CHLAMYDOMonas AS A MODEL ORGANISM

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Abstract The unicellular green alga *Chlamydomonas* offers a simple life cycle, easy isolation of mutants, and a growing array of tools and techniques for molecular genetic studies. Among the principal areas of current investigation using this model system are flagellar structure and function, genetics of basal bodies (centrioles), chloroplast biogenesis, photosynthesis, light perception, cell-cell recognition, and cell cycle control. A genome project has begun with compilation of expressed sequence tag data and gene expression studies and will lead to a complete genome sequence. Resources available to the research community include wild-type and mutant strains, plasmid constructs for transformation studies, and a comprehensive on-line database.

CONTENTS

INTRODUCTION ................................................ 364
THE GENUS CHLAMYDOMonas ................................. 364
Major Taxonomic Features ........................................ 364
Laboratory Strains of *C. reinhardtii* ............................. 366
CELL ARCHITECTURE AND FUNCTIONAL SYSTEMS .......... 368
The Cell Wall .................................................. 368
The Nucleus and the Nuclear Genome ............................. 368
Contractile Vacuoles and Osmotic Regulation ................... 368
Mitochondria .................................................. 369
The Chloroplast ................................................. 369
Flagella ....................................................... 375
Basal Bodies ................................................... 376
The Eyespot and Phototaxis ....................................... 379
MITOTIC AND MEIOTIC LIFE CYCLES ......................... 380
Vegetative Cell Growth ......................................... 380
The Sexual Cycle ............................................... 382
Tetrad Analysis ................................................ 384
PHYSIOLOGICAL PROCESSES .................................... 385
Nutrient Update and Metabolic Stress ............................ 385
Light-Mediated Responses ....................................... 386
Circadian Rhythms ............................................... 387

1040-2519/01/0601-0363$14.00 363
INTRODUCTION

At the Fifth International Chlamydomonas Conference in 1992, the venerable phycologist Ralph Lewin delivered a keynote address with the title, “The Cloaked One Emerges from Obscurity.” Lewin’s talk referred to the development of research on the unicellular green alga *Chlamydomonas* (Greek *chlamys*, a cloak; *monas*, solitary), beginning with nineteenth-century morphological descriptions and the first genetics studies in the early twentieth century. Pascher (1916, 1918, cited in 86) demonstrated the suitability of *Chlamydomonas* species for genetic analysis and pointed out the advantages of a haploid system in which all four products of meiosis could be recovered and analyzed. His investigations were not continued, but interest was soon renewed in this alga as a eukaryotic organism whose life cycle could be controlled in the laboratory. The work of Franz Moewus in the 1930s demonstrated that mutants could be isolated and characterized, but was clouded by irreproducibility of some of the reported results; it was only with the work of Lewin himself, Ruth Sager, and others in the 1940s and 1950s that a few *Chlamydomonas* species, in particular *C. reinhardtii* and *C. eugametos*, began to be developed as laboratory organisms (see 86 for review). The past 50 years have indeed seen the emergence of this organism from obscure beginnings into one of the premier model systems for diverse areas of cell and molecular biology.

Lewin’s 1992 talk concluded with the arrival of his subject in the new age of molecular biology. Transformation of the chloroplast, nuclear, and mitochondrial genomes had been achieved, and the research presented at that meeting gave clear evidence that rapid progress lay ahead in developing new technologies for isolation and manipulation of genes. We are now embarking on a new era, as *Chlamydomonas* enters the age of genomics. Sequencing, gene expression studies, and molecular mapping projects are under way, and prospects are good for a complete genome sequence of *C. reinhardtii*. A review of the main features of this organism and its laboratory manipulation seems most appropriate at this time.

THE GENUS CHLAMYDOMonas

Major Taxonomic Features

Historically, species of *Chlamydomonas* have been defined based solely on morphological criteria. The genus comprises unicellular chlorophyte algae with two anterior flagella, a basal chloroplast surrounding one or more pyrenoids, and a distinct cell wall (Figure 1). Species within the genus have been distinguished by...
Figure 1  A semidiagrammatic representation of an interphase *Chlamydomonas* cell. Cell length, 10 µm; BB, basal bodies; Chl, chloroplast; Cv, contractile vacuole; Cw, cell wall; Er, endoplasmic reticulum; Es, eyespot; F, flagella; G, Golgi apparatus; L, lipid body; Mi, mitochondria; N, nucleus; No, nucleolus; P, pyrenoid; r, ribosomes; S, starch grain; v, vacuole. From (85), originally adapted from a figure by H Ettl, courtesy of John Harper.
differences in overall size and body shape, shape and position of the chloroplast and pyrenoids, flagellar length, number and position of contractile vacuoles, and more subtle structural features visible at the light microscope level. Ettl (1976, cited in 86) recognized 459 species, which he consigned to nine major morphological groups. Although many of these species are represented in culture collections, only a few have found significant roles as laboratory research organisms.

*Chlamydomonas reinhardtii* has emerged as the predominant laboratory species of *Chlamydomonas*, primarily owing to its ability to grow nonphotosynthetically with acetate as its sole carbon source, and is discussed at length below. Some research studies have utilized the interfertile species pair *C. eugametos* and *C. moewusii*. *C. eugametos*, of European origin, derives ultimately from Moewus and has been used particularly for investigation of sexuality, where it forms a useful contrast to *C. reinhardtii*. Gowans isolated a number of nutritional and resistance mutations in the *C. eugametos* background, but these are not under active investigation at present. *C. moewusii* was isolated in New York in 1948 by Provasoli and was used soon afterwards by Lewin for selection of flagellar mutants. One of the most active current topics of research with *C. eugametos* and *C. moewusii* is phospholipid-mediated signal transduction (see 119, 152 and references cited therein). Sequence of the nuclear genes encoding 18S ribosomal RNA, the nuclear ribosomal DNA spacer ITS2, and chloroplast ribosomal RNAs place *C. moewusii* and *C. eugametos* in a group more closely allied to *Haematococcus* and *Chlorogonium* than to the *C. reinhardtii* cluster (see 35). The predicted evolutionary distance between *C. reinhardtii* and *C. eugametos/C. moewusii* is consistent with the marked differences in chloroplast architecture and overall cell morphology and steps in the mating reaction, and with the inability of *C. eugametos* and *C. moewusii* to use acetate as their sole carbon source, presumably a very fundamental physiological difference. One suspects that the characters that place all these species within the same genus (two flagella, cell wall, presence of a pyrenoid) are not sufficient to define the genus *Chlamydomonas* as a phylogenetic entity. *Volvox carteri*, *Pandorina morum*, and some other colonial Volvocales used as research organisms appear to be closely allied with *C. reinhardtii* based on molecular criteria (21, 35, 131).

*C. monoica* is a homothallic *Chlamydomonas* species that has been used to investigate the processes of mating, chloroplast gene inheritance, and zygospore formation (228, 230). Some experimental work has also been done in various laboratories on *C. geitleri*, *C. segnis*, *C. chlamydogama*, and several other species (see 86).

Except as otherwise indicated, the remainder of this article focuses on *C. reinhardtii*.

**Laboratory Strains of *C. reinhardtii***

The principal laboratory strains of *C. reinhardtii* are thought to derive from isolates made by GM Smith in 1945 from soil collected near Amherst, Massachusetts. Smith gave cultures to Sager, Lewin, Hartshorne, and perhaps others, and three
main lineages deriving from Smith’s collection have been separate since approximately 1950 (see Harris, Chapter 1 in 168). Although analyses of transposon insertion sites and chloroplast DNA restriction digests strongly suggest that all these strains do have a common origin, especially when compared with interfer- tile isolates from other localities (see 35 for citations), they are distinguished by inability of some of the strains to assimilate nitrate and by variability in light requirements for gametogenesis (202). AW Coleman (personal communication) has found significant differences among several of the Smith-derived strains in sequence of the ribosomal spacer ITS2 and has advanced the hypothesis that Smith may have distributed more than one original isolate. The situation has been complicated further by later crosses among representatives of these three lineages and by poorly documented transfers of strains among laboratories.

It is only now, as sequencing of the entire genome begins, that nucleotide variations among descendants of the Smith strains are becoming significant. Sager’s strain 21 gr (Chlamydomonas Genetics Center strain CC-1690) has been chosen as the primary target for sequencing efforts. Extensive EST (expressed sequence tag) data are also available from strain C9 (4), equivalent to 21 gr but separate from it since approximately 1955, and from CC-125, Smith’s 137C strain as used by Levine and Ebersold, which differs from 21 gr and C9 in lacking nitrate reductase activity.

Strains S1 D2 and S1 C5 are isolates from soil collected in Plymouth, Minnesota (CH Gross et al, 1988, cited in 35). Although fully interfertile with 21 gr and 137C, these strains are distinguished from the Smith isolates by extensive nucleotide polymorphisms, especially in noncoding regions. The genomes appear to be colinear, however, without major chromosome rearrangements. A cross between 21 gr and S1 C5 has been used as the foundation of the molecular map (<http://www.biology.duke.edu/chlamy-genome/maps.html>), and EST data are also being obtained from this strain. Preliminary analyses indicate less than 1% sequence divergence between S1 D5 and the Smith strains in coding regions but as much as 7% in 3′ untranslated regions (CR Hauser, personal communication).

Isolates of C. reinhardtii have also been made from Quebec, Pennsylvania, North Carolina, and Florida, and may provide an additional source of diversity in future (see 35 for citations). A second Massachusetts isolate made by Smith in 1946, designated C. smithii mating type plus by Hoshaw & Ettl, should also be considered as part of the C. reinhardtii group based on interfertility (86). However, the strain identified by Hoshaw and Ettl as C. smithii mating type minus (CC-1372, from Santa Cruz, California) does not belong with the C. reinhardtii group based on its lack of full fertility and on DNA sequence criteria that suggest it is more closely related to C. culleus (35). All authentic C. reinhardtii isolates to date thus appear to derive from North America east of the Rocky Mountains.

