dieckmann, unpublished observations). Additionally, some cells were observed to possess more than two eyespots. Electron microscopy of \textit{mlt1-3} cells demonstrated that all of the eyespots observed in this strain had a structure similar to wildtype eyespots, except for the unusual placement of the secondary eyespot.

The last mutants described by Lamb et al. (1999) are the mini-eyed mutants, \textit{min1}. These strains have a smaller than wildtype eyespot which is generally located in the wildtype position near the equator of the cell. Electron microscopy of the \textit{min1-2} mutant showed variability in the organization of the mutant eyespot. The smaller eyespot showed an essentially wildtype morphology (although smaller in size) when the strain was grown in acetate medium, but was more disordered when cells were grown in acetate-free medium. The disorganization of the eyespot pigment granules in \textit{min1-2} in acetate-free medium was manifest as an increase in the spacing between the plasma membrane and the chloroplast envelope, and this observation was interpreted as supporting evidence for a role for the \textit{MIN1} gene in organization of the entire visual apparatus.

To facilitate the isolation of the wildtype genes corresponding to the mutant loci described by Lamb et al. (1999), a mutant screen was performed to generate tagged eyespot assembly mutants. Transformation of \textit{Chlamydomonas} results in the random integration of the transforming DNA (Kindle, 1992), and the random integration can be used to generate mutant strains in which genes have been ablated by the integrated
plasmid. Using the *Chlamydomonas ARG7* or *CRY1* genes to select for transformants, non-phototactic mutants were screened for eyespot abnormalities (Quinton and Dieckmann, unpublished observations; Lamb et al., unpublished observations). Three tagged strains were recovered. *minl-5* was found to be mini-eyed, and tightly linked to the *MINI* locus, while *eye2-2* was eyeless and tightly linked to the *eye2-1* mutation. Another eyespot assembly mutant, *min2-1* was obtained by disruption with *CRY1*. This mutant is also mini-eyed and non-phototactic, but the insertion is unlinked to the mutant phenotype. For *eye2-2* and *minl-5*, the inserted *ARG7* marker segregated with the eyespot-defective phenotype, indicating that the inserted DNA was in or near the eyespot assembly gene that had been affected. The recovery of tagged eyespot assembly mutants made the isolation of the wildtype genes feasible, leading to a new understanding of the mechanism of eyespot assembly in *Chlamydomonas*.

It is anticipated that genes affecting eyespot assembly might fall into three functional classes. The first class of proteins involved in eyespot assembly would have structural roles, involved in either positioning the eyespot granules in the appropriate cellular location by recognizing the four membered microtubule rootlet emanating from the daughter basal body, or perhaps involved in packaging of the carotenoid-filled lipid granules inside the chloroplast. The second class of proteins involved in eyespot assembly would be ones that were involved in regulating other genes which function in eyespot assembly. This class might include transcriptional activators or repressors which would directly control expression of eyespot-specific genes. Alternatively, proteins of this class
could function to directly regulate other proteins which assemble eyespot components. The final class of proteins involved in eyespot assembly would be those proteins which synthesize components required for eyespot formation. This class might contain proteins which modify structural components of the eyespot, or enzymes which synthesize specific compounds necessary for eyespot formation.

To understand more about the process of eyespot assembly in *Chlamydomonas reinhardtii*, behavioral characterization of the eyespot assembly mutants was done. Additionally, the *EYE2* gene was cloned, and a partial cDNA clone was recovered. Attempts to elucidate the function of the EYE2 protein and its role in eyespot assembly were unsuccessful.
Chapter 2: Materials and methods

*Chlamydomonas* strains and media. *Chlamydomonas* strains (Table 1) were grown in modified Sager and Granick medium I with Hutner’s trace elements (Harris, 1989), either with 0.1% (w/v) sodium acetate (R) or without acetate (M). Gamete induction was performed in acetate-free low nitrogen medium (M-N/5) (Harris, 1989). Arginine-requiring strains were grown with supplemental arginine at a final concentration of 0.2 mg/mL (Harris, 1989). Cultures were typically grown at 21°C, usually under continuous light except as noted, where strains were grown on a 12 hour light:12 hour dark photoperiod. Strains grown for the electrophysiological measurements, phototaxis and photoshock assays were grown in liquid M medium on a 14 hour light:10 hour dark photoperiod, and supplemented with the bubbling of a mixture of CO₂ and air at a final concentration of 5% CO₂. Solid media contained 1.5% washed agar.

*Chlamydomonas* genomic DNA isolation. Nuclear DNA was isolated from *Chlamydomonas* following the protocol of Rochaix (1980), with the omission of the second CsCl gradient required for the purification of chloroplast DNA.

*Chlamydomonas* transformation. *Chlamydomonas* strains were transformed according to the silicon carbide whisker method of Dunahay (1993), with the following
**Table 1. Chlamydomonas strains used in this study.**

<table>
<thead>
<tr>
<th>Strain name</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>137c Mr</td>
<td>wildtype Mt</td>
<td>Harris, 1989</td>
</tr>
<tr>
<td>137c Mt</td>
<td>wildtype Mt</td>
<td>Harris, 1989</td>
</tr>
<tr>
<td>10-18</td>
<td>eye2-1 arg7-2 Mt</td>
<td>Lamb et al., 1999</td>
</tr>
<tr>
<td>12-12</td>
<td>minl-2 arg7-2 Mt</td>
<td>Lamb et al., 1999</td>
</tr>
<tr>
<td>12-18</td>
<td>mtl1-1 arg7-2 Mt</td>
<td>Lamb et al., 1999</td>
</tr>
<tr>
<td>H6-2 (minl-5)</td>
<td>MIN1::ARG7 arg7-2 Mt</td>
<td>Quinton and Dieckmann</td>
</tr>
<tr>
<td>H9-8 (eye2-2)</td>
<td>EYE2::ARG7 arg7-2 Mt</td>
<td>Quinton and Dieckmann</td>
</tr>
<tr>
<td>A3-6-1 transformants</td>
<td>(eye2-1 arg7-2 Mt)::A3-6-1</td>
<td>This study</td>
</tr>
<tr>
<td>3-10 transformants</td>
<td>(eye2-1 arg7-2 Mt)::3-10</td>
<td>This study</td>
</tr>
<tr>
<td>7-8-1 transformants</td>
<td>(eye2-1 arg7-2 Mt)::7-8-1</td>
<td>This study</td>
</tr>
<tr>
<td>8-3 transformants</td>
<td>(eye2-1 arg7-2 Mt)::8-3</td>
<td>This study</td>
</tr>
<tr>
<td>C/B-10 transformants</td>
<td>(eye2-1 arg7-2 Mt)::C/B-10</td>
<td>This study</td>
</tr>
<tr>
<td>B-15 transformants</td>
<td>(eye2-1 arg7-2 Mt)::B-15</td>
<td>This study</td>
</tr>
<tr>
<td>B-42 transformants</td>
<td>(eye2-1 arg7-2 Mt)::B-42</td>
<td>This study</td>
</tr>
<tr>
<td>EY2CYS2 transformants</td>
<td>(eye2-1 arg7-2 Mt)::EY2CYS2</td>
<td>This study</td>
</tr>
<tr>
<td>pEY2BIO transformants</td>
<td>(eye2-1 arg7-2 Mt)::pEY2BIO</td>
<td>This study</td>
</tr>
<tr>
<td>pEY2MYC transformants</td>
<td>(eye2-1 arg7-2 Mt)::pEY2MYC</td>
<td>This study</td>
</tr>
</tbody>
</table>
modifications. 5 mL cultures were grown overnight in acetate medium, containing arginine, and used to start 250 mL R+ arginine cultures for use in transformation. Three such 250 mL cultures were started, staggering the inoculation of each by one day. After the third culture was started, all cultures were grown at least three days at 21°C in continuous light, with constant agitation. Since the doubling time of strains grown on acetate is about eight hours, the three cultures should differ about ten-fold in the number of cells/mL. When the density of the middle culture attained between $4 \times 10^6$ and $7 \times 10^6$ cells/mL, all cultures were transformed with approximately 10 µg of linearized DNA, and plated on acetate medium lacking arginine, to select for transformants. Typically, the middle culture gave the highest number of transformants, usually on the order of a few hundred per transformation.

**Light microscopy.** Cells were scraped from fresh plates into liquid medium and when necessary, fixed with one twentieth volume of tincture of iodine (Harris, 1989). The presence of eyespots was confirmed by observation at 1000x magnification under oil immersion.

**Flanking sequence plasmid rescue from tagged eyespot mutant strains.** Approximately 5 to 10 µg of genomic DNA from tagged insertion mutant strains was digested to completion with SalI (which should regenerate the bacterial plasmid containing flanking *Chlamydomonas* genomic DNA; for a more detailed discussion see chapter four),
ligated in a total volume of 500 μL with T4 DNA ligase (Boehringer Mannheim, Indianapolis, IN) and transformed into bacteria.

**RNA isolation from Chlamydomonas.** Chlamydomonas total RNA was prepared using TRIzol reagent (Gibco BRL, Gaithersburg, MD), using a modification of the manufacturer's instructions. Cells were lysed in the TRIzol mix at 65°C for twenty minutes, followed by extractions with a 1:1 (v/v) phenol (pH 5.0)/chloroform mixture until the interface was clear. RNA was then precipitated using one tenth volume of 3M sodium acetate and two volumes of ice cold ethanol. Poly(A)^+ RNA was isolated using oligo-dT cellulose column chromatography by standard methods (Ausubel et al., 1992).

**Total protein isolation from Chlamydomonas.** Chlamydomonas total protein extracts were prepared by taking approximately 100 μL of liquid cultures at approximately 10^6 to 10^7 cells/mL and pelleting them by microcentrifugation. Cell pellets were resuspended in 20% trichloracetic acid with glass beads and vortexed for two minutes. Samples were incubated on ice for one hour. Proteins and glass beads were pelleted by centrifugation and the supernatant was removed and discarded. Excess acid was neutralized by the addition of 1 M Tris-HCl (pH 8.0). The protein pellet was dissolved by addition of Laemmli buffer and heat treatment at 100°C for ten minutes for use on SDS-polyacrylamide gels. SDS-polyacrylamide gels were performed by standard methods (Ausubel et al., 1992) using a 10% resolving gel, and a 4% stacking gel prepared from a
29.2.0.8 acrylamide/bis-acrylamide stock solution.

**Western analysis.** Western blotting was performed by standard methods (Ausubel et al., 1992). Detection of MYC-tagged EYE2 proteins was performed by incubating with the anti-MYC monoclonal antibody 9E5 (Babco, Berkeley CA) at a dilution of 1:100 at room temperature in BLOTTO (5% non-fat dry milk in TBS + 0.1% Tween 20) for one hour. Secondary detection of the mouse primary antibody was performed using horseradish peroxidase conjugated sheep anti-mouse IgG at a dilution of approximately 1:10000. Bands were visualized with Supersignal Chemiluminescent Substrate (Peirce, Rockford, IL) according to the manufacturer's instructions.

**DNA sequencing.** Automated DNA sequencing was performed at the DNA sequencing facility, Laboratory of Molecular Systematics and Evolution, University of Arizona, Tucson, AZ.

**Southern analysis.** Southern analyses were performed according to standard methods (Ausubel et al., 1992), using uniformly labeled DNA fragments as probes. All hybridizations and prehybridizations were performed at 65°C. High stringency washes were performed at room temperature in 5 mM Tris-HCl (8.0). For Southern analysis of *Chlamydomonas* DNA, approximately 10 μg of digested genomic DNA was loaded on the gel. For Southern analysis of PCR reactions, one tenth of the volume of the PCR reaction
was run on the gel.

**PCR amplifications.** PCR amplifications were performed according to standard methods in a total volume of 100μL (Ausubel et al., 1992). In general, the final template concentration in reactions was 1 ng/100μL for plasmids, and 100 ng/100μL for genomic DNA. For PCR amplifications using libraries as template, the reaction typically contained approximately 100 ng of template, and contained DNA from approximately 10^6 clones. Reactions generally contained 25 pmol of each primer, and the annealing temperature was chosen as 2°C lower than the calculated T_m of the primer with the lower T_m.

**Oligonucleotide synthesis.** The oligonucleotides used in this study are shown in Tables 2 (EYE2 sequencing), 3 (EYE2 PCR) and 4 (mutagenesis and cloning). The oligonucleotides used in this study were synthesized by National Biosciences Inc., Plymouth, MN, or Genosys Biotechnologies, The Woodlands, TX.

**Site-directed mutagenesis.** Plasmids CYS2 and Stop7 were generated by the method of Kunkel, (1985) using the single stranded DNA from the plasmid pKS+C/B-10R5/H as template, with oligonucleotides Newdcys2 or Newdstop as replication primers. Mutated plasmids were verified as correct by restriction analysis and by sequencing the mutated region.
Table 2. Oligonucleotides used in this study. *EYE2* sequencing primers.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>Location in <em>EYE2</em> gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Argbam</td>
<td>CGGGTGTGACAGAGTTAC</td>
<td>---</td>
</tr>
<tr>
<td>Pbrcla</td>
<td>GTGGTATATCCAGTGATT</td>
<td>---</td>
</tr>
<tr>
<td>Argbam1</td>
<td>GGACACAGAGCAATCCTGG</td>
<td>429-448</td>
</tr>
<tr>
<td>Pbrcla1</td>
<td>ACAAGAATCATTGCAGCACC</td>
<td>3537-3516</td>
</tr>
<tr>
<td>Argbam3</td>
<td>GCTGTATGTGAGGTATAG</td>
<td>697-717</td>
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<tr>
<td>Pbrcla3</td>
<td>GCGATTAACTCTGTACCTCG</td>
<td>3209-3190</td>
</tr>
<tr>
<td>Argbam4</td>
<td>TCTGTGCGAATGGCATGCGT</td>
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<tr>
<td>Pbrcla4</td>
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<tr>
<td>Argbam5</td>
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<tr>
<td>Pbrcla5</td>
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<tr>
<td>Argbam6</td>
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</tr>
<tr>
<td>Pbrcla6</td>
<td>GCTCAAAACTCCAGACAGTCCA</td>
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<td>Argbam7</td>
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<td>Pbrcla7</td>
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<td>PCA3</td>
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<td>ABA5</td>
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<td>ABA2</td>
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Table 3. Oligonucleotides used in this study. *EYE2* cDNA PCR primers.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5' to 3')</th>
<th>Location in <em>EYE2</em> gene</th>
</tr>
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<tbody>
<tr>
<td>R1E1A</td>
<td>AGGAGCAAGCACCTCGGCGGAC</td>
<td>2985-2965</td>
</tr>
<tr>
<td>R1E1S</td>
<td>GTCGCCGAGGTGCTTGCTCCT</td>
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</tr>
<tr>
<td>R1E2A</td>
<td>TGTGCCACGGCGGCACGAGCA</td>
<td>2727-2705</td>
</tr>
<tr>
<td>R1E2S</td>
<td>TGCTGCTGCGCGCAGGGGACA</td>
<td>2705-2727</td>
</tr>
<tr>
<td>R2E1A</td>
<td>CGGCCCTTGTACTTGTAAGA</td>
<td>2253-2233</td>
</tr>
<tr>
<td>R2E1S</td>
<td>CTTCTCAAGTACAAGGGGCG</td>
<td>2233-2253</td>
</tr>
<tr>
<td>R3E1A</td>
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<td>R3E1S</td>
<td>GAGGGGCTGTCGCCAGGCA</td>
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<td>R4E1A</td>
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<tr>
<td>R5E1A</td>
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<td>1109-1130</td>
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<td>CB3250S</td>
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<td>CB840A</td>
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<td>CB3350S</td>
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Table 3 (continued). *EYE2* cDNA PCR primers.

<table>
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<tr>
<th>Primer</th>
<th>Sequence (5' to 3')</th>
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<td>CB3480S</td>
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Table 4. Oligonucleotides used in this study. *EYE2* mutagenesis\(^a/\)cloning.

<table>
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<tr>
<th>Primer</th>
<th>Sequence (5' to 3')</th>
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</thead>
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<tr>
<td>Newdcys(^b)</td>
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<tr>
<td>Newdstop(^c)</td>
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<td>Mycup</td>
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<tr>
<td>Mycdn</td>
<td>AAAAAAGCTTTACAGTAGGGCGAATTGGGT</td>
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</tr>
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<td>Newchtop</td>
<td>PO(_4^--)CGACCGGGATTC</td>
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</tr>
<tr>
<td>Newchbot</td>
<td>PO(_4^--)AGCTGAATCCCGGT</td>
<td>---</td>
</tr>
<tr>
<td>Bio2up</td>
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<tr>
<td>Bio2dn</td>
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<tr>
<td>63395</td>
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</table>

\(^a\) Nucleotides which vary from the wildtype *EYE2* sequence are underlined.

\(^b\) This oligonucleotide introduces change of *EYE2* codon 193 from cysteine to serine.

\(^c\) This oligonucleotide introduces change of the *EYE2* stop codon to a *ClaI* site.
**Plasmid construction.** The plasmids used in this study are shown in Table 5. Plasmids were constructed by standard methods (Ausubel et al., 1992). All plasmids were verified as being correct by restriction analysis and either by sequencing across cloning junctions, or by sequencing the entirety of the cloned inserts. Flanking-sequence containing plasmids pBR329-10C#1 and pBR329-5B#1 were obtained by the plasmid rescue method of Tam and Lefebvre (1993). Plasmid pKS-10C S/P was constructed by subcloning a 1.2 kb *SalI/PstI* fragment from the *EYE2* flanking sequence in pBR329-10C#1 into *SalI/PstI* digested pBluescriptII-KS(+). *EYE2* containing cosmids A3-6-1, 3-10, 7-8-1 and 8-3 were isolated from a *Chlamydomonas* cosmid library (Purton and Rochaix, 1995). This library was prepared by partially digesting genomic DNA from a *cw15* strain with *MboI* and ligating size-selected fragments to the cosmid vector pARG7.8cos. This vector contains the *Chlamydomonas* selectable marker *ARG7* in the vector backbone.

Subclones of cosmid 3-10 containing the *EYE2* gene were generated by partial digestion of cosmid 3-10 with either *Sau3A1* or a combination of *Sau3A1* and *TaqI*. Cosmid fragments were size selected and ligated to either *BamHI* digested (B-15 and B-42) or *BamHI/ClaI* digested pARG7.8 (C/B-10). Plasmid pKS+C/B-10R5/H was prepared by isolating the 2.2 kb *EcoRV/HindIII* fragment from the *EYE2* gene in C/B-10, and ligating that fragment to pBluescriptII-KS(+) digested with *HindIII* and *HincII*.

Plasmids containing amplified *EYE2* cDNA products, pGEM+3400 and pGEM+670, were constructed by ligating PCR products from a synchronized cDNA
Table 5. Plasmids used in this study.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Purpose</th>
<th>Reference / Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBluescriptII-KS(+)</td>
<td>Cloning vector</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pBR329-10C#1</td>
<td>Plasmid rescue from eye2-2</td>
<td>This study</td>
</tr>
<tr>
<td>pBR329-5B#1</td>
<td>Plasmid rescue from minI-5</td>
<td>This study</td>
</tr>
<tr>
<td>pKS-10C S/P</td>
<td>Subclone of EYE2 flanking DNA</td>
<td>This study</td>
</tr>
<tr>
<td>YEP352-BIO6</td>
<td>Source of Bio tag</td>
<td>Ackerman et al., 1992</td>
</tr>
<tr>
<td>pMPY-3xMYC</td>
<td>Source of 3xMYC tag</td>
<td>Schneider et al., 1995</td>
</tr>
<tr>
<td>A3-6-1</td>
<td>EYE2 containing cosmid</td>
<td>This study</td>
</tr>
<tr>
<td>3-10</td>
<td>EYE2 containing cosmid</td>
<td>This study</td>
</tr>
<tr>
<td>7-8-1</td>
<td>EYE2 containing cosmid</td>
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</tr>
<tr>
<td>8-3</td>
<td>EYE2 containing cosmid</td>
<td>This study</td>
</tr>
<tr>
<td>pARG7.8</td>
<td>Chlamydomonas selectable marker</td>
<td>Debuchy et al., 1989</td>
</tr>
<tr>
<td>C/B-10</td>
<td>Subclone of EYE2 from 3-10</td>
<td>This study</td>
</tr>
<tr>
<td>B-15</td>
<td>Subclone of EYE2 from 3-10</td>
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<td>B-42</td>
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<tr>
<td>pKS+C/B-10R5/H</td>
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<td>This study</td>
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<td>T/A cloning vector for PCR products</td>
<td>Promega</td>
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</tr>
<tr>
<td>pGEM+670</td>
<td>EYE2 3' end cDNA clone</td>
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</tr>
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<td>CYS2</td>
<td>Site-directed mutant of EYE2</td>
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</tr>
<tr>
<td>Stop7</td>
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<tr>
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<td>Cys2 mutant in EYE2 gene</td>
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<td>SLB14</td>
<td>Bio tag at C-terminus of EYE2</td>
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</tr>
<tr>
<td>SLM7</td>
<td>3xMYC tag at C-terminus of EYE2</td>
<td>This study</td>
</tr>
<tr>
<td>pEY2BIO</td>
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<td>This study</td>
</tr>
<tr>
<td>pEY2MYC</td>
<td>3xMYC tagged EYE2 gene</td>
<td>This study</td>
</tr>
</tbody>
</table>
library using primers R2E1A and CB3400S (pGEM+3400) or R5E1S and CB670A (pGEM+670) to the pGEM-Teasy vector.

The plasmid fusing the bacterial BIO tag at the C-terminus of EYE2, SLB14, was generated by HindIII digestion of an amplified product of the BIO tag from YEP352-BIO6 using primers Bio2up and Bio2dn. This fragment was ligated along with annealed linkers Newchtop/Newchbot to C/al digested Stop7. Likewise, the 3xMYC fusion to EYE2, plasmid SLM7, was generated by HindIII digestion of a PCR product from amplification of plasmid pMPY-3xMYC with primers Mycup and Mycdn. This fragment was ligated along with annealed linkers Newchtop/Newchbot to C/al digested Stop7.

Plasmids EY2CYS2, pEY2BIO and pEY2MYC were generated by replacing the wildtype Sgf/BglII fragment of the EYE2 gene in C/B-10 with the SgfUBglll fragments from plasmids CYS2, SLB14 and SLM7 respectively. These plasmids reintroduce EYE2 constructs into the context of the genomic EYE2 clone, either the C193S mutation, the BIO-tag or the 3xMYC tag respectively.

**Population-based phototaxis assays.** Strains to be tested for phototaxis were grown on fresh R plates for a minimum of five days at 21°C in constant light. Cells were then scraped into liquid medium at low density and allowed to sit for at least four hours on the benchtop to recover motility. Tubes were then placed into a box with a slit at the bottom (Lamb et al., 1999) and illuminated with a 20 W fluorescent bulb placed 42 cm away from the slit. Positively phototactic strains were scored by migration to the slit.
region within twenty minutes after the start of the illumination period.

**Camera flash photoshock assays.** Cells were grown on fresh acetate plates for at least five days in constant light. Cells were then scraped into fresh liquid medium, and placed in complete darkness overnight to recover motility and adapt to the absence of light. Cells were then placed on a microscope slide, viewed under red-filtered light, and subjected to high intensity camera flashes. Motile cells were scored as positive for the photoshock response if the strains displayed a transient stop response directly following the light flash.

**Electrophysiology.** Cells were grown to early log phase in M or R medium bubbled with 5% CO₂ on a 14 hour light: 10 hour dark photoperiod. Aliquots of cells were dark adapted for three hours, centrifuged, washed two or three times in 50 mL of 10mM HEPES (7.5)/0.1mM CaCl₂ and placed into an "electrophysiology chamber" in a dark room for analysis (Sineshchekov et al., 1992; Pazour et al., 1995). The electrophysiology chamber is a 3 mL cell with two electrodes attached to a recording device. The population of cells is added to the chamber, and the chamber is sealed inside a box with a small opening which allows light flashes to penetrate. The light source for this experiment was a white-light generating camera flash, with no pre-orientation of the population of cells in the chamber. Amperage changes in the chamber were recorded for a duration of 50 ms, and the cells were subjected to a high intensity camera flash 5 ms into the recording
period. Recording was performed using the pClamp5.5 software package (Axon Instruments, Foster City, CA). Photocurrents were recorded five times for each strain, and the cells were allowed three minutes in between light flashes for recovery (Zacks et al., 1993).

Phototaxis assays (motion analysis). Cells were grown to early log phase in M or R medium bubbled with 5% CO₂ on a 14 hour light:10 hour dark photoperiod. Aliquots of cells were dark adapted for three hours, and placed into a “phototaxis chamber” in a dark room for analysis (Moss et al., 1995). The phototaxis chamber consists of two 18 mm x 18 mm coverslips mounted about 2 mm apart on a microscope stage. Cell suspensions are added between the two coverslips. Light is delivered by a fiber-optic cable which illuminates the coverslips from one side. Cells were subjected to light flashes from a non-actinic laser (500 +/- 10 nm) in which the laser beam was passed through neutral density filters to alter the intensity of the stimulus beam. The individual swimming paths of cells were recorded by the ExpertVision motion analysis system (Motion Analysis Corp., Santa Rosa, CA). The recording only captures paths of cells in the x and y directions, as cells swimming in the z direction drift out of the focal plane of the microscope and are no longer detected by the motion analysis system.

The duration of the stimulus beam was twenty seconds, and recording of cell swimming began ten seconds after the laser was turned on. The ten second duration of recording comprised the final ten seconds of the duration of the laser stimulus.
ExpertVision software was used to calculate the mean swimming direction for each cell path during the duration of the recording. Data for swimming direction was collected at a series of differing light intensities, with a minimum of a hundred cell paths collected for a given light intensity. Cells were allowed two minutes of dark adaptation in between stimuli.

Photoshock assays (motion analysis). Cells were grown to early log phase in M or R medium bubbled with 5% CO₂ on a 14 hour light:10 hour dark photoperiod. Aliquots of cells were dark adapted for three hours, and placed into a “phototaxis chamber” in a dark room for analysis (Moss et al., 1995). Cells were subjected to unfiltered light flashes from a non-actinic laser (500 +/- 10 nm). The individual swimming paths of cells were recorded by the ExpertVision motion analysis system (Motion Analysis Corp., Santa Rosa, CA). Recording of cell swimming was for a duration of three seconds, with a laser flash of 100 ms beginning 1.5 seconds into the recording period. ExpertVision software was used to calculate the mean swimming speed for each cell’s path during the duration of the recording. A minimum of a hundred cell paths collected for each strain. Cells were allowed two minutes of dark adaptation in between stimuli.
Chapter 3: Photobehavior and ion influx assays of eyespot assembly mutants

Abstract

It is thought that the presence of eyespot pigment granules is not required for the assembly of a functional visual apparatus. The experiments outlined in this chapter were designed to answer three questions about our eyespot mutant strains: first, do our eyespot assembly mutants have the capacity for photobehavior? Second, if the eyespot assembly mutants can respond to external light stimulus, do the strains possess properly asymmetrically localized photoreceptors? Finally, if the mutant strains do have asymmetrically localized photoreceptors, what is the basis for the phototaxis defects in these strains?

All mutant strains tested (Lamb et al., 1999; Lamb et al., unpublished observations) were able to undergo photoshock, although different mutants appear to have different threshold sensitivities for photoshock. The observation that all strains are capable of photoshock indicates that these strains possess a functional visual system. In phototaxis assays, all strains but mltl-3 displayed phototactic behavior, though the strains displayed different sensitivities to light as well. Therefore, all mutants but mltl-3 have properly asymmetrically localized photoreceptors, as this is a requirement for any phototactic response. Ion influx assays were performed on mutant cell populations, and all eyespot assembly mutant strains were observed to have a reduced photoreceptor
current (PRC), and the flagellar current (FC) was reduced in all strains. Thus, the basis for the loss of phototactic behavior in these mutant strains is mostly due to the loss of the signal enhancement provided by the eyespot. The observation that the strains can still undergo the photoshock response indicates that all other components required for photobehavior are present, and the ability to undergo phototaxis indicates a near wildtype organization of the photoreceptive machinery in these strains.

Introduction

A central question of eyespot assembly is how the photoreceptive apparatus is assembled into a fully functional system in vivo. Because the apparatus is thought to be composed of components in the plasma membrane, cytoplasm, and the chloroplast (Witman, 1993), coordinated positioning of these components must be achieved to produce a functional visual system. Two hypotheses had been proposed to explain the assembly of the photoreceptive system. One idea was that the placement of the photoreceptor molecules, either in the plasma membrane or the outer chloroplast membrane, dictates the placement of the eyespot pigment granules within the chloroplast stroma (Deininger et al., 1995). Another possibility is that some cytoplasmic component such as the four-membered microtubule rootlet emanating from the daughter basal body (Holmes and Dutcher, 1993) serves to position both elements (Walne 1966; Gruber and Rosario, 1974).
Experimental evidence was suggestive that the carotenoid-filled pigment granules do not position other components of the eyespot. Immunolocalization of the presumptive photoreceptor in a wildtype strain as well as the eyeless mutant *lts-I-30*, which is completely deficient in pigmented eyespot granules, indicated that the chlamyopsin photoreceptor was localized within a small patch at the surface of the cell (Deininger et al., 1995). These observations led to the conclusion that the appropriate localization of eyespot pigment granules was not necessary for the localization of the photoreceptor. Stronger evidence that the placement of eyespot pigment granules is not required for the localization of the photoreceptor is the observation that eyeless (*eye l-3*) mutants are still able to undergo phototaxis, although at a reduced efficiency compared to wildtype (Morel-Laurens and Feinleib, 1983). Because phototactic orientation is thought to require a photoreceptor precisely localized relative to the flagella (Foster and Smyth, 1980), this is good evidence that, for the *eye l-3* mutation at least, the presence of eyespot pigment granules is unnecessary for photoreceptor localization. Additionally, the multi-eyed mutant *ptx4-1* (Pazour et al., 1995) exhibits the photoshock response. However, as this is essentially a saturation response, the photoreceptor is not necessarily required to be localized in a small patch; in *ptx4-1*, the photoreceptor could be located anywhere within the cell.

A critical test for the appropriate localization of the photoreceptor in our eyespot mutants would be to immunolocalize the chlamyrhodopsin *in vivo*. However, this experiment has not been done because the immunolocalization procedure of Deininger and
colleagues (1995) has proven difficult to reproduce (Roberts and Dieckmann, unpublished observations).

Results

Photoshock behavior of the eyespot mutants

To test whether the phototaxis-deficient eyespot mutants have a functional photoreceptive apparatus, and to test whether the organization of the photoreceptive apparatus might vary with different growth conditions, photoshock tests were performed using our mutant strains. Cells taken from fresh plates were resuspended in liquid media, overnight in the dark, with acetate (R) and without acetate (M), or minimal low nitrogen media (M-N/5). These conditions allow the cells to recover motility by assembling flagella, and also allow the cells to become adapted to low light levels. It was thought that the mutant strains might show different behavioral responses in differing media because previous observations indicated that eyespots in wildtype cells were larger in M grown cells than R grown cells (Dieckmann, unpublished observations) and that M grown cells were more sensitive than R grown cells in the population-based phototaxis assay (Dieckmann, unpublished observations). Additionally, gametes of *Chlamydomonas* are known to exhibit the highest degree of light sensitivity (Zacks et al., 1993), so each strain was acclimated to gamete induction medium, M-N/5.
Acclimated cells were observed under the microscope with red-filtered light (which does not induce photoresponses), and subjected to high-intensity light flashes, using a camera flash. Motile cells were scored for the ability to transiently stop swimming after the light flash. Essentially, all motile cells for all strains tested (wildtype, eye2-1, min2-1, mltl-3, minl-2 and eye3-10) displayed the photoshock response (Table 6). These data are suggestive that in the eyespot mutants tested, the presence of eyespot pigment granules (eyeless strains) or the presence of a wildtype sized eyespot (mini-eyed strain) is not required for assembly of the components of the photoreceptive apparatus. However, these results do not allow for speculation about the localization of the photoreceptors, because the response does not require a precisely localized patch of photoreceptor molecules.

Interestingly, the minl-2 mutant was observed to photoshock conditionally in one experiment. The ability of this mutant to photoshock was evident in acetate (R) adapted cells, but not in cells that were shifted to acetate-free (M, M-N/5) media. This observation correlates with earlier data which demonstrated that the eyespot in the mini-eyed mutant became more disordered in cells shifted to media without a carbon source (Lamb et al., 1999). These observations are suggestive that the photoreceptive apparatus in the mini-eyed mutant might lose some component required for eyespot integrity when shifted to media without a carbon source. Alternatively, the observation that minl-2 cells could photoshock in a second experiment may demonstrate a difference in the photo-acclimation state of the cells between the two experiments.
Table 6. Photoshock responses of eyespot mutant strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Acetate Medium</th>
<th>Camera flash shock response</th>
<th>Laser shock response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wildtype</td>
<td>+</td>
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<td>--</td>
</tr>
<tr>
<td>Wildtype</td>
<td>-</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Wildtype</td>
<td>-, LN</td>
<td>Yes</td>
<td>--</td>
</tr>
<tr>
<td>eye2-1</td>
<td>+</td>
<td>Yes</td>
<td>--</td>
</tr>
<tr>
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<td>-</td>
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<td>No</td>
</tr>
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<td>--</td>
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<td>-, LN</td>
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<tr>
<td>mlt1-3</td>
<td>-, LN</td>
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<td>--</td>
</tr>
</tbody>
</table>

aData presented are the results of three independent experiments.

b Yes indicates that 100% of the motile cells observed displayed the response. Yes/No indicates that no cells were observed to respond in one experiment, while 100% were observed to respond in the other experiment. The flash intensity is approximately 3x10^7 erg/cm^2/s. The mutant strains tested in this experiment were \emph{arg7-2 Mt^+} derivatives, requiring supplemental arginine in the medium. LN indicates low nitrogen medium.

c Yes indicates that 100% of the motile cells observed displayed the response at a flash intensity of 3x10^5 erg/cm^2/s. The mutant strains tested in this experiment were \emph{ARG^7} did not require supplemental arginine for growth.
In a more quantitative photoshock assay, dark adapted cells of various strains were subjected to high intensity non-actinic (wavelength 500 +/- 10 nm) laser flashes, and the mean swimming speed of the population was monitored. Strains were scored as positive for the photoshock response if they displayed a transient stop response, resulting in a drop in the mean swimming speed for the population. Figure 11 shows the results of such an experiment using a wildtype strain and the minl-2 strain grown on minimal medium. Swimming paths for both strains were recorded for three seconds and the cells were subjected to an unfiltered non-actinic laser beam (500 +/- 10 nm) for 100 ms starting 1.5 seconds into the recording. For the wildtype strain shown in Figure 11 panel A, the mean swimming speed of the cells is about 60 μm/s for the 1.5 s prior to the light flash, and when the laser is turned on at 1.5 s, the mean swimming speed of the cells drops to about 20 μm/s. This drop in mean swimming speed is indicative that the cells are undergoing the photoshock response. Most cells in the population stop swimming in response to the light flash, and the mean swimming speed for the population decreases. The duration of the stop response of the population lasts for about 750 ms. However, the stop responses of individual cells is likely to be around 500 ms (Hegemann and Bruck, 1989). Thus, the mean swimming speed never drops to zero because the cells do not shock in synchrony during the 100 ms stimulus.

While the wildtype strain clearly displays a shock response under these conditions, the minl-2 mutant grown in minimal medium (Figure 11, panel B) does not. The mean swimming speed is about 40 μm/s and stays at that level for the duration of the
**Figure 11.**

Photoshock behavior of wildtype and *minl-2* strains grown in minimal media.

Both panels show the mean swimming speed in μm/s of dark-adapted populations of cells of either wildtype (panel A) or *minl-2* (panel B) during the course of three second of observation period. The light stimulus used for photoshock was the full intensity non-actinic laser (500 +/- 10 nm, 30000 erg/cm²/s); this stimulus was applied for 0.1 s starting 1.5 s into the course of observation, and is marked by the thick black line just below the 1.5 second time point on the abscissa. Strains are positive for the photoshock response if a transient stop response is observed following the light stimulus. This is observed as a temporary drop in the mean swimming speed of the population. For reference, the scale of the ordinate is not the same between panels A and B.
recording, even following the light stimulus at 1.5 s. This is indicative that, at least for this light intensity, the *min1-2* mutant is incapable of undergoing the photoshock response. This corroborates the earlier camera flash photoshock experiment where in one of the trials, the *min1-2* mutant failed to shock when grown on minimal medium.

In contrast to the earlier camera flash photoshock assay, not all mutants were found to be capable of photoshock under these conditions. The *min2-l* mutant and the *eye2-l* mutant did not show the stop response, while the *eye3-l0* mutant, and the acetate-grown *min1-2* mutant did (Table 6). This observation is indicative that the mutants actually have differing thresholds for photoshock, and that the response needs to be measured over a range of stimulus intensities to find out the intensity required to cause the shock response for each mutant strain. However, the high-intensity (approximately $3 \times 10^7$ erg/cm$^2$/s) camera flash photoshock experiment demonstrates that all the eyespot mutant strains in the collection have the capacity to undergo photoshock. As previously noted, the ability of the mutant strains to photoshock does indicate that they have all the components required for the visual signal transduction pathway, but it does not allow for any inference as to the organization of those components in the absence of wildtype eyespots. To attempt to infer the organization of the photoreceptor patch in our eyespot mutant strains, phototaxis assays were performed.

**Phototactic behavior of the eyespot mutants**
To attempt to infer the organizational state of the photoreceptors in our eyespot assembly mutant strains, quantitative phototaxis assays were performed. Because phototactic responses are thought to require a properly asymmetrically localized photoreceptor (Foster and Smyth, 1980), the ability of our eyespot mutant strains to undergo phototactic responses would indirectly be suggestive that the strains possess asymmetrically localized photoreceptors.

The phototaxis assay involves taking dark-adapted strains and placing them in a "phototaxis chamber" (Moss et al., 1995) in which they are subjected to a directional stimulus of known intensity. The paths of swimming cells are then recorded (using light of wavelengths that does not induce phototaxis), and the angles of the paths relative to the direction of the light stimulus is a measure of the phototactic state of the population. To elaborate, Figure 12 considers two cases of a cell responding with a directional swimming path in response to a light stimulus. If the incident light was coming in from the left-hand side of the page, then the cell swimming along the path in part A is swimming at an angle defined as +40°. If the cell had traveled directly toward the light, this would have defined a swimming angle of zero, and the behavior of the cell would be characterized as positively phototactic. In part B, the intensity of the light has been increased, and the cell is swimming in a direction that is more toward the right hand side of the page. This angle between the incident light and the cell's path is -160°. If the cell had been shown swimming directly away from the light, the subsequently defined angle between light direction and direction of travel would be + (or -) 180°, and the behavior of
Figure 12.

Representation of cell swimming behavior in the phototaxis assay.

A. If a low intensity light source is on the left, and the cell swims in the indicated direction as a response to that light, then the angle between the two vectors defines the direction of travel, in this case +40°. For reference, a cell that was swimming directly into the light would have a direction of travel of 0°.

B. If the light intensity were raised, and the cell responded with the indicated path, the direction of travel would be -160°. For reference, a cell that was swimming directly away from the light would have a direction of travel of + (or -) 180°.
A

Light Direction

Direction of travel
\[ \theta = +40^\circ \]
Cell swimming path

B

Direction of travel
\[ \theta = -160^\circ \]

Light Direction
Cell swimming path
the cell would be considered negatively phototactic. In the course of our phototaxis assays, a population of cells was placed in the "phototaxis chamber", and cell swimming paths were recorded for twenty seconds at a time. The filtered laser was turned on at the start of the recording period, but directional data were only retained for the final ten seconds of recording to ensure that photoshock responses at higher light intensities would not be recorded. Swimming path data were collected until at least a hundred cell paths were recorded for each strain at each light intensity used. A total of nine different light intensities were tested for each strain. ExpertVision motion analysis software (Motion Analysis Corp., Santa Rosa, CA) was used to determine the mean swimming direction of the path of each cell during the course of the experiment. The results of such an experiment for the wildtype strain and the eye2-l mutant are displayed in the polar histograms shown in Figure 13.

In part A, the polar histograms of the responses of populations of wildtype and the eye2-l mutant are shown for the lowest intensity of light tested. The wildtype strain is essentially showing random swimming direction in response to the light stimulus, but may have a slight bias toward the direction of the incident light. The eye2-l mutant clearly shows a random direction of travel at this intensity. As the light intensity is increased tenfold from part A to part B, the wildtype strain clearly shows positive phototaxis; approximately 50% of the population is swimming at angles near 0°. The eye2-l mutant population still swims randomly at this intensity. As the light intensity is further increased tenfold from part B to part C, a higher fraction of the wildtype
Figure 13.

Phototactic orientation of wildtype and *eye2-l* at various light intensities.

Represented are polar histograms of the mean swimming direction a population of cells subjected to a directional non-actinic laser stimulus of varied intensity, with the direction of the incoming light defined as 0°. The bin size in each plot is twenty degrees, with the middle angle of each bin indicated by the angle at the terminus of each radial line. For example, the bin labeled 170° represents the total number of cell paths traveling at angles from 160° to 180°. Cells traveling with a mean angle of 0° are positively phototactic, while cells with paths of angles near 180° are negatively phototactic. The black bars indicate the number of paths for a wildtype strain traveling at the angles defined by the twenty degree bin, while the grey bars indicate the same for the *eye2-l* mutant strain. Each annulus of the histogram represents ten percent of the population. The length of each bar represents the percentage of the population contained in the defined bin.

Panel A represents the lowest light intensity used in the experiment, and each new panel from A to E shows the behavior of the population at a tenfold higher intensity of light than the previous panel. Panel I is the response of the populations at a light intensity tenfold higher than in Panel E, while the light intensity increases from panels E to F, F to G, G to H, and H to I are approximately 2, 1.5, 1.5 and 2-fold respectively. Panel I is the response of the populations of cells stimulated by the full intensity laser beam.
A 0.3 erg/cm²/s

B 3 erg/cm²/s
C 30 erg/cm^2/s

D 300 erg/cm^2/s
E 3000 erg/cm$^2$/s

F 6000 erg/cm$^2$/s

WT

eye2-l
G 10,000 erg/cm²/s

H 15,000 erg/cm²/s
I 30,000 erg/cm²/s

WT

eye2-1
population is swimming toward the light; almost 50% of the population is swimming at angles between -20° and 20°. The eye2-1 mutant population appears to be randomly orienting, although there may be a slight bias for swimming toward the light. As the light intensity is again increased tenfold from part C to part D, the wildtype population is even more strongly oriented toward the light source, while the eye2-1 mutant population shows orientation toward the light; the cells are essentially evenly distributed between the bins at all angles between -80° and +80°. This is strongly suggestive that the eye2-1 mutant is not only about a hundred-fold less sensitive to light than the wildtype strain, is also is much less precise than wildtype in its ability to orient to the light direction. As the light intensity is further increased tenfold from part D to part E, the wildtype population still retains the same degree of positive phototactic orientation. The eye2-1 mutant population still displays an overall positive orientation, it is still imprecise in its orientation.

The next step up in light intensity, from part E to part F, represents a twofold increase. Evident in both strains is the reversal of sign of phototaxis. About 15% of the wildtype population swim away from the light at this intensity, while the eye2-1 mutant population has also begun to orient and swim away from the light. Again, the eye2-1 mutant population displays less precision in its orientation than the wildtype strain, the eye2-1 strain exhibits a wider range of angles about +/- 180° even when swimming away from the light. The next increase in light intensity is one-and-a-half fold from part F to part G. A higher proportion of the wildtype population has started to swim away from the light, although there are still cells undergoing positive phototaxis at this intensity. The
eye2-l mutant population exhibits an increase in the proportion of cells swimming away from the light, about 15% are swimming directly away from the light source. As the light intensity is further increased one-and-a-half fold from part G to part H, the orientation of both strains becomes more precise. The wildtype population is essentially evenly distributed between positively and negatively phototactic swimming directions, while the eye2-l mutant population is strongly oriented away from the light, almost 40% of the cells are swimming at angles between -160° and +160°. The final increase in light intensity, from part H to part I, is a twofold increase, in which the cells are subjected to the full strength laser. Both wildtype and eye2-l mutant populations display almost exclusively negatively phototactic orientations, although the eye2-l mutant is again not as precise in its orientation as wildtype.

All other eyespot assembly mutants in our collection were subjected to the same assay, and selected histograms for mtl-3, minl-2, min2-l, and eye3-10 are shown in Figure 14. Part A shows the responses of the populations of four strains at the intensity which corresponds to the same light intensity used for part C of Figure 13. The wildtype population shows a clear positively phototactic orientation at this intensity, while mtl-3 and min2-l are essentially randomly oriented. The response of the eyeless mutant, eye3-10, is interesting, as the population clearly shows a positively phototactic orientation at this intensity, although the orientation of this strain is less precise than wildtype. The minl-2 mutant may show a slight positive orientation at this intensity. Note that at this
Figure 14.

Phototactic orientation of various mutant strains at selected light intensities.

Represented are polar histograms of the mean swimming direction for each cell in a population as described in the legend to Figure 13.

Panel A corresponds to the same stimulus intensity as was used for panel C of Figure 13, while panel B corresponds to the unfiltered full intensity laser stimulus shown in panel I of Figure 13.
WT  A 30 erg/cm²/s  mlt1-3

min1-2

eye3-10  min2-1
B 30,000 erg/cm²/s

WT

mlt1-3

min1-2

eye3-10

min2-1
intensity (Figure 13, part C) the eyeless strain, eye2-l, was randomly oriented. Part B of figure 14 shows the responses of the same strains at the full strength laser intensity which corresponds to the same light intensity used for part I of figure 13. The wildtype population displays a clear negatively phototactic response, as does the eye3-l0 strain and to a lesser extent, min2-l. The response of the mlt1-3 mutant clearly exhibits a random orientation, even at this high light intensity. While the significance of this observation will be discussed later, the result is suggestive that mlt1-3 cannot orient properly at any light intensity. The min1-2 mutant was also assayed in this manner. This strain shows a clear negative orientation at this intensity, indicating that the strain is competent for directional sensing.

Having discovered that our mutant strains had altered capacities to undergo photobehaviors, it became desirable to attempt to find an explanation for the decreased photosensitivity of our strains. We hypothesized that in eyespot mutants, the lack (or reduction) in reflected light reaching the photoreceptors would result in reduced sensitivity during behavioral responses, which was observed in the course of the phototaxis assay. If this lack (or reduction) in the activation of the photoreceptors is the basis for the decreased light sensitivity of our eyespot mutants, then this decreased level of photoreceptor activation should be manifested as a decreased ability of these strains to generate the calcium currents involved in signal transduction during the visual processes. To ascertain the ability of our eyespot mutants to generate the calcium influxes necessary for visual responses, we assayed cell populations in response to high light flashes for their
ability to generate the photoreceptor current.

**Signal transduction analysis of the eyespot mutant collection**

To determine the abilities of our eyespot mutant strains to generate the photoreceptor current, we utilized the population-based assay (Sineshchekov et al., 1992; Pazour et al., 1995; Matsuda et al., 1998). This method records the net change in ion influx into a population of cells when stimulated by light (for a more detailed description, see Materials and Methods, electrophysiology section). Because the PRC and the FC are primarily carried by calcium (Harz, et al., 1992; Holland et al., 1996), the cells are resuspended in medium containing $10^{-4}$ M calcium.

Cells were grown to mid to late-log phase, and assayed for net ion influx following a high intensity camera flash. Recordings of the change in current in the measuring chamber were performed for a duration of 50 ms, and the applied light flash was triggered 5 ms into the recording period. Representative electrical traces are shown in Figure 15. Trace A is the recording of the wildtype strain. The downward spike which follows the light flash (downward arrow) is the photoreceptor current, the small magnitude upward peak is the flagellar current. Trace B shows a recording of the response of the eyeless mutant, *eye2-l*, following the light flash. This mutant has a substantially reduced PRC. It seems possible that the FC for this strain is undetectable. The background noise for this trace is fairly substantial, and the magnitude of the PRC is so small that the small upward peak following the PRC may or may not represent an FC.
Figure 15.

Electrophysiology of selected mutant strains in response to a high intensity light flash.

Panels A, B, and C are representative recordings of the net ion influx of a population of cells stimulated by a high intensity light flash. The ordinate represents the measured current in the chamber, shown in nanoamperes (the spacing between successive ticks denotes a 2 nA difference for all panels), while the abscissa indicates the time in milliseconds. The light flash was triggered 5 ms after the start of recording, and the time of the light flash is marked in each trace by a small downward arrow. Panel A shows the response of a wild type strain of Chlamydomonas to the light flash. The downward spike represents the photoreceptor current (PRC), while the slight upward peak is the flagellar current (FC). Panel B shows the recording for a population of eye2-1 cells, and panel C shows the recording for a population of mlh1-3 cells. Note that the strength of the current obtained strongly depends on the number of cells in the chamber, and direct comparison of peak heights can only be done by normalizing to the number of cells in the chamber (as shown in Table 7). However, the number of cells used to generate these recordings differs by no more than five-fold.
for this strain. Trace C shows the response of the multi-eyed mutant, \textit{mltI-3}, following the light stimulus. This trace is interesting, as it has a strong, clear PRC, followed by an equally clear FC which is reversed in sign from the wildtype FC. The sign reversal of the FC has been previously observed in another multi-eyed mutant, \textit{ptx4-1}, (Pazour et al., 1995) and the sign reversal was attributed to a bias in the positioning of the supernumerary eyespot in that strain (Pazour, et al., 1995). The observation of a reversed sign FC in the \textit{mltI-3} mutant is indicative of some bias in the positioning of the extra eyespot in that strain. This is in agreement with previous work (Anderson and Dieckmann, unpublished observations) in which light microscopy of \textit{mltI-3} observed a bias in the positioning of the extra eyespot toward the anterior (flagellar) end of the cell.

It should be noted that quantitative comparisons of peak heights cannot be made directly from these electrical traces; as a consequence of the experimental design, the magnitude of the peaks will be dependent on the number of cells in the recording chamber. Thus, to make a quantitative comparison of the amplitudes of the PRC between strains, the photoreceptor currents were normalized to a standard number of cells. The results of this normalization are shown in Table 7. The mean PRC/10$^7$ cells for wildtype is about 2.44 nA. The \textit{mltI-3} mutant has a PRC that is about one-twentieth the amplitude of wildtype, it has a mean PRC/10$^7$ cells of 0.13 nA. \textit{min2-1} also has a reduced amplitude PRC, about one-eighth of wildtype. Both eyeless mutants have a substantially reduced PRC compared to wildtype. \textit{eye3-10} has a mean PRC/10$^7$ cells of 0.04 nA, about sixty-fold less than the PRC amplitude of wildtype, and the \textit{eye2-1} mutant is even more
Table 7. Photoreceptor currents of eyespot mutant strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>PRC (nA)/10^2 cells</th>
<th>SD</th>
<th># of observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>2.44</td>
<td>.68</td>
<td>33</td>
</tr>
<tr>
<td>mlt1-3</td>
<td>0.13</td>
<td>0.09</td>
<td>10</td>
</tr>
<tr>
<td>min2-1</td>
<td>0.29</td>
<td>0.14</td>
<td>15</td>
</tr>
<tr>
<td>eye3-10</td>
<td>0.04</td>
<td>0.02</td>
<td>3</td>
</tr>
<tr>
<td>eye2-1</td>
<td>0.02</td>
<td>0.01</td>
<td>11</td>
</tr>
<tr>
<td>mini-2</td>
<td>0.71</td>
<td>0.32</td>
<td>23</td>
</tr>
</tbody>
</table>
inefficient at the generation of the PRC. *eye2-l* has a mean PRC/10^7 cells of 0.02 nA, one hundred and twenty-fold less than the PRC amplitude of wildtype. The *minl-2* mutant also has a reduced amplitude PRC; the mean PRC/10^7 cells for this strain is about one-fourth that of wildtype.

These results are indicative that the inefficient photobehaviors demonstrated by the *minl-2, and min2-I* strains are probably to be due to the reduced ability of these strains to generate the photoreceptor current required for the behavioral responses. The interesting case is the *mlt1-I* mutant, where the mean normalized PRC is not as drastically reduced, but the phototactic behavior of this mutant is completely absent. It seems that multiple input signals coming from the multiple eyespots in this strain are detrimental to its ability to phototax. The reduced PRC observed from the eyeless mutants, *eye2-l* and *eye3-I0* reflects a loss of ability of these strains to block photoreceptor activation of light entering the cell from the side opposite the eyespot, and is probably not due to any inherent inability to generate the PRC.

**Discussion**

Because the visual system is composed of elements in the plasma membrane, cytoplasm and the chloroplast (Witman, 1993), and because the efficiency of the visual system depends on the precise arrangement of those elements relative to each other, the discovery of new mutants with defects in eyespot assembly (Lamb et al., 1999; Lamb et
al., unpublished observations) allows for the order of assembly of eyespot components in *Chlamydomonas* to be addressed. Previous work had indicated that for the *eye* 1-3 mutant, the absence of eyespot pigment granules did not affect the localization of the photoreceptor molecules, since the *eye* 1-3 mutant was still able to show phototactic responses, although at a reduced efficiency compared to wildtype (Morel-Laurens and Feinleib, 1983). To determine the capacity of our mutant strains to carry out light-induced behavioral responses, photoshock assays were performed. For reference, the strains used in these assays, as well as other strains which have been described previously are shown in Table 8.

The photoshock assays provide a rapid test for the ability of the eyespot mutant strains to undergo any sort of photobehavior. Since the photoreception pathways for phototaxis and photoshock are thought to share common photoreceptor and signal transduction components, at least for the early part of the pathway (Foster et al., 1984; Uhl and Hegemann, 1990; Kröger and Hegemann, 1994) the demonstration of a photoshock response in our mutant strains is an indication that the mutants still contain all the components necessary for photoreception. Two main conclusions may be drawn from our photoshock assays. One is that all of the isolated eyespot assembly mutants have the capacity for photoshock. This is a clear indication that they have both the photoreceptors and the signal transduction machinery necessary for photoshock. However, since the photophobic response is not thought to depend on light direction, there is no requirement that the photoreceptors be organized asymmetrically in the cell for this response. Thus,
Table 8. Mutant strains used for discussion of photobehavior

<table>
<thead>
<tr>
<th>Eyeless strains</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>eye1-1, eye1-2, eye1-3</em></td>
<td>Hartshorne, 1952, Morel-Laurens, 1987</td>
</tr>
<tr>
<td><em>eye2-1</em></td>
<td>Lamb et al., 1999</td>
</tr>
<tr>
<td><em>eye3-10</em></td>
<td>Lamb et al., 1999</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mini-eyed strains</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>min1-2</em></td>
<td>Lamb et al., 1999</td>
</tr>
<tr>
<td><em>min2-1</em></td>
<td>Lamb, unpublished observations</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Multi-eyed strains</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ptx4-1</em></td>
<td>Pazour et al., 1995</td>
</tr>
<tr>
<td><em>mlt1-3</em></td>
<td>Lamb et al., 1999</td>
</tr>
</tbody>
</table>
the eyespot assembly mutants do have the requisite photoreceptors and signaling mechanisms, but the organizational state of those components remains unknown.

The second conclusion is that the eyespot assembly mutant strains have different threshold sensitivities for the photoshock response. For example, the eye2-I mutant was able to photoshock in the camera flash experiment, but was not observed to photoshock in the motion analysis experiment. Since the camera flash is approximately one hundred-fold higher light intensity than the laser used for the motion analysis experiment, the threshold for photoshock in the eye2-I mutant lies somewhere in between these two intensities. The eye3-10 mutant was observed to photoshock in both experiments, thus the threshold intensity for photoshock in this strain is at some intensity below that provided by the laser. min2-I did not photoshock when stimulated by the laser, but did photoshock with the higher intensity camera flash. mtl-3 did photoshock when stimulated by the camera flash, but has not been tested for photoshock with the laser. Thus, for both min2-I and mtl-3, it is clear that these strains can undergo the photophobic response, but no information is available about the intensity required to trigger this response.

The case of the mini-eyed min1-2 mutant is most interesting. Previous electron micrographs of this mutant indicated that the organizational state of the eyespot was conditional. When min1-2 was grown in medium containing acetate (R), the eyespot was more organized, while when grown in minimal medium (M), the eyespot was more disorganized (Lamb et al., 1999). Most notably, this mutant shows an essentially wildtype spacing between the plasma membrane and the chloroplast envelope, as well as a regular
wildtype spacing between lipid granules within the chloroplast when grown on R medium. When grown in M, this mutant shows an increase in the spacing between the plasma membrane and the chloroplast outer membrane, as well as a decreased organization of the pigment granules themselves. Because the spacing between membranes and the organization of the pigment granules are critical for establishing the proper reflective properties of the eyespot, this observation led us to hypothesize that the \textit{minJ-2} mutant might exhibit conditional behavioral responses as well. If the \textit{minJ-2} mutant contains the photoreceptors and signal transduction components involved in visual responses, the ability of this strain to respond to light stimulus should be reduced when the strain is grown in minimal medium, due to the decreased organization of the eyespot.

The results of the camera flash photoshock assay would appear to substantiate this hypothesis; at least in one experiment, the \textit{minJ-2} strain did not photoshock when grown in minimal medium, while the \textit{minJ-2} strain grown in acetate medium did photoshock. However, when the experiment was repeated, the strain was observed to photoshock under both conditions. The observation that \textit{minJ-2} cells grown in minimal media could photoshock in one trial of the camera flash photoshock experiment may reflect a difference in the photo-acclimation state of the cells between the two experiments. The cells may have been in a different growth state in one experiment, or the degree of dark adaptation for each strain may have been different. Photoshock assays (at approximately one hundred-fold lower intensity than the camera flash) with the motion analysis system would appear to confirm that there is a conditional difference in the ability of \textit{minJ-2} to
photoshock. Cells of this strain grown in M medium did not photoshock, while cells
grown in R medium did. The simplest explanation for these observations is that there is a
conditional difference in the photoshock threshold for the \textit{minl-2} mutant. The variability
of the response of the photoautotrophically grown \textit{minl-2} strain between trials in the
camera flash photoshock experiment probably reflects a difference in the light adapted
state of the strain for that experiment. Because the absolute intensity at which
photobehaviors are seen is sensitive to many environmental parameters (Stavis and
Hirschberg, 1973; Nultsch and Throm, 1975; Nultsch 1977; Morel-Laurens, 1987;
Takahashi and Watanabe, 1993), differences in the environmental state of the strain prior
to the experiment may give different results. A more conclusive test that should resolve
this, as well as providing more information about the shock threshold of other mutant
strains, would be an experiment in which the capacity of each strain to photoshock was
monitored over a wide range of light intensities. It is predicted that this experimental
design would reveal a clear conditional difference in the threshold photoshock capacity for
the \textit{minl-2} strain. It also seems possible that the other mutant strains will have different
photoshock threshold intensities (relative to wildtype) as well.

Because the photoshock assay tests only for the presence or absence of the
photoreceptive machinery, the observation that all eyespot mutant strains (save \textit{min2-1})
can photoshock does not allow for any inference about the organizational state of the
visual apparatus. Previous experiments indicated that the \textit{eye1-3} mutant still retained the
capacity for phototactic responses, (although the orientation was less precise than
wildtype). This was taken as evidence that the photoreceptors in this strain were still localized asymmetrically within the cell, and that the presence of the eyespot pigment granules was not required for appropriate localization of the photoreceptor molecules. Thus, to attempt to deduce the organizational state of the photoreceptors in our eyespot mutant strains, phototaxis assays were performed.

Both eyeless strains, eye2-l and eye3-10, tested in our phototaxis assays displayed a reduced sensitivity for undergoing positive phototaxis, showing a positively phototactic response at one hundredfold and tenfold higher light intensity than wildtype, respectively. Moreover, for both these mutants, the orientation of these strains was less precise than wildtype. Interestingly, both mutant strains displayed negatively phototactic behavior starting at the same light intensity as wildtype. This may be an indication that the presence of the pigment granules is much more important for extending the sensitivity of the eyespot at low light intensity, and that the absence of the pigment granules is not as detrimental for orientation when the light intensity is high.

Three conclusions can be drawn from these observations: first, the fact that both eyeless strains display phototactic responses is indicative that they both possess some degree of organization of the photoreceptors even in the absence of the eyespot pigment granules, because an asymmetrically localized photoreceptor is required for phototactic behavior (Foster and Smyth, 1980).

Second, the lack of eyespot pigment granules decreases the sensitivity of the visual apparatus. The fact that it requires more intense light than wildtype to cause positive
phototaxis is suggestive that the reflection provided by the eyespot is critical for signal
enhancement at lower light intensities. Alternatively, this difference in threshold sensitivity
for positive phototaxis could be due to a quantitative difference in the number of
photoreceptor molecules present in these strains, resulting in a decreased sensitivity to
light. The latter possibility seems unlikely, because if a decreased number of
photoreceptors were the cause of the decrease in sensitivity at lower light intensities, then
these strains should also be less sensitive at higher light intensities as well. The
observation of a wildtype threshold for negative phototaxis in the eyeless mutant strains is
indicative that these strains do not have a decreased number of photoreceptor molecules.

Third, the presence of eyespot pigment granules enhances the ability of
*Chlamydomonas* to orient itself with respect to the direction of the external light source.
Both the *eye2-l* and the *eye3-10* mutants clearly display a much broader range of
swimming angles than wildtype, even when the eyeless populations are clearly phototactic
(for reference, see Fig. 13, parts D and I, and figure 14).

The *min2-I* (mini-eyed) mutant did not display any clear positively phototactic
response, although it was capable of negative phototaxis. The orientation of this strain
was less precise than wildtype (Fig. 14). This again is indicative that this strain does
possess asymmetrically localized photoreceptors, allowing phototactic responses. For the
phototaxis assays, this mutant was cultured in minimal medium. The lack of positive
phototaxis in this strain may reflect a conditional difference in the organization of the
visual apparatus in this strain, similar to what was described for the *min1-2* mutant.
Alternatively, this strain may always possess a disorganized visual apparatus. In the absence of any ultrastructural data for this mutant, the appression of the membranes in the eyespot region and the organization of the eyespot pigment granules in this strain cannot be predicted.

Phototaxis assays with the minI-2 mutant, are indicative that this strain is about one hundredfold less sensitive than wildtype in the intensity required to produce positive phototaxis, and its intensity threshold for negative phototaxis is similar to that of wildtype. It is currently unclear if there is a conditional difference in the phototactic ability of this strain.

The final mutant strain analyzed by the phototaxis assay produced the most interesting results. The multi-eyed mutant (mltI-3) failed to show any degree of photoorientation at any light intensity (Figure 14). Because the mltI-3 mutant has the capacity to photoshock, it clearly possesses all of the components necessary for visual responses. The lack of directional orientation displayed by this strain is suggestive of a defect in the asymmetric placement of the photoreceptors in this strain. There are two probable explanations for the behavioral data.

First, this strain may be defective in co-localization of its photoreceptors with its eyespots; the photoreceptor molecules might be distributed throughout the cell. If this were the case, the photoreceptors could still sense light intensity, and trigger the calcium influx during photoshock, but the lack of asymmetric placement of the photoreceptors would preclude any directional sensitivity, and thus the strain could not undergo
phototactic responses. Alternatively, the photoreceptors might be co-localized with the eyespots, but the presence of an extra eyespot with photoreceptors might lead to a "confused" cell. If both eyespots produced signals concerning light direction and intensity, the flagella might respond inappropriately for phototactic steering. A cell with more than one photoreceptive eyespot should still be able to photoshock, as this response is independent of any ability to sense light direction.

Because the mutants all display some kind of defect in their photobehavioral ability, electrophysiological measurements of cellular ion influxes were performed. In photobehaviors, the main ion influxes into the cell following a light stimulus are carried by calcium (Holland et al., 1996; Nonnengäßer et al., 1996). The results of this experiment are a good indication of the reason behind the altered photobehavioral abilities of the eyespot mutant strains.

All of our eyespot assembly mutants display a PRC of reduced amplitude. min2-1 is the most interesting, as it showed the least propensity for the phototactic response, yet it produced a PRC that was quite large, one-eighth the amplitude of wildtype. It is not altogether surprising that this strain is not wildtype for the generation of the PRC, as a mini-eyed cell probably will not generate a wildtype PRC due to a lesser degree of photoreceptor activation due to the reduced reflectivity one would predict for a smaller eyespot. Thus, it is surprising that this strain has such imprecise phototactic response, given the fairly large PRC that this strain generates. This may reflect a defect in eyespot assembly in this strain that also affects some other component of the signal transduction
pathway downstream of the generation of the PRC.

The drastically reduced PRC generated by the *eye2-1* and *eye3-10* mutants does not reflect any inherent inability to generate the PRC, but rather is a consequence of the method used to measure the ion influxes in the assay. The orientation of the cells relative to the light flash has an effect on the resultant current which are measured (Sineshchekov, et al., 1992; Pazour et al., 1995). When the population is randomly oriented prior to the light flash, as in our assays, the population will contain cells whose eyespot both face the incoming light and are facing away from that light. The PRC measured for the first orientation would have an amplitude of one sign, while the PRC measured for the second orientation would have an amplitude of the opposite sign. So, for a strain with wildtype eyespots assayed in this manner, cells with eyespots facing the light will be greatly stimulated, and produce a PRC peak of high amplitude. Wildtype cells with eyespots facing away from the light source will not be as fully stimulated, due to the attenuation of light reaching the photoreceptors by the cell body and the pigment granules. Cells oriented in this manner would produce a greatly reduced PRC compared to those oriented facing the light, and it would be of opposite sign. Thus the net PRC for the population being measured in the assay would mostly be due to the PRC of cells with eyespots facing the light. In the case of the eyeless mutants, the loss of attenuation by the pigment granules would result in a much larger contribution to the net PRC from cells with photoreceptors activated by light passing through the cell body; this would almost completely cancel the PRC generated from cells facing the light. The net PRC in this case
is greatly reduced, but is still present, as the attenuation of light by the cell body would result in some loss of PRC in cells facing away from the light source. Therefore, the reason for the greatly reduced PRC for the two eyeless strains is due to the lack of pigment granule attenuation of light passing through the cell body, and does not reflect an inability of these strains to generate calcium influx necessary for photobehavior.

The *min-l-2* mutant also displays a reduced amplitude PRC, about one-quarter that of wildtype, and this strain also displays a reduced phototactic sensitivity. The behavioral defect of this strain is likely to be a combination of a reduction in the amount of light activating photoreceptors due to decreased eyespot reflection and an additional component may be a signal transduction defect downstream of the generation of the PRC.

The multi-eyed mutant, *mlt-l-3*, displays an interesting phenotype with respect to the generation of the PRC. The mean amplitude of the PRC for this strain is about one-twentieth that of wildtype, however, the direction of the FC in this strain is reversed. As previously mentioned, this is indicative of a non-random distribution of the supernumerary eyespot (Pazour et al., 1995). A similar trace was observed for the multi-eyed mutant *ptx-1-l*, and it was proposed that the secondary eyespots in this strain were biased slightly toward the anterior of the cell. This is known to be the case for *mlt-l-3* as well (Anderson and Dieckmann, unpublished observations). More importantly, this sign reversal of the flagellar current could only come about if the second eyespot in this strain was capable of generating a distinct PRC. This is strongly suggestive that the photobehavioral defect in this strain is due to "confusion"; the cell is receiving input signals from both eyespots, and
never swims in the appropriate direction. If this hypothesis is correct, examination of individual cell swimming paths observed in the motion analysis phototaxis experiment would be useful. Cells in the population might show paths that “zig-zagged” as the cell switched from responding due to input signal from one eyespot to the other. As the motion analysis histograms shown in figure 14 calculated a mean swimming direction for the path of each cell, this pattern of swimming would not be detected, as the “zig-zags” would be averaged out.

Additionally, the fact that the placement of the supernumerary eyespot varies from cell to cell, it might be that subsets of cells in the population show characteristic swimming paths for a certain placement of the extra eyespot. To elaborate, a cell with both eyespots on the same side might have a different type of swimming path from a cell in which the extra eyespot was on the opposite side of the cell.
Chapter 4: Cloning of the EYE2 gene from Chlamydomonas reinhardtii

Abstract

While several mutants with abnormal eyespot development have been described in Chlamydomonas, none of the genes affected have been isolated. Thus, the central aim of this chapter is to isolate and analyze one of these genes. This study used a tagged allele of the EYE2 locus to recover the sequence adjacent to the site of insertion, and used a portion of that sequence to isolate clones containing this region from a genomic library. Genomic clones were identified which were able to rescue the defect in eye2-l strains, indicating that the genomic clones isolated contained the wildtype EYE2 gene. Smaller fragments were identified that were still capable of rescuing the defect in the eye2-I strain, and the smallest clone was sequenced. A partial cDNA for the EYE2 gene was amplified by RT-PCR and sequenced; the deduced protein encoded by the EYE2 gene has only one sequence motif which resembles other known proteins, a thioredoxin active-site motif. Thus, the function of the EYE2 protein in eyespot production may be to regulate other eyespot assembly proteins via oxidation and/or reduction of disulfide bonds.

Introduction

Precise phototactic orientation in Chlamydomonas requires the presence of the
eyespot (Morel-Laurens and Feinleib, 1983). However, little is known about the assembly of eyespots, or the genes involved in the eyespot assembly pathway. Previous investigations have described four eyespot assembly mutants, eyeless mutants eye1-1, eye1-2, and eye1-3 (Hartshorne, 1952; Morel-Laurens 1987), all alleles of eye1 (Lamb et al., 1999) and the multi-eyed mutant ptx4-l, (Pazour et al., 1995). Although both mutant types have been characterized at the physiological level, neither gene has been isolated. The aim of the present study is to characterize mutants of Chlamydomonas that are defective for eyespot formation, and to isolate and characterize the genes involved in eyespot assembly.

A mutant screen was undertaken to identify new Chlamydomonas eyespot assembly mutants. UV-mutagenesis of wildtype Chlamydomonas resulted in mutations that define four loci causing aberrant eyespots (Lamb, et al., 1999). Two types of mutants (eye2 and eye3) were completely eyeless when observed by light microscopy, while mutants at two other loci were mini-eyed (min1) and multi-eyed (mlt1). These mutations were all found to be recessive and displayed Mendelian inheritance, indicating they affect nuclear genes rather than chloroplast genes. After the UV-mutagenized strains were isolated, it became possible to efficiently transform Chlamydomonas with exogenous DNA (Kindle, 1992; Dunahay, 1993). As the transforming DNA was found to integrate into the genome at random, it was possible to generate tagged mutants with the desired phenotype, facilitating isolation of the mutant gene. The mutant screen was repeated using a marker to disrupt genes involved in the eyespot assembly pathway. Two tagged eyespot assembly
mutants were recovered (Quinton and Dieckmann, unpublished results). minl-5 was found to be mini-eyed, and tightly linked to the previously isolated minl-2 mutant. eye2-2 was found to be eyeless, and tightly linked to the previously isolated eye2-1 mutant.

Results

Plasmid rescue of flanking DNA from eye2-2 and minl-5

The isolation of tagged mutants with aberrant eyespots allowed attempts to clone the corresponding wildtype genes. Due to the lack of complementing auxotrophic markers and the low frequency of obtaining unselected diploids in crosses of Chlamydomonas, true complementation tests could not be performed between the ARG7 tagged mutants and the original UV-induced collection. However, linkage analysis demonstrated that the insertional mutant eye2-2 (which has an eyeless phenotype) was tightly linked to the eyeless UV-induced mutant eye2-1, and the insertional mutant minl-5 (which has a mini-eyed phenotype) was tightly linked to the mini-eyed UV-induced mutant minl-2 (Lamb and Dieckmann, unpublished observations). These data indicated that the insertional events in eye2-2 and minl-5 were located in or near the same genes that were mutated in the UV-induced mutant strains eye2-1 and minl-2. The generation of tagged eyespot assembly mutants that show tight linkage of the Arg marker and the eyespot phenotype allowed the scheme shown in Figure 16 for recovery of genomic
Figure 16.

Insertional mutagenesis and recovery of flanking DNA in Chlamydomonas.

An arg7 strain (labeled WT) of Chlamydomonas is transformed with a linear plasmid bearing the wildtype ARG7 gene (labeled pARG7.8). This plasmid integrates into the genome at random, and is generally mitotically stable. These integration events usually inactivate the genes at the site of integration, either by integrating within the gene or by integrating in enhancer sequences. Mutants that no longer require arginine for growth can then be screened for the phenotype of interest, in this case the loss of the ability to positively phototax due to eyespot aberrations. The insertion event shown here has integrated within the EYE2 gene, inactivating it. As the original plasmid had an origin of replication and a selectable marker for E. coli from the plasmid pBR329, an appropriate restriction digest, such as with SalI, and subsequent intramolecular ligation of the digested genomic DNA should yield a plasmid bearing the sequence of the genome adjacent to the site of insertion.
**W.T.**

- Sall

**pARG 7.8**

- HindIII
- Sall

**Insertion Mutant**

- Sall

**Recovered plasmid**

- Sall
DNA adjacent to the insertion sites (Tam and LeFebvre, 1993) in *eye2-2* and *minl-5*. Genomic DNA was isolated from both *eye2-2* and *minl-5*. Samples were digested with SalI, which cuts between the pBR329 and ARG7 sequences in the pARG7.8 plasmid, ligated in a large volume and transformed into *E. coli*. Thirteen ampicillin resistant transformants were recovered from *eye2-2* genomic DNA, and two from *minl-5* genomic DNA. The recovered plasmids appeared to contain genuine flanking DNA from *eye2-2* and *minl-5* because the restriction maps for the plasmids recovered from *eye2-2* genomic DNA were identical (Fig. 17), and the restriction maps for both plasmids recovered from *minl-5* genomic DNA were identical, but different from those recovered from the *eye2-2* strain. Additionally, the plasmids were all confirmed as intramolecular ligations because restriction mapping verified a single SalI site in all plasmids.

Interestingly, restriction analysis also revealed that the recombination endpoints in the pBR329 portion of the inserting DNA were different for the two sets of plasmids recovered from *eye2-2* and *minl-5*. Integration of linearized exogenous DNA into the nuclear genome of *Chlamydomonas* typically does not leave the integrating DNA completely intact (Weeks, personal communication; this study). Restriction mapping revealed the recombination endpoint for the *eye2-2* insertion was approximately 1000 bp away from the HindIII site used to linearize the integrating pARG7.8 plasmid, and the endpoint for the *minl-5* insertion was approximately 350 bp away from that Hind III site. These data were suggestive that the recovered plasmids contained flanking DNA from the sites of insertion for both *eye2-2* and *minl-5*. One plasmid containing the recovered
Figure 17.

Restriction maps of the recovered flanking sequence plasmids

The plasmid pARG7.8 was linearized with HindIII and used to transform an $arg^7$-2 Chlamydomonas strain, generating strains $eye^2$ (eyeless) and $min^1$ (mini-eyed). Genomic DNA from both strains was digested with SalI, diluted, ligated and transformed into E. coli. Both recovered plasmids from $min^1$ genomic DNA were identical 6 kb plasmids. All plasmids recovered from $eye^2$ genomic DNA were identical.
pARG 7.8

HindIII  Sall  Sall  XmnI  HindIII

ARG7  AmpR/OriC

pBR329-5B#1

Sall  XmnI  Sall

AmpR/OriC  minl-5 flanking DNA

pBR329-10C#1

Sall  XmnI  Sall

AmpR/OriC  eye2-2 flanking DNA
flanking sequence from eye2-2 was named pBR329-10C#l. Similarly, the plasmid containing the flanking sequence from min1-5 was named pBR329-5B#l.

Tests of recovered min1-5 flanking DNA as probes for genomic DNA

In order to clone the genes that had been inactivated in the insertional mutants, it was necessary to find a fragment from the flanking DNA that would uniquely identify the region of the genome containing the gene that had been inactivated by the insertion. The nuclear genome of *Chlamydomonas reinhardtii* contains a high proportion of repetitive elements (Wakarchuk et al., 1992; Hails et al., 1993), such that prior to screening a wildtype genomic library for the gene of interest, it is necessary to verify that the probe sequence being used does not contain repetitive DNA.

**Figure 18** shows the results of one such experiment using a probe from the min1-5 flanking sequence. Plasmid pBR329-5B#1 and genomic DNA from an arg7-2 strain (lane WT), min1-5 (lane Δ), and the progeny from a cross of min1-5 to wildtype (lanes A-D) were digested with *Hind*III, and subjected to Southern analysis using a uniformly labeled 400 bp *Apa*1/*Hpa*I fragment from the flanking sequence contained in plasmid pBR329-5B#1. The probe fragment clearly hybridized to the plasmid from which it was derived (lane P). However, it failed to detect any specific sequence in *Chlamydomonas* genomic DNA. The *Hind*III digestion should reveal a restriction fragment length polymorphism (RFLP) between min1-5 and the wildtype genomic DNA, with the
Figure 18.

*mini-5* flanking sequence probe fails to identify a unique sequence in the Chlamydomonas genome.

Approximately 3 ng of *Hind*III digested pBR329-5B#1, from which the probe sequence was derived (lane P), and approximately 10 μg of *Hind*III digested genomic DNA from wildtype (lane WT), *min2-1* (lane Δ), and progeny from a backcross of *mini-5* to wildtype (lanes A-D), were resolved on an agarose gel, blotted onto Nytran, and probed with a random primed 400 bp *ApaI/HpaI* fragment from plasmid pBR329-5B#1. The plasmid DNA has been incompletely digested.
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fragment detected in the minI-5 strain being larger by the size of the integrated pARG7.8 plasmid (~11 kb). The observed pattern of hybridization is identical for both minI-5 and the wildtype genomic DNA, and this is indicative that this probe does not recognize a unique genomic sequence. The hybridization pattern of the progeny from the backcross to wildtype confirms this, as the strains that are Arg⁺/Ptx⁺/Eye⁺ (lanes A and D), like the minI-5 parent, and the strains that are Arg⁻/Ptx⁻/Eye⁻ (lanes B and C), like the wildtype parent, display the same hybridization pattern. Similar results were obtained with other non-overlapping probe sequences tested from the flanking DNA from minI-5, and this project was abandoned.

Tests of recovered flanking eye2-2 DNA as probes for genomic DNA

To find a unique probe near the EYE2 gene, fragments from the recovered flanking DNA from pBR329-10C#1 were used to probe wildtype genomic DNA. Southern analysis demonstrated that a flanking sequence probe from pBR329-10C#1 was unique in the wildtype genome (Fig 19). Approximately 10 µg of HindIII-digested genomic DNA from wildtype (lane WT), the eye2-2 insertion (lane DEL), and progeny from a cross of eye2-2 to wildtype were probed with a uniformly labeled 1200 bp SalI/PstI fragment from a subclone of the flanking sequence from pBR329-10C#1, pKS-10C S/P (10C S/P probe). The probe hybridized to a wildtype fragment which is approximately 2.5 kb.
Figure 19.

*eye2-2* flanking sequence probe identifies a unique band in wildtype genomic DNA.

Approximately 10 μg of *HindIII*-digested genomic DNA from wildtype (lane WT), *eye2-2* (lane DEL), and progeny from a backcross of *eye2-2* to wildtype (lanes backcross WT and DEL) were resolved on an agarose gel, blotted onto Nytran, and probed with a random primed 1.2kb *SalI/PstI* fragment from pKS-10C S/P. Both the positions of the wildtype 2.5 kb band and the RFLP band indicating the integration of the pARG7.8 plasmid are indicted with arrows. Note, that only three of the four progeny from the backcross are shown; while the fourth progeny had an Arg'/Ptx'/Eye- phenotype, it failed to yield enough DNA for the Southern.
### Phenotype:

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<td>4.3</td>
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</table>

**Backcross progeny**

- RFLP Band

**WT Band**
Additionally, the RFLP between \textit{eye2-2} (lane DEL) and wildtype (lane WT) is clear, as the probe hybridizes to a fragment which is approximately 11 kb in the \textit{EYE2} insertional mutant strain \textit{eye2-2}. This result is consistent with the fact that the detected fragment from \textit{eye2-2} genomic DNA should have the pARG7.8 plasmid insertion in the wildtype sequence, resulting in the detection of a fragment which is approximately 9 kb larger than the fragment from wildtype genomic DNA. Also clear is the segregation of the insertion event with the Ptx'/Eye'/Arg' phenotype in the backcross. Two of the three progeny shown from a cross of \textit{eye2-2} to the parent strain have a fragment, similar to wildtype (2.5 kb) which hybridizes to the probe. This observation is consistent with the wildtype Arg'/Ptx'/Eye' phenotype exhibited by these strains. Similarly, the remaining progeny has a fragment at approximately 11 kb, similar to \textit{eye2-2}, which hybridized to the probe sequence. The size of this fragment is consistent with the mutant Arg'/Ptx'/Eye' phenotype of this strain. The fourth strain from this tetrad also had the Arg'/Ptx'/Eye' phenotype, but failed to yield enough DNA for Southern analysis. The discovery of a probe which hybridized to a specific fragment in wildtype genomic DNA made possible a genomic library screen.

\textbf{Genomic library screen for \textit{EYE2}}

It was decided to screen a genomic library for several reasons. Most important was the fact that the only way to verify clones obtained from the screen as containing the \textit{EYE2} gene was by using them to complement the defect in the original mutant strains.
This criterion could not be met with a clone found from a cDNA library, because successful complementation using a cloned cDNA has not been reported in *Chlamydomonas*. Moreover, the tagging of genes by insertional mutagenesis has been reported to cause deletions of up to 10 kb at the site of insertion. Thus, the probe sequence used to identify a cDNA clone could actually be hybridizing to a gene up to 10 kb away from the one inactivated. Additionally, there was no evidence that the flanking sequence probe tested was actually contained in a transcribed region.

These reasons led to the decision to screen the pARG7.8-cos library (Purton and Rochaix, 1995). This library contains the *ARG7* gene in the vector backbone, which allows direct selection for the cosmid molecules in *Chlamydomonas*. Additionally, this genomic cosmid library has an average insert size of 40 kb, so the probability of finding a clone containing both the probe sequence and the *EYE2* gene is higher than in standard phage genomic libraries.

Cosmids were first screened by subdividing the cosmid library into pools of between 500 and 1000 cosmid clones per pool. Twenty pools (A-J and 1-10), representing a total of three genome equivalents, were screened by Southern analysis of *HindIII*-digested pools using the 10C S/P probe (Fig. 20). The probe hybridized to a 2.5 kb *HindIII* fragment in pool A (lane A), which is identical to the 2.5 kb *HindIII* band from wildtype genomic (lane WT). Pools B through I (lanes B-I) do not contain this band. The probe also hybridized non-specifically to the vector backbone, detecting the approximately 14 kb band in all pools.
Figure 20.

Identification of the wildtype genomic region in cosmid pools.

Approximately 2 μg of HindIII-digested DNA from pARG7.8cos cosmid pools, containing between 500 and 1000 individual cosmid molecules (lanes A-I), or approximately 10 μg of wildtype 137c genomic DNA (lane WT) were resolved on an agarose gel, blotted onto Nytran, and probed with a uniformly labeled 1.2 kb SacI/PstI fragment from pKS-10C S/P.
The results of this experiment were suggestive that pool A harbored a cosmid containing the *eye2-2* flanking DNA, while pools B through I did not. Similarly, three other pools (#3, #7 and #8) from the original twenty were observed to contain the *eye2-2* flanking DNA (data not shown). Dilutions of the cosmid pools were plated, and approximately 2000 single colonies from each pool were patched and screened by colony hybridization using oligonucleotide #63395, designed from a sequenced portion of the 1200 bp *SalI/PstI* fragment of the *eye2-2* flanking sequence. Eight individual cosmids hybridizing to the oligonucleotide probe were then analyzed by Southern blotting (Fig. 21). Approximately 1 μg of each cosmid clone was digested with *HindIII*, resolved on an agarose gel, and blotted onto Nytran. The blot was probed with the uniformly labeled 10C S/P probe. For reference, the wildtype *HindIII*-digested genomic band detected by this probe is approximately 2.5 kb. The *eye2-2* flanking sequence probe hybridizes to an approximately 2.5 kb band in cosmids A3-6-1, 3-10, 7-8-1, and 8-3, indicating that these cosmids contain the *eye2-2* flanking sequence. Because the oligonucleotide sequence used for screening the cosmid colonies is contained within the 1200 bp probe, the failure of the 1200 bp probe to detect any bands in cosmids 3-1, 3-6, 7-6 and 7-8-2 indicates that these cosmids were false positives detected by non-specific hybridization of the oligonucleotide probe. Restriction mapping of the cosmids indicated that the inserts from cosmids containing the 2.5 kb band were all related but non-identical, while the inserts from cosmids that did not contain the band appeared to be unrelated, both to each other and to the set containing the wildtype band. Because they contained the wildtype 2.5 kb
Figure 21.

Identification of single cosmids containing the wildtype genomic region.

Approximately 0.5 μg of HindIII-digested DNA from single pARG7.8cos cosmids (lanes 8-3, 7-8-2, 7-8-1, 7-6, 3-10, 3-6, 3-1, and A3-6-1), were resolved on an agarose gel, blotted onto Nytran, and probed with a uniformly labeled 1.2 kb Sal/PstI fragment from pKS-10C S/P. For reference, the wildtype HindIII genomic band which hybridizes to this probe is approximately 2.5 kb.
HindIII band, cosmids A3-6-1, 3-10, 7-8-1 and 8-3 were chosen for further analysis.

Restriction mapping of the four cosmids demonstrated insert sizes of approximately 40-45 kb, and approximately 15 kb of common sequence among all four isolates (data not shown). Pairwise comparisons of sets of cosmids indicated that 3-10 and 7-8-1 were most similar, and that cosmids A3-6-1 and 8-3 probably represented the ends of the contiguous sequence for the region. The isolated cosmids containing the wildtype genomic region identified by the eye2-2 flanking sequence probe were then used to attempt to rescue the defect in the eye2-l UV-induced mutant.

**Complementation of the eye2-l defect with isolated cosmids**

Because the four cosmids all contained the wildtype 2.5 kb HindIII band, which was identified using a probe from the genomic DNA adjacent to the eye2-2 (eyeless) mutation, it seemed probable that some or all of the inserts from these cosmids might contain the EYE2 gene. Since the cosmid vector contained the ARG7 gene, this marker allows the direct selection for integration of the cosmids in an arg7-2 strain. It was desirable to attempt to complement the defect in strain eye2-2, as the flanking DNA and probe sequence were isolated from that strain. However, due to the pARG7.8 insertional mutagenesis which generated the eyeless mutation in eye2-2, the strain no longer requires supplemental arginine. Due to the tight linkage of the eye2-2 mutation to the original UV-induced eye2-l mutant, it was concluded that the mutations in the two strains were likely to be affecting the same gene. This fact allowed for transformation of the cosmids into an
arg7-2 strain harboring the eye2-1 mutation.

Because linearized molecules integrate with higher efficiency in *Chlamydomonas* (Dunahay, 1993), cosmids A3-6-1, 3-10, 7-8-1 and 8-3 were partially digested with EcoRI to maximize the production of singly cut molecules. Linearized cosmids were then transformed into the eye2-1 arg7-2 strain. Colonies growing in the absence of supplemental arginine were patched onto arginine-free acetate plates, and the patched cells were tested for the ability to phototax. Cosmids 3-10 and 7-8-1 both generated Arg⁺ transformants that were phototactic, albeit at low frequency (Table 9). Cosmids A3-6-1 and 8-3 did not produce transformants which could phototax. The transformant strains which were found to positively phototax were examined by light microscopy, and all were observed to have eyespots. It should be noted that the low frequency of phenotype restoration in the cosmid transformants is to be expected. The selection during integration is only for the ARG7 gene in the cosmid backbone, and there is no requirement for integration of the other contiguous DNA contained in the cosmid inserts. This makes the likelihood that any gene present in the cosmid insert will also be integrated along with the ARG7 marker strongly dependent on the physical distance between the two genes. Additionally, it is to be expected that some fraction of the partially digested cosmids might be linearized within the potential complementing gene, making such molecules incapable of complementation. The discovery of cosmids capable of restoring eyespots was indicative that cosmids 3-10 and 7-8-1 were likely to contain the wildtype EYE2 gene.
Table 9. Results of transformation of cosmid clones into *eye2-1 arg7-2*

<table>
<thead>
<tr>
<th>Cosmid isolate</th>
<th># Arg&lt;sup&gt;+&lt;/sup&gt; tested</th>
<th># Ptx&lt;sup&gt;+&lt;/sup&gt;</th>
<th># Eye&lt;sup&gt;+&lt;/sup&gt;</th>
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<tr>
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<td>67</td>
<td>0</td>
<td>N.D.&lt;sup&gt;a&lt;/sup&gt;</td>
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<sup>a</sup> N.D., not determined

<sup>b</sup> Yes indicates that all Ptx<sup>+</sup> transformants possessed eyespots
The probable causes of the Arg$^+$/Eye$^+$ phenotype of the transformant strains are as follows. The Arg$^+$ phenotype could be due to either a reversion of the original arg7-2 mutation, or to a genuine integration of the ARG7-bearing cosmid. Because a reversion event at the arg7-2 allele has never been observed (Weeks, personal communication), all Arg$^+$ colonies are probably to be transformants. It seemed likely that the best explanation for the Eye$^+$ phenotype in these transformants was a genuine complementation by the wildtype gene in the cosmids (for a more detailed analysis, see Discussion). The observation of cosmids that appeared to complement the eye2-l defect led us to attempt to identify a smaller fragment from the cosmid inserts of 3-10 and 7-8-1 which would rescue the defect in the eye2-l strain.

Subcloning of cosmid inserts into pARG7.8

The identification of cosmids 3-10 and 7-8-1 as candidates which probably harbored the EYE2 gene, allowed for narrowing down the DNA sequence containing the gene to a smaller fragment of the cosmid insert. Subclones of cosmid 3-10 were generated by partial digestion. Populations of fragments were size-selected and cloned into pARG7.8. Because cosmids 3-10 and 7-8-1 were observed to cause restoration of the eye2-l defect, it was reasoned that the EYE2 gene was present in the region of common sequence between the two clones. Therefore, subclones were analyzed by Southern blotting using probes generated from the region of overlap between the two cosmids. Those subclones containing inserts from the region of overlap between 3-10 and
7-8-1 were then subjected to further analysis.

**Phenotype rescue by cosmid subclones**

Eighteen subclones that had inserts from the sequence common to 3-10 and 7-8-1 were partially digested with EcoRI to maximize the production of linear molecules and transformed into the eye2-1 arg7-2 strain. Arginine prototrophs were selected, and between 20 and 40 individual transformants for each subclone were analyzed for the ability to phototax and the presence of eyespots. *Table 10* shows the frequency of phenotype restoration by subclones of 3-10. Transformants generated from three subclones (C/B-10, B-15 and B-42) were able to complement the phototaxis defect in strain eye2-1, and all transformant strains which could positively phototax were found by light microscopy to possess eyespots. Additionally, transformants from fifteen other individual subclones were not observed to produce transformants capable of phototaxis. These observations are indicative that the three subclones C/B-10, B-15 and B-42 contain the *EYE2* gene. Another observation is that the frequency of eyespot phenotype restoration in Arg\(^+\) subclone transformants is increased relative to the frequency observed in cosmid transformants (*Table 9*). This result is to be expected, because the subcloning scheme should place genes in the subclone insert in closer physical proximity to the *ARG7* gene in the vector backbone, increasing the probability that any transformant integrating the *ARG7* gene would also integrate the *EYE2* gene contained in the insert sequence.
Table 10. Results of transformation of cosmid subclones into eye2-I arg7-2

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<th>Cosmid subclone</th>
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<td>B-15</td>
<td>19</td>
<td>12</td>
<td>Yes&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>B-42</td>
<td>32</td>
<td>11</td>
<td>Yes&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>All others&lt;sup&gt;b&lt;/sup&gt;</td>
<td>366</td>
<td>0</td>
<td>N.D.&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Yes indicates that all Ptx<sup>+</sup> transformants possessed eyespots

<sup>b</sup> Fifteen different subclones tested

<sup>c</sup> N.D., not determined
Restriction mapping of subclones C/B-10, B-15 and B-42 indicated that the inserts in these plasmids were nearly identical. The inserts of C/B-10 and B-15 were found to be approximately 4 kb each, while the insert in B-42 was found to be 6 kb. Mapping analysis also indicated that the 6 kb insert in subclone B-42 contained almost all of the insert sequence contained in subclones C/B-10 and B-15. The inserts from subclones C/B-10 and B-15 were indistinguishable at the resolution level of restriction mapping. Because the subclones all appeared to contain the \textit{EYE2} gene, and because comparison of the three subclones did not eliminate any portion of the insert sequences as candidates for the location of the gene, it was decided to sequence the insert from subclone C/B-10.

\textbf{Sequencing the 4 kb insert of C/B-10}

Primers were designed against the known sequence of the pARG7.8 vector flanking either side of the 4 kb insert. Because the insert of C/B-10 was cloned into pARG7.8 using the vector restriction sites \textit{Clal} and \textit{BamHI}, one oligonucleotide primer designed against the pBR329 portion of the vector near the \textit{Clal} site was named Pbrcla (arbitrarily designated the top strand), while the other oligonucleotide primer designed against the \textit{ARG7} sequence near the \textit{BamHI} site was named Argbam (bottom strand).

These two primers were used to sequence the C/B-10 insert at the LMSE DNA Sequencing Facility, University of Arizona. Sequence data was obtained from both reactions, and the novel sequence obtained was used to design new oligonucleotide primers for continued sequencing. The new primers were designed approximately 400 bp
away from the Pbrcla or Argbam primers and were named Pbrcla3 and Argbam3. These new primers were then used to sequence the C/B-10 insert. The new sequence data was examined for its overlap with the sequence data from the first cycle, and the contiguous sequence was assembled. New sets of primers were synthesized near the ends of the contiguous sequence, and the process was reiterated until the contiguous sequence of the top strand was observed to overlap with the contiguous sequence from the bottom strand. Once overlap between the two sequences was detected, oligonucleotide primers which were complementary to all of the original sequencing primers were used to sequence the complement of the original sequence of the C/B-10 insert. This strategy allowed the completion of the sequence of the 4 kb insert such that each nucleotide on both strands of DNA had been sequenced at least once, minimizing the probability of obtaining sequencing errors. The complete sequence of the bottom strand corresponding to the direct Argbam sequence of the 4 kb insert of C/B-10 is shown in Fig 22. Inspection of the sequenced insert of C/B-10 did not yield a conclusive prediction about the organization of the $EYE2$ gene within the 4 kb region. It was then decided to find the $EYE2$ cDNA, using its information to elucidate the organization of the $EYE2$ gene in the genomic clone.

**Gene prediction using GCG Codonpreference and Genemark**

Numerous attempts were made to identify the $EYE2$ cDNA by conventional
Figure 22.

Sequence of the bottom strand of the 4 kb insert from plasmid C/B-10.

The sequence of the bottom strand of the C/B-10 insert is shown in the 5' to 3' direction. Singly underlined sequences represent the potential TATA boxes for candidate promoters in the 4 kb region. Doubly underlined sequences represent potential polyadenylation signals for the bottom strand.
Figure 22. Bottom strand sequence of the 4 kb insert of subclone C/B-10

1 GCCGTTTCAN CGGATCCGNC CATGGATTAAGGATC AAGTCCGGG TGCCAGCAGCA GGTAGAGC TGCTGGCACGT TG GGGCAGTC
81 GGTACTGTTG CCGGCGCAGG CATCGTGCAGGG CGTGGCAGGCA GCCAGCAGCA CACAGGCTCA CAGCGTCA CAGCTGTCCT
161 CTTGATGAGA CAAAGCTCACCT TCTCATCGCTT GCTCCAGGCG ACTCCCGGCG GCCGCGGGCG GGAAGGCGCGC
241 CTGCCCAGCTG AGCCCCAGCG GCGGTCCCCG CTTTATTAGTG GGGGGCTGTA ACCTGCTCCTC GCTGCTCTCT ACTGCTTTGAA
321 TACGCGGCTA AAGTATCCATG GGAAGGGCGG GCTAGACCGA GCGGCGAGGC TCCAGACGCA AAACATTCTC AACAGCGGCT
401 ACGTTTCTGC ACAAATCTCGG GTTCCCAGGG ACCACACGAC AATCTGCGG TACACAAGAC TATAGTAGAT CTTTTGGCGT
481 TATGACAAAAC CCGAATGCAGTT TCCATGCGTG ACGGCGGCGTG CCCGCTCGG GCCGCTGCTG TACAGAGACG
561 CTGCTATAG CCGCTATAGT AATATGCTAT GCTGTATAGG TCCTGTATAT CCGGTTGACAG GCGGCCAGGC CACCTGTTAT
641 GGGCAGCTTG CAGCTGCTGAC AAGCTGCTGT CACGGCTGCTC AGGTGCTATAG CCGGCTGCTC AACGCTGCTG TATATAGACA
721 AGGGACATTC ACAAGCTCAC AATCGACGCC TCCAGCACCA GCACTAGCCG GCGAAGGCGC TACAGCTCTG ACGGCTGACG
801 CAGCCTGTATG AAACCTCACT TGGTGATATT CTTGAGTCTAC ATCGCTGCTG CACCGCGGAG CACCGCTGCTCACGCTGCTG
881 TCCGATGCGCC ACGCTTCCCTG ACGGCCTCAG GCCAGCTGTA GCTGGCTGCTGTAAGG TATATAGACA
961 TGGGTTCATG TCTGTGCTTAC ACCAGCTGCTGTTTGACG GGTGCTGTTAC ATGGTACGCTG ACGGCTGCTG
1041 GCGCACAGCTG AGCCGCTGCTG CCGGCGAGGA CACCCAGCCC GCCATCGTGA ACTACAGGTC ACGGAGGCTC ACGGGAAGGC
1121 GACACTATG GAGAGCTGCA GGGGTGCTTAC GACGCTGCTG ACGGAGCTCT CAAAGGCTCA GCGGAGGGCA
Figure 22 (continued). Bottom strand sequence of the 4 kb insert of subclone C/B-10

2561 GGCATGTCGC CCAGCGCTCT GCCGCGCTGGC GTTGGCTCCG CGGCGCTGGG ACTGGTGGGC ATTGCGGCTT CCTGCGCAGCG
2641 CGGCAGCACCAG TGAGTGGCCGA GACGCTGACAG CCCGCGCTGGC GTTGGGCTGGC CGGCGCTGGC GAGCTGGGCG
2721 GGGCAGCTCC TGGTGGCTGGCT CGGCGCTGGC GTTGGGCTGGC CGGCGCTGGC GAGCTGGGCG
2801 CGGCGCTGGC GCCAGCGCCGG GAGCTGGGCG GCCAGCGCCGG GAGCTGGGCG GCCAGCGCCGG
2881 CGGCGCTGGC GCCAGCGCCGG GAGCTGGGCG GCCAGCGCCGG GAGCTGGGCG GCCAGCGCCGG
2961 GCCGCTGGG CAGGCGGCTGG GAGCTGGGCG GCCAGCGCCGG GAGCTGGGCG GCCAGCGCCGG
3041 CAGGCGGCTGG GAGCTGGGCG GCCAGCGCCGG GAGCTGGGCG GCCAGCGCCGG GAGCTGGGCG GCCAGCGCCGG
3121 GGGCACACGC GTGGCGCTGG TGCTGGGGCT GGGCCGCAAC GAGGAGGCAC AGGCCGCAGC CGCAGTGGTC GCAGCCGTGC
3201 GTGAAATGCGC GAGGGGTGCT GAGGGGTGCT GAGGGGTGCT GAGGGGTGCT GAGGGGTGCT GAGGGGTGCT GAGGGGTGCT
3281 GGATGGTGGC CGGGCTGGG GCCGCTGGG GCAGCTGGG CGGCTGGG CGGCTGGG CGGCTGGG CGGCTGGG
3361 GATGCTGGGC ATATGGGATG ATATGGGATG ATATGGGATG ATATGGGATG ATATGGGATG ATATGGGATG ATATGGGATG
3441 GCTTTCAAAT GGGAGTGGCT GGGAGTGGCT GGGAGTGGCT GGGAGTGGCT GGGAGTGGCT GGGAGTGGCT GGGAGTGGCT
3521 GGGCGGCTGG GGGCGGCTGG GGGCGGCTGG GGGCGGCTGG GGGCGGCTGG GGGCGGCTGG GGGCGGCTGG GGGCGGCTGG
3601 GGGCGGCTGG GGGCGGCTGG GGGCGGCTGG GGGCGGCTGG GGGCGGCTGG GGGCGGCTGG GGGCGGCTGG GGGCGGCTGG
3681 GGGCGGCTGG GGGCGGCTGG GGGCGGCTGG GGGCGGCTGG GGGCGGCTGG GGGCGGCTGG GGGCGGCTGG GGGCGGCTGG
3761 GGGCGGCTGG GGGCGGCTGG GGGCGGCTGG GGGCGGCTGG GGGCGGCTGG GGGCGGCTGG GGGCGGCTGG GGGCGGCTGG
3841 GGGCGGCTGG GGGCGGCTGG GGGCGGCTGG GGGCGGCTGG GGGCGGCTGG GGGCGGCTGG GGGCGGCTGG GGGCGGCTGG
3921 GGGCGGCTGG GGGCGGCTGG GGGCGGCTGG GGGCGGCTGG GGGCGGCTGG GGGCGGCTGG GGGCGGCTGG GGGCGGCTGG

158
cDNA library screening. These attempts all failed, presumably due to the low abundance of the *EYE2* cDNA. Further attempts to identify the *EYE2* cDNA by RT-PCR also failed, most probably because of the rarity of the message and the lack of PCR primers known to lie within the coding sequence or untranslated regions of the message. *Chlamydomonas* genes are known to be comprised of many small exons and introns (Mages, unpublished observations; Purton and Rochaix, 1995). It was decided that the most rational way to find the *EYE2* cDNA was by RT-PCR using primers designed against regions of high coding potential, as predicted by computational algorithms.

Two programs were used to predict coding potential for the *EYE2* genomic sequence. GCG Codonpreference (Genetics Computer Group, University of Wisconsin) and GeneMark (Georgia Technical University) were used to analyze the 4 kb sequence from C/B-10. Both programs use a database of known genes to predict coding regions in novel genomic sequence. While the algorithms used by the programs differ, both use a sliding window to assess coding potential. A *Chlamydomonas* codon usage training set was provided by Dave Mitchell (State University of New York, Health Sciences Center, Syracuse, NY) and used with GCG Codonpreference to predict the coding potential of the 4 kb insert sequence from the plasmid C/B-10. The default *Chlamydomonas* codon usage training sets provided by the GeneMark program were used for the GeneMark prediction. Additionally, default parameters for both window size and step size were used for both programs. The output for the coding potential of the three reading frames of the bottom strand of the C/B-10 genomic sequence is shown in Fig. 23.
**Figure 23.**

Predictions of coding potential by the programs GCG Codonpreference and GeneMark for the 4 kb genomic sequence of C/B-10.

The 4 kb sequence of C/B-10 was analyzed using the program GCG Codonpreference, trained with a set of *Chlamydomonas reinhardtii* genes provided by Dr. Dave Mitchell (State University of New York, Health Sciences Center, Syracuse, NY). The sequence was also analyzed using the GeneMark program at Georgia Technical University. In both cases, default settings for window size and step size were used. Only the three reading frames for the bottom strand sequence are displayed; these are indicated by the number at the lefthand side of each row.

For Codonpreference, the solid line indicates coding potential, while the dashed line indicates third position GC bias. Values above the threshold lines (even dashed horizontal line for coding potential and staggered dashed horizontal line for third position GC bias) are considered candidates for exons. For GeneMark, the dark bars indicate regions of high probability of being exons, while the spikes indicate a narrower window of high coding potential.

The schematic in the middle of the figure represents the organization of the five high potential coding regions predicted by the GeneMark program within the context of the 4 kb insert of plasmid C/B-10. The locations of potential promoter and poly(A) addition elements are indicated.
For the Codonpreference program, the solid (peaking) line indicates the coding potential at each codon of the 4 kb sequence. The dashed (peaking) line indicates the third position GC bias exhibited for each codon along the 4 kb. The evenly-spaced (flat) dashed line indicates the threshold value for coding potential. Values above that line are likely to be coding sequence regions. Additionally, the irregularly-spaced (flat) dashed line indicates the threshold value for third position GC bias. Values above that line have bias similar to the genes in the training set. Boxed regions below the prediction in each reading frame denote the position of ORFs in that frame, while the vertical lines below the boxed regions indicate the positions of rare *Chlamydomonas* codons in the reading frame.

The GeneMark program displays the predicted coding potential for the same three reading frames from the bottom strand of the C/B-10 sequence. Each reading frame has spikes that denote regions of high coding potential for that reading frame. Along each reading frame are horizontal lines that denote regions of uninterrupted coding sequence. The positions of common initiation codons (ATG) or rare initiation codons (GTG) are marked as upward ticks along the ORF. The positions of stop codons are represented by the downward ticks at the end of each horizontal line. Additionally, a less stringent prediction of candidate exons is represented by the solid black boxes.

The output of the Codonpreference program did not yield a convincing prediction of the organization of the *EYE2* gene on the bottom strand of the 4 kb sequence. Likewise, a prediction for the three reading frames from the top strand yielded a similarly ambiguous prediction. The output of the GeneMark program is much clearer. Five
regions of the bottom strand C/B-10 sequence are predicted to be of high coding potential. The regions span nucleotides from approximately 900-1500 (region 5) and 2100-2500 (region 2) in the first reading frame, from 1450-1850 (region 4) and 2500-3400 (region 1) in the second reading frame, and from 1900-2100 (region 3) in the third reading frame. It was concluded that these might represent candidate exons from the bottom strand sequence for the 4 kb insert of C/B-10. Additional analysis of the coding potential of the top strand of the C/B-10 genomic sequence using the GeneMark program failed to yield any regions of high coding potential for the three reading frames from that strand. These data were indicative that the coding information for the EYE2 protein was probably on the bottom strand of the sequenced clone. Moreover, that the high coding potential peaks jump between reading frames along the 4 kb sequence is strongly suggestive that, if the prediction is correct, the gene is composed of at least four introns, as these must be present to splice the discontinuous reading frames together. Also possible are additional introns that might splice a predicted coding region onto itself, for instance, the two peak areas in region 1 might be joined together by an in-frame splice. Additionally, the GeneMark program is inefficient at detecting exons of less than 100 nt in *Chlamydomonas* genes (Borodovsky and Lukashin, unpublished results; see also http://genemark.biology.gatech.edu/GeneMark/hum.cgi), so other small exons might be contained in the coding sequence in addition to those GeneMark has predicted. It was then decided to design primers for RT-PCR using the predictions of the GeneMark program to position the oligonucleotides within regions that are candidate exons.
RT-PCR of wildtype *Chlamydomonas* mRNA

Pairs of complementary oligonucleotide primers were designed based on the output of bottom strand coding potential predicted by the GeneMark program. Coding potential spikes above the threshold value were used in the design of the oligonucleotide primer pairs. The primers which were designed based on the peak for region 5 are R5E1S from nucleotides 1109-1130 (sense primer) and R5E1A from 1130-1109 (antisense primer) of the C/B-10 bottom strand sequence shown in Figure 22. Likewise, other primers based on the predicted peak regions are R4E1S (1584-1601) and R4E1A (1601-1584) for region 4, R3E1S (1956-1975) and R3E1A (1975-1956) for region 3, and R2E1S (2233-2253) and R2E1A (2253-2233) for region 2. Based on the coding potential peaks for region 1, it seemed possible that this region might comprise two distinct exons. Therefore, two pairs of complementary primers were designed for region 1, R1E2S (2705-2727) and R1E2A (2727-2705), and R1E1S (2965-2985) and R1E1A (2985-2965).

Having designed primer pairs that were likely to fall within the coding sequence of the *EYE2* cDNA, it was decided to amplify fragments of the cDNA by RT-PCR using *Chlamydomonas* poly(A)\(^+\) mRNA. The location of the primers used for the amplifications, the sizes of predicted genomic amplification products, the observed amplified cDNA products and deduced intron sizes can be seen in Fig. 24A and 24B. If the mRNA is spliced between the two primer positions, the resultant PCR product will be
Figure 24.

Identification of EYE2 PCR products amplified from a wildtype Chlamydomonas reverse transcribed mRNA sample.

A. Schematic indicating the PCR strategy to identify specific EYE2 PCR products from the reverse transcribed mRNA sample. Primers were designed from predicted coding regions from the GeneMark program. One primer, A1, was chosen as an anchor, and then antisense primers within that candidate exon (S2) and in the other candidate exons (S3, S4, S5, S6) were used for PCR. The prediction was that each amplified product from the next downstream primer would be bigger than the last product, but smaller than the expected genomic product by the size of the introns excised. This process was then reiterated using a different anchor primer, A3 or A4, with the appropriate downstream primers, to confirm the observations made from the first PCR series.

B. Sizes of expected genomic and observed cDNA PCR products.

C. PCR products from primer pairs indicated by the schematic in A, with approximately 100 ng of first strand cDNA synthesized from poly(A)^ mRNA prepared as template from wildtype Chlamydomonas grown on acetate for seven days and then shifted to gamete-inducing media for four hours. PCR products were resolved on a 0.8% agarose gel, blotted onto Nytran, and probed with a random primed 2.3 kb HindIII/EcoRV fragment from C/B-10. Bands which are genuine amplified EYE2 cDNA products are surrounded by a black or white box.
A

B  PCR products

| Expected | 0.28 | 0.75 | 1.02 | 1.40 | 1.87 | 0.29 | 0.67 | 1.14 | 0.39 | 0.86 |
| Observed | - | - | 0.7 | 0.8 | 1.1 | 0.1 | ? | 0.5 | ? | ? |
| Deduced Intron size | - | - | 0.3 | 0.2 | 0.2 | 0.1 | ? | 0.2 | ? | ? |

C  

| Sense primer | S2 | S3 | S4 | S5 | S6 | S4 | S5 | S6 | S5 | S6 |
| Antisense primer | A1 | A1 | A1 | A1 | A1 | A3 | A3 | A4 | A4 |

Size, kbp

- 1.078
- 0.872
- 0.603
- 0.310
smaller than the expected genomic product. The size difference between the expected genomic PCR product and the observed cDNA PCR product will be the size of the intron which has been excised. This process was repeated with several combinations of primers to identify the positions and sizes of all introns in the mRNA.

It was decided to use the primer pairs to amplify first-strand cDNAs that were reverse-transcribed from mRNA from a wildtype strain, as well as an existing *Chlamydomonas* wildtype cDNA library. This method allowed comparison of the two sets of products to eliminate artifactual bands amplified in either reaction. As an additional test to eliminate PCR artifacts, amplified fragments were verified as genuine *EYE2* cDNA products by Southern analysis of the PCR fragments with C/B-10 genomic probes. The results with various primer combinations can be seen in Fig. 24C for the reverse transcribed mRNA, and Fig. 25C for the cDNA library. All observed fragment sizes from the blot and all inferred intron sizes are approximate.

Southern analysis of the PCR products amplified from the reverse transcribed mRNA sample from wildtype cells, using the primer combinations R1E1A and R1E2S (lane A1/S2 in Fig. 24A) or primers R1E1A and R2E1S (lane A1/S3) reveals a failure to amplify a specific product from the cDNA sample. This failure is probably a result of these primers hybridizing to the template inefficiently, and therefore yielding no product, since subsequent sequencing of the amplified *EYE2* cDNA indicated that this sequence is present in the cDNA.

Summarizing the results from the reverse transcribed mRNA sample (Fig. 26),
Figure 25.

Identification of EYE2 cDNA PCR products from a wildtype Chlamydomonas cDNA library.

A. Same schematic as in Figure 24.

B. Sizes of expected genomic and observed cDNA PCR products.

C. PCR products from primer pairs indicated by the schematic in A. 100 ng of DNA representing approximately $5 \times 10^6$ cDNA clones from a synchronized population (1 hour into dark phase) of wildtype Chlamydomonas as template. PCR products were resolved on a 0.8% agarose gel, blotted onto Nytran, and probed with a random primed 2.3 kb HindIII/EcoRV fragment from C/B-10. The cDNA library used as template for this procedure was the gift of Drs. Greg Pazour and George Witman. The larger of the two bands present in the S4/A3 lane is not the expected cDNA product. Bands which are genuine amplified EYE2 cDNA products are shown with a white underline.
A

B  PCR products

Expected 0.28 0.75 1.02 1.40 1.87 0.29 0.67 1.14 0.39 0.86

Observed - - 0.7 0.8 1.1 0.1 0.3 0.5 0.1 0.3

Deduced Introns size

0.2 0.2 0.2 0.2 0.2 0.2 0.2 0.2 0.2 0.2

C

Sense primer S2 S3 S4 S5 S6 S4 S5 S6 S5 S6 S6

Antisense primer A1 A1 A1 A1 A1 A3 A3 A3 A4 A4

Size, kbp

1.078

0.872

0.603

0.310
Figure 26.

Summary of PCR on wildtype RT-mRNA and Pazour cDNA library

A. The arrow indicates the presumed direction of the transcription unit of the \textit{EYE2} gene within the 4 kb sequence of the C/B-10 insert.

B. The organization of the five regions predicted to be of high coding potential by the GeneMark program and their position in the 4 kb sequence of the C/B-10 insert.

C. Organization of the \textit{EYE2} cDNA. Approximate positions of the four presumed introns. The known ends of the partial cDNA are defined by the positions of the primers R5E1S and R1E1A. Sequence known to lie within the cDNA comprises approximately the middle two kb of the 4 kb insert, and approximately 760 bp are removed by splicing.
B  EYE2  
Genomic

C  EYE2  
partial  
cDNA
PCR amplifications are indicative that the *EYE2* gene contains at least four introns, with a 150 bp intron between primers R2E1S and R1E1A, another 150 bp intron between primers R3E1S and R2E1A, a 260 bp intron between primers R4E1S and R3E1A, and a 200 bp intron between primers R5E1S and R4E1A. This prediction for the organization of the *EYE2* gene represents a minimally complex organization for the arrangement of exons. More complicated arrangements are possible, for example, having the 260 bp intron between primers R4E1S and R3E1A represent the existence of two introns with a total size of 260 bp.

Analysis of the pattern of amplified products from reactions using a cloned cDNA library as template confirmed the observations from PCR of reverse transcribed mRNA (Fig. 25). The library was prepared from a synchronous population of *Chlamydomonas*. *Chlamydomonas* cells divide synchronously when grown in medium without a carbon source, when stimulated using a 12 hour light:12 hour dark photoperiod (Harris, 1989). Cell division begins about an hour into the dark phase, and is completed at a time about eight hours into darkness (Morel-Laurens and Bird, 1984). The library was prepared from a population one hour into the dark phase of the photoperiod, because the transcript for another gene involved in phototaxis (*PTX2*) was observed to accumulate at this time (Pazour and Witman, unpublished observations). It was hypothesized that *EYE2* mRNA might be more abundant at this time as well. This library was used as a template for PCR amplification using the same primer combinations as the reverse transcribed mRNA sample used in Fig. 24. As before, amplified products were subjected to Southern analysis using
a uniformly labeled *EYE2* genomic probe. It should be noted that the hybridization intensities of PCR products from the cloned cDNA library are much stronger than those products from the reverse transcribed mRNA sample. This difference reflects a difference in the amount of products obtained from the library PCR reactions, due to the inclusion of approximately tenfold more template DNA in the cDNA library PCR reactions.

The identification of mRNA and cDNA populations that contained the *EYE2* cDNA, allowed for the utilization of several strategies to isolate the complete *EYE2* cDNA. First, a screen of the synchronized (Pazour) library by standard plaque *in situ* hybridization methods was undertaken. Two different primary screens, using an *EYE2* genomic probe or an *EYE2* amplified cDNA product from the 3' portion of the coding sequence, each of which surveyed over four million cDNA clones, failed to identify a plaque which hybridized to the probes tested. This result was indicative of the low frequency of the *EYE2* cDNA in the Pazour library.

Repeated attempts to amplify the 5' and 3' ends of the cDNA from the Pazour library, using various combinations of both a primer in the *EYE2* cDNA and a primer in the phage vector backbone, failed to give PCR products that would hybridize to *EYE2* genomic probes. Attempts to perform secondary amplifications using nested primers with the primary PCR reaction as template also failed to amplify products that hybridized to *EYE2* genomic probes. Numerous other attempts to amplify the 5' and 3' ends of the *EYE2* cDNA with *EYE2* primer/vector primer combinations using the reverse transcribed mRNA sample, the synchronized cDNA library, or other cDNA libraries prepared from
asynchronously dividing *Chlamydomonas* strains (supplied by Quinn and Merchant, Goldschmidt-Clermont, Ferris) as templates failed to amplify the 5' or 3' ends of the *EYE2* cDNA. These amplification experiments seem likely to have failed due to the low abundance of the *EYE2* cDNA in the template populations. Since it was clear that standard methods were not working to identify the complete *EYE2* cDNA, it was decided to continue to amplify the partial *EYE2* cDNA, with the aim of extending the PCR products obtained to encompass the entire transcribed sequence.

**Extension of the *EYE2* cDNA sequence**

Primers designed to extend the PCR products from the *EYE2* cDNA were used to amplify products from the Pazour cDNA library. To identify the 5' region of the *EYE2* cDNA (Fig. 27A), primer R2E1A was used in combination with primers with 5' most nucleotides of 804 (primer G), 763 (F), 666 (E), 616 (D), 589 (C), 575 (B) or 538 (A) to amplify the *EYE2* cDNA from the Pazour cDNA library. Products were verified as being genuinely derived from the *EYE2* cDNA because the anticipated reaction products were expected to be 610 bp less then the genomic PCR product using the same primers, due to the presence of the 200 bp, 260 bp, and 150 bp intron(s) known to lie between the R2E1A primer and the previously identified 5' end of the cDNA at nt 1111. Amplification of products of the expected cDNA size were observed for the R2E1A primer combined with primers with 5' most nucleotides 786, 746, 646, and 596. Primers more to the 5' side of
Figure 27.

Extension of 5' and 3' borders of EYE2 cDNA by PCR

For both diagrams, the positions of the five exons in the EYE2 gene are represented within the context of the EYE2 genomic clone.

A. Extension of the 5' end of the amplified EYE2 cDNA

PCR amplification was performed using primer R2E1A in combination with the indicated primers. Primers are noted by the most 5' nucleotide at the indicated positions in the EYE2 genomic sequence 804 (G), 763 (F), 666 (E), 616 (D), 589 (C), 575 (B) or 538 (A). The Pazour synchronized cDNA library was used as the template. The seven primers (A-G) span a 230 nt interval from nt 516 to nt 804 of the sequence of Fig 22.

B. Extension of the 3' end of the amplified EYE2 cDNA

PCR amplification was performed using primer R5E1S in combination the indicated primers. Primers are noted by the most 5' nucleotide at the indicated positions in the EYE2 genomic sequence 3093 (G), 3155 (F), 3324 (E), 3367 (D), 3386 (C), 3395 (B) or 3417 (A). The Pazour synchronized cDNA library was used as the template. The seven primers (A-G) span a 324 nt interval from nt 3093 to nt 3417 of the sequence of Fig. 22.
all failed to amplify a product in combination with the R2E1A primer. This observation led us to conclude that the 5' end of the cDNA was probably between nucleotide 565 and nucleotide 596. An alternative explanation, however, might be that the primers more 5' than nucleotide 596 are actually contained within the EYE2 cDNA, but fail to amplify the appropriate PCR product for unknown reasons. The longest 5' end product obtained, a 1043 bp product obtained from the amplification using primers CB3400S (with 5' most nucleotide 596) and R2E1A, was then gel purified and sequenced.

A similar strategy was used to extend the 3' end of the partial EYE2 cDNA. Primer R5E1S was used in combination with primers with the 5' most nucleotides at positions 3093 (G), 3155 (F), 3324 (E), 3367 (D), 3386 (C), 3395 (B) or 3417 (A) to amplify products from the Pazour cDNA library template. Products were verified as being genuine EYE2 cDNA amplification products because the reaction products were expected to be 760 bp less than the genomic PCR product using the same primers, due to the presence of the 200 bp, 260 bp, and the two 150 bp introns known to lie between the R5E1S primer and the previously identified 3' end of the cDNA at nt 2984. Amplification of products of the expected size was observed for the combinations of R5E1S with primers with 5' most nucleotides 3093, 3155 and 3324. Combinations of the R5E1S primer with all other primers further to the 3' side of nucleotide 3324 failed to generate a PCR product. This result led to the conclusion that the 3' end was likely to lie between nucleotides 3324 and 3367, although the possibility remains that primers more 3' of nucleotide 3324 might actually fall within the EYE2 cDNA and fail to amplify a PCR
product from the cDNA library for unknown reasons. Regardless, the longest 3' end PCR product obtained, a 1437 bp product of the reaction amplified by primers CB670A (with 5' most nucleotide 3324) and R5E1S, was gel purified and sequenced. The sequence of the R5E1S/CB670A product was examined for its overlap with the 5' EYE2 cDNA product amplified with the R2E1A/CB3400S primers, and the contiguous partial EYE2 cDNA sequence was assembled.

**Sequence of the partial EYE2 cDNA**

The assembled sequence of the amplified cDNA product is shown in Fig. 28. Examination of the sequence of the partial cDNA confirmed the prediction of four introns within the EYE2 gene. The doubly underlined nucleotide pairs at positions 723-724, 866-867, 962-963 and 1172-1173 indicate the position of the observed splice junctions in the EYE2 cDNA, with the first nucleotide of the pair representing the last nucleotide of the preceding exon, and the second nucleotide of the pair representing the first nucleotide of the following exon. The removal of these four introns causes the assembly of a major open reading frame (ORF). The deduced protein product for the ORF is shown just below the nucleotide sequence. This ORF encodes a protein of 503 amino acid residues, and we hypothesize that this ORF is the one which encodes the EYE2 protein.

Interestingly, examination of the EYE2 cDNA sequence reveals the existence of
Figure 28.

Sequence of the partial EYE2 cDNA and predicted EYE2 protein sequence

The sequence shown is the sense strand of the amplified EYE2 partial cDNA. The sequence is shown in the 5' to 3' direction. Nucleotide 1 is the eighth nucleotide of the primer CB3400S, and the last nucleotide (1950) shown is the complement of the 5' end of the primer CB670A. The predicted EYE2 protein sequence is shown on the lines just below the major ORF in the cDNA sequence, with the single letter amino acid code positioned just below the first nucleotide of the codon for that residue. The initiation codon ATG at nucleotide 270 and the termination codon TGA at nucleotide 1779 are shown in bold. The italicized nucleotides represent the upstream ORF (uORF) that is found in the 5' untranslated region of the EYE2 cDNA. The stop and start codons for the uORF are singly underlined. The positions of the splice sites are represented by the doubly underlined dinucleotides found at positions 723-724, 866-867, 962-963, and 1172-1173. The first nucleotide of these pairs is the last nucleotide of the preceding exon, while the second doubly underlined nucleotide is the first nucleotide of the subsequent exon. The singly underlined protein sequence identifies the thioredoxin active site motif contained in this protein.
Figure 28. Partial EYE2 cDNA sequence and predicted EYE2 protein

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Figure 28 (continued). Partial EYE2 cDNA sequence and predicted EYE2 protein

781  CCCAGTGGATCCGGCCCAAACAGAAACAGACAGCTGGTGGCTGGTACGCGCCCTGGTGCC 840
172  QCDRANSQNTLVLYAAPWP 191
841  CGCAGTGAGAGATGGAGAGAGAGCTGGAGCGGCTGGCGGAGGGGCTGTCGCACGAGC 900
192  HCREMEDELRLEAGLSEHEQ 211
901  AGACCGGGTGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGT

181
Figure 28 (continued). Partial EYE2 cDNA sequence and predicted EYE2 protein

1501 GGTCGATGCCGCAAGAGTTGCGCACAGCCGCCACAGCAGCATGGCCACCGCGGC
1561 AGCTGCTGAGGCCTATCCGGTTGCTGTCGCGAAGTTGCTTGGCTCTTGGCGGC CGCGGC
1620 AGCTGCTGAGGCGCCTATCCCGGTGGTGGTCGCCGAGGTGCTTGCTCCTGCGG CGCCCGC
1680 GCCTGTGGACGCCGCTGCCGCCGCGGCCGGCGCGCCCCGTCAACAGCGAATGGCAACGG
1740 CGTGGGGACGACAGCAGCAGCAGCTGCTGCTGCGGACGGCTGCTGCTGCTGCGGCGGC
1800 GCTGACGCGGCTGACCGATGACGAGTTATCGCGCTGGTGAACAGTGATCCAGATCTGGA
1860 CAAGGTGCTGGCGGGCTGGGAGACAAGTGATGGGAGGGGCGGATGATGATGGGCCC
1920 TGGGTAGGATGGGCTGAAAGCGATCGTG

1 1 f. 1 ^ +
H + 1 + + 1-
WGRQQRPHLLLLGRLLLRRR 491
RQCQQPLRLRAS* 503
GCTGACGCGGCTGACCGATGACGAGTTAATCGCGCTGGTGAACAGTGATCCAGATCTGGA 1860
CAAGGTGCTGCTGGCGGGCTGGGAGACAAGTGATGGGAGGGGCGGATGATGATGGGCCC 1920
TGGGTAGGATGGGCTGAAAGCGATCGT 1948
another ORF whose stop codon is located 96 nt upstream of the initiator ATG of the presumed EYE2 protein coding region. Such upstream ORFs (or uORFs) have been observed in some mRNAs from other organisms (Hinnebusch, 1984; Kozak, 1991; Geballe 1996; Vilela et al., 1998). In cases where the function of these uORFs has been elucidated, a role has been shown for the uORFs in determining either the efficiency of translation initiation at the coding ATG (Kozak, 1991; Pinto et al., 1992), or in the control of the stability of the mRNA due to translation of the uORF (Ruiz-Echevarria et al., 1996; Linz et al., 1997; Vilela et al., 1998). The position of the uORF is shown by the italicized nucleotides and extends from the ATG at nt 55 to the TAG at nt 172.

Figure 29 shows the organization of the EYE2 gene within the sequenced genomic clone of C/B-10, and this is also represented schematically in figure 30. The presumed start codon for the EYE2 protein is the bold, underlined ATG at nucleotide 872. The presumed stop codon for the EYE2 protein is the bold, underlined TAG at nucleotide 3155. The deduced amino acid sequence of the EYE2 protein is shown under the nucleotide sequence, and the slashes represent the splice junctions found in the partial cDNA obtained from the Pazour library.

The introns all contain 5' splice site and 3' splice site sequences that match the consensus for Chlamydomonas splice junctions. The 5' splice site consensus for Chlamydomonas is G/GTG[A or C or G]G (Purton and Rochaix, 1995). The observed 5' junctions for the four EYE2 introns are either G/GTGAG or G/GTGGG. The 3' splice site consensus is more degenerate than the 5' consensus, with the proposed 3' splice site
Figure 29. Organization of the EYE2 gene in the C/B-10 genomic sequence

The genomic sequence of the 4 kb insert of the C/B-10 plasmid is displayed. The 5' and 3' ends of the EYE2 cDNA verified from PCR amplification of the Pazour cDNA library are in bold, and have asterisks below them. The position of the 5' end of the partial EYE2 cDNA is at nucleotide 603, while the position of the 3' end of the partial EYE2 cDNA is at nucleotide 3324. The positions of the verified splice junctions are in bold, with double underline. Possible promoter elements are singly underlined, and possible poly(A) addition signal sequences are doubly underlined. The position of the presumed initiation codon for the EYE2 protein is at nucleotide 872 and the ATG is labeled in bold and underlined. The presumed stop codon is also bold and underlined, and is located at nucleotide 3155. The nucleotide sequence of the upstream ORF has been italicized and the stop and start codons for the uORF are in bold italics. The deduced amino acid sequence of the uORF is provided on the lines just below the nucleotide sequence. The deduced amino acid sequence of the EYE2 ORF is also below the nucleotide sequence which encodes it, and the amino acid numbers are in bold at the beginning of each amino acid row. The slashes represent the borders between intron and exon junctions.
Figure 29. Organization of the EYE2 gene in the C/B-10 genomic sequence
Figure 29 (continued). Organization of the EYE2 gene in the C/B-10 genomic sequence

1201 ACCTTTCTC AGAGGAGGGC AGCAGCTGCT GTGTACCAGGC TCCGGATGAT CCACAGCTG CGGCTGCGCA
1181 PLL KEGQ QLL VPA SGIS QLA VME APAT
1281 GCGACACCC CTGACATCCC CGCGCAGCGAT GTGTGCA CTGACATCCC CGGCGCTGCC
138 TTP VLG PSVAPRA GTMWWV/
1361 TACCACAGT ATGGCCGATA GTGACGACTT GTGCTGCTG CTGACAGGCA AGCCGAGATG ACCAGTATC
1441 ACCTCCACTC TCCCGGTTGC CTCACACAG CCGGCGGCT CTCACCAGCC TCGCCCGCTT CCACCTTGC A
1521 TCCCGGCTCG CTCTTTCTGTG CAGCTGCGA CGAGTAGGGG CCGATCTGGG CATTCTCGGA CAGGGGGGAC GGCCTGCTCGA
153 GSEYVLATISDAQRQLYS
1601 CCCAGGCTCA CGCCGCAACG AGCCGAGAGC CACTTGGTCT GTGTAGTGGGT CCGCTGGGAG GAGATGGGAG
157 QCDRANSQONTLLVLPWCIPCHREME
1681 GAGCGTGGG GGAGGGACA AATGGAGGACA GGGAGGACAA GGGAGGGAAC GGGGAGGGGAC TGTGCTGCGC
198 DE /
1761 GTGACTGCTT GCTGACGTGC TCCGCGCTTGC GACGTTATGT TGCGCTGACG AGCCAGACG
1841 ACCTCCCTCTC ACGGCAGCTCA GTGTAGTGGGT TATTCAGCA TGCTGCTGAC ACTTGCCAGAA TCATTCTTGT TATGTCCCA
1921 TCATTCTGTG CTCCTTCTCAG CAGCTGCGA CGGACGGGAGC TGGCGGCTG ACCTGCTGCG CAGGACTAAC GGGAGGAGAC
200 GSMPEGRIFSERE/
2081 AGACACGGAG TACCTTGGTTT GGAATACCAA TCTGGACGGC CCCCGTAAAC CAGACGTTGC TCTCACCC
2161 CCACTCCTAT CACCCGAGGTC GTGGCGGTGC GTACTTACCD TCCAAATTTT CTTCCTTTGC GACTCAGGCA ACCTTCTACA
232 VLGAYPSIIISPFEHSTRYK
2401 AGTACAGGAG CGCCGACGAG CAGCGGAGAT CGCCGAGCGAT CTGGTCTGCC ATGGAGATGGT GAGATGGGAAG
2241 AGTACAGGAG CGCCGACGAG CAGCGGAGAT CGCCGAGCGAT CTGGTCTGCC ATGGAGATGGT GAGATGGGAAG
254 YKGRQRDLERASLLRFBNMTCCSARRED
232 CAGATGTGGA CGCTGCCGCGC CGGCGACACA CGCTCGACGT TGCGGCTGAA GAGGGCGAGC AGGACTGTTG GTGTATAT
280 QMMWTLRPA GTSTRTVAAALPGAST/
2401 TCAAGGAGGTT GGTAGAGCCTT AGACCATCTT GGGTGCTGCGGCCCGGCGAC AGCGGAGCTG GGGGCGGCTC CGGCAGTGC
Figure 29 (continued). Organization of the EYE2 gene in the C/B-10 genomic sequence
Figure 29 (continued). Organization of the *EYE2* gene in the C/B-10 genomic sequence

3841 TCTCAAGCGC TCTTTGCTG TTGGCCCTAT CGGGCGCTACC TGCAGCTCTG ACAGAAAGCT GTAGAGCCAT ACTCAATGCA

3921 ACTGTGCCAG GAAACAACAG CCTTTGGTC GATGATAAGC TGTCAACAT CAGGAGCTCC GGAAGCTAAA ATGGA
Figure 30.

Representation of the layout of the EYE2 gene in the four kb genomic clone.

The exons of the EYE2 gene are represented by the dark boxes. The probable start and stop codons for the EYE2 protein are noted by the ATG and the TAG at the start of the first exon and the end of the fifth exon, respectively. The 5' and 3' UTRs of the EYE2 gene are shown as white boxes. The uORF in the 5'-UTR of the EYE2 gene is shown as a grey box. The introns are shown by the lines linking the 3' end of each exon to the 5' end of the subsequent one. The position of the likely promoter for the EYE2 gene is shown by the arrow denoted TATA. The position of the likely polyadenylation signal is shown by the line marked Poly(A).
consensus as NNNCAG/N, where N can be any nucleotide (Purton and Rochaix, 1995). The four 3' splice sites observed for the *EYE2* gene are all different, having the sequences GTGCAG/T, TCTCAG/T, GCCCAG/G, and GCGCAG/T. The splice junctions sequences are listed in Table 11.

The possible promoter elements for the *EYE2* gene are underlined in Figure 29. Proposed TATA boxes for *Chlamydomonas* are typically defined as a stretch of six or more adenine or thymine nucleotides (Brunke et al., 1985; Fukuzawa et al, 1990; Kropat et al., 1995), although TATA boxes containing cytosine have been proposed as well (Silflow et al., 1985). Candidate TATA boxes for the *EYE2* gene were identified using a consensus that allowed a stretch of Ts or As that could be interrupted by a single G or C. Eight such sequences can be found in the region upstream of the presumed initiation codon for the *EYE2* protein (Table 11, Fig. 29), at positions 272-278, 329-336, 380-389, 411-416, 530-537, 568-574, 582-588, and 711-716. Any of the candidate TATA boxes could possibly be the *EYE2* promoter, although the spacing between the element at nt 582-588 and the observed 5' end of the partial *EYE2* cDNA at nt 603 is closest to the spacing typically observed between other eukaryotic promoters and the site of transcription initiation (Guarente, 1984). Given that the start of transcription is usually 20 to 40 nt downstream of the TATA box, the other promoter candidates are much farther away from the known 5' end of the partial cDNA than would be expected. Therefore, we predict that the TATA-like elements at either nt 568-574 or nt 582-588 are the *in vivo* promoter for the *EYE2* gene.
Table 11. Predicted and experimentally verified non-coding sequence information for the *EYE2* gene

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The signal for mRNA polyadenylation in *Chlamydomonas* is well conserved. All known mRNAs but one contain the sequence TGTAA 20 to 30 nt upstream of the site of poly(A) addition (Silflow et al., 1985; Merchant and Bogorad, 1987). The only known exception to this rule is the actin mRNA, which contains the close sequence variant TGTAG (Sugase, et. al, 1996) 13 nt upstream of the poly(A) addition site. Two candidates for poly(A) signal sequences can be found 3’ to the end of the partial *EYE2* cDNA (Table 11). In Fig. 29, the potential poly(A) signal sequences are doubly underlined. The most proximal signal is located at nt 3352-3361, and this signal is similar to the one observed for the *Chlamydomonas* actin mRNA. If this is the *in vivo* poly(A) signal sequence used for the *EYE2* mRNA, then the Pazour library cDNA PCR reactions with primer having the 5’ nucleotide of the primer at the indicated position of the *EYE2* genomic clone 3093, 3155, 3324, 3367 and possibly 3386 and 3395 should have amplified a cDNA product when paired with R5E1S (Fig. 27, part B). As previously indicated, only the first three of those primers amplified a cDNA product when paired with R5E1S. Similarly, the other potential poly(A) signal sequence observed in the *EYE2* 3’ region is located at nt 3741-3745, and this sequence is a perfect match to the canonical *Chlamydomonas* poly(A) signal sequence. However, if this signal were the *in vivo* poly(A) addition site used for the *EYE2* mRNA, then all seven of the primer combinations tested in the PCR extension of the *EYE2* 3’ end shown in Fig 27 (part B) should have amplified products, as all the primers would lie within the 3’ untranslated region of the *EYE2* cDNA. Therefore, it can be concluded that one of these sites is the *in vivo* poly(A)
signal for the *EYE2* mRNA and the previously discussed PCR amplifications have failed for unknown reasons, or alternatively, some novel poly(A) signal sequence is used for the *EYE2* mRNA. We hypothesize that the signal at 3352-3361 is the genuine signal for the *EYE2* mRNA, based upon its proximity to the observed 3' end of the partial *EYE2* cDNA.

**Discussion**

The experiments described in this chapter were designed to isolate the first eyespot assembly gene described in any green alga. Using insertional mutagenesis of *Chlamydomonas*, (Quinton and Dieckmann, unpublished observations) tagged insertions in or near both the *EYE2* and *MINI* genes were obtained. In this study we used the technique of plasmid rescue (Tam and Lefebvre, 1993) to recover the sequence adjacent to the site of the integration which inactivated the *EYE2* gene. Using a portion of that sequence, we were able to isolate clones from a genomic library which were capable of rescuing the original *eye2-1* defect. The smallest genomic clone capable of rescuing the defect was sequenced, and an algorithm was used to predict regions of likely coding sequence for the genomic clone. An almost complete cDNA for the *EYE2* gene was isolated.

The results of the plasmid rescue experiment were interesting from a technical standpoint. Many mutants have been generated in *Chlamydomonas* by the method of insertional mutagenesis, using an *E.coli* plasmid carrying an endogenous *Chlamydomonas*
gene as the integrating DNA. The original procedure of Tam and Lefebvre (1993) recovers the inserted DNA as an *E. coli* plasmid which contains a portion of the *Chlamydomonas* genome adjacent to the site of insertion. In most cases, however, this is not so straightforward, and the inserted DNA is not integrated in a completely intact fashion. A requirement of the Tam and Lefebvre method is that the *E. coli* plasmid replication origin and the bacterial selectable marker must remain intact, and in many cases this does not happen. In our experiments, however, the *E. coli* origin and ampicillin resistance gene were intact, and thus a bacterial plasmid containing *Chlamydomonas* genomic DNA flanking the insertion site was recoverable. Recovery of the inserting DNA revealed that the recombination endpoint of the inserting plasmid in the *minl*-5 strain was approximately 350 bp away from the restriction site used to linearize the inserting DNA, while in the *eye2*-2 strain, the recombination endpoint of the inserting plasmid was approximately 1000 bp away from the site used to linearize the integrating DNA. In the case of the *eye2*-2 strain, this recombination endpoint is probably within 50 to 100 bp away from the end of the ampicillin resistance gene. If much more of the inserting plasmid had been lost, the plasmid rescue experiment would not have worked. Thus, for future insertional mutagenesis screens, it might be useful to engineer a vector in which there is more distance between the site used to linearize for transformation and the plasmid sequences necessary for the recovery. This might improve the chances of a successful plasmid rescue experiment.

Once the flanking sequence was obtained, fragments from both the *minl*-5 and
eye2-2 flanking DNA were used to probe wildtype *Chlamydomonas* genomic DNA. A unique probe adjacent to the inactivated gene was sought to screen a genomic library for clones containing the corresponding wildtype genes. For the eye2-2 flanking DNA, a large (1.2 kb) flanking sequence probe was found to be unique. However, using many fairly large flanking sequence probes from minl-5 flanking sequence, no unique probe was found. It is possible that the insertion inactivating MINI in this strain has integrated in a region of the genome which has a higher proportion of repetitive elements than the sequence of the genome adjacent to the insertion site in eye2-2. Recently, however, a small 200 bp probe from the minl-5 flanking DNA was found to be unique, and was used to screen a *Chlamydomonas* BAC library for clones which are likely to contain the MINI gene (Sibayan and Dieckmann, unpublished observations).

Since the eye2-2 flanking sequence probe was unique, the probe was able to identify four cosmids from a *Chlamydomonas* genomic library. Two of the four cosmids were able to rescue the defect in an eye2-1 mutant, although the frequency of phenotypic rescue was low. This low frequency of obtaining the Eye^ phenotype could be explained by a number of events. First, there could be complementation by introduction of the wildtype *EYE2* gene in the cosmid insert. Second, there could be a reversion of the original eye2-1 UV-induced mutation. This possibility seems unlikely, because there should be no selective pressure to recover the ability to phototax when the cells are grown on plates. Cells growing on plates do not assemble flagella completely, and without motility, photobehaviors cannot occur. Third, the eye2-1 mutation could be suppressed by
the introduction of a copy of a gene which is not $EYE2$ in the cosmid insert. This explanation is possible, but less likely than true complementation, as the cosmids are derived from the region of the genome near the mutations $eye2-2$ and $eye2-l$, and thus are likely to contain the wildtype gene. Fourth, suppression of the $eye2-l$ defect could occur by the loss of a gene (or genes) deleted or inactivated by the integrating cosmid. This possibility is also unlikely. Since the transforming DNA integrates at random, the observed frequency of eyespot restoration seems too high to be caused by suppression due to a loss of gene(s) at the insertion site. Finally, it might be possible to acquire an extragenic suppressor which restores eyespot formation independent of any cosmid-linked event. However, this possibility is not probable due to the expected low selection pressure for recovery of photobehavior on cells maintained on plates. Thus, by far the most probable explanation for the restoration of eyespots in the transformed strains is complementation by the wildtype $EYE2$ gene.

Once smaller subclones from cosmids 3-10 and 7-8-1 were found to rescue the $eye2-l$ defect, the smallest was sequenced. Upon recovering the sequence of the entire insert from this subclone, it was evident that there were many possibilities for what might constitute the $EYE2$ gene. After several attempts at conventional cDNA library screens, it was necessary to use a prediction of coding potential to design primers for RT-PCR. The GeneMark algorithm turned out to be remarkably accurate in its predictions. The program predicted that the $EYE2$ gene would contain five exons, and that prediction was confirmed by the sequence of the $EYE2$ cDNA. The $EYE2$ cDNA obtained probably contains the
entire coding sequence of the EYE2 protein. The presence of a stop codon at codon 504 of the long ORF contained in the cDNA makes this the obvious choice for the stop codon used *in vivo*. Likewise, the start codon for the EYE2 protein, is probably the one used *in vivo*, because of the presence of stop codons in all three reading frames in the cDNA upstream of this ATG.

Comparisons of the EYE2 protein sequence to several sequence databases revealed no candidate sequence homologs for the entire EYE2 polypeptide. The only part of the EYE2 protein which shows any homology is a short sequence in exons two and three, which comprises a putative thioredoxin-like active site sequence. Thus, it may be that the EYE2 protein is involved in control of the eyespot assembly process through the action of this sequence. EYE2 might control other proteins by oxidizing or reducing cysteine residue pairs in selected targets. The target proteins could have functions in the structure of the pigment granules, the proteins which position the visual apparatus at the four membered microtubule rootlet emanating from the daughter basal body, or it might control transcription factors specific for eyespot assembly genes through the action of this motif. Alternatively, this motif might be involved in the synthesis of some compound required for eyespot assembly.
Chapter 5: Functional analysis of the EYE2 gene and protein

Abstract

EYE2 is the first eyespot assembly gene to be isolated from any green algae. As a check to be certain that the gene we had isolated actually was responsible for the eyespot assembly defect in the eye2-I strain, PCR amplification of part of the isolated gene from the eye2-I mutant, and subsequent sequencing of the PCR product demonstrated a single nucleotide change from G to A at the first nucleotide of the first intron, indicating that the gene isolated was actually defective in the eye2-I mutant strain. Additionally, the introduction of a construct with an early stop codon in the second exon of the presumed coding sequence failed to restore eyespots when transformed into the eye2-I mutant strain defective in splicing. Thus, the rescue of the eyespot assembly defect in the eye2-I mutant strain is actually due to complementation by the introduction of the isolated EYE2 gene.

In an effort to determine the functional role of the EYE2 protein in eyespot assembly, a sequence homology search was performed. This search demonstrated that the only identifiable sequence homology in EYE2 to any known proteins was a thioredoxin active site motif. A mutated gene which harbored the amino acid change C193S still rescued the eye2-I defect, indicating that the potential redox activity of the EYE2 protein may not be required for its function in eyespot assembly. An epitope-tagged EYE2 construct was still able to rescue the eye2-I defect, indicating that the additional C-
terminal amino acids do not interfere with eyespot assembly. Epitope-tagged EYE2 was undetectable by Western analysis and in vivo immunofluorescence. Thus, the localization of the EYE2 protein and the function of the EYE2 protein remain unknown.

Introduction

This study represents the first description of a gene involved in eyespot assembly from a green alga. Based upon previous work, it was anticipated that genes affecting eyespot assembly might fall into three functional classes. The first class of proteins involved in eyespot assembly would have structural roles, involved in either positioning the eyespot granules in the appropriate cellular location by recognizing the four membered microtubule rootlet emanating from the daughter basal body, or perhaps involved in packaging of the carotenoid-filled lipid granules inside the chloroplast. The second class of proteins involved in eyespot assembly would be ones that were involved in regulating other genes which function in eyespot assembly. This class might include transcriptional activators or repressors which would directly control expression of eyespot-specific genes. Alternatively, proteins of this class could function to directly regulate other proteins which assemble eyespot components. The final class of proteins involved in eyespot assembly would be those proteins which synthesize components required for eyespot formation. This class might contain proteins which modify structural components of the eyespot, or enzymes which synthesize specific compounds necessary for eyespot formation.
While no conclusive evidence for the function of EYE2 has been obtained, it seemed that based upon the rarity of the mRNA for EYE2, the protein was also likely to be rare, and that might preclude its function as a structural component of the eyespot. However, recent experiments with purified eyespots from *Spermatozopsis similis* (Kreimer, unpublished observations) indicate that the proteins found in the pigment granules are of low abundance. Therefore, this observation does not preclude a structural eyespot assembly function for EYE2. Thus, a functional role for the EYE2 protein was unknown, and it remained possible that its function might fall into any of the three classes listed above. The observation that EYE2 contained a putative thioredoxin active site sequence allowed for the hypothesis that this sequence might be necessary for EYE2 function, and that it might serve as an element where either EYE2 might be regulated, or might regulate other proteins.

**Results**

**Demonstration of the mutation in the eye2-l strain**

To determine the defect in the *eye2-l* strain, PCR amplification and sequencing of the products were performed. Genomic DNA from the *eye2-l* strain was amplified with primers designed to non-overlapping one kb fragments of the four kb genomic clone. Two of the four reactions successfully amplified the *EYE2* genomic products. Subsequent
sequencing of the two amplified genomic PCR products from the eye2-l mutant strain revealed a mutation of G to A at nucleotide 1326 in one of the products. Repeated amplification of this region consistently produced products with this change, indicative that this is likely to be a genuine mutation. The altered nucleotide is the first nucleotide of the first intron, and in other introns, mutations of this conserved nucleotide completely abolish splicing (Parker and Siliciano, 1993). This observation is indicative that the isolated gene in plasmid C/B-10 which rescues the eyespot assembly defect of the eye2-l strain is actually the gene that is mutated in that strain. Based on the nature of this mutation, it seems probable that the eye2-l strain cannot produce the EYE2 protein, since the message cannot be spliced correctly.

**Demonstration that the 503 aa ORF is the likely coding region for EYE2**

The EYE2 ORF encodes a protein of 503 amino acid residues, and we hypothesize that this ORF is the one which encodes the EYE2 protein. This hypothesis was supported by the mutagenesis of the genomic EYE2 sequence to introduce a premature stop codon at amino acid 186 (a change of TAC to TAG). Introduction of this mutant gene into an eye2-l strain resulted in 40 Arg^+ transformant strains which failed to produce eyespots, while the wildtype EYE2 gene transformed in this manner typically gives around sixteen Eye^+ out of about 40 Arg^+ transformants. This result is indicative that the long ORF encodes the EYE2 protein.
Sequence comparison of the EYE2 protein to the TRX family

The analysis of the partial EYE2 cDNA sequence was indicative that the 503 aa EYE2 ORF was likely to be the complete coding sequence for the EYE2 protein for two reasons. First, the presence of stop codons in all three reading frames in the region of the EYE2 cDNA upstream of the initiator methionine (starting at nucleotide 270 of the cDNA) is indicative that this methionine is the probable start signal. Additionally, no paired splice sites can be observed in the region 5' of the methionine codon at nt 270 indicative that there are no potential introns in this region, and that the partial cDNA which has been isolated is not representative of a partially spliced mRNA. This argument can also be made in reasoning that the stop codon for the 503 amino acid ORF is the real stop codon for the EYE2 mRNA. Therefore, we conclude that the 503 amino acid ORF is the complete coding sequence for the EYE2 protein.

Inspection of the amino acid sequence revealed that the protein was rich in arginine and proline residues. The theoretical pI of the protein is approximately 11.3, and the theoretical molecular weight of the protein is 56.6 kDa. Interestingly, the EYE2 protein also contains a repeated sequence motif, RCRRRPQRLTLRRCRSMRPQR (where the repeated sequence is in bold) from amino acids 397-416 in the fifth exon. Database searches using this motif and EYE2 sequences which contained this motif failed to detect any database homologs containing this region. Thus, while it is an unusual sequence, and may be important for EYE2 function, no specific functional assignment for this sequence can be made. Further sequence analysis of the EYE2 protein failed to reveal any
characteristic localization motifs for the protein. No organellar localization motif was observed for EYE2, nor does it contain any regions which look like transmembrane spanning helices. BLAST analysis (Altschul et al., 1997; http://www.ncbi.nlm.nih.gov/BLAST/) of the EYE2 protein sequence indicated no highly probable sequence homologies, although one small region of the EYE2 protein (amino acids 150-200) had similarity to several other proteins in the non-redundant database. This region was determined to be similar to the active site consensus sequence of the thioredoxin superfamily (Eklund et al., 1991). The thioredoxin superfamily is composed of proteins which catalyze redox reactions through an active site that contains two cysteine residues. These cysteines can undergo reversible oxidation and reduction, and serve to participate in a number of cellular processes including enzymatic regulation (thioredoxin, Thelander and Reichard, 1979), protein folding (protein disulfide isomerase, Freedman et al., 1994), sulfur metabolism (adenylyl (phospho) sulfate reductases, Setya et al., 1996; Bick et al., 1998), and have been proposed to play a role in control of developmental processes (deadhead, Salz, et al., 1994; Pellicena-Palle et al., 1997). For the EYE2 protein, the best similarity was observed with a family of plant adenylyl (phospho) sulfate reductase enzymes, although similarity was also found to the family of protein disulfide isomerases from a variety of organisms.

A multiple sequence alignment of the proteins found by BLAST searching to be most homologous to EYE2 can be found in Fig. 31. The program GCG Pileup was used to generate the alignment, using selected parts of both the EYE2 protein and the database
homologs identified in the BLAST search. Additionally, other arbitrarily chosen members of the thioredoxin superfamily which were not identified as EYE2 homologs by BLAST were included in the alignment. When alignments were performed using more of the EYE2 sequence than just amino acids 153-210, the similarity of EYE2 to the other proteins became undetectable, and the Pileup program was no longer able to align EYE2 with the other proteins. This indicates that it is only the small region of EYE2 found in exons 2 and 3 that is similar to the thioredoxin family, and that the rest of the EYE2 protein is unrelated to the family.

In Figure 31, the underlined sequences designate the thioredoxin active site consensus, with the two catalytically active cysteine residues shown in bold. The sequence of the EYE2 protein is displayed on the bottom row. The thioredoxin signature sequence is best described by the consensus sequence of VDFWAPWCGPCK. The EYE2 protein has a variant of this from amino acids 183-194. The sequence present in EYE2, LVLYAPWCPHCR, is somewhat unusual in that the two amino acids between the putative active site cysteines are different from those seen in most thioredoxin family members. EYE2 has the sequence Pro-His at this position, while thioredoxins typically have Gly-Pro. Protein Disulfide Isomerases typically have Gly-His, while APSR enzymes have Pro-Phe. Interestingly the CPHC sequence observed in EYE2 is found in one thioredoxin superfamily member, the *E. coli* protein DsbA. This protein functions to introduce disulfide bonds into proteins in the periplasmic space (Zapun et al., 1993;
Figure 31. Multiple sequence alignment of the TRX active site motif of the EYE2 protein with Thioredoxin superfamily members

The Pileup program from GCG (Genetics Computer Group, University of Wisconsin) was used to align a selected region of the deduced EYE2 protein with regions of other thioredoxin family proteins identified by a BLAST search, as well as selected other superfamily members. The thioredoxin active site consensus is underlined, and the active site cysteine pair is in bold. Only 100 (or fewer) amino acids of the aligned proteins are shown. The proteins displayed in the lineup are the 5' adenylyl (phospho)-sulfate reductase- like proteins ssapr2at (AC000375, amino acids 337-436; A. thaliana), aprsrat2 (AF016282, aa 337-436; A. thaliana), prh43 (U53866, aa 336-435; A. thaliana), apsredat (U96045, aa 338-437; A. thaliana), apr3at (AF016284, aa 341-440; A. thaliana), prh26 (U53865, aa 341-440; A. thaliana), apr1 (U43412, aa 348-446; A. thaliana), prh19 (U53864, aa 348-447; A. thaliana), papsrcr (U63784, aa 346-445; C. roseus); thioredoxins trxr (X62335, aa 1-89; C. reinhardtii), trxmcrl (X78821, aa 15-111; C. reinhardtii), trxm (X51462, aa 70-163; S. oleracea), trxa (M54881, aa 1-90; E. coli), trxh (Z14084, aa 20-90; A. thaliana), trxhr1 (X80887, aa 1-96; C. reinhardtii), trxf (P09856, aa 77-130; S. oleracea); protein disulfide isomerases pdinc (Q92249, aa 13-112; N. crassa), pdian (Q00216, aa 12-111; A. nidulans), pdimouse (P08003, aa 510-590; M. musculus), pdicorn (G1709619, aa 371-468; Z. mays); others sprot (X81994, aa 120-215; P. coerulescens) dsba (M77746, aa 12-66; E. coli), and ey2protl (this study, aa 153-210; C. reinhardtii).
Figure 31. Multiple sequence alignment of EYE2 and TRX superfamily members

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Figure 32. Multiple sequence alignment of EYE2 and TRX superfamily members

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Wunderlich et al., 1993). The identity of the amino acids between the two active site cysteines is thought to influence the redox potential of the enzyme (Eklund et al., 1991), so it can be concluded that if EYE2 is a redox enzyme its redox potential is probably most similar to that of DsbA. It is not expected that EYE2 will be an extracellular protein in vivo, as it is difficult to imagine how an extracellular protein could influence the production of the intracellular eyespot.

It is also unlikely that the EYE2 protein is an A(P)SR-like enzyme in *Chlamydomonas*, because these enzymes are chloroplast-localized in plants, and the EYE2 protein lacks a recognizable chloroplast transit peptide. It is also clear that EYE2 is not likely to be a typical protein disulfide isomerase. PDI is typically localized within the endoplasmic reticulum (Freedman et al., 1994). PDIs have the C-terminal KDEL signal sequence for ER retention, and the EYE2 protein has no such sequence at its C-terminal end. An additional factor which makes it unlikely that EYE2 is a protein disulfide isomerase is that PDIs generally contain more than one TRX active site sequence, and EYE2 has only one.

EYE2 is probably not a genuine thioredoxin because EYE2 is 503 amino acids, and thioredoxins typically are about 100 amino acids long. This comparison is indicative that, while EYE2 has a signature sequence for a thioredoxin-like active site, it is unlikely to be a protein whose activity has already been characterized. That this protein does contain a variant of the conserved thioredoxin signature sequence is suggestive that the EYE2 protein may be involved in eyespot assembly through the use of this motif. It is
possible that the activity of the EYE2 protein in eyespot assembly is regulated through this motif; alternatively, the EYE2 protein may act to regulate other proteins involved in eyespot assembly through this redox active site. Redox control of developmental processes has been proposed recently. The *Drosophila deadhead* protein is a thioredoxin homolog, and mutations in the gene encoding this protein result in developmental abnormalities (Salz, et al., 1994; Pellicena-Palle et al., 1997).

**Site-directed mutagenesis of the EYE2 protein**

To test whether the thioredoxin active site motif was required for the eyespot-assembly function of the EYE2 protein, it was decided to undertake site-directed mutagenesis of the region. Previous work has generally indicated that for thioredoxin-like proteins to function, both cysteine residues need to be present (Oblong et al., 1994), although this is not true for all cases (Tachibana and Stevens, 1992; Zapun et al., 1994; Wunderlich et al., 1995; Walker et al., 1996). We hypothesized that if the thioredoxin motif was required for EYE2 function in eyespot production the change of a cysteine residue in the thioredoxin motif would inactivate the protein, resulting in cells with no eyespots.

The change of cysteine to serine at amino acid 193 (the second cysteine of the thioredoxin motif, denoted Cys2 mutant) was introduced into the *EYE2* gene in plasmid C/B-10. This plasmid was then transformed into the *eye2-l arg7-2* strain, selecting for arginine prototrophs. Colonies able to grow in the absence of supplemental arginine were
then tested for their ability to phototax and observed for the presence of eyespots. Of the
34 Arg\(^+\) transformants generated with the Cys2 mutant plasmid, 20 were able to positively
phototax. All twenty of the Ptx\(^+\) transformant strains were observed to have eyespots.
Recalling the earlier data for the complementation of the eye2-l mutation with the
wildtype C/B-10 plasmid, the wildtype gene introduced in this manner gave 16 Ptx\(^+\)/Eye\(^+\)
transformants out of 39 Arg\(^+\) transformants tested. Because the EYE2 gene is not under
selection during this transformation method, not all transformants will have integrated the
entire gene, and this is why not all Arg\(^+\) transformants are Ptx\(^+\)/Eye\(^+\). Interestingly, the
proportion of Ptx\(^+\)/Eye\(^+\) transformants bearing the Cys2 mutation is similar to the
proportion observed for the integration of the wildtype EYE2 gene in the C/B-10 plasmid,
indicative that the cysteine to serine change at amino acid 193 in EYE2 has no major
effect on the production of eyespots.

This result is indicative that the thioredoxin motif is not required for the EYE2
protein to make eyespots, or that the mutant EYE2 protein may be compromised in its
ability to make eyespots, but only in some subtle way which does not affect the gross
structure and function of the eyespot. It is also possible that the EYE2 protein actually
plays some other unknown role in the cell, and that the mutation of the thioredoxin motif
has affected that unknown function without perturbing the EYE2 function in eyespot
assembly. A final possibility is that the thioredoxin motif is important for EYE2 function
in eyespot assembly, but that the mutation of the second cysteine to serine in the motif is
not enough to impair the eyespot assembly function, similar to what has been seen for
other thioredoxin family members (Tachibana and Stevens, 1992; Zapun et al., 1994; Wunderlich et al., 1995; Walker et al., 1996).

**Epitope tagging of the EYE2 protein**

Because the homology search and targeted mutagenesis failed to reveal a function for the EYE2 protein in eyespot assembly, it was decided to undertake a new approach. One way to address a functional role for the EYE2 protein would be to know its subcellular location. An expected subset of eyespot assembly genes might have a function in direct packaging of eyespot pigment granules, or might play a structural role in sandwiching the granules between the thylakoid inner chloroplast envelope and the thylakoid membrane. A protein in this class would be expected to be localized within the chloroplast. Another possible role for eyespot assembly genes is the regulation of other genes which are involved in eyespot assembly. While the mechanisms which control gene expression are numerous, and the proteins involved in such regulatory mechanisms could be located almost anywhere in the cell, it is expected that at least one type of control of eyespot assembly could be caused by transcription factors which regulate eyespot assembly genes. It is expected that proteins of this type should be localized within the nucleus. One additional class of eyespot assembly proteins would be structural proteins which dictate the placement of the eyespot pigment granules adjacent to the daughter four-membered microtubule rootlet. Proteins of this type should display a specific localization at or near the distal tip of this microtubule rootlet.
To determine if the EYE2 protein displayed a localization pattern expected from these types of eyespot assembly proteins, immunofluorescence detection of the EYE2 protein was undertaken. Rather than raise antibodies specific to the EYE2 protein itself, it was decided to use epitope tags to mark the EYE2 protein with a specific amino acid sequence, the c-MYC epitope, to which commercial antibodies are available. Previous work had established that the detection of rare proteins could be enhanced by the introduction of tandemly repeated epitope sequences into the tagged protein (Schneider et al., 1995). In this case, three repeats of the MYC epitope were used. In addition to this method, it was also decided to fuse the EYE2 gene to a fragment of a gene encoding a transcarboxylase from a bacterium (P. shermanii). This fragment targets the bacterial transcarboxylase protein for the covalent attachment of biotin, through the action of the enzyme biotin ligase (Cronan, 1990). Yeast proteins fused to the bacterial transcarboxylase have been demonstrated to be biotinylated in vivo (Ackerman et al., 1992), and have been used for detection and subsequent purification of the fusion proteins using the biotin-binding protein avidin (Ackerman et al., 1992; Roberts and Dieckmann, unpublished observations).

Fusion proteins were constructed by site directed mutagenesis of the presumed stop codon for EYE2 (nt 3155 of the genomic sequence, (Fig. 29) to a restriction site. Primers with unique restriction sites at the 5' ends were designed to amplify the 3xMYC tag and the BIO tag and these amplified fragments were cloned into the site at the end of the EYE2 coding sequence using linkers. Both constructs generate in-frame translational
fusions of the EYE2 coding sequence to the tag sequences, and for both fusions, the stop codon for the fusion is provided by the attached tag sequence. This means that if eyeless strains transformed with these constructs are found to produce eyespots, they are likely to have the intact tag sequence. Fusion constructs were then cloned into the intact EYE2 gene in plasmid C/B-10 and transformed into the eye2-I arg7-2 strain, selecting for transformants able to grow in the absence of supplemental arginine.

For the 3xMYC tagged EYE2 construct, out of 25 Arg\(^+\) transformants examined, twelve were Ptx\(^+\). Of the twelve Ptx\(^+\) transformants for the EYE2-3xMYC fusion, seven were conclusively observed to have eyespots. The other five Ptx\(^+\) transformants were difficult to grow on acetate plates, and the cells looked unhealthy when observed by light microscopy, so it is unclear whether they actually possessed eyespots. This growth defect could potentially occur because the integration of the transforming DNA affected genes necessary for cell survival. It also may be possible that the transformants were not scored properly, being counted as Ptx\(^+\) when they were actually Ptx\(^-\). Regardless, the existence of Ptx\(^+/\)Eye\(^-\) MYC-tagged EYE2 strains is indicative that the introduction of the 3xMYC tag at the C-terminus of the EYE2 protein does not affect the eyespot assembly function of the protein in any severe way.

For the transcarboxylase-EYE2 fusion, the results are less clear. Out of 38 Arg\(^+\) transformants tested, eleven were scored as Ptx\(^+\), but only two were observed to have eyespots. For the two strains that did have eyespots, it was generally difficult to find cells in the population which had them. It is unclear if this observation is indicative of genuine
eyespot formation by the EYE2-BIO fusion strains, or if this represents an improper scoring of the Ptx/Eye phenotypes. It may be possible that the introduction of the transcarboxylase fusion at the C-terminal end of the EYE2 protein impairs its eyespot assembly function, but not completely, such that some fraction of cells in the population can make eyespots. Another possibility is that the bacterial transcarboxylase sequence is recognized as non-Chlamydomonas sequence, and silenced in a fraction of the population. Epigenetic silencing of a nuclearly-integrated foreign gene has been observed in Chlamydomonas (Cerutti et al., 1997), and the larger size of the BIO-tag (about 400 bp) may make it more prone to silencing. Whatever the explanation for this observation, the clear observation of eyespots in the MYC-tagged EYE2 transformants is indicative that the MYC tag is potentially useful for further study.

Western analysis of the Ptx\(^{-}\)/Eye\(^{+}\) 3xMYC-tagged EYE2 transformants (Fig. 32) was indicative that the fusion protein was undetectable in approximately 100 \(\mu\)g of total cellular protein for all transformants. The fusion protein should have been apparent at a molecular weight of about 62 kDa, but no band was observed in this size range. Two large proteins were detected with the anti-MYC antibody, having apparent molecular weights of about 100 and 200 kDa. These were also observed in extracts of both the UV-induced mutant eye2-1 (with no MYC-tagged construct), lane UV, and the eyeless insertion mutant eye2-2 (also with no MYC-tagged construct), lane DEL, as well as the two Ptx\(^{-}\)/Eye\(^{+}\) 3xMYC-tagged EYE2 transformants shown (lanes A and B). This result is
Figure 32.

EYE2-3xMYC fusion protein is undetectable in whole cell extracts.

Approximately 100 μg of total cell protein from *eye2* strains *eye2-1* (lane UV), *eye2-2* (lane DEL) and two Ptx^-/Eye^- transformants of *eye2-1* with the EYE2-3xMYC fusion plasmid (lanes A and B) were run on an SDS-polyacrylamide gel and blotted onto nitrocellulose. The blot was incubated with an mouse anti-MYC monoclonal antibody, followed by a secondary incubation with a sheep anti-mouse IgG antibody conjugated to horseradish peroxidase. Proteins were visualized by ECL. The expected size of the EYE2-3xMYC fusion is approximately 62 kDa.
suggestive that the anti-MYC antibody cross-reacts with endogenous *Chlamydomonas* proteins. Native *Chlamydomonas* proteins which cross-react with anti-MYC antibodies have been previously observed (Finst and Quarmby, personal communication); purified basal bodies were observed to contain three bands of approximately 200, 100 and 21 kDa which were detected with anti-MYC antibodies by Western analysis.

The fact that the EYE2 protein is undetectable in whole cell extracts is not surprising, considering the rarity of the *EYE2* mRNA. However, it still may be possible that the protein, although rare, is concentrated at a specific location in the cell. To test this possibility, immunofluorescence using the anti-MYC antibody was performed. Repeated attempts to detect the MYC-tagged EYE2 protein by immunofluorescence were unsuccessful, showing no conclusive localization for the protein, and demonstrating no differences in staining between the MYC-tagged strains and untagged controls. Thus it appears likely that the EYE2 protein is rare, and/or not concentrated in a specific cellular location. However, the antibody against the MYC epitope is monoclonal. Monoclonal antibodies typically have a lower affinity for their cognate antigens than do polyclonal antibodies, so it remains possible that the EYE2 protein is just below the threshold of detection with the anti-MYC antibody, and could still be detected with polyclonal antibodies.

**Discussion**
Two experiments provide a more concrete demonstration that the restoration of the eyespot assembly defect in the eye2-1 strain by the four kb insert sequence of C/B-10 is actually due to complementation by the wildtype EYE2 gene. The first experiment was the determination of the mutation in the eye2-1 strain. We hypothesized that if the 4 kb insert sequence of the plasmid C/B-10 actually contained the wildtype EYE2 gene, then a mutation in that four kb region of the eye2-1 genome should be detectable. By PCR amplification of one kb regions of the four kb insert, and sequencing of the amplified products, a point mutation which corresponded to nucleotide 1326 of the four kb insert sequence was discovered. The nucleotide at this position was a change of G to A in the sense strand, and this is the first nucleotide of the first intron. This change should completely abolish the splicing of this intron, as mutagenesis of this conserved nucleotide abolishes splicing in other systems (Parker and Siliciano, 1993). It would be desirable to verify the prediction of an altered splicing pattern in the eye2-1 mutant, but it is probable that if the splicing of the first intron is compromised, the EYE2 transcript in this strain would be unstable. Given the difficulty in detecting the wildtype EYE2 mRNA, no experiments to test the prediction of a splicing defect in the eye2-1 mutant strain have been done.

The second experiment which confirms that the four kb insert of C/B-10 is likely to harbor the wildtype EYE2 gene is one in which a premature stop codon was introduced into the second exon of the EYE2 gene. This mutant construct failed to rescue the eye2-1 defect when used for transformation. This is indicative that the 503 aa ORF observed in
the partial cDNA is the *EYE2* protein coding sequence.

Since it was probable that the 503 aa ORF encoded the *EYE2* protein, the protein sequence was analyzed. Two regions of the *EYE2* protein were notable, one which contained an unusual sequence repeat motif, and one in which a thioredoxin active site sequence was observed. The tandemly repeated RCRxxPQR sequence occurred in the highly repetitive fifth exon, and no database homologs containing this repeated sequence were found. The observation of this repeated sequence in *EYE2* makes the sequence a desirable candidate for mutagenesis. If the sequence is important for *EYE2* function, then its deletion should have an effect on the ability of the mutant construct to restore eyespots in the *eye2-I* strain. Such a large deletion might compromise *EYE2* function simply due to misfolding of the protein, thus a more subtle mutagenesis of the region might also be undertaken.

A similar strategy was used in the analysis of the thioredoxin active site sequence in *EYE2*. The thioredoxin active site motif is most similar to that of the bacterial protein, DsbA, while the region around the LVLYAPWCPHCR sequence in *EYE2* is most similar to the adenylyl (phospho) sulfate reductase family from higher plants. Because this sequence was the only conserved motif in the *EYE2* protein, we predicted that this motif in *EYE2* might be important for its function in eyespot assembly. However, mutagenesis of the second cysteine in the motif failed to interfere with the ability of the *EYE2* gene to restore eyespots in when transformed into the *eye2-I* strain. There are several possible explanations for this observation. First, the thioredoxin active site motif may not be
required for the EYE2 protein to function in eyespot assembly. This hypothesis essentially means that the occurrence of the thioredoxin motif in EYE2 is due to chance, and that it has no function for this protein. A second hypothesis is that while the catalytic function of the thioredoxin motif might be dispensable for EYE2 function, the actual sequence could be important for the structure of the EYE2 protein. A third hypothesis is that the mutation of the second active site cysteine in EYE2 has affected its function in eyespot assembly, but only in some subtle manner. Our assays for the effects of the mutation of the second cysteine of the TRX motif only check for the recovery of the ability of the mutant strains to phototax, and whether or not the transformant strains possess eyespots when observed by light microscopy. Whether or not the mutagenized EYE2 construct transformants have more subtle defects might be resolved by looking at the ultrastructure of the eyespots by electron microscopy, as well as by using the motion analysis system to obtain more detailed information about the photobehavioral capacities of the transformants. A fourth hypothesis is that the EYE2 gene might have multiple functions. The EYE2 protein may have another unknown role in the cell, and mutation of the thioredoxin motif has affected the unknown function without perturbing the EYE2 function in eyespot assembly. A fifth possibility is that the thioredoxin active site motif does have a function in eyespot assembly, but that the mutation of the second active site cysteine is insufficient to abolish its function. The tolerance for substitutions at the second cysteine of the thioredoxin active site motif has been observed for other thioredoxin superfamily members (Tachibana and Stevens, 1992; Zapun et al., 1994; Wunderlich et al.,
1995; Walker et al., 1996), so this may be the most plausible explanation for our data.

Another experimental approach to determine the function of the EYE2 protein in eyespot assembly was to attempt to localize the protein in vivo and in vitro. It is expected that eyespot assembly mutants describe several different types of proteins which would display characteristic localization patterns. To determine the localization of the EYE2 protein, a MYC epitope tag was engineered as an in-frame translational fusion at the C-terminus of the EYE2 protein. While this fusion was functional, as evidenced by the ability of this construct to restore eyespots to the eye2-l strain, the protein could not be localized in vivo by immunofluorescence, and could not be detected in vitro in extracts of total cellular protein. The failure to detect the EYE2 protein fusion could be due to the rarity of this protein. Alternatively, the failure could be due to a combination of the rarity of the protein and the lower affinity of the monoclonal antibody used for detection of the fusion protein. Why EYE2 was not detected will become more clear when polyclonal antisera against EYE2 are produced.

The rarity of the protein and transcript is interesting, and may be an observation which has bearing on future experiments. Several distinct lines of experiments are all suggestive that the EYE2 transcript and the EYE2 protein are rare in asynchronous populations. The failure to detect the cDNA in five different libraries by hybridization of various EYE2 genomic and cDNA derived probes might be due to two reasons. Either these failures indicate an inefficiency in the ability of these EYE2 probes to detect complementary sequences, or the failures are indicative of a low frequency of the
complementary target molecule in the cDNA populations. Both explanations could also apply when considering the repeated failure to detect the transcript by Northern analysis, and Western analysis. From PCR amplifications using cDNA libraries as template, it was clear that the yield of amplified fragments were generally quite low. The low yield could be indicative of a rare template molecule in the reaction, or the low yield might be an indication that the primers hybridize to the target template inefficiently. The fact that the low yield of amplified products was generally independent of the primers or the reaction conditions used for the amplification, would seem to indicate that the \textit{EYE2} transcript (and thus the cDNA and probably the protein) is rare.

If the reason for the indicated experimental results is that the \textit{EYE2} mRNA and protein are rare, rather than being caused by experimental inefficiency, then this a meaningful result with respect to the timing of \textit{EYE2} expression. It is expected that new components involved in making eyespots will be synthesized primarily during the time when new eyespots are being made. When new eyespots are assembled, the synthesis of new eyespot components will occur shortly after cell division (Morel-Laurens and Bird, 1984; Holmes and Dutcher, 1989; Gaffal et al., 1993). Thus, in the asynchronous populations used for the production of most of the cDNA libraries used in this study, and which were used for both the preparation of mRNA for Northernns and for the preparation of total protein for Westerns, only a fraction of the cells in the population might be expected to be making components for the production of new eyespots. Therefore, if the \textit{EYE2} protein has a function in either the assembly or positioning of the eyespot, or in the
regulation of other components involved in eyespot formation, its pattern of expression might be expected to be periodic as a function of the cell cycle, and it might be more abundant around the time of cytokinesis. Thus, for future work, the examination of the temporal expression pattern of EYE2 should be a priority. It seems quite possible that EYE2 will have a cell-cycle dependent expression pattern. If this is true, it should enhance the possibility of detecting both the EYE2 mRNA and the EYE2 protein.
Chapter 6: General discussion and prospective future studies

The findings of this study will lead to a more detailed understanding of how *Chlamydomonas* is able to make its eyespot, and to assemble a fully functional visual system from components that reside in three distinct cellular locations. The first gene discovered affecting eyespot assembly in green algae is the EYE2 gene. The lack of sequence homology in the EYE2 protein does not allow for any clear functional assignment of the role of the EYE2 protein in eyespot production, nor do the experiments presented in this study lead to a conclusive localization for EYE2. Nevertheless, several potential lines of experiments follow logically from the study presented here; the results of these future experiments should greatly enhance our knowledge of eyespot assembly at the molecular level.

The expression pattern of the EYE2 gene and protein

The first set of experiments which should be done is to try to determine the expression pattern of the EYE2 gene and the protein. Previous work (Morel-Laurens and Bird, 1984; Holmes and Dutcher, 1989; Gaffal et al., 1993) has established that new eyespot components are probably synthesized directly after cell division is complete. Thus, if the EYE2 protein controls production of new eyespot materials, or if it is a structural component of the eyespot, then the abundance of the transcript and/or the
protein is likely to be periodic as a function of the cell cycle. If the EYE2 protein functioned to repress genes (or inactivate proteins) involved in producing eyespots, then the message and protein should be present at almost all times, and then disappear around the time of cytokinesis. On the other hand, if the EYE2 protein functions to activate the synthesis of, or directly synthesize eyespot components, then the message and protein should accumulate at the time of cytokinesis. This could be verified by performing Northern and Western analysis of synchronous populations at various timepoints. This experiment might be more easily performed by fusing the 5' region of the \textit{EYE2} gene to the endogenous \textit{Chlamydomonas} reporter gene arylsulfatase (since foreign reporters probably would not be expressed), and analyzing the amount of reporter enzyme activity detected during a time course in a synchronous population.

One could also look at the effects of environmental conditions on the levels of \textit{EYE2} message and protein, testing to see if these levels change in response to the presence or absence of acetate or light. However, because the eyespot is always present in wildtype cells regardless of changes in these parameters, it is not expected that \textit{EYE2} protein or message abundance will be affected.

The results of these experiments should be interpreted with caution. While a change in the absolute levels of the \textit{EYE2} transcript or protein might be important, in the absence of a functional assay for the \textit{EYE2} protein, such a change might be misleading. For instance, the levels of the \textit{EYE2} protein might not change over the course of the cell cycle, but the protein could become activated or inactivated during that same time period.
The location of the EYE2 protein in *Chlamydomonas*

One clue to the possible function of the EYE2 protein might lie in its localization. A structural component of the eyespot might localize with the pigment granules, or with the four membered microtubule rootlet emanating from the daughter basal body, while a transcription factor which controls eyespot-specific genes should localize to the nucleus. Some experiments designed to address this would include immunofluorescence using a polyclonal antisera generated against EYE2, or a biochemical fractionation of *Chlamydomonas* wildtype strains, looking for nuclear, chloroplast or cytoplasmic localization. Preparation of eyespot fractions would be desirable, but due to the placement of the eyespot within the chloroplast, and the presence of a cell wall, purification of eyespots from *Chlamydomonas* is difficult. An alternative to this is to perform Westerns with purified eyespot preparations from the related green alga *Spermatozopsis similis*, from which eyespot purification is technically easier. At least one visual protein, chlamyopsin, is known to have an immunologically related homolog in *S. similis* (Calenberg et al., 1998), so antisera against the *Chlamydomonas* EYE2 protein might recognize a homolog from Spermatozopsis.

Identification of other proteins which interact with the EYE2 protein

It is expected that the EYE2 protein might interact with other proteins *in vivo* in
Chlamydomonas. If the EYE2 protein is a structural component of the eyespot, or is involved in recognition of the four membered microtubule rootlet emanating from the daughter basal body (which is thought to position the eyespot appropriately in the cell), then it is possible that EYE2 will bind other cellular proteins in fulfilling its function. To look for other proteins which might interact with EYE2, either immunoprecipitations with the polyclonal antisera against EYE2 could be performed, or a two-hybrid screen with a Chlamydomonas cDNA library could be performed. Both of these experiments will be difficult to interpret. If a protein is found which co-immunoprecipitates with EYE2, it cannot be known whether this is artifactual or represents a real *in vivo* interaction. Additionally, a two-hybrid screen with Chlamydomonas will be difficult as well, the high GC content of the Chlamydomonas genome may preclude the expression of some fusion cDNAs due to the codon bias of *S. cerevisiae*.

**Screening for suppressors of an eye2 mutation**

Perhaps the best approach to identify other genes which could interact with EYE2 would be to screen for dominant suppressors of *eye2* mutations. A method that would be likely to yield suppressors in genes encoding proteins which interact with EYE2 would require the isolation of a *ts* allele of EYE2. The conditional allele could be mated to the *eye2*-2 deletion strain. The resultant diploid from this cross would then be mutagenized, and dominant suppressors could be obtained. This approach for obtaining suppressors in
interacting partners has been successful in the past (Chen and Dieckmann, 1997; Islas-Osuna and Dieckmann, unpublished observations; Sandrock et al., 1997) Any dominant suppressors obtained in this manner could be isolated by constructing genomic libraries from the suppressor strain, and transformation of the conditional eye2 allele. If the dominant suppressor had a phenotype of its own, it could be cloned by complementation

The role of the thioredoxin active site motif in the EYE2 protein

The presence of a thioredoxin active site motif in the EYE2 protein is the only clear sequence motif which allows for speculation about the function of EYE2. However, mutagenesis of the second active site cysteine failed to demonstrate an effect on the eyespot assembly. This result could be explained in a number of ways. First, the thioredoxin active site sequence may not play a role in the function of EYE2 in eyespot assembly, but instead might affect some other heretofore unknown function of EYE2. Multiple functions for EYE2 seems unlikely, but one could examine the cells of this strain for other phenotypes which may be compromised. This could be difficult, as the introduction of the mutant construct may have inactivated other genes during its integration. Since integration is thought to occur randomly, multiple insertion strains should be examined for alternate phenotypes. Additionally, the original eye2-1 mutant strains could be examined for secondary phenotypes, since the thioredoxin active site function of EYE2 is most likely absent in those strains as well.
The second possibility to explain the failure of the thioredoxin active site mutation to affect the eyespot assembly function of EYE2 is that the phenotype exhibited by these mutant strains could be subtle, and below the threshold of sensitivity of the assays used to detect eyespot and phototaxis deficiencies. Light microscopy demonstrated that the C193S transformants possessed eyespots, but electron microscopy of the transformants might reveal a more subtle defect in these strains. Likewise, these strains could have subtle defects of phototactic orientation which detailed motion analysis (Pazour et al., 1995; this study) could resolve, but our population-based phototaxis assay could not. Additionally, measurements of the photoelectric responses of these transformants would reveal any defects in the ability to generate the PRC or FC.

A third possibility which would explain the failure of the thioredoxin active site mutation to affect the eyespot assembly function of EYE2 is that the thioredoxin motif might be genuinely important for eyespot assembly, but that the single cysteine to serine change is not sufficient to affect the redox function of EYE2. This effect has been observed in the protein DsbA, (Zapun et al., 1994) where the replacement of the second cysteine in the thioredoxin motif of DsbA with serine only reduced the $k_{cat}$ of the enzyme twofold. Another study replaced the second cysteine of DsbA with alanine and observed wild type levels of catalysis in the mutant (Wunderlich et al., 1995). Replacement of the second cysteine in the thioredoxin active site motif of PDI with serine revealed that the second cysteine was not essential for the rearrangement of disulfide bonds in a substrate protein, but that the mutant was 2 to 8 fold less efficient at catalysis (Walker et al., 1996).
One further implication that the second cysteine in the thioredoxin active site motif can be dispensable for enzyme activity comes from the complementation of a yeast strain deficient in PDI with a gene encoding another ER-localized protein, \textit{EUGI} (Tachibana and Stevens, 1992). \textit{EUGI} contains a motif related to the thioredoxin active site which does not contain the second cysteine, the motif in this protein is CXXS. Thus, the thioredoxin active site motif in \textit{YE2} may still be functional, even when the second cysteine has been replaced by serine.

An entirely different approach could be undertaken to test for redox activity through the thioredoxin active site motif in \textit{YE2}. By overexpression of the \textit{YE2} cDNA, purification of the overexpressed protein could be performed, and assays for redox activity of the overexpressed protein could be done. If the protein was found to have redox activity, this would support a hypothesis that the thioredoxin active site motif was functional in the \textit{YE2} protein. Based on the observation that a PDI with the second active site cysteine changed to serine accumulated a trapped enzyme-substrate intermediate complex (Walker et al., 1996), \textit{Chlamydomonas} cell extracts could be added to the overexpressed CXXS mutant enzyme, and substrates could be identified. Alternatively, immunoprecipitation of \textit{YE2} from the CXXS mutant strain could also reveal substrates for \textit{YE2}.

\textbf{The role of the RCRxxPQR repeat motif in the \textit{YE2} protein}
While the RCRxxPQRxxxxRCRxxPQR repeated motif (aa 397-416) has no sequence homologs in the non-redundant protein database, it remains an intriguing possibility for a functionally important region of the EYE2 protein. By targeting this sequence for mutagenesis, one could determine whether this sequence is necessary for the eyespot assembly function of EYE2. By transforming the mutant constructs into an eye2-1 strain, the ability of these mutant strains to produce eyespots will demonstrate the necessity of this sequence. The first step to address the function of this sequence would be to delete the entire region from EYE2, if this interferes with the eyespot assembly function, then more subtle single amino acid changes could be made. However, because this sequence is not a defined protein functional motif, even a successful mutagenesis will not allow for a functional assignment for the EYE2 protein. This experimental scheme will only test the requirement of this sequence for the eyespot assembly function of EYE2.

The role of the uORF in control of EYE2 expression

Translation initiation of nuclearly encoded genes in eukaryotes is thought to occur by ribosome assembly at the 5' cap, followed by scanning to the initiator methionine. For a typical mRNA, the first AUG from the 5' end is usually the start codon for the protein to be translated. Exceptions to this generalization have been observed, mammalian (Kozak, 1991; Geballe 1996), yeast (Hinnebusch, 1984; Vilela et al., 1998) and plant (Lukaszewicz et al., 1998; Martinez-Garcia et al., 1998; Wang et al., 1998) mRNAs have been found to
contain upstream open reading frames (uORFs) that are more 5' than the ORF encoding the protein. Approximately ten percent of all yeast genes contain uORFs, and in the cases where the function of the uORF is known, translation of the uORF affects both translation initiation at the coding AUG (Kozak, 1991; Pinto et al., 1992), and stability of the mRNA (Ruiz-Echevarria et al., 1996; Linz et al., 1997; Vilela et al., 1998).

Since the 5'-UTR of the EYE2 cDNA contains a uORF, we hypothesize that the uORF might play a role in the control of translation of the EYE2 protein. To investigate whether the translation of the uORF might regulate translation initiation at the EYE2 start codon, mutations can be made which remove the ATG of the uORF, so that there can be no initiation until the EYE2 ATG. Thus, if translation of the uORF inhibited initiation at the EYE2 start codon, more EYE2 protein should be produced in this strain. Alternatively, if translation of the uORF activated translation of the EYE2 ORF, then less EYE2 protein would be produced. This possibility cannot be determined, since the wildtype protein cannot be detected in asynchronous populations. Another possibility for the function of the uORF is to control the stability of the EYE2 mRNA. This could be assayed by Northern analysis of pulse-labeled mRNA, although with the rarity of the EYE2 message in wildtype cells this could be difficult. Quantitative RT-PCR might then be a reasonable strategy to test this hypothesis.

A simpler method may also work to assay for the effects of uORF mutations. The EYE2 5'UTR plus and minus uORF mutations could be fused to the arylsulfatase reporter gene. The assay for arylsulfatase activity would provide a more sensitive method for
quantitating the effects of uORF mutations.

It is possible that translation of the uORF does not allow sufficient time for reinitiation at the downstream ATG. Another mutagenesis experiment to test this hypothesis would be to increase the spacing between the stop codon of the uORF and the next start codon, which should increase the amount of protein produced from translation of the second ORF because initiation factors would have more time to reassemble before the EYE2 start codon. If the spacing is important for the probability of reinitiation at the second ATG, then increasing the spacing between mutant uORFs and the ATG of the reporter gene should result in an increased probability of reinitiation, and more reporter enzyme activity. In this manner, the role of the uORF in control of EYE2 expression can be elucidated.

These experiments should further our understanding of eyespot assembly in *Chlamydomonas*, and more specifically, the role of the EYE2 protein in the generation of eyespots. It is clear that *Chlamydomonas* is now a useful system for the study of the control of cell organization, both temporally and spatially. The study of eyespot assembly provides an excellent archetype for answering questions about the coordinated assembly of complex systems in which the functional unit requires that components in different cellular locations be organized. It is quite clear that strictly reductionist views of cells are inadequate to explain the high degree of organization that cells contain. The study of eyespot assembly in *Chlamydomonas* can only further our understanding of how cells can generate complex, organized systems.
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