

Genome-Wide Annotation and Expression Profiling of Cell Cycle Regulatory Genes in *Chlamydomonas reinhardtii*^{1[w]}

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Eukaryotic cell cycles are driven by a set of regulators that have undergone lineage-specific gene loss, duplication, or divergence in different taxa. It is not known to what extent these genomic processes contribute to differences in cell cycle regulatory programs and cell division mechanisms among different taxonomic groups. We have undertaken a genome-wide characterization of the cell cycle genes encoded by *Chlamydomonas reinhardtii*, a unicellular eukaryote that is part of the green algal/land plant clade. Although *Chlamydomonas* cells divide by a noncanonical mechanism termed multiple fission, the cell cycle regulatory proteins from *Chlamydomonas* are remarkably similar to those found in higher plants and metazoans, including the proteins of the RB-E2F pathway that are absent in the fungal kingdom. Unlike in higher plants and vertebrates where cell cycle regulatory genes have undergone extensive duplication, most of the cell cycle regulators in *Chlamydomonas* have not. The relatively small number of cell cycle genes and growing molecular genetic toolkit position *Chlamydomonas* to become an important model for higher plant and metazoan cell cycles.

It is well established that eukaryotic cell cycles are controlled by a conserved set of proteins. Central among these are cyclin-dependent kinases (CDKs; Nasmyth, 1996; Sherr, 1996; Johnson and Walker, 1999; Mironov et al., 1999; Pavletich, 1999). CDKs are subject to multiple regulatory mechanisms that cause their activity and substrate specificity to oscillate and thereby drive cell cycle phase transitions. These regulatory mechanisms include binding of cyclins that are obligatory activating subunits of CDKs, binding of inhibitory proteins, and activating or inhibitory phosphorylation (Morgan, 1995). Research over the past two decades has highlighted the remarkable conservation of these mechanisms, indicating that eukaryotes share a fundamentally conserved cell cycle (Morgan, 1997; Obaya and Sedivy, 2002; De Veylder et al., 2003; Dewitte and Murray, 2003).

Despite the unity in underlying principles of cell cycle regulation, there are some important differences that remain to be elucidated. First, cell cycles that deviate in some way from the best characterized

G1-S-G2-M progression occur in some unicellular eukaryotes and are essential for the proper development of most multicellular eukaryotes, but the mechanisms required to generate altered cell cycles are not fully understood (Joubes and Chevalier, 2000; Moser and Russell, 2000; Schwab et al., 2000; Edgar and Orr-Weaver, 2001; Coffman, 2004). Second, the mechanics of cell division in different taxa vary considerably, particularly the mechanisms used for cytokinesis (Guertin et al., 2002). It is not known to what extent CDKs became adapted to their new substrates, or vice versa, as separate division mechanisms evolved. Third, it is not clear why multicellular eukaryotes, such as plants and animals, contain multiple CDKs, whereas in budding yeast (*Saccharomyces cerevisiae*) and fission yeast (*Schizosaccharomyces pombe*), a single CDK is sufficient to drive the cell cycle. Until a broader spectrum of unicellular eukaryotes is examined, this question cannot be addressed.

The cell cycle regulators from the sequenced genomes of animals, plants, and fungi show distinct patterns of divergence, duplication, and gene loss. Higher plants are interesting because the CDK and cyclin family proteins have duplicated and diverged in them, thus giving rise to a novel CDK, CDKB (Mironov et al., 1999; Joubes et al., 2000; Vandepoele et al., 2002), and several new cyclin families, including a plant-specific D-type cyclin family that appears to be functionally related to the animal D-cyclins (Meijer and Murray, 2000; Oakenfull et al., 2002). Unlike the fungi that lack the retinoblastoma (RB) pathway, homologs of RB and its binding partners E2F/DP are found in higher plants and probably function in a similar manner to those of animals (Xie et al., 1996;

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Huntley et al., 1998; Ramirez-Parra et al., 1999; Albani et al., 2000; Boniotti and Gutierrez, 2001; Mariconti et al., 2002). The extensive duplications within higher plant cell cycle gene families have complicated the task of reverse genetics. It is therefore of great interest to determine the extent to which cell cycle regulators in higher plants are conserved in simpler representatives of the plant kingdom.

Chlamydomonas reinhardtii is a chlorophyte alga that has served as a model for plant cell biology and physiology (Goodenough, 1992; Rochaix, 1995; Gutman and Niyogi, 2004), and whose genome has recently been sequenced (<http://genome.jgi-psf.org/chlre2/chlre2.home.html>). Cell division in *Chlamydomonas* occurs by a noncanonical mechanism, termed multiple fission (Fig. 1; Setlik and Zachleder, 1984; Donnan et al., 1985; John, 1987). The multiple fission cell cycle is characterized by a prolonged G1 period during which cells may grow to many times their original size. This growth phase is followed by a rapid series of (n) alternating S (DNA synthesis) and M (mitotic) phases (Coleman, 1982), producing 2^n daughter cells of uniform size. Size homeostasis is maintained by two related mechanisms. A mitotic sizer governs mother cell division number (n) so that daughter cell size is similar regardless of mother cell size. A second sizer operates during early G1 to control passage through Commitment (Donnan and John, 1983, 1984), a cell cycle control point that is conceptually similar to Start in yeasts or the Restriction Point in animal cells (John, 1984, 1987). Cells that have passed Commitment will divide at least one time during the S/M phase. A major difference between Commitment and Start/Restriction is that, after passage through Commitment, *Chlamydomonas* cells remain in G1 for an additional 5 to 8 h, whether or not they continue to grow, and only begin the S/M phase after this delay. Under physiological conditions of alternating light and dark periods, *Chlamydomonas* cells become highly syn-

chronized so that growth occurs during the light period and S/M occurs during a brief interval in the dark period (Fig. 2).

Given its specialized cell cycle, its unicellular life-style, and its estimated divergence time from higher plants (*Chlamydomonas* and *Arabidopsis* [*Arabidopsis thaliana*] may have shared a common ancestor approximately 1.1 billion years ago [Hedges, 2002]), it might be expected that the cell cycle genes in *Chlamydomonas* would have diverged considerably from those in higher plants and other eukaryotes. In this article, we have identified and profiled the expression of the core cell cycle regulators from *Chlamydomonas*. Contrary to our initial expectation, we have found that *Chlamydomonas* encodes orthologs of the major plant CDK and cyclin families. *Chlamydomonas* also encodes two CDK subtypes and two cyclin subtypes that are not found in higher plants, fungi, or animals. Besides cyclins and CDKs, we have found orthologs of *wee1* kinase, a negative regulator of CDKs, *CKS1/suc1*, a CDK subunit, and potential *CDC25* homologs. As expected from the presence of an RB-related gene, *MAT3* (Umen and Goodenough, 2001), we have also identified *Chlamydomonas* E2F and DP orthologs. Unlike higher plants and animals, most of the core cell cycle regulatory genes in *Chlamydomonas* are present in single copy. We discuss these results in light of their evolutionary implications and with respect to *Chlamydomonas* as a model for higher plant and metazoan cell cycles.