Two additional isolates have been placed with C. reinhardtii (UG Schlösser 1984, cited by 35) based on their susceptibility to the C. reinhardtii vegetative cell lytic enzyme or autolysin, although they do not appear to be cross-fertile with the authentic C. reinhardtii strains (58). Originally identified as C. incerta (SAG
7.73, supposedly from Cuba), and *C. globosa* (SAG 81.72, supposedly from the Netherlands), these isolates appear to be identical based on restriction fragment analysis of chloroplast DNA (EH Harris et al 1991, cited by 35), the ribosomal ITS sequences (35), HindIII and PstI digests of total DNA probed with the beta-tubulin gene (59), and intron sequences from the *Ypt4* gene (59, 131).

CELL ARCHITECTURE AND FUNCTIONAL SYSTEMS

The Cell Wall

The wild-type *C. reinhardtii* cell (Figure 1) averages about 10 µm in diameter (with significant variation through the cell cycle) and is enclosed within a wall consisting primarily of hydroxyproline-rich glycoproteins that resemble plant extensins. Contrary to a few erroneous early reports that have unfortunately been perpetuated in some textbooks, the *C. reinhardtii* cell wall does not contain cellulose. The wild-type wall comprises seven principal layers (241). Genes for some wall components have been cloned and sequenced, and many mutants with defects in cell wall biogenesis have been isolated. Most of these mutants seem to make the precursor proteins of the wall in normal amounts but fail to assemble them into complete walls (232). Cell wall mutants have found widespread use as recipients for transformation with exogenous DNA, a process that is much more efficient with wall-less cells.

The Nucleus and the Nuclear Genome

The cell nucleus and nucleolus are prominent in cross-sections of *Chlamydomonas* cells. The nuclear membrane is continuous with the endoplasmic reticulum, and one to four Golgi bodies are situated nearby. Chromosome cytology is poor, with only eight discrete chromosomes being consistently visible by light microscopy in metaphase cells. Electron microscopy of synaptenemal complexes suggested 16 or more chromosomes, a number that is consistent with the 17 linkage groups now defined by genetic analysis. Attempts to separate chromosomes electrophoretically have not been fully successful (82). Vegetative cells are normally haploid, but stable diploids can be selected using auxotrophic markers. The nuclear genome size is estimated at approximately 1 × 10⁸ base pairs (86, 194). It is GC-rich, approximately 62% overall in denaturation studies; sequence analysis gives a similar figure. This high GC content may produce difficulties in cloning genes. Amplification is improved by selection of primer sequences with 45% to 50% G-C content, and by including c7dGTP in the PCR reaction mixture (see 189 for methods).

Contractile Vacuoles and Osmotic Regulation

Two contractile vacuoles are located at the anterior end of the *C. reinhardtii* cell. Mutants requiring hyperosmotic media for survival (136) may provide a starting
point for a study of genetic control of vacuole structure and function. Salt-sensitive mutants have also been isolated but are thought to affect ion transport across the plasma membrane and have not been implicated directly in vacuole function (179).

Mitochondria

Mitochondria are dispersed throughout the cytosol and are sometimes seen in electron micrographs as elongated or branching structures. The difficulty of purifying mitochondria free of chloroplast contamination has limited biochemical research on *Chlamydomonas* mitochondria, and methods for purification of active mitochondria have been developed only recently (14, 53). The 15.8-kb mitochondrial genome is linear and contains only a few genes: *cob*, *cox1*, five subunits of mitochondrial NADH dehydrogenase, the mitochondrial rRNAs (which are fragmented in the DNA sequence); three tRNAs, and an opening reading frame that resembles a reverse transcriptase (see GenBank Accession number U03843 for complete sequence and citations). Mutants that delete the *cob* gene are unable to grow on acetate in the dark but are viable when grown phototrophically. Point mutations in the *cob* gene can confer myxothiazol resistance. Nuclear mutants with respiratory deficiencies and a dark-dier phenotype have also been obtained (see 45 for citations).

The Chloroplast

A single cup-shaped chloroplast occupies the basal two thirds of the cell and partially surrounds the nucleus. Thylakoid membranes are arranged in well-defined appressed and non-appressed domains whose composition and functional organization have been extensively investigated in wild-type and mutant strains of *Chlamydomonas* (Olive & Wollman, in 196). A distinctive body within the chloroplast, the pyrenoid, is the site of CO₂ fixation and the dark reactions of photosynthesis. Starch bodies surround the pyrenoid and are also seen dispersed throughout the chloroplast under some conditions of growth. Presence or absence of a pyrenoid distinguishes *Chlamydomonas* from the genus *Chloromonas*, and within the genus *Chlamydomonas*, the number and arrangement of the pyrenoids is an important species character in traditional taxonomy.

Sequencing of the 195-kb chloroplast genome is nearly complete (DB Stern and colleagues, personal communication). The chloroplast genomes of all *Chlamydomonas* species examined have an inverted repeat structure reminiscent of that of most land plants, but gene order differs markedly from the plant model and cannot be accounted for by any simple scheme of rearrangements or inversions. The gene content of the chloroplast genome largely does resemble that of land plants, however, with only a few significant differences (19).

*Chlamydomonas* became known early on as an excellent model system in which to study both photosynthesis and biogenesis of the chloroplast. The y1 mutant, originally isolated by Sager, loses chlorophyll and forms only a rudimentary proplastid when cultured on acetate-containing medium in the dark. On exposure to light,
y1 cells become green and form a complete chloroplast structure over the course of approximately 8 h. Although not precisely analogous to greening of an etiolated plant seedling in terms of how this process is regulated, y1 re-greening has nevertheless proved to be a powerful and accessible system for studying synthesis and assembly of the photosynthetic machinery, and for investigation of the relative roles of nuclear and chloroplast genomes in this process (237 and references cited therein).

One avenue of research arising from these early studies has been investigation of the genetic control of chlorophyll synthesis (see 12 for review of chlorophyll biosynthesis in general). Besides y1, there are several additional, nonallelic, mutants that show a yellow-in-the-dark phenotype, including some temperature-sensitive alleles (see 23). C. reinhardtii has two pathways for conversion of protochlorophyllide to chlorophyllide. The y mutants are blocked in the light-independent pathway, homologous to the protochlorophyllide reductase seen in organisms ranging from purple bacteria through gymnosperms (23, 126). The core enzyme in Chlamydomonas consists of three subunits coded by the chloroplast genes chlB, chlL, and chlN. The nuclear loci Y1 and Y5 through Y10 in C. reinhardtii all appear to be involved in expression of these chloroplast genes and/or assembly of their products (23). The pc1 mutant is blocked in light-mediated protochlorophyllide conversion because of a deletion in the gene encoding NADPH:protochlorophyllide oxidoreductase (130), which is equivalent to the light-dependent enzyme found in angiosperms (126). Other mutants in the chlorophyll biosynthetic pathway in Chlamydomonas include strains in which formation of Mg protoporphyrin from protoporphyrin IX is blocked and mutants specifically deficient in chlorophyll b. A signal transduction pathway for light-induced expression of glutamate 1-semialdehyde aminotransferase, an early enzyme in synthesis of both chlorophyll and heme, has been analyzed by Im & Beale (101).

In the 1960s, Levine’s laboratory at Harvard produced a series of papers demonstrating that the photosynthetic electron transfer chain was amenable to genetic dissection. Nonphotosynthetic mutants of C. reinhardtii were isolated, and identified by the prefix ac, for acetate-requiring. Many of these mutants were assigned to specific processes—water oxidation, photosynthetic electron transport, ATP synthesis, CO2 fixation—but identification of lesions in specific proteins was not possible with technology available at that time. Investigation in the early years was limited to mutations in nuclear genes. A breakthrough was made in 1979, however, with development of methods to select nonphotosynthetic mutations by using 5-fluorodeoxyuridine to reduce the number of copies of the chloroplast genome prior to mutagenesis (HS Shepherd et al 1979, cited in 86). The list of photosynthetic genes cloned and marked by mutations is now impressive (41) (Table 1). Chloroplast transformation with exogenous DNA occurs by homologous replacement (see below), thereby potentially permitting analysis by site-directed mutagenesis of every chloroplast gene. More than 50 nuclear gene loci affecting chloroplast biogenesis and photosynthetic functions are marked by mutations, and nearly all the structural genes for chloroplast components known in land plants have also been identified in Chlamydomonas either by complete sequencing or as ESTs.
<table>
<thead>
<tr>
<th>Component</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Photosystem II reaction center and water-splitting complex</td>
<td></td>
</tr>
<tr>
<td>Deletions of chloroplast-encoded ( psbA ) gene</td>
<td>See (86)</td>
</tr>
<tr>
<td>Site-directed mutations in ( psbA ) affecting photosynthesis</td>
<td>(87, 137, 220, 249)</td>
</tr>
<tr>
<td>Herbicide-resistance mutations in ( psbA )</td>
<td>See (86)</td>
</tr>
<tr>
<td>Nuclear mutations that cause accumulation of excess D1 protein or make D1 unstable at high light intensity</td>
<td>(254)</td>
</tr>
<tr>
<td>Nuclear mutations that destabilize ( psbB ) mRNA</td>
<td>(225)</td>
</tr>
<tr>
<td>Mutation in chloroplast-encoded ( psbC ) gene</td>
<td>(195)</td>
</tr>
<tr>
<td>At least four nuclear mutations affecting translation of ( psbC )</td>
<td>(195, 253)</td>
</tr>
<tr>
<td>Induced and site-directed mutations in chloroplast-encoded ( psbD ) gene</td>
<td>(107)</td>
</tr>
<tr>
<td>At least three nonallelic nuclear mutations affecting ( psbD ) translation</td>
<td>(18, 155)</td>
</tr>
<tr>
<td>Chloroplast ( psbD ) mutation producing unstable mRNA; three nuclear loci have been identified that suppress this mutation ( psbE ) null mutant</td>
<td>(150)</td>
</tr>
<tr>
<td>Disruption of chloroplast-encoded ( psbH ) gene and site-directed mutations in this gene</td>
<td>(163, 219)</td>
</tr>
<tr>
<td>Disruption of chloroplast ( psbl ) gene; can grow photosynthetically but is light sensitive</td>
<td>See (219)</td>
</tr>
<tr>
<td>Disruption of chloroplast ( psbK ) gene</td>
<td>See (219)</td>
</tr>
<tr>
<td>Disruption of chloroplast ( ycf8 ) gene; impairs PS II function under stress conditions</td>
<td>See (194)</td>
</tr>
<tr>
<td>Transposon insertion in ( PsbO ) gene</td>
<td>See (194)</td>
</tr>
<tr>
<td>Two allelic nuclear mutants deficient in OEE2 protein</td>
<td>(200)</td>
</tr>
<tr>
<td>State-transition mutations affecting LHCII phosphorylation</td>
<td>(118)</td>
</tr>
<tr>
<td>Cytochrome b6/f complex</td>
<td></td>
</tr>
<tr>
<td>Site-directed point and deletion mutations in chloroplast-encoded ( petA ) gene</td>
<td>(11, 31, 32, 36)</td>
</tr>
<tr>
<td>Five allelic nuclear mutations affecting ( petA ) mRNA stability and/or maturation</td>
<td>(41)</td>
</tr>
<tr>
<td>Point and deletion mutations in chloroplast-encoded ( petB ) gene</td>
<td>(41, 257)</td>
</tr>
<tr>
<td>A nuclear mutation affecting ( petB ) mRNA stability and/or maturation</td>
<td>(41)</td>
</tr>
<tr>
<td>Induced, null and site-directed mutations in the nuclear-encoded ( PetC ) gene</td>
<td>(41)</td>
</tr>
<tr>
<td>Numerous induced and site-directed mutations in chloroplast-encoded ( petD ) gene</td>
<td>(41, 92, 256)</td>
</tr>
<tr>
<td>Two nuclear mutations affecting ( petD ) mRNA stability and/or maturation</td>
<td>(41)</td>
</tr>
<tr>
<td>Several allelic mutations in nuclear-encoded ( PetE ) gene</td>
<td>(129)</td>
</tr>
<tr>
<td>Deletion of chloroplast-encoded ( petG ) gene</td>
<td>See (41)</td>
</tr>
<tr>
<td>A nuclear mutation affecting ( petG ) mRNA stability and/or maturation</td>
<td>(41)</td>
</tr>
</tbody>
</table>

(Continued)
At least four nuclear mutations affecting heme attachment to cytochrome b$_6$ Disruptions of chloroplast ycf7 (petL) gene; distabilization of the cytochrome b$_6$/f complex

Deletion of petO gene

Photosystem I reaction center
Deletion or disruption of chloroplast-encoded psaA gene
Site-directed point mutations in psaA
Disruption of tscA, a 430-nt RNA involved in psaA trans-splicing
At least 5 nuclear loci affecting psaA exon 2–3 splicing
At least 2 nuclear loci affecting both trans-splicing steps
At least 7 nuclear loci affecting exon 1–2 trans-splicing
Frame-shift and site-directed mutations in chloroplast psaB gene
Mutation in a nuclear gene that blocks a post-transcriptional step in psaB expression
Site-directed mutations in chloroplast psaC gene
Mutations in nuclear PsaF gene
Disruptions of chloroplast ycf3 and ycf4 genes; produce PS I deficiency
Site-directed mutations in ycf3 and ycf4
Insertional mutations in nuclear Crd1 gene; blocked in response to copper deficiency, fail to accumulate PS I and LHC I

Xanthophyll cycle and photoprotection
At least three nonallelic nuclear mutants blocked in xanthophyll cycle
Several nonallelic nuclear mutants resistant to very high light intensities

Photophosphorylation
Site-directed mutations in chloroplast atpA gene
Nuclear mutation affecting translation of chloroplast atpA gene
Nuclear mutation that destabilizes atpA mRNA
Nuclear mutation that destabilizes atpB mRNA
Many point and deletion mutations in chloroplast atpB gene
Site-directed mutations in atpB
e.g. (29, 30, 99)
Site-directed mutations in nuclear AtpC gene
Induced mutations in chloroplast atpE, atpF and atpI genes
Nuclear mutation affecting expression of the chloroplast-encoded atpH and atpI genes

CO$_2$ uptake
Mutation in nuclear CAH3 gene encoding intracellular carbonic anhydrase
Additional mutants that require high levels of CO$_2$ for growth

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**TABLE 1 (Continued)**

<table>
<thead>
<tr>
<th>Component</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Five nuclear gene loci involved in synthesis of chloroplast c-type</td>
<td>(102, 246)</td>
</tr>
<tr>
<td>cytochromes</td>
<td></td>
</tr>
<tr>
<td>Chloroplast mutations in the ccsA (ycf5) gene, encoding a protein</td>
<td>(246, 247)</td>
</tr>
<tr>
<td>required for heme attachment of cytochrome c</td>
<td></td>
</tr>
<tr>
<td>At least four nuclear mutations affecting heme attachment to cytochrome b$_6$</td>
<td>(120)</td>
</tr>
<tr>
<td>Disruptions of chloroplast ycf7 (petL) gene; distabilization of the cytochrome b$_6$/f complex</td>
<td>(222)</td>
</tr>
<tr>
<td>Deletion of petO gene</td>
<td>(83)</td>
</tr>
<tr>
<td>Photosystem I reaction center</td>
<td></td>
</tr>
<tr>
<td>Deletion or disruption of chloroplast-encoded psaA gene</td>
<td>(36)</td>
</tr>
<tr>
<td>Site-directed point mutations in psaA</td>
<td>(55)</td>
</tr>
<tr>
<td>Disruption of tscA, a 430-nt RNA involved in psaA trans-splicing</td>
<td>(195)</td>
</tr>
<tr>
<td>At least 5 nuclear loci affecting psaA exon 2–3 splicing</td>
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</tr>
<tr>
<td>At least 2 nuclear loci affecting both trans-splicing steps</td>
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</tr>
<tr>
<td>At least 7 nuclear loci affecting exon 1–2 trans-splicing</td>
<td></td>
</tr>
<tr>
<td>Frame-shift and site-directed mutations in chloroplast psaB gene</td>
<td>(63)</td>
</tr>
<tr>
<td>Mutation in a nuclear gene that blocks a post-transcriptional step in</td>
<td>(195, 215)</td>
</tr>
<tr>
<td>psaB expression</td>
<td></td>
</tr>
<tr>
<td>Site-directed mutations in chloroplast psaC gene</td>
<td>(63)</td>
</tr>
<tr>
<td>Mutations in nuclear PsaF gene</td>
<td>(62, 93)</td>
</tr>
<tr>
<td>Disruptions of chloroplast ycf3 and ycf4 genes; produce PS I deficiency</td>
<td>(20, 41)</td>
</tr>
<tr>
<td>Site-directed mutations in ycf3 and ycf4</td>
<td>(20)</td>
</tr>
<tr>
<td>Insertional mutations in nuclear Crd1 gene; blocked in response to copper deficiency, fail to accumulate PS I and LHC I</td>
<td>(151)</td>
</tr>
<tr>
<td>Xanthophyll cycle and photoprotection</td>
<td></td>
</tr>
<tr>
<td>At least three nonallelic nuclear mutants blocked in xanthophyll cycle</td>
<td>(161)</td>
</tr>
<tr>
<td>Several nonallelic nuclear mutants resistant to very high light intensities</td>
<td>(65)</td>
</tr>
<tr>
<td>Photophosphorylation</td>
<td></td>
</tr>
<tr>
<td>Site-directed mutations in chloroplast atpA gene</td>
<td>(44)</td>
</tr>
<tr>
<td>Nuclear mutation affecting translation of chloroplast atpA gene</td>
<td>See (41)</td>
</tr>
<tr>
<td>Nuclear mutation that destabilizes atpA mRNA</td>
<td></td>
</tr>
<tr>
<td>Nuclear mutation that destabilizes atpB mRNA</td>
<td></td>
</tr>
<tr>
<td>Many point and deletion mutations in chloroplast atpB gene</td>
<td>See (86)</td>
</tr>
<tr>
<td>Site-directed mutations in atpB</td>
<td>e.g. (29, 30, 99)</td>
</tr>
<tr>
<td>Site-directed mutations in nuclear AtpC gene</td>
<td>(114, 199)</td>
</tr>
<tr>
<td>Induced mutations in chloroplast atpE, atpF and atpI genes</td>
<td>See (44)</td>
</tr>
<tr>
<td>Nuclear mutation affecting expression of the chloroplast-encoded atpH and atpI genes</td>
<td></td>
</tr>
<tr>
<td>CO$_2$ uptake</td>
<td></td>
</tr>
<tr>
<td>Mutation in nuclear CAH3 gene encoding intracellular carbonic anhydrase</td>
<td>(71, 218)</td>
</tr>
<tr>
<td>Additional mutants that require high levels of CO$_2$ for growth</td>
<td>(110, 166)</td>
</tr>
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</table>
Among the greatest strengths of *Chlamydomonas* as a model organism in which to study chloroplast biogenesis has been its use to identify nuclear genes that regulate the expression of genes encoded in the chloroplast. Many of the mutants isolated in Levine’s laboratory have turned out to be involved in processing of chloroplast mRNAs or other regulatory steps. For example, the *ac115* mutant, isolated by Gillham in 1960 and initially described as lacking several proteins of photosystem II (PS II), has finally revealed its true nature (190). The *Ac115* gene product, a small basic protein with a potential membrane-spanning domain at the carboxyl terminus, is required for translation of mRNA for the chloroplast-encoded *psbD* gene, encoding the D2 protein of the photosystem II reaction center.

### TABLE 1 (Continued)

<table>
<thead>
<tr>
<th>Component</th>
<th>References</th>
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<tr>
<td>Disruption of chloroplast <em>ycf10</em> gene; produces inefficient carbon uptake into chloroplast ntl1-tagged mutants affecting CO₂ uptake</td>
<td>(197)</td>
</tr>
<tr>
<td><strong>Carbon fixation</strong></td>
<td>See (191)</td>
</tr>
<tr>
<td>Many mutations in chloroplast <em>rbcL</em> gene</td>
<td>(125)</td>
</tr>
<tr>
<td>Site-directed mutation in <em>rbcL</em> that alters its specificity for Rubisco activase</td>
<td>(98)</td>
</tr>
<tr>
<td>Nuclear mutation that inhibits <em>rbcL</em> expression; second-site suppressors of this mutation</td>
<td>(7)</td>
</tr>
<tr>
<td>Point mutation in structural gene for phosphoribulokinase</td>
<td>(23)</td>
</tr>
<tr>
<td><strong>Chlorophyll biosynthesis</strong></td>
<td>(E Chekunova, personal communication)</td>
</tr>
<tr>
<td>Deletion mutation in gene encoding NADPH:protochlorophyllide oxidoreductase</td>
<td>(130)</td>
</tr>
<tr>
<td>Nuclear mutations in at least six loci affecting expression of the chloroplast-encoded <em>chlB</em>, <em>chlL</em>, and <em>chlN</em> genes and/or assembly of the protochlorophyllide reductase complex</td>
<td>(86)</td>
</tr>
<tr>
<td>Two allelic nuclear mutants in the nuclear gene encoding Mg chelatase</td>
<td>(194)</td>
</tr>
<tr>
<td><strong>Chloroplast protein synthesis and protein translocation</strong></td>
<td>(H Naver, personal communication)</td>
</tr>
<tr>
<td>Many antibiotic resistance mutations in 16S and 23S ribosomal RNA genes</td>
<td>See (86)</td>
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<tr>
<td>Nuclear mutation that blocks processing of chloroplast rRNA</td>
<td>(96)</td>
</tr>
<tr>
<td>Additional nuclear mutations that result in deficiency in chloroplast ribosomes</td>
<td>See (125)</td>
</tr>
<tr>
<td>Nuclear mutations that suppress site-directed alterations in thylakoid signal sequences</td>
<td>(15)</td>
</tr>
<tr>
<td>Disruption of chloroplast-encoded <em>clpP</em> protease</td>
<td>See (194)</td>
</tr>
<tr>
<td>Nuclear mutation affecting LHC assembly, probably at the level of a chaperone protein</td>
<td>(H Naver, personal communication)</td>
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</table>
The original \textit{ac115} allele is a nonsense mutation near the 5′ end of this gene. The nuclear \textit{NAC1} and \textit{NAC2} loci, unlinked to \textit{AC115}, are also involved in \textit{psbD} translation, and a mutation that suppresses both \textit{ac115} and \textit{nac1} has been found (HY Wu & MR Kuchka, cited in 190). In \textit{nac2} mutants, the \textit{psbD} mRNA is unstable, leading to failure of assembly of the PS II reaction center. The \textit{Nac2} gene product is a 140-kDa hydrophilic polypeptide containing nine tetratricopeptide repeats (18). Additional nuclear mutants have been isolated that suppress a site-directed mutation within the 5′ untranslated region of the \textit{psbD} gene (155).

The chloroplast \textit{psaA} gene presents an even more complex example of interactions between nuclear and chloroplast genes in \textit{Chlamydomonas}. This gene is split into three separately transcribed exons in \textit{C. reinhardtii}, and the mRNA is assembled by \textit{trans}-splicing. At least 14 different nuclear genes affect the \textit{trans}-splicing process, and a small chloroplast-encoded RNA molecule (\textit{tscA}) is also required (81, 155, 173).

Synthesis and assembly of components of the cytochrome b\textsubscript{6}/f complex have been investigated in several laboratories (e.g. 11, 32, 120, 257). As is also true for the reaction centers of photosystems I and II, this complex contains both chloroplast and nuclear gene products, and additional nuclear genes may be required for control of chloroplast gene expression. For example, one chloroplast locus and at least four nuclear loci are required for heme attachment to the cytochrome \textit{c} apoprotein (246).

Wollman et al (cited in 32) have proposed a general model for stoichiometric accumulation of chloroplast-encoded proteins based on the concept of “control of epistasy of synthesis” or CES. Synthesis of some chloroplast-encoded subunits of the cytochrome b\textsubscript{6}/f complex (“CES subunits”) is strongly attenuated if other subunits of the complex (“dominant subunits”) are absent. For example, mutants deficient in either cytochrome b\textsubscript{6} or subunit IV show a greatly reduced rate of translation of cytochrome \textit{f}, encoded by the chloroplast \textit{petA} gene (32). However, in mutants lacking cytochrome \textit{f}, cytochrome b\textsubscript{6} and subunit IV are synthesized at normal rates and then degraded. Choquet et al (32) showed that the 5′ untranslated region of the \textit{petA} mRNA regulates its own translation by interaction, either directly or through an intermediary protein, with the C-terminal domain of the unassembled cytochrome \textit{f} protein.

Merchant and colleagues have studied regulation of the copper-containing protein plastocyanin (129), and the \textit{c}-type cytochromes (144, 248). Copper deficiency in \textit{Chlamydomonas} results in degradation of plastocyanin and induction of cytochrome \textit{c} \textsubscript{6} and coproporphyrinogen oxidase, and copper-responsive sequences have been identified within the promoters of the \textit{Cyc6} and \textit{Cpx1} genes (185, 186).

Mutants at the newly identified \textit{CRD1} locus are chlorophyll deficient in the absence of copper and have defects in photosystem I (151). Restoration of copper rescues both phenotypes. The \textit{Crd1} gene product is a 47-kDa hydrophilic protein with a carboxylate-bridged di-iron binding site, and it appears to be required for adaptation to either copper or oxygen deficiency.

These are only a few examples of the ways in which \textit{Chlamydomonas} is currently being used for investigation of the chloroplast and photosynthesis. For a much
more comprehensive treatment, the recent book edited by Rochaix, Goldschmidt-Clermont & Merchant (196) is highly recommended. Rochaix (194) and Davies & Grossman (38) have reviewed the use of Chlamydomonas for elucidation of photosynthetic processes. Niyogi (159) has reviewed photoprotection and photoinhibition, a process for which Chlamydomonas is proving to be a very useful model (9, 65, 90, 104, 139, 160, 161, 210). Xiong et al (250) have published a three-dimensional model for the photosystem II reaction center of Chlamydomonas in a paper that thoroughly reviews the literature on components of this complex. For an assessment of photosystem I function and its indispensability, see Redding et al (192). There is also a substantial literature on transcriptional and translational control of chloroplast gene expression in Chlamydomonas (44, 57, 73, 88, 116, 141, 252) and on processing of introns in chloroplast rRNA and psbA genes (95). Goldschmidt-Clermont (76) has reviewed coordinated expression of nuclear and chloroplast genes in plant cells, including Chlamydomonas, and Nickelsen & Kück (156) have reviewed the use of C. reinhardtii as a model system for study of chloroplast RNA metabolism. Chloroplast DNA replication has also been studied in several laboratories (28 and references cited therein).

Flagella

Two anterior flagella, 10 to 12 µm in length, protrude through specialized collar regions in the cell wall. The structure of the flagellar axoneme has been described thoroughly (147), and more than 250 component proteins have been resolved by two-dimensional electrophoresis. From the very beginning, Chlamydomonas has been among the very best organisms for research on flagellar function and assembly. Mutants with defects in motility were among the first to be isolated both in C. moewusii and C. reinhardtii. Many of these early mutants are extant and are finally revealing their precise defects.

Mutations have been identified that affect nearly all of the principal components of the flagellar axoneme. More than 75 genetic loci have been identified in C. reinhardtii that affect flagellar assembly and/or function, and more than 40 genes for flagellar components have been cloned and sequenced. Some of these mutants have parallels in mutations affecting animal cilia and sperm cells (33, 167–169). Complementation in the transient dikaryons formed after mating can be used to identify specific proteins of the radial spokes, central pair, and other complexes. Fusion of gametes of opposite mating type in C. reinhardtii produces a cell with two nuclei and four flagella. This quadriflagellate cell remains motile for about two hours before the flagella are resorbed and formation of the zygospore wall begins. During this motile period, flagellar assembly continues, and polypeptides contributed by both parental gametes are incorporated into all four flagella. Thus mating of two nonallelic paralyzed mutants usually results in restoration of full motility in this transient dikaryon, as each partner supplies a wild-type copy of the defective flagellar protein produced by the other gamete. The simple observation of restoration of motility makes a very nice laboratory exercise.
for students. Luck and colleagues (cited in 46, 86) used radioactive labeling and
two-dimensional gel electrophoresis to identify the specific proteins that were re-
stored by mating labeled mutant cells to unlabeled wild-type cells. The reviews by
Dutcher (46) and Mitchell (147) of the genetics of flagellar assembly and structure
are highly recommended as an introduction to this field of research for the non-
specialist. Johnson (106) has reviewed flagellar beating motility and its structural
correlates. Detailed reviews have also been published on dyneins (176), radial
spokes (37), the central apparatus (213), kinesins (16), and intraflagellar transport
(198).

In addition to mutants with primary defects in flagellar components, many
second-site suppressor mutations have been found that restore partial or complete
motility, and these mutations have permitted identification of additional structural
components. Mutants with unusually long or short flagella are also known (5), and
the regulation of flagellar length through the cell cycle in wild-type cells is being
elucidated (224).

Gliding motility, by means of movement of the flagellar membrane, has long
been known, and non-gliding mutants have been isolated (RA Bloodgood in 43).
This gliding motion can also be visualized by transport of adherent particles or
polystyrene beads along the rigid, extended flagella of certain mutants with defects
in the central pair microtubules (e.g. pf18; 17).

Flagellar assembly depends on yet another type of motility, intraflagellar
transport (IFT), first identified in Chlamydomonas by Rosenbaum and colleagues
(34, 117, 198). This process involves bi-directional movement of protein com-
plexes (“rafts”) along the flagella. Transport toward the flagellar tip is mediated by
a kinesin, first identified as the site of the flagellar assembly mutation fla10 (117).
The return transport of rafts back toward the cell body is dependent on cytoplas-
mic dynein 1b and dynein light chain LC8 (34, 175). Mutants in which anterograde
IFT is disrupted may have short or “stumpy” flagella and may accumulate flagellar
proteins in the cell body, whereas mutants in which retrograde IFT is defective
display a bulge in the flagellar membrane (34, 175). One of the components of
IFT complex B shows homology to a mammalian protein implicated in a form of
polycystic kidney disease, known from a mouse mutant in which renal cilia are
abnormal. The IFT system is also implicated in retinitis pigmentosa, where retinal
photoreceptor cells, whose outer sector is a modified cilium, are progressively lost
(33, 34, 198).

Basal Bodies

The flagella arise from a pair of basal bodies located just beneath the apical end
of the cell, surmounting the cell nucleus. The basal bodies are connected to one
another by a distal striated fiber (Figure 2) and are attached at their proximal ends
to four sets of microtubules that extend around the anterior portion of the cell.
Proximal fibers connect the basal bodies to the nucleus, and a cruciate fibrous root
is located directly beneath the basal bodies.
Figure 2  Flagellar root system of Chlamydomonas. B, basal bodies; P, pro-basal bodies; DSF, distal striated fiber; PSF, proximal striated fiber; SMAF, striated microtubule-associated fiber; 2MTR and 4MTR, 2- and 4-membered rootlet microtubules. Figure by Andrea Preble, courtesy of Susan Dutcher.

In cross-section, the basal bodies show a progression from a ring structure through a series of cartwheel configurations also seen in centrioles of animal cells, to the final 9 + 2 microtubule structure characteristic of eukaryotic flagella and cilia (Figure 3). Chlamydomonas has proved an especially favorable system in which to investigate the formation and function of basal bodies and flagella. Centrin, the 20-kDa contractile protein of the distal striated fiber and the nucleus-basal body connector, was discovered in Chlamydomonas (see 204), as was delta-tubulin, required for assembly of triplet microtubules in the basal body (49). Three additional centriole-associated proteins, BAp90, BAp95, and striated fiber assemblin, were also first identified in green algae (127).

Prior to mitosis, the basal bodies assume their alternative role as centrioles, components of the microtubule organizing center as in animal cells (138). The
connections from the flagella to the basal bodies are lost early in mitosis, and the flagella are resorbed. The basal bodies (centrioles) duplicate in late G1 phase by forming a new partner next to each pre-existing one, and by prophase the cell has a pair of centrioles at each spindle pole, each pair consisting of an old and a new centriole. After cytokinesis, the basal bodies return to the cell anterior, and new flagella are formed. Recent studies indicate that centrioles are able to form de novo (49, 138).

When *Chlamydomonas* cells are treated with weak organic acids, mastoparan, calmodulin antagonists, detergent, or various other stimulants, the flagella are severed at the level of the transition region between the basal body and the flagellar axoneme (see Figure 3). The process is thought to be similar to katanin-mediated severing of cytoplasmic microtubules in mitotic *Xenopus* and starfish oocytes (see 132, 133, 182). Investigation of this phenomenon in *Chlamydomonas* has revealed a complex signal transduction pathway that is proving to be amenable to genetic analysis. Mutants at two genetic loci fail to sever their flagella in response to any stimulus and are thought to be defective in the calcium-activated severing process. The product of one of these loci is a 171-kDa protein with an N-terminal coiled-coil domain and three Ca\(^{2+}\)/calmodulin-binding domains (60). Mutants at a third locus are blocked in acid-stimulated deflagellation but respond to nonionic
detergent plus Ca\(^{2+}\) and are proposed to affect protein-stimulated Ca\(^{2+}\) influx (61).

Deflagellation is followed by active transcription of the genes encoding tubulin and other flagellar components, and regeneration of the flagella over a 3-h period. This has proved an excellent system both for purification of axonemes and for study of the assembly process [see articles by SM King and PA Lefebvre in (43) for methods]. Deflagellation induces both transcription of genes for flagellar proteins and changes in mRNA half-life (79, 109, 172). In the presence of inhibitors of protein synthesis, assembly of half-length flagella still occurs, suggesting that there is a cytoplasmic pool of flagellar precursors (66). Pre-assembled complexes move from the cytoplasm to the flagellar compartment, where they are attached to microtubule-associated docking sites (162).

The Eyespot and Phototaxis

The eyespot, or stigma, appears bright orange at the light microscope level, owing to a high concentration of carotenoid pigments. Electron microscopy reveals it as a region of electron-dense granules located just inside the chloroplast membrane at the cell equator. The carotenoid-containing granules of the eyespot are thought to act as a quarter-wave plate to direct light to the true photoreceptor (KW Foster & RD Smyth, cited in 206), located in the overlying plasma membrane and now identified as a retinal-binding rhodopsin homologue (chlamyopsin; 42, 89). The complex functions as a directional antenna that enables swimming cells to orient themselves with respect to unidirectional light. Mutants lacking the eyespot structure show reduced efficiency of phototaxis but may still be able to perceive light through the photoreceptor. Mutants with defects in eyespot assembly have been grouped into six complementation groups, several of which map to a closely linked cluster of loci (123; DGW Roberts, MR Lamb & CL Dieckmann, submitted). Mutants deficient in the signal transduction pathway essential to the phototactic response have also been isolated (115, 170, 201). Methods for assaying phototaxis have been described by Moss et al (contained in 43).

The position of the eyespot gives the *Chlamydomonas* cell an inherent asymmetry, because it is associated with the distal end of one of the flagellar roots, specifically the four-membered rootlet emanating from newly formed basal body from the previous mitosis. This basal body is referred to as *cis* (relative to the eyespot), whereas the parent basal body is *trans*. The flagellar beat in normal forward swimming is a breast-stroke action that propels the cell in a helical path, with constant orientation of the eyespot relative to the helical axis. However, the *cis* and *trans* flagella respond differently to phototactic signals, thus effecting a turning response (205). The position of the eyespot is established during mitosis, when the old eyespot disappears and a new one is formed, invariably opposite the site of the cleavage plane.

Two distinct photoresponses have been observed in *Chlamydomonas*, the oriented movements of phototaxis in response to a constant unidirectional light and the photophobic or stop response to sudden light flashes. The same photoreceptor
appears to mediate both responses (P Kröger & P Hegemann 1994, cited in 205). Analysis of mutants that show phototactic but not photophobic responses suggests that the photophobic response requires a calcium-dependent all-or-none electric current induced by photoreceptor-mediated depolarization of the flagellar membrane (140). Phototaxis also depends on calcium-induced changes in flagella beating, but at a lower molarity of calcium that shifts the balance in beating strength of the two flagella.

MITOTIC AND MEIOTIC LIFE CYCLES

Vegetative Cell Growth

Wild-type *C. reinhardtii* is easily grown in defined liquid or agar media at neutral pH, and has no requirements for supplementary vitamins or other co-factors (see 86). Strains in the Ebersold/Levine 137C background cannot assimilate nitrate and therefore require a reduced nitrogen source (usually NH₄Cl). Acetate can be used as a carbon source by wild-type strains, with the consequence that growth can occur in the dark, and mutants blocked in photosynthesis are viable if acetate is provided. Other intermediates in the citric acid cycle do not support growth in the dark, nor do various pentose or hexose sugars, ethanol, glycerol, or other organic compounds.

For wild-type strains, growth in light either with or without acetate is faster than dark growth and is therefore recommended. Optimal growth temperature is from 20° to 25°. At 25°, in minimal medium and with adequate light (200–400 µEinstein/m²sec photosynthetically active radiation), an average doubling time of 6 to 8 h should be achieved.

When grown on a 12:12, 14:10, or 16:8 light-dark cycle, cells remain in G1 throughout the light phase and divide during the dark phase, usually with two or sometimes three mitotic divisions taking place in rapid succession. Four daughter cells are retained within a common mother cell wall and released simultaneously on secretion of a specific lytic enzyme. Commitment to divide appears to be determined at a specific point in G1 phase, thought to be analogous to the START event in the yeast cell cycle (see 85 for review). Beyond this point, division will still occur even if light and nutrients are removed from the culture. The number of successive divisions that take place in a given cycle depends on the cell size reached during G1. A circadian oscillator may also be involved in determining timing of division (78).

The progress of mitosis in *C. reinhardtii* was described in 1968 by Johnson and Porter in a classic paper (cited in 86). Since that time, computer-assisted analysis of serial sections and immunofluorescence techniques have added to our understanding of the changes in cytoplasmic microtubules, actin, and the chloroplast during mitosis (51, 85).

Conditional mutants blocked at specific points in the cell division cycle at restrictive temperature were described more than 25 years ago by Howell and colleagues (reviewed in 85), but the state of knowledge of the cell cycle at that
time did not permit full characterization of their defects. New conditional cell cycle mutants have been isolated by John and colleagues (203, 242), and the old mutants have been subjected to further study as well. The results now emerging indicate that Chlamydomonas has great potential as a system for genetic analysis of cell cycle control.

A nonconditional mutant defective in size control has been found to have a deletion in a gene encoding a homologue of the retinoblastoma family of tumor suppressors (3; J Umen, unpublished). This protein appears to function at two points in the cell cycle, in G1 and again in S phase. The result of its deletion in Chlamydomonas is impairment both in timing of commitment to divide and in control of the number of divisions that eventually occur.

Mutants of C. moewusii described as “twins” and “monsters” were reported by Lewin in 1952 (reviewed in 85). The twinning mutant is now thought to be defective in formation of the cleavage furrow. Cultures of monster mutants have a significant proportion of cells that are blocked in division but continue to grow. The cyt1 mutant of C. reinhardtii resembles the monster mutants in its failure to complete cytokinesis in many cells of a culture, with the consequence that cells may be multinucleate, large, and multilobed (JR Warr, cited in 85). Ehler & Dutcher (51) found that cyt1 cultures often produce cells with incomplete cleavage furrows. They also isolated an insertional allele of cyt1 and a mutant at a second locus, cyt2, which makes additional, misplaced, cleavage furrows. The number of flagella on a given cell is correlated with the number of nuclei. Similar mutants (oca1 and oca2, for occasional cytokinesis arrest) have been described by Hirono & Yoda (94).

Mutants of C. reinhardtii with abnormalities in the basal body cycle have proved to be particularly amenable to study. Several classes of mutants were first identified by their variable number of flagella. Vf11 cells have lost control of the timing and placement of basal body and flagellar formation and show abnormalities in the direction of the flagellar beat, as well as structural abnormalities in several of the doublet microtubules of the flagellar axoneme (223). New basal bodies can appear at any point during G1 phase, and the flagellar insertions can be anywhere on the cell surface. Vf12 mutants have alterations in the structural gene for centrin (221) and show random segregation of the basal bodies. Pedigree analysis of mitotic progeny from a vf12 mutant has been used to demonstrate de novo centriole assembly (138). Vf13 mutants are defective in placement of the probasal bodies and have been shown to have defects in straited fibers (HJ Hoops, RL Wright, JW Jarvik & GB Witman 1984, and RL Wright, Chojnacki & JW Jarvik, 1983, cited in 86). Uni mutants were so named because the majority of cells in a culture have only a single flagellum. The uni1 and uni2 mutants both show alterations in the transition zone (Figure 3, level 7) but are distinguishable at the ultrastructural level (49). The uni3 mutant may have zero, one, or two flagella and exhibit aberrant cytokinesis. In this mutant, the C tubule is missing in the triplet microtubule of the basal body (Figure 3, level 3). The defect in uni3 has been traced to delta-tubulin, a protein first identified when the Uni3 gene was cloned from Chlamydomonas (49).
Bld ("bald") mutants lack flagella altogether. One such mutant, bld2, lacks functional basal bodies and has defects in cytokinesis consistent with loss of centrioles (52). The cleavage furrow loses its precise orientation with respect to the mitotic spindle. Another mutation affecting cytokinesis is fla10, originally isolated as a temperature-conditional flagellar assembly mutant, but subsequently shown to have a defect in a kinesin-homologous protein that functions in basal bodies both in flagellar assembly and in organization of the mitotic spindle (117, 231).

The Sexual Cycle

C. reinhardtii cells are normally haploid and are of one of two genetically fixed mating types, designated plus (mt+) and minus (mt−). The mating-type locus is a complex region of recombinational suppression on linkage group VI, comprising approximately 1 megabase and containing genes involved in cell recognition and fusion, zygospore maturation, and in the mating-type controlled inheritance of organelle genes, as well as some additional closely linked loci that have no apparent role in the sexual cycle (58, 59). Additional genes that map elsewhere in the genome but whose expression is sex-limited have also been identified (25, 77, 122, 226).

When deprived of nitrogen, cells of both mating types differentiate into sexually competent gametes (Figure 4). Some strains have an additional requirement for blue light to progress from a pregamete state to mating competence (13, 74, 165, 202). Plus and minus gametes pair initially along the lengths of their flagella in a reaction mediated by sex-specific agglutinin proteins. Flagellar pairing initiates a cAMP-mediated signal transduction cascade, which has been investigated extensively (181, 239). Pairing is followed by a morphological change ("activation") in the flagellar tips and by dissolution of the cell walls of the mating partners by a gamete-specific lytic enzyme. Flagellar agglutination, activation of the flagellar tips, and wall lysis can all be by-passed by supplying exogenous dibutryl cAMP and isobutyl methylxanthine (SM Pasquale & UW Goodenough, cited in 86; this observation has been exploited as a means of genetic analysis of mutants lacking flagella). Fusion of the mating partners begins at sex-specific structures at the anterior ends of the cells (238, 240), and continues laterally from anterior to posterior. The newly formed diploid zygote remains motile for several hours as a quadriflagellate cell.

In C. moewusii, C. eugametos, C. monoica, and some other Chlamydomonas species, flagellar contact is followed initially by fusion only at the extreme anterior ends of the cells, and the partner cells swim about for several hours as a "vis-à-vis" pair before full cell fusion occurs (229). This distinction in the mating process is probably a very fundamental character separating different ancestral lineages within the green algae. Sexual cycles have been described in relatively few Chlamydomonas species, so this has not been used as a character in traditional taxonomy. The overall process of recognition, signal transduction, and cell fusion in C. eugametos resembles that of C. reinhardtii in many ways, however, and a substantial body of literature exists on the sexual cycle in this species (see 229).
Figure 4  The sexual cycle of *Chlamydomonas reinhardtii*. Courtesy of William Snell.
Zygote-specific transcripts appear within minutes of gamete fusion (121 and references cited therein). Formation of a hard, impermeable zygospore wall begins, chloroplasts appear to disintegrate, with loss of chlorophyll, and lipid bodies accumulate over the ensuing 4 to 6 days. The zygospore wall in sexual species of *Chlamydomonas* affords protection against adverse environmental conditions. Like the vegetative cell wall, the zygospore wall of *C. reinhardtii* contains hydroxyproline-rich glycoproteins, some of which are marked by (SerPro)x repeats. Zygospores can remain viable in soil for many years. However, under laboratory conditions only a few days are required for zygospore maturation before germination can be induced by restoration of nitrogen in the presence of light. Meiosis occurs, with the subsequent release of the four haploid meiotic products. Under some conditions, a mitotic division follows meiosis prior to opening of the zygospore wall, with the resulting release of eight rather than four progeny cells; predilection for release of eight rather than four cells may be strain dependent (86).

A small percentage of mated pairs fail to initiate the zygospore maturation pathway and begin instead to divide mitotically as stable vegetative diploids (see 86). These can be deliberately selected using complementing auxotrophic markers, and can be recognized 3 or 4 days after mated cells have been plated on agar, as bright green hemispherical colonies visible using a dissected microscope, on a lawn of unmated gametes and immature zygospores.

**Tetrad Analysis**

Separation of all meiotic products from a single zygospore is the foundation of traditional *Chlamydomonas* genetics by tetrad analysis (see SK Dutcher in 43 and 86 for methods). Nuclear genes show 2:2 inheritance in crosses and are scored by their segregation in parental ditype, nonparental ditype, or tetratype tetrads. Chloroplast and mitochondrial genes show primarily uniparental inheritance, from the plus and minus mating types, respectively. Elucidating the mechanism by which uniparental inheritance is achieved was one of the earliest challenges in *Chlamydomonas* research. Although the mysteries are still not entirely solved, the tools of molecular biology are now being brought to bear on this problem (146, 158).

Mutations are readily induced in the nuclear genome of haploid *Chlamydomonas* cells by UV or chemical mutagenesis (86), or by insertional mutagenesis in which transformation with exogenous DNA results in disruption of nuclear genes (LW Tam & PA Lefebvre in 43). This technique is becoming increasingly common as a preliminary step in cloning genes for which a mutated phenotype is known or predictable and is discussed further below.

The spectrum of mutants available in *C. reinhardtii* includes nonphotosynthetic, nonmotile, and nonphototactic strains, auxotrophs, mutants resistant to antibiotics or herbicides, and other phenotypes (see 86, 128). As of this writing, nearly 200 nuclear gene loci have been identified by mapped mutations.
PHYSIOLOGICAL PROCESSES

Nutrient Update and Metabolic Stress

Most wild-type strains of *C. reinhardtii* can assimilate nitrogen as nitrate, nitrite, ammonium, or other small molecules such as urea, acetamide, etc. Strains in the 137C background carry two mutations blocking nitrate reductase activity. The *Nia1* gene (*NIT1* locus) is the structural gene for nitrate reductase (108); the *Nia2* gene (*NIT2*) encodes a regulatory protein in the nitrate assimilation pathway (207). The nitrate transport system has been characterized in some detail and several component genes have been cloned (153, 184, 255). Nitrite is transported by a separate system, regulated by blue light (187). The structural gene for nitrite reductase (*Nii1*) maps to a cluster that also includes nitrate assimilation genes and a light-regulated gene (183). Genes for some additional enzymes involved in nitrogen metabolism have also been cloned and are under investigation.

Arginine is the only amino acid for which auxotrophic mutations are readily isolated in *Chlamydomonas*. Mutations at six loci affect the arginine biosynthetic pathway (see 86). Only the argininosuccinate lyase gene has been completely sequenced (6), but ESTs have been found corresponding to ornithine transcarbamoylase (the step blocked by the *arg4* mutation) and argininosuccinate synthase (*arg8*). Efforts to obtain a broader spectrum of amino acid auxotrophs have not been successful, possibly owing to lack of active transport systems for these compounds. Some mutants resistant to amino acid analogs have been isolated, however. Auxotrophic mutants are also known for thiamine, nicotinamide, and para-aminobenzoic acid.

Carbon assimilation involves a complicated pathway with multiple forms of carbonic anhydrase (218), the genes for several of which have been cloned from *C. reinhardtii*. Expression of the periplasmic enzyme is regulated by acetate and pH (227). Carbonic anhydrase activity associated with the chloroplast (2, 71) is required for photosynthesis at ambient concentrations of CO₂. A novel 29.5-kDa alpha-type carbonic anhydrase associated with the thylakoid membrane has been cloned from *Chlamydomonas* by Karlsson et al (110). Mitochondrial carbonic anhydrase is induced at low CO₂ concentrations (54). Mutants blocked in carbonic anhydrase activity are dependent on high CO₂ levels for growth (69, 112, 214).

Limitation for CO₂ in the light or for acetate in the dark results in accumulation of large amounts of starch (amylopectin) by *Chlamydomonas* cells (22). Mutants deficient in amylase biosynthesis resemble waxy mutants of maize and other plants in accumulating an aberrant amylopectin. A mutant blocked in ADP-glucose pyrophosphorylase has also been isolated.

Sulfur deprivation induces expression of a high-affinity transport system (251) and a periplasmic arylsulfatase that has found utility as a reporter gene (164). Mutants at three loci show aberrant responses to sulfur deprivation; two of these loci have been shown to correspond to genes for regulatory proteins (39, 40).
Phosphorous starvation induces expression of several phosphatases (1, 8, 188) and of the Psrl gene, encoding a regulatory protein (245).

When grown under anaerobic conditions in the light, *Chlamydomonas* cells are capable of producing molecular hydrogen. Sulfur deprivation enhances hydrogen production by repressing photosynthetic oxygen evolution, and under laboratory conditions, cultures can be maintained that cycle between photosynthesis and hydrogen production as sulfur is alternately removed and resupplied (142). The wild-type hydrogenase enzyme is very sensitive to inhibition by oxygen, but efforts are under way to select mutants with a more oxygen-tolerant enzyme (T Flynn & M Ghirardi, in preparation). The process is potentially of enormous value as a source of renewable fuel.

Another potential commercial application of *Chlamydomonas* is in removal of heavy metal wastes from the environment. Tolerance for cadmium and other metals by *Chlamydomonas* cells can be altered by growth conditions, by mutation (177, 234), by treatment with phytochelatin inhibitors (24), or by genetic engineering (R Sayre, personal communication).

As our knowledge increases of genes involved in individual metabolic pathways, studies of the integration of these pathways is becoming feasible. Huppe & Turpin (100) provide a good summary of the relationships between carbon and nitrogen metabolism. Wykoff et al (244) have assessed effects of nutrient deprivation on photosynthesis. Rochaix (194) has discussed state transitions, chlororespiration and interactions between the chloroplast and mitochondria. The study of stress responses in *Chlamydomonas* is in fact emerging as an integrated discipline, encompassing nutrient limitation, excess light, heavy metal contamination, osmotic stress, and heat responses. Bell and colleagues have published a series of papers dealing with genetic fitness and response of *Chlamydomonas* cells to experimentally controlled environmental fluctuations (75, 111 and references cited therein).

**Light-Mediated Responses**

As mentioned in connection with the sexual cycle, blue light photoreceptors may be involved in regulation of gametogenesis in *Chlamydomonas* (74, 165). The blue-light-responsive *LRG5* locus encodes a protein rich in arginine, lysine, and alanine with a putative nuclear-localization signal at its C-terminal end. No significant sequence homology was found to proteins from other organisms, but DNA hybridization experiments suggest some conservation of related sequences in other Volvocales and in land plants. The blue-light signaling pathway in gametogenesis appears to involve consecutive action of a phosphatase and a kinase resembling protein kinase C (165). Disruption of the *LRG6* gene, encoding a protein with significant homology to the yeast membrane transport facilitator YJR124p, eliminates the requirement for blue light in gametogenesis (G Dame, G Glöckner & CF Beck, personal communication). Blue light has also been implicated in cell cycle regulation in *Chlamydomonas* (233), in chlorophyll biosynthesis (91),
and in repair of UV-induced DNA damage (174). An early report that antibodies to plant phytochrome reacted with a *Chlamydomonas* protein was misleading; Bonenberger et al (1994, cited in 74) have shown that a monoclonal antibody to pea phytochrome reacts with a totally unrelated protein in *C. reinhardtii*, and there is no evidence to date for any phytochrome-mediated responses. The absence of red/far-red responses increases the utility of *Chlamydomonas* for the study of other light-regulated responses. A cryptochrome photoreceptor, CPH1, has recently been cloned from *C. reinhardtii* (NA Reisdorph & GD Small, in preparation). Although cryptochrome proteins have been implicated in molecular clock mechanisms in some organisms, the CPH1 gene of *C. reinhardtii* does not seem to show circadian regulation. Promoter regions of light-responsive genes in *Chlamydomonas* do not have the conserved control elements seen in higher plants but do appear to have characteristic light-regulated *cis* sequences (80).

**Circadian Rhythms**

Like many other algae, *Chlamydomonas* does have a circadian clock system, however, and its potential for genetic analysis increases its utility as a model for this area of research. Circadian rhythms of phototactic aggregation were observed by Bruce and coworkers in the early 1970s, and mutants with altered rhythms were obtained (see 86). Mergenhagen isolated additional mutants and explored the nature of the timer, or zeitgeber, under various environmental conditions, including zero gravity. Circadian rhythms have also been found in abundance of mRNAs for a number of genes involved in nitrogen metabolism and in photosynthesis (68, 103), and in UV sensitivity (157). Circadian rhythm phases can be shifted by brief pulses of light given during the dark period. The action spectrum for this response shows peaks at 520 and 660 nm, but is not far-red reversible (T Kondo et al 1991, cited in 103). Mittag (1994, cited in 148) discovered a regulatory factor in the dinoflagellate *Gonyaulax polyedra* that binds to a *cis* element, a UG repeat in the 3′ untranslated region of the gene encoding the luciferin-binding protein in that alga. Further investigation revealed that *Chlamydomonas* has an analogous clock-controlled RNA-binding protein (148, 149) whose target sequence is also a UG repeat. This sequence appears in the 3′ untranslated region of many *Chlamydomonas* genes, including several involved in nitrogen metabolism and previously reported to exhibit temporal expression (H Waltenberger, C Schneid, J Grosch, A Bariess & M Mittag, submitted).

**TECHNIQUES AND RESOURCES**

**The Molecular Tool Kit**

Over the past 12 years transformation of the nuclear and chloroplast genomes with exogenous DNA has become routine (see 113 for review). The first successful
transformations were accomplished using biolistic™ bombardment with tungsten or gold particles. Vortex-mixing with DNA-coated glass beads or silicon carbide whiskers is also effective, especially for nuclear gene transformation, as is electroporation (211). The glass bead method is especially recommended, as it requires no specialized equipment or expensive supplies. Highest frequencies are obtained with glass bead transformation and electroporation when cell walls are removed prior to transformation, and when steps are taken to improve plating efficiency of cells after transformation (180). Wall-deficient mutant strains can be used, or the walls of wild-type cells can be removed prior to transformation with a preparation of the gamete lytic enzyme (113). With all methods, cotransformation with two different plasmids occurs at a high frequency (216). When selection is made for one of the introduced genes, most transformants are found to carry the unselected DNA as well. This observation suggests that the critical event in transformation is in sustaining a nonlethal “hit” by the particles, glass beads, fibers, etc, and that cells that survive such a hit are likely to assimilate whatever DNA was present at the moment of impact.

Chloroplast transformation usually occurs by homologous replacement and has been extensively used to study proteins of the photosynthetic complexes by site-directed mutagenesis (e.g. 63, 99, 124, 137, 193, 249, 257). Selectable markers for cotransformation include antibiotic resistance mutations in the chloroplast ribosomal RNA genes, the bacterial antibiotic resistance genes \textit{aadA} (56, 57, 64) and \textit{aphA-6} (10), \textit{uidA} (GUS; 57), and \textit{Renilla} luciferase (145). Transformation of the mitochondrial genome has also been reported (B Randolph-Anderson et al, 1993, cited in 45).

In contrast to the organelles, nuclear gene transformation generally occurs by nonhomologous insertion into the genome of one or (usually) more copies of the transforming DNA. Transformation with glass beads usually results in integration of fewer copies of the transforming DNA than does particle bombardment (79). Insertion usually results in deletion of 10 to 20 kb or more of DNA at the integration site (128). When this event disrupts a nonessential gene, the insertion can be used as a probe for hybridization to clone the gene (LW Tam & PA Lefebvre, in 43). More than 50 genes have already been cloned using this technique. Because the insertion nearly always disrupts the gene function, creating a null mutation, this method is not useful for cloning essential genes. However, insertion frequencies are sufficiently high that the method is a very efficient means of obtaining populations of mutant cells to screen for specific phenotypes, e.g. loss of motility or photosynthetic function (128).

Insertional mutagenesis has usually been accomplished by transformation of a mutant deficient in either nitrate reductase (\textit{nit1}) or argininosuccinate lyase (\textit{arg7}) with the corresponding wild-type gene. Transformants are selected by restoration of ability to grow on nitrate as sole N source (\textit{NIT1}), or on minimal medium (\textit{ARG7}), and can then be screened for other phenotypes resulting from nonhomologous insertion of the transforming DNA.
Transposon tagging of *Chlamydomonas* genes has also been used as a cloning strategy, although less extensively to date than *ARG7* or *NIT1* insertions. Transposable elements characterized in *C. reinhardtii* include a class I retrotransposon (*TOC*), three class II elements (*Gulliver; Pioneer*, and *TOC2*); and three elements, *Tcr1* through *Tcr3*, which are characterized by inverted terminal repeat sequences [for review, see (236)].

Other constructs that have been used for nuclear transformation include several selectable markers that can confer inhibitor resistance on wild-type cells. The *CRY1* and *ALS* markers are endogenous *Chlamydomonas* genes from mutant cells resistant to emetine and sulfometuron methyl, respectively (JAE Nelson & PA Lefebvre, in 43; 70). Several bacterial antibiotic resistance genes have also been expressed successfully in *Chlamydomonas* (27, 212, 217).

The nuclear transforming constructs used to date generally include a native *C. reinhardtii* promoter and 3′ untranslated region, most commonly from the *RbcS2* gene encoding the small subunit of ribulose bisphosphate carboxylase. Improved expression of nuclear genes has been achieved by including the first intron of *RbcS2* (135). Further enhancement has been obtained with a construct that fuses the promoter of the *HSP70A* (heat shock protein) gene upstream of the promoter and first intron from *RbcS2* (208).

Failure to achieve satisfactory expression of some introduced genes has been attributed to posttranscriptional gene silencing, codon bias, and incomplete promoters, enhancers, or other regulatory sequences. Finally, these problems seem to be yielding to creativity, hard work, and persistence. Cerutti and colleagues (26) have investigated silencing of the bacterial *aadA* gene after transformation into *Chlamydomonas* in a construct where it is flanked by the *RbcS2* 5′ and 3′ non-coding regions. Expression of *aadA* was found to be unstable in approximately half the transformants recovered. Inactivation of the gene was epigenetic, i.e. no alterations were observed in the presence or sequence of the transforming DNA, and the changes were reversible in selected clones transferred to and from selective conditions (spectinomycin). Spectinomycin resistance always cosegregated with the integrated construct in crosses. Direct analysis of *aadA* expression showed that the gene was inactivated at the transcriptional level and that this inactivation was not correlated with cytosine methylation or with accessibility of the integrated construct to restriction enzymes (which would have suggested silencing by chromatin condensation). Insertional mutagenesis has been used to recover suppressors of this epigenetic silencing (243). Although methylation was not implicated in the *aadA* silencing, work with *Volvox* suggests that CpG methylation does have a role in expression of introduced genes in this alga, and by extension may be expected to have significance in *Chlamydomonas* as well (P Babinger, I Kobl, W Mages & R Schmitt, submitted).

*C. reinhardtii* nuclear genes show a pronounced codon bias, a consequence of the GC-rich genome. The search for foreign genes that can be used as selectable markers has led to bacterial genes whose codon usage is similar to that of *Chlamydomonas*. The *ble* gene from *Streptoalloteichus hindustanus*, conferring
phleomycin resistance, and the *aphVIII* gene of *Streptomyces rimosus*, conferring resistance to aminoglycoside antibiotics, were chosen for this reason (212, 217). Expression of the green fluorescent protein gene was improved by resynthesizing it with a codon set more typical of native *C. reinhardtii* genes (67).

The molecular tool kit for *Chlamydomonas* includes several additional constructs useful for analyzing gene expression (also see 128, 134, 216). The arylsulfatase gene (*Ars1*), whose synthesis is induced when cells are starved for sulfur, can be used as a reporter for promoter function (164). The enzyme is assayed using a chromogenic sulfate substrate. Haring & Beck (84) have adapted insertional tagging to create a promoter trap system. Cells of a double mutant, *arg7 pf14*, are transformed with the *Arg7* gene and a promoter-less *Rsp3* gene (radial spoke protein 3, complementing the *pf14* mutation). Transformants are selected for ability to grow on minimal medium, and then screened for restoration of motility resulting from integration of the *Rsp3* gene downstream of an endogenous promoter. The gene whose promoter is now tagged with *Rsp3* can thus be cloned. Auchincloss et al (6) have reported development of a shuttle vector based on the cDNA for the *Arg7* gene that is able to complement both the *C. reinhardtii* *arg7* mutant and the *E. coli* *argH* mutant. Constructs have also been made to permit epitope tagging of cloned genes (e.g. 105).

Although targeted disruption of *Chlamydomonas* nuclear genes by transformation has succeeded only rarely owing to the rarity of homologous insertion (154), RNAi is showing promise as an alternative means of targeting and inactivating expression of specific genes (50, 209). For example, expression of chlamyopsin (the photoreceptor protein) has been studied in constructs driven by the *Hsp70A* and *RbcS2* promoters (M Fuhrmann, S Rank, E Govorunova & P Hegemann, in preparation). When the complete *Cop* gene encoding this protein, including introns, is expressed from these promoters, chlamyopsin levels are 5 to 10 times higher than in untransformed cells. Replacing the *Cop* gene with an inverted gene sequence capable of forming a double-stranded RNA structure reduced expression to as little as 10% of the wild-type level.

Fortunately, lack of directed homologous integration has not prevented rescue of mutants by transformation with cosmid or BAC clones derived from *C. reinhardtii* (71, 74, 189), and this technique is now becoming routine. In a variation of this technique suitable for genes with no selectable phenotype, Purton & Rochaix (180) prepared a cosmid library in a vector carrying the wild-type *Arg7* DNA. Arginine-independent transformants were selected and then screened for complementation of the original mutation. Rescue of mutant phenotypes by genes from other organisms may become feasible as better expression of heterologous genes is achieved.

A BAC library (http://www.biology.duke.edu/chlamy/ChlamyGen/libraries.html) with approximately 10- to 12-fold coverage of the *Chlamydomonas* genome is commercially available from Incyte Genomics and is being used to generate a molecular map (P Kathir, PA Lefebvre & CD Silflow, in preparation). Positional cloning using individual BAC clones together with this map has already been
successful (CD Silflow & PA Lefebvre, personal communication) and will undoubtedly become more practical as the density of markers on the molecular map increases. As BAC clones are identified that hybridize to molecular markers, they can be used to assemble overlapping contigs that will eventually cover the entire genome.

The current molecular map contains 240 markers, with an average physical spacing of 400 to 500 kb. All molecular markers analyzed to date have been placed on 17 linkage groups, suggesting that this is indeed the definitive number of linkage groups for *C. reinhardtii*. For nearly all of the linkage groups, the molecular and genetic maps can be anchored and oriented to each other by markers in common.

**Sources of Strains and Information**

The Chlamydomonas Genetics Center at Duke University provides cultures of wild-type and mutant strains of *C. reinhardtii*, *C. eugametos*, and *C. moewusii*. The web site is at http://www.biology.duke.edu/chlamy; mailing address c/o Elizabeth H. Harris, DCMB Box 91000, Duke University, Durham, NC 27708-1000. Wild-type strains of other genera are available from several major algal collections worldwide, including Culture Centre of Algae and Protozoa (CCAP; Freshwater Biology Association, The Ferry House, Ambleside, Cumbria, LA22 0LP, UK; http://wiua.nwi.ac.uk/ccap/ccaphome.html); Institute of Applied Microbiology, Tokyo (IAM; The University of Tokyo, 1-1-1 Yayoi, Bunkyou-ku, Tokyo 113, JAPAN); Sammlung von Algenkulturen (SAG; Pflanzen physiologisches Institut, Universität Göttingen, Nikolausberger Weg 18, D-3400 Göttingen, Germany (http://www.gwdg.de/~botanik/phykologia/epsag.html)); University of Texas Algal Collection (UTEX; Department of Botany, Austin, TX 78713-7640, Section of Molecular Cell and Developmental Biology, University of Texas, Austin, TX 78713 (http://www.bio.utexas.edu/research/utex/); University of Toronto Culture Collection (UTCC; Department of Botany, University of Toronto, Toronto, Ontario MSS 3B2, Canada (http://www.botany.utoronto.ca/utcc/). ChlamyDB is a comprehensive database for information on *Chlamydomonas* and other algae in the Volvocales, including publications, sequence citations, gene descriptions, and genetic maps. This is available in a web presentation through the USDA-ARS Center for Bioinformatics and Comparative Genomics at Cornell University (http://arsgenome.cornell.edu/cgi-bin/WebAce/webace?db=chlamydb). Recommendations for nomenclature of Chlamydomonas genetic loci have been published by Dutcher (47) in the *Trends in Genetics Genetic Nomenclature*

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1In early genetic mapping studies, 19 linkage groups were identified and numbered. Subsequent analysis (48) has shown that linkage groups XII and XIII in fact are colinear, as are groups XVII and XVIII. The UNI linkage group, or ULG, previously reported by Ramanis & Luck to be a circular linkage group having a specific association with the basal body (see 235), now appears to be a linear nuclear linkage group like the others and is now designated as group XIX (97).
Guide. The Chlamydomonas Genetics Center coordinates availability of names for loci and mutant alleles; contact Elizabeth Harris (chlamy@duke.edu) for assistance. The bionet.chlamydomonas newsgroup (http://www.bio.net:80/hypermail/CHLAMYDOMONAS/) provides a moderated forum for discussion of Chlamydomonas and other algae. The 10th International Conference on the Cell and Molecular Biology of Chlamydomonas will be held at the University of British Columbia (UBC) Conference Center in Vancouver, BC, Canada, June 11–16, 2002.

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