RESULTS

Annotation Strategy

The gene families for which we carried out comprehensive searches were CDK, cyclin, RB-related, E2F/DP, *CKS1/suc1*, *wee1*, *CDC25*, and CDK inhibitors (CKIs). We first made use of automated annotations based on genome-wide BLAST searches carried out as part of the *Chlamydomonas* genome project and available on the genome Web site (<http://genome.jgi-psf.org/chlre2/chlre2.home.html>). This initial search yielded a few high-scoring positives but mostly low-scoring or misannotated genes. A more productive strategy involved the use of low-stringency reciprocal BLAST searches with representatives from each gene class to query the conceptually translated *Chlamydomonas* genome sequence: Typically, one or more BLAST hits from *Chlamydomonas* could be found, and then the BLAST search was repeated with the *Chlamydomonas* candidate genes against the National Center for Biotechnology Information (NCBI) nonredundant protein database and against the *Chlamydomonas* genome to identify potential duplications or additional family members. When possible, gene model and expressed sequence tag (EST) evidence were used to assemble predicted coding regions in order to improve the search. Reverse transcription

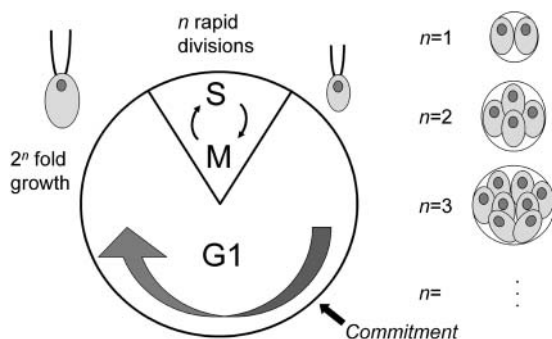


Figure 1. Diagram of the multiple fission cell cycle. A clock-type diagram depicts the phases of the *Chlamydomonas* cell cycle. Most of the cycle is spent in G1, which is divided into two periods demarcated by the Commitment point (see text for details). At the end of G1, a rapid series of alternating S and M cycles generates 2^n daughter cells of uniform size. The value of n is variable and is related to mother cell size. Postmitotic mother cells with different values of n are depicted to the right with 2, 4, 8, etc., daughters.

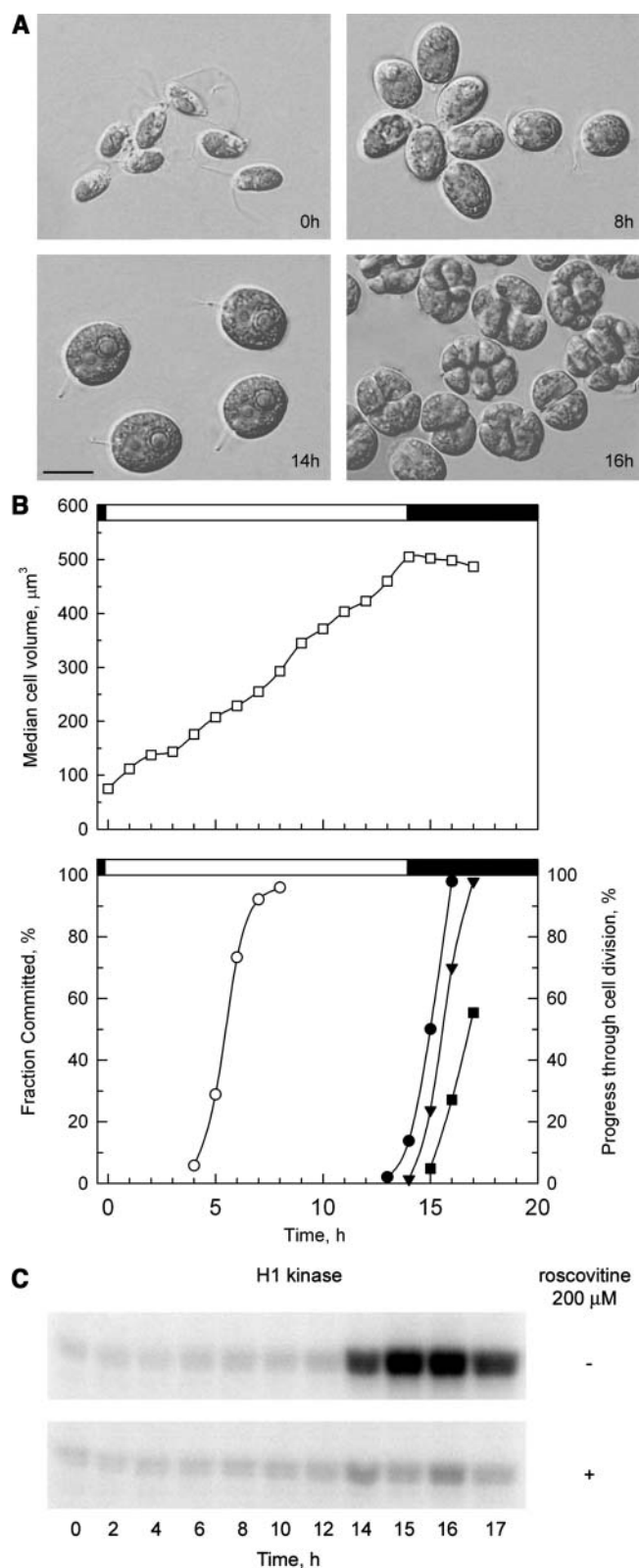


Figure 2. Synchronization of the cell cycle with alternating light and dark periods. A, Photomicrographs of cells at different time points with a scale bar in the bottom left corresponding to 10 μm . B, Graph of synchronized cultures. The top image depicts cell size (white squares). The bottom image depicts the fraction of cells that have passed

(RT)-PCR was used in some cases to confirm gene models and to test for expression. Using these strategies, we found that a reciprocal orthology relationship could be established for most of the cell cycle genes. For example, the *CDKB* genes from Arabidopsis show the highest similarity to a single gene model in Chlamydomonas, and that gene model in Chlamydomonas shows the highest degree of similarity to the *CDKB* genes from higher plants (Fig. 3; Table I). To complement this approach, we also carried out BLAST searches against the conceptually translated Chlamydomonas EST database that includes more than 100,000 sequences. No cell cycle genes were identified in our EST search that had not already been identified in the sequenced genome, suggesting that our search is essentially complete. Low-stringency Southern blots of several genes were also used to confirm that the key cell cycle regulators are single copy (Supplemental Fig. 1). Phylogenetic analyses were carried out to more objectively place each family member within its proper clade. The results from individual gene families are discussed below, and the results of our annotation are summarized in Table I. For a more complete description of gene models and annotation evidence, see Supplemental Table I.

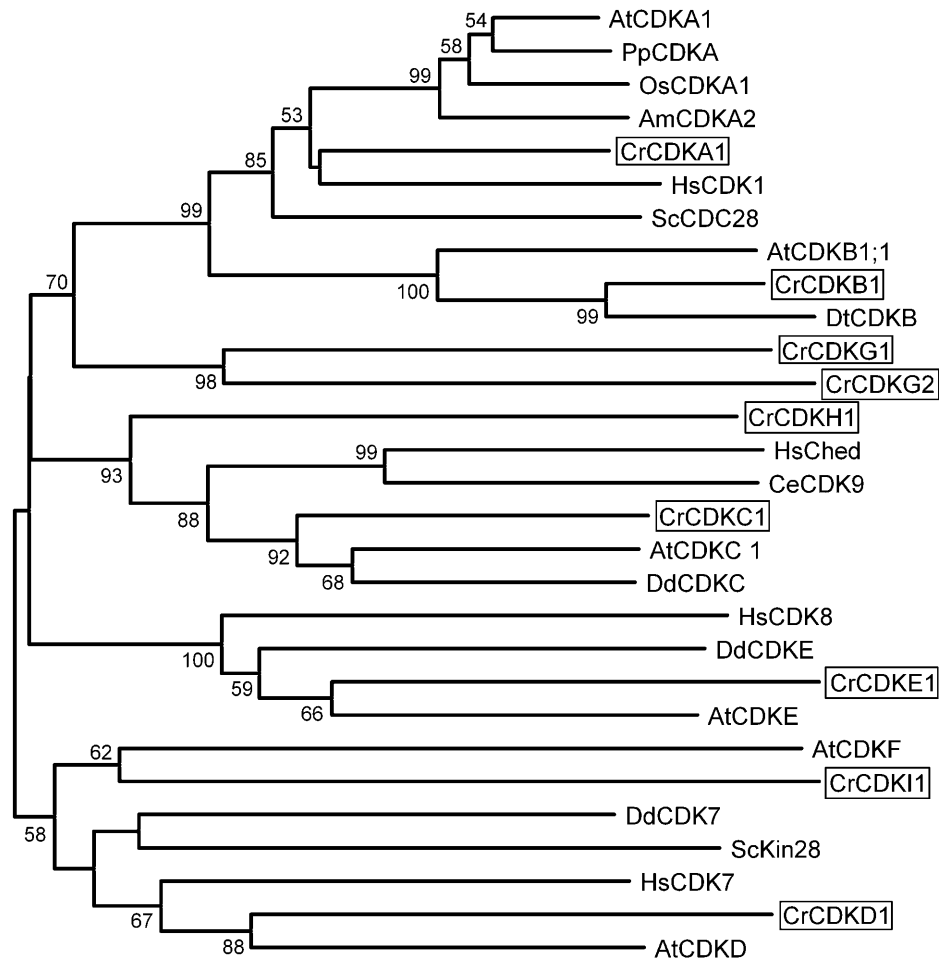
Synchronization and Expression Profiling

A major advantage of using Chlamydomonas to investigate cell cycle regulation is the ease with which cultures can be synchronized under physiological conditions. By growing cells phototrophically in alternating periods of light and dark, we were able to synchronize our cultures so that they passed Commitment during the middle of the light period, entered the S/M phase of the cell cycle at the end of the light period, and completed division during the dark period (Fig. 2).

We used semiquantitative RT-PCR to examine the expression pattern of a subset of the genes predicted to be cell cycle regulators. Expression levels for each gene were determined in RNA samples prepared from synchronous cultures. The cultures from which RNA was prepared were simultaneously monitored for cell size (Fig. 2, A and B), passage through Commitment (Fig. 2B), passage through mitosis (Fig. 2, A and B), and histone H1 kinase activity (Fig. 2C). Besides the annotated cell cycle regulatory genes, we also examined expression of some genes encoding proteins required for S phase that were expected to be cell cycle regulated.

Commitment (white circles), and progression through cell division with mother cells that have undergone one (black circles), two (black triangles), and three (black squares) rounds of division. Division was complete by 17 h with approximately 60% of the cells dividing three times into eight daughters and approximately 40% dividing twice into four daughters. The light-dark phasing is indicated by the white or black bars above the graphs. C, CKS1-purified histone H1 kinase activity assayed from extracts prepared at different time points. Each image shows an autoradiograph indicating the extent of histone H1 phosphorylation in the presence and absence of the CKI roscovitine.

Figure 3. Neighbor-joining tree of CDKs. Bootstrap values of 50% or higher are shown for each clade. Am, *Antirrhinum majus*; At, *Arabidopsis*; Cr, *C. reinhardtii*; Ce, *Caenorhabditis elegans*; Dd, *Dictyostelium discoideum*; Dt, *Dunaliella tertiolecta*; Hs, *Homo sapiens*; Os, *Oryza sativa*; Sc, budding yeast; Sp, fission yeast; Pp, *Physcomitrella patens*. See Supplemental Table II for GenBank accession numbers.



CDK Family

CDKs are Ser-Thr kinases that function in cell cycle regulation and in other processes such as transcription. The most widely conserved CDKs possess a canonical PSTAIRE motif in the C-helix (De Bondt et al., 1993). In both fission and budding yeast, a single PSTAIRE CDK is sufficient to regulate all the cell cycle phases (Mendenhall and Hodge, 1998; Moser and Russell, 2000). By contrast, metazoans and plants encode several variant CDKs with functions in cell cycle regulation. In humans, there are three PSTAIRE CDKs (CDK1/*cdc2*, CDK2, and CDK3) and a variant CDK4/6 subfamily with a P(I/L)ST(V/D)RE motif, all of which function in cell cycle regulation (Meyerson et al., 1992; Pines, 1995; Reed, 1997; Lee and Yang, 2003). Higher plants encode only one PSTAIRE CDK, designated CDKA, that can functionally substitute for its yeast orthologs, *cdc2*/CDC28 (Ferreira et al., 1991; Hirt et al., 1991). Plant CDKs C, D, and E are variants that have metazoan counterparts CDK9, CDK7, and CDK8, respectively (Mironov et al., 1999; Joubes et al., 2000; Vandepoele et al., 2002). Plant-specific kinase CDKB has been found in all plants and CDKF only in *Arabidopsis*. B-type CDKs are expressed during the

G2/M transition when they are thought to function (Mironov et al., 1999; Porceddu et al., 2001; Lee et al., 2003; Boudolf et al., 2004). D- and F-type CDKs are CDK-activating kinases (CAK) that serve to activate A-type CDKs (Umeda et al., 1998; Yamaguchi et al., 1998, 2000; Shimotohno et al., 2003). CDKD binds cyclin H to form a CAK complex (Yamaguchi et al., 2000), while CDKF, a distant relative of CDKD, is able to function without any binding partner. Animal CDK8 and CDK9, as well as plant C-type CDKs, have been implicated in transcriptional control (Oelgeschlager, 2002; Barroco et al., 2003), and no function has yet been determined for E-type CDKs in plants (Magyar et al., 1997).

Chlamydomonas encodes a single ortholog for each of the plant-type CDKs A, B, C, D, and E (genes were designated *CDKA1*, *B1*, *C1*, *D1*, and *E1*, respectively) but does not encode an F-type CDK (Fig. 3; Table I; Supplemental Fig. 1). In addition, *Chlamydomonas* encodes four novel members of the CDK family that are not orthologous to any known CDKs in plants or other CDKs in databases. Two of these novel CDKs encoded by genes designated *CDKG1* and *CDKG2* are related to each other, but are significantly diverged in their predicted C-helices (SDSTIRE and AASTLRE,

Table 1. Summary of annotated cell cycle genes in *Chlamydomonas*

Gene model names are according to *Chlamydomonas* genome version 2 (<http://genome.jgi-psf.org/chlre2/chlre2.home.html>). na, Not applicable; Yes, successful amplification and subcloning of RT-PCR fragment; No, amplification was tried but was not successful; nd, RT-PCR was not done.

Chlamydomonas Gene Model	Gene Name	Protein Family	Protein Subfamily	Signature Motif	Putative Function(s)	EST	RT-PCR ²
C_1630009	<i>CDKA1</i>	CDK	CDKA	PSTAIR	G1/S, G2/M	Yes	Yes
C_1340024	<i>CDKB1</i>	CDK	CDKB	PSTTLRE	G2/M	Yes	Yes
C_1730005	<i>CDKC1</i>	CDK	CDKC/CDK9	PITAIR	Unknown	Yes	Yes
C_700049	<i>CDKD1</i>	CDK	CDKD/CDK7	DPTLARE	CAK	Yes	Yes
C_1700010/11	<i>CDKE1</i>	CDK	CDKE/CDK8	SPTAIR	Unknown	No	Yes
C_270112	<i>CDKG1</i>	CDK	Novel	SDSTIRE	Unknown	Yes	Yes
C_1020028	<i>CDKG2</i>	CDK	Novel	AASTLRE	Unknown	Yes	No
C_980026	<i>CDKH1</i>	CDK	Novel	PVSTIRE	Unknown	No	Yes
C_740039	<i>CDK11</i>	CDK	Novel	PDVVVRE	Unknown	Yes	nd
C_120118	<i>CYCA1</i>	Cyclin	A	<u>LVEVSEEY</u>	S/G2/M	Yes	Yes
C_1420018	<i>CYCB1</i>	Cyclin	B	<u>HLKF</u>	G2/M	Yes	Yes
C_460084	<i>CYCC1</i>	Cyclin	C		Transcription	No	No
C_140186	<i>CYCD1</i>	Cyclin	D		G1/S	No	No
C_290120	<i>CYCD2</i>	Cyclin	D	<u>LQCDE</u>	G1/S	Yes	Yes
C_1460039	<i>CYCD3</i>	Cyclin	D	<u>LFCGE</u>	G1/S	Yes	Yes
C_570097	<i>CYCL1</i>	Cyclin	L		Unknown	Yes	nd
C_320095	<i>CYCM1</i>	Cyclin	Novel		Unknown	Yes	nd
C_660038	<i>CYCU1</i>	Cyclin	U		Unknown	Yes	nd
C_1740016	<i>CYCT1</i>	Cyclin	T		Unknown	No	nd
C_1630021	<i>CYAB1</i>	Cyclin	Novel A/B related		Unknown	No	Yes
C_1840020	<i>MAT3</i>	RBR	na		G1/S, S/M	Yes	Yes
C_180138	<i>E2F1</i>	E2F	E2F		G1/S, S/M	No	Yes
C_570078	<i>DP1</i>	E2F	DP		G1/S, S/M	Yes	Yes
C_210071	<i>E2FR1</i>	E2F	E2F?		Unknown	No	No
C_980002	<i>WEE1</i>	WEE1	na		G2/M	Yes	Yes
None ^a	<i>CKS1</i>	CKS1	na		CDK subunit	Yes	Yes
C_980016	<i>RDP1</i>	cdc25-like	na	<u>HCHGSKVRGP</u>	G2/M	No	Yes
C_410005	<i>RDP2</i>	cdc25-like	na	<u>HCMFSQQRGP</u>	G2/M	No	Yes
C_100077	<i>RDP3</i>	cdc25-like	na	<u>HCHFSSKVRGP</u>	G2/M	Yes	nd

^aGene is located between gene models C_860084 and C_860085.

respectively). The third locus that we have designated *CDKH1* encodes a protein with a PVSTIRE motif and forms a sister group with the CDKC family (Fig. 3). However, *CDKH1* is more distantly related to *Chlamydomonas* *CDKC1* than are the plant, metazoan, and slime mold *CDKC* orthologs, indicating that its duplication preceded the divergence of these taxa. The fourth CDK, encoded by the *CDK11* gene, is the most diverged CDK that we identified and is very distantly related to *Arabidopsis* *CDKF*, but it does not appear to be a *CDKF* ortholog. It does not contain the N-terminal insertion that is characteristic of *CDKF*, and it does not show a reciprocal best-hit relationship with *CDKF* in BLAST searches.

The expression profiles of *Chlamydomonas* CDKs are similar to those of their plant counterparts. mRNA for *CDKA1* was present constitutively during the cell cycle with expression increasing as cells entered the growth phase at the beginning of the light period and increasing further around the time of S/M phase (Fig. 4). Abundance of presumed *Chlamydomonas* CDKA protein (reacting with anti-PSTAIR antibody) was relatively constant during the cell cycle with phosphorylation-induced isoforms appearing during

S/M phase (John et al., 1989). Higher plant CDKA message and protein levels are also relatively constant during the cell cycle (Martinez et al., 1992; Hemerly et al., 1993; Magyar et al., 1997; Richard et al., 2001; Sorrell et al., 2001; Menges et al., 2003). mRNA for *CDKB1* shows two peaks of expression, one corresponding to passage through commitment and a second, very strong peak during S/M phase (Fig. 4). Elevated expression at the time of mitosis was described for other members of the CDKB subfamily in plants (Magyar et al., 1997; Richard et al., 2001; Sorrell et al., 2001; Menges et al., 2002; Menges and Murray, 2002) and is consistent with a role for CDKB in the regulation of the G2/M transition (Porceddu et al., 2001; Lee et al., 2003; Boudolf et al., 2004).

CDKC1 and *E1* are expressed constitutively as are their orthologs in higher plants and in animals (De Luca et al., 1997; Magyar et al., 1997; Garriga et al., 1998). The *Chlamydomonas*-specific CDKs, *G1* and *H1*, are also expressed constitutively at a low level, with *CDKG1* message levels rising somewhat after Commitment (Fig. 4). Although we were able to amplify *CDKD1*, its abundance appears to be extremely low, and the variability of amplification prevents us

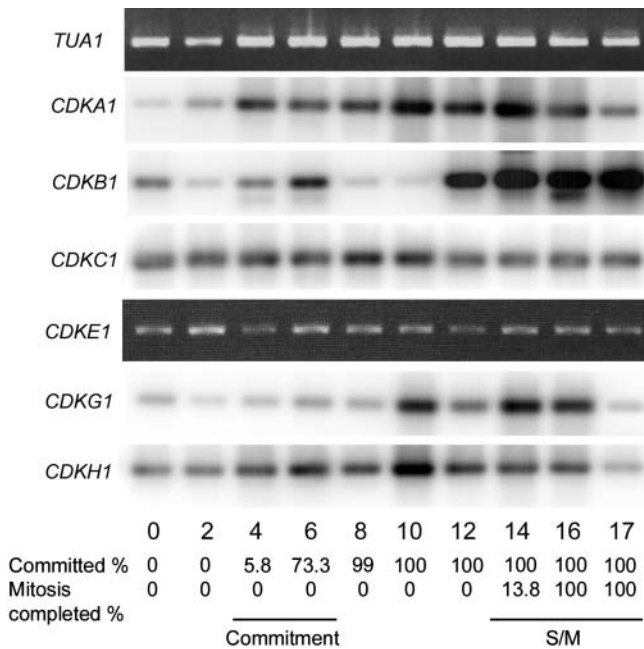


Figure 4. Expression of *Chlamydomonas* CDK homologs. RT-PCR products were detected by Southern blotting (*CDKA1*, *B1*, *C1*, *G1*, *H1*) or ethidium bromide staining (*CDKE1*). Fraction of cells that had passed Commitment and completed cell division when the sample was prepared is indicated at the bottom. α -Tubulin transcript (*TUA1*) detected by ethidium bromide staining serves as an internal control.

from drawing any conclusions about its expression pattern during the cell cycle. We have not detected expression of *CDKG2* by RT-PCR, but there is a single EST that corresponds to the predicted *CDKG2* mRNA (Supplemental Table I). We have not examined expression of *CDK11*, but there is EST evidence supporting its expression (Supplemental Table I).

Cyclin Family

Cyclins bind CDKs, and formation of cyclin-CDK heterodimers is essential for kinase activity. In plants and animals, there are three main classes of cyclins that regulate the cell cycle. D-type cyclins function in G₁, A-type cyclins function in S phase, and B-type cyclins function in mitosis. The plant A and B cyclins have orthologs in animals, while the D-type cyclins from plants and animals are divergent (for review, see Renaudin et al., 1996; Murray, 2004). However, the D-cyclins of plants and animals share some structural similarities in that they contain a conserved N-terminal LXCXE motif. The LXCXE peptide binds to the pocket region of RB-related proteins (RBRs) and targets RBRs for CDK phosphorylation. In animals, D-cyclins associate with CDK4/6, whereas in plants they associate with CDKA (Xiong et al., 1992; Bates et al., 1994; Meyerson and Harlow, 1994; Nakagami et al., 1999; Boniotti and Gutierrez, 2001; Healy et al., 2001).

We identified a single *Chlamydomonas* ortholog for each of the A- and B-type cyclins, designated *CYCA1*

and *CYCB1*, respectively, and three D-type cyclins, *CYCD1*, *CYCD2*, and *CYCD3* (Fig. 5; Table I; Supplemental Fig. 1). Both *CYCA1* and *CYCB1* have a destruction box (D-box) in their N-terminal domains (RAMLGDLTN and RRALGDLSN, respectively; Supplemental Table I). The D-box is a characteristic feature of A- and B-type cyclins that targets their degradation by ubiquitin-dependent proteolysis (Glotzer et al., 1991). The three *Chlamydomonas* D-type cyclin homologs cluster with the plant D-type cyclin group but are not closely related to any of the higher plant D-type cyclin subtypes, indicating that the *Chlamydomonas* D-type cyclin family probably arose as a result of lineage-specific gene duplication. Both *CYCD2* and *CYCD3* have a LXCXE motif in their N-terminal domains that supports their classification as D-type cyclins. *CYCD1* does not have an LXCXE motif in its N-terminal domain, but it is possible that this is due to misannotation. There is an LXCXE coding sequence approximately 500 bp upstream from the annotated start for *CYCD1* that is not part of a predicted exon; however, we have not yet been able to determine whether *CYCD1* is expressed and therefore have no information on its putative mRNA structure.

There is a novel cyclin in *Chlamydomonas* that we have designated *CYCA1* (Fig. 5; Table I; Supplemental Fig. 1). This novel cyclin does not have signature motifs that place it definitively within the A or B cyclin families, but its closest homologs are in the cyclin A and cyclin B clades. Like A and B cyclins, *CYCA1* has a D-box motif (RATLVDWLSE) in its N-terminal domain, indicating that it may undergo destruction via the ubiquitination pathway.

Poorly characterized groups of cyclins include L, T, C, and H families from plants and animals, and some divergent groups of plant cyclins designated SOLO DANCERS (Azumi et al., 2002), J (Abrahams et al., 2001), and either U (Wang et al., 2004) or P (Torres Acosta et al., 2004). Some of the U/P family members can bind CDKA in a yeast two-hybrid assay (Torres Acosta et al., 2004), but there is no direct evidence for their cell cycle involvement. Cyclin C can bind CDK3 and direct phosphorylation of pRb in humans (Ren and Rollins, 2004), and cyclin H forms a CAK complex with CDK7/CDKD (animals/plants; Fisher and Morgan, 1994; Makela et al., 1994; Yamaguchi et al., 2000). *Chlamydomonas* encodes one representative of each of the L, T, C, and U/P classes that we have designated *CYCL1*, *CYCT1*, *CYCC1*, and *CYCU1*, respectively (Fig. 6). We have not identified an H-type cyclin, the significance of which is discussed below. *Chlamydomonas* encodes one highly divergent cyclin that does not appear to be related to any previously characterized group and has been designated *CYCM1* (Fig. 6).

The expression profiles of both *CYCA1* and *CYCB1* resemble that of *CDKB1* with elevated levels of mRNA appearing at Commitment and during S/M phase (Fig. 7). Whereas the expression during S/M phase is expected based on the predicted function of these

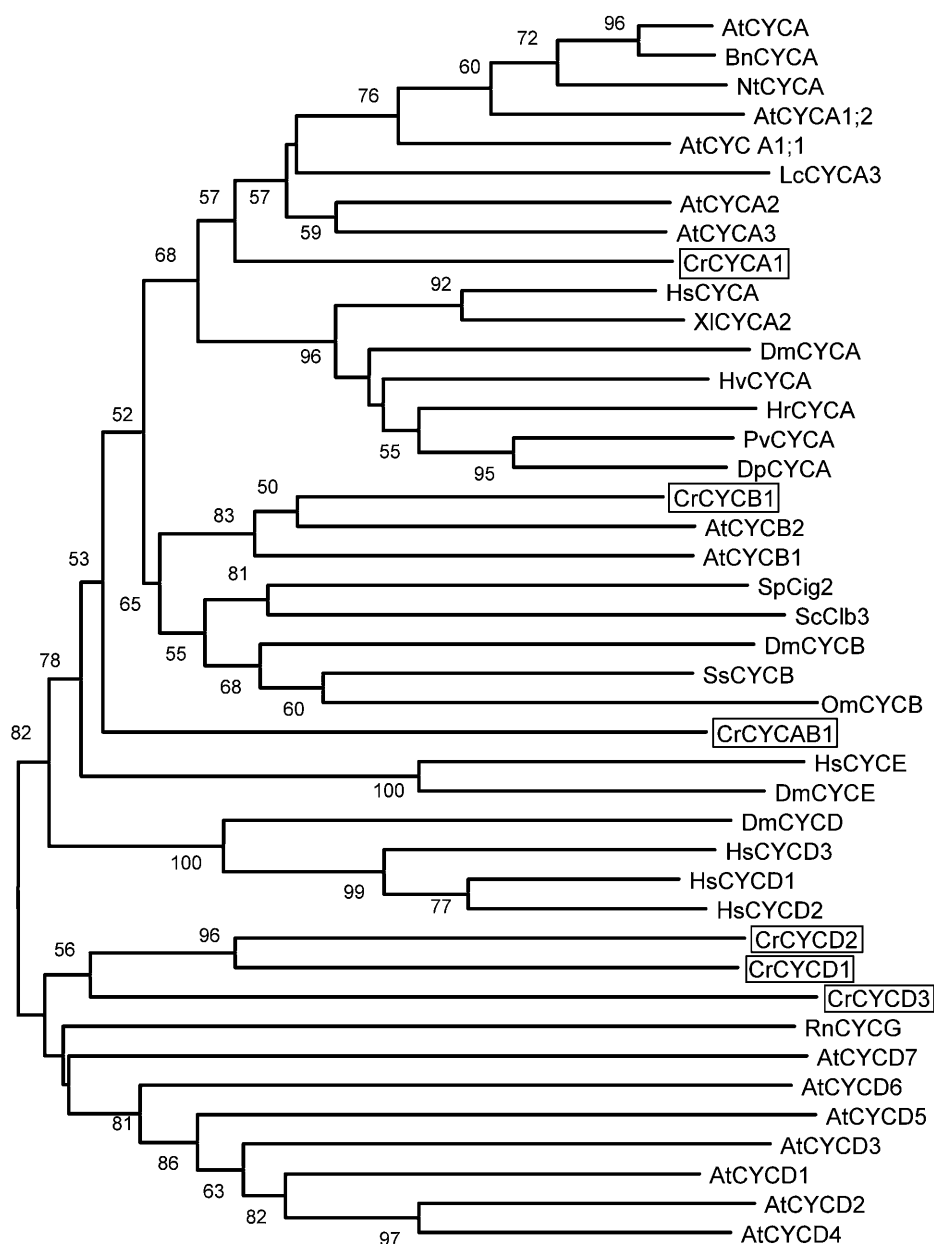


Figure 5. Neighbor-joining tree of A-, B-, and D-cyclin groups. Bootstrap values of 50% or higher are shown for each clade. At, *Arabidopsis*; Cr, *Chlamydomonas reinhardtii*; Bn, *Brassica napus*; Dp, *Dreissena polymorpha*; Dm, *Drosophila melanogaster*; Hv, *Hydra viridis*; Hr, *Helobdella robusta*; Hs, *Homo sapiens*; Lc, *Lycopersicon esculentum*; Nt, *Nicotiana tabacum*; Om, *Oncomorhynchus mykiss*; Os, *Oryza sativa*; Pv, *Patella vulgata*; Rn, *Rattus norvegicus*; Sc, budding yeast; Sp, fission yeast; Ss, *Spisula solidissima*; Xl, *Xenopus laevis*. See Supplemental Table II for GenBank accession numbers.

genes, the early G1 increase and then suppression of expression after Commitment was not anticipated. This G1 expression pattern may be related to the cell cycle regulatory mechanisms used by *Chlamydomonas* (see "Discussion"). We analyzed expression of *CYCD2* and *CYCD3* but were unable to amplify *CYCD1*. *CYCD2* and *CYCD3* are expressed constitutively during the entire cell cycle (Fig. 7), as has been observed for some plant and animal D-cyclins (Fuerst et al., 1996; Sorrell et al., 1999; Riou-Khamlichi et al., 2000; Menges and Murray, 2002; Sa et al., 2002; Sherr, 2002). *CYCAB1* expression was difficult to detect, suggesting that the mRNA may be very low in abundance. *CYCAB1* cDNA could only be amplified from S/M phase samples (Fig. 7).

We have not looked at expression of any of divergent *Chlamydomonas* cyclins, but EST evidence indicates that *CYCL1*, *CYCM1*, and *CYCU1* are expressed (Supplemental Table I).

CDK/Cyclin-Binding and Regulatory Proteins

CKS (cyclin-dependent kinase subunit) proteins are orthologs of the fission yeast protein *suc1*^{P13} that mediate interactions of CDK with their substrates and with other regulatory proteins (Endicott and Nurse, 1995). Budding yeast CKS was recently shown to be involved in promoting mitosis by activating the APC ubiquitin ligase (Morris et al., 2003). CKS proteins are also widely used as a means to purify CDKs.

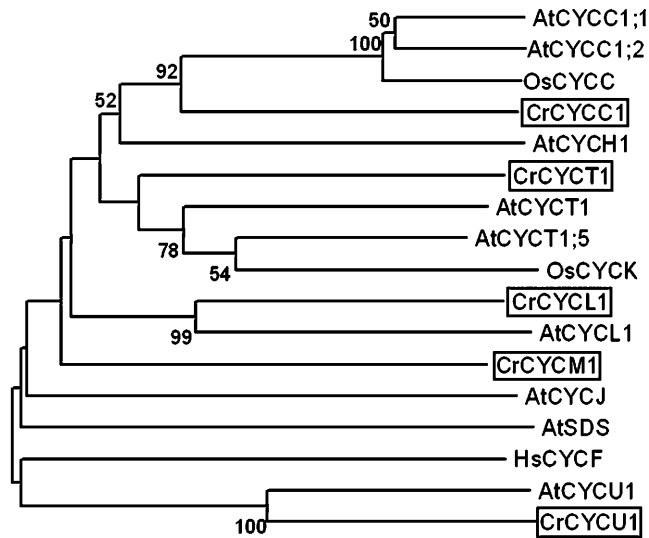


Figure 6. Neighbor-joining tree of divergent cyclin groups (C, H, L, T, J, F, U, SDS). Bootstrap values of 50% or higher are shown for each clade. At, Arabidopsis; Cr, *Chlamydomonas reinhardtii*; Hs, *Homo sapiens*; Os, *Oryza sativa*. See Supplemental Table II for GenBank accession numbers.

Chlamydomonas has a single CKS ortholog, encoded by the *CKS1* gene (Fig. 8), whose expression is constitutive during the cell cycle (Fig. 13). The presence of this protein was previously inferred from the existence of a *Chlamydomonas* protein that cross-reacted with antibodies specific for fission yeast *suc1^{P13}* (John et al., 1991). We used recombinant *Chlamydomonas* CKS1 to purify complexes of CDKs from crude protein extracts. The CKS1-purified CDK activity peaked during S/M phase and displayed sensitivity to the CKI roscovitine (Fig. 2C).

CDK/cyclin complexes are negatively regulated by *wee1* kinases that phosphorylate a conserved Tyr residue of the CDK subunit. This negative regulation is necessary for the proper timing of mitosis (Gould and Nurse, 1989; Jin et al., 1996). *Chlamydomonas* has a single *WEE1* ortholog encoded by the *WEE1* gene (Fig. 9), whose expression is up-regulated during S/M phase but not detectable at earlier stages (Fig. 13).

The *CDC25* genes encode dual-specificity phosphatases that activate CDKs by opposing the activity of *wee1* family kinases (Russell and Nurse, 1986; Kumagai and Dunphy, 1991). *Ostreococcus tauri* was found to encode a functional *CDC25* that shows a close match to the *CDC25* consensus indicating that the gene is present in the green algal/land plant lineage (Khadaroo et al., 2004), but no obvious *CDC25*-encoding genes are present in the *Chlamydomonas* or *Arabidopsis* genome sequences. Recently, however, a small *CDC25*-like dual-specificity phosphatase was found in *Arabidopsis*. The *Arabidopsis* *CDC25*-like protein contains a functional phosphatase domain with a rhodanese fold, but not an N-terminal regulatory domain that is typical for fungal and metazoan *CDC25s* (Landrieu et al., 2004). Three predicted proteins in the *Chlamy-*

domonas genome sequence, designated RDP1, RDP2, and RDP3 (rhodanese domain phosphatase), show homology to the conserved rhodanese domains from the *CDC25* family and contain the core catalytic motifs (HCXXSXXRGP and aspartic acid residue) necessary for phosphatase activity (Denu and Dixon, 1995; Fauman and Saper, 1996; Fig. 10B). A closer analysis of RDP1 and RDP2 using the KOG database (Koonin et al., 2004) revealed that these two *Chlamydomonas* proteins are homologous to small phosphatases in fission yeast, *Arabidopsis*, and other plants (data not shown). A phylogenetic analysis indicates that RDP1 to RDP3 are closely related to *CDC25* proteins and form a clade with them (Fig. 10A). We were able to amplify cDNAs for both *RDP1* and *RDP2* and found that transcript levels were constitutive during the cell cycle (data not shown). EST evidence indicates that *RDP3* is also expressed (Supplemental Table I).

We searched for homologs of the conserved CKI family represented by *Cip/Kip* proteins in mammals and *ICK/KRP* proteins in plants (Sherr and Roberts, 1995, 1999; Wang et al., 1997; Stals et al., 2000; De Veylder et al., 2001), but we have yet to identify any likely candidates in *Chlamydomonas*.

RBR, E2F/DP, and S-Phase Genes

RBRs and the E2F/DP family of transcription factors are a central part of the pathway regulating DNA

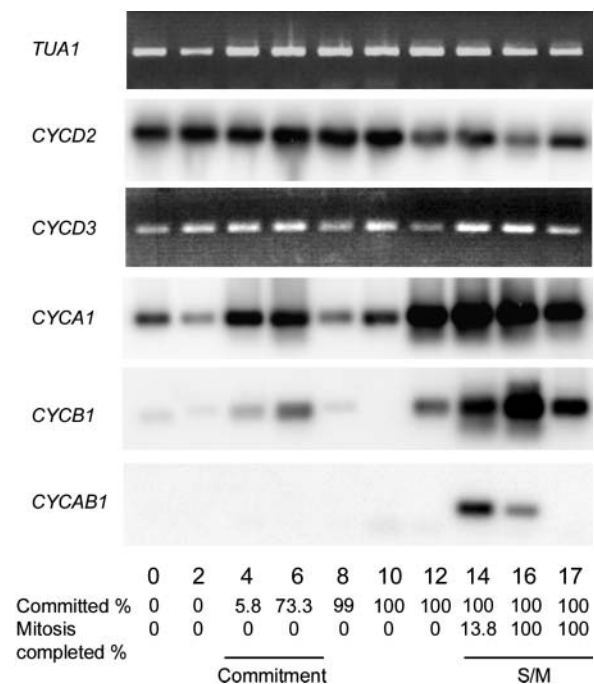


Figure 7. Expression of *Chlamydomonas* cyclins. RT-PCR products were detected by Southern blotting (*CYCD2*, *A1*, *B1*, *AB1*) or ethidium bromide staining (*CYCD3*). Fraction of cells that had passed Commitment and completed cell division when the sample was prepared is indicated at the bottom. α -Tubulin transcript (*TUA1*) detected by ethidium bromide staining serves as an internal control.

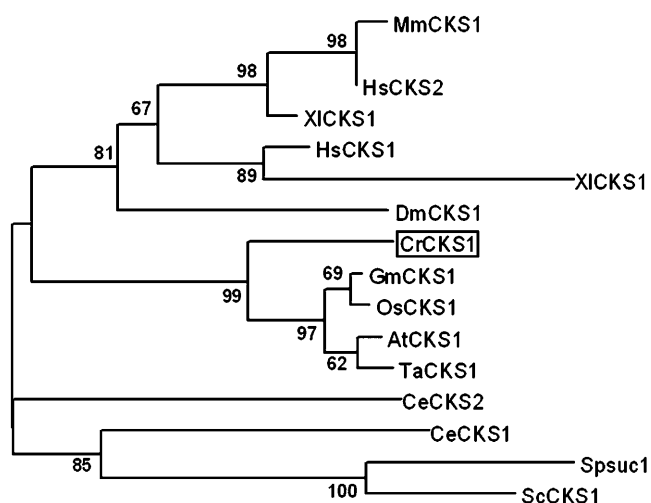


Figure 8. Neighbor-joining tree of CKS1 homologs. Bootstrap values of 50% or higher are shown for each clade. At, Arabidopsis; Cr, *Chlamydomonas reinhardtii*; Ce, *Caenorhabditis elegans*; Dm, *Drosophila melanogaster*; Gm, *Glycine max*; Hs, *Homo sapiens*; Mm, *Mus musculus*; Os, *Oryza sativa*; Sc, budding yeast; Sp, fission yeast; Ta, *Triticum aestivum*; Xi, *Xenopus laevis*. See Supplemental Table II for GenBank accession numbers.

replication in animals and likely play a similar role in plants (Weinberg, 1995; Gutierrez, 1998). E2F and DP form a heterodimeric transcription factor that binds to promoters of many S-phase-regulated genes. These target genes include those necessary for initiation of and progression through S phase. Hypophosphorylated RB binds to E2F/DP complexes at promoters and inhibits transcription. At the G1/S-phase transition, RB is hyperphosphorylated by CDKs and released from promoter complexes, thereby allowing the activation of E2F/DP target genes. Animals and plants encode one or more RBRs and multiple E2F/DP family members (Gutierrez, 1998, 2002; Harbour and Dean, 2000; Classon and Harlow, 2002; De Veylder et al., 2002, 2003; Dewitte and Murray, 2003). E2Fs can serve as transcriptional activators or repressors depending on the presence or absence of an activation domain (Muller and Helin, 2000; Bracken et al., 2004). Another class of E2F/DP-related proteins is represented by DEL (DP-E2F-like) in plants (Kosugi and Ohashi, 2002; Vandepoele et al., 2002) and E2F7 in animals (de Bruin et al., 2003; Di Stefano et al., 2003; Logan et al., 2004). DEL/E2F7 proteins contain a tandem DNA-binding domain and no RBR interaction or transcriptional activation domains. DEL/E2F7 proteins are thus thought to function as transcriptional repressors (Di Stefano et al., 2003; Ramirez-Parra et al., 2004).

Chlamydomonas has a single RBR encoded by the *MAT3* gene (Fig. 11). Loss of *MAT3* leads to deregulated cell size and cell cycle control (Umen and Goodenough, 2001). The *MAT3* message is expressed constitutively during the cell cycle (Fig. 13), and the protein level is also constant (K. Bisova, unpublished data).

In searching the *Chlamydomonas* genome, we found three loci that can encode proteins with DNA-

binding domains related to the E2F/DP family. One of these, designated *E2F1*, encodes an E2F ortholog, and a second one, designated *DP1*, encodes a DP ortholog (Fig. 12A; Supplemental Fig. 1). The third locus, designated *E2FR1*, potentially encodes a protein with partial similarity to E2F/DP-like DNA-binding domains, but with no other domains that are found in the E2F and DP families (Fig. 12B). Interestingly, neither *E2F1* nor *DP1* displays recognizable sequence homology to the conserved C-terminal RBR-binding and transcriptional activation domains present in plant and animal E2Fs. Nonetheless, genetic evidence places both *E2F1* and *DP1* downstream of *MAT3* as activators of cell cycle progression (S.-C. Fang and J.G. Umen, unpublished data). mRNAs for both *E2F1* and *DP1* are present constitutively at low abundance and do not appear to be cell cycle regulated (Fig. 13). The mRNA of *E2FR1* was very low abundance and also present constitutively during the cell cycle (data not shown). We have found no DEL/E2F7 homologs in *Chlamydomonas*.

We examined the expression of several *Chlamydomonas* genes whose plant or animal orthologs are under the control of the RB-E2F pathway and whose expression is up-regulated during S phase (Ramirez-Parra et al., 2003; Bracken et al., 2004). These include ribonucleotide reductase large subunit (*RNR1*), DNA polymerase delta subunit (*POLD1*), DNA primase (*POLA4*), proliferating cell nuclear antigen (*PCNA1*), and minichromosome maintenance protein 2 (*MCM2*). Most of the genes showed an increase in message levels at the time of Commitment. Up-regulation was seen again during the S/M phase, although none of these genes was as strongly regulated as *CYCA1*, *CYCB1*, and *CDKB1* (Fig. 14).

DISCUSSION

This work represents a comprehensive description and expression profiling of cell cycle regulators in the green algae. The only comparable expression and annotation information available for cell cycle regulators from the plant kingdom was from Arabidopsis (Richard et al., 2001; Menges et al., 2002, 2003; Menges and Murray, 2002; Vandepoele et al., 2002; Hennig

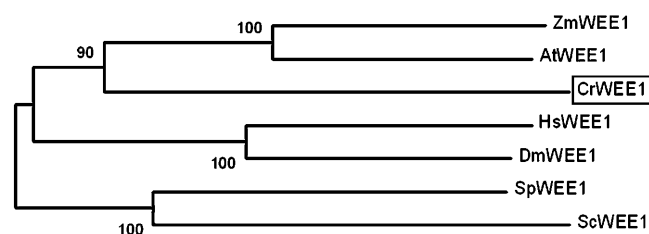


Figure 9. Neighbor-joining tree of *wee1* kinases. Bootstrap values of 50% or higher are shown for each clade. At, Arabidopsis; Cr, *Chlamydomonas reinhardtii*; Dm, *Drosophila melanogaster*; Hs, *Homo sapiens*; Mm, *Mus musculus*; Os, *Oryza sativa*; Sc, budding yeast; Sp, fission yeast; Zm, *Zea mays*. See Supplemental Table II for GenBank accession numbers.

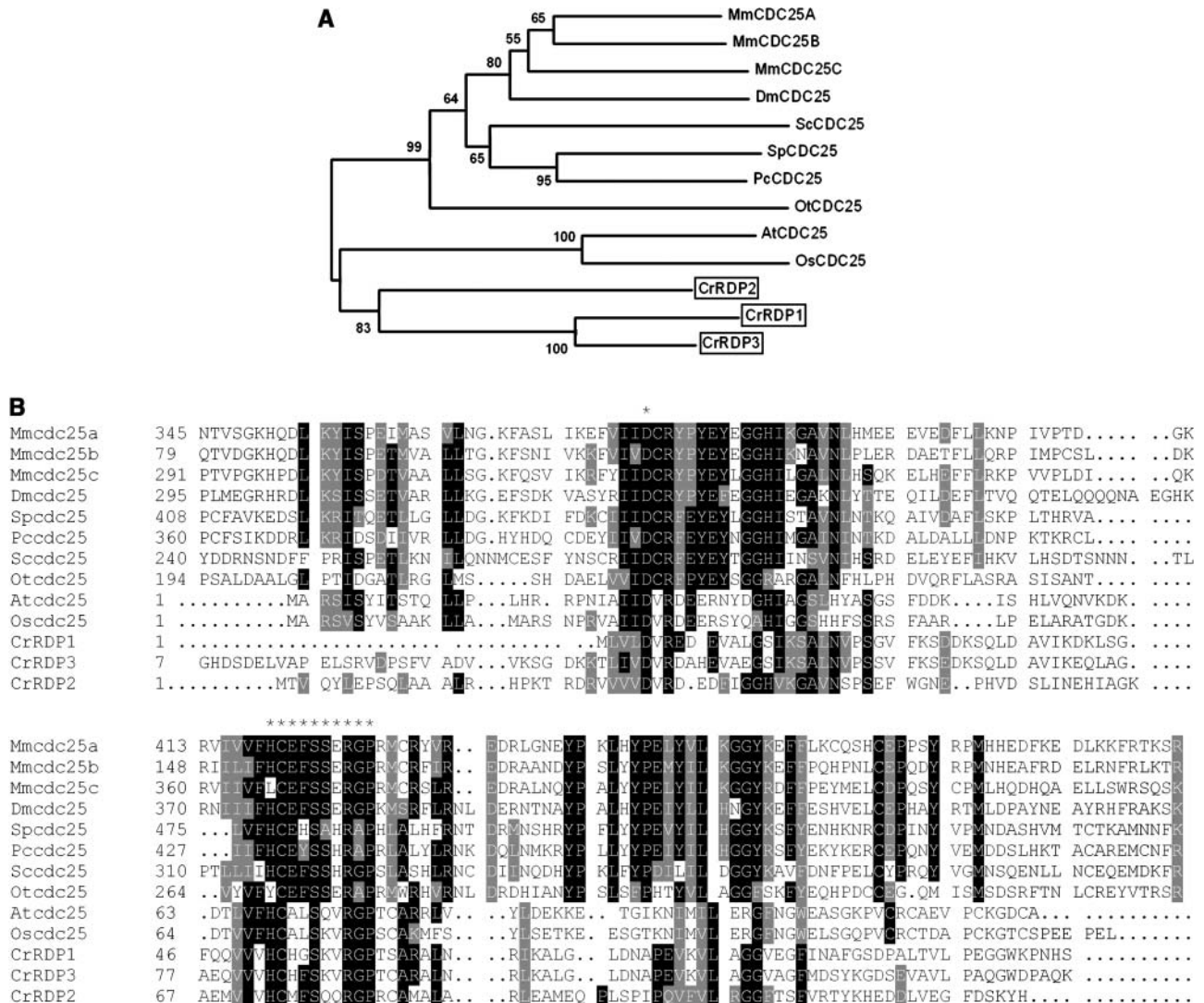


Figure 10. A, Neighbor-joining tree of *cdc25* phosphatase. Bootstrap values of 50% or higher are shown for each clade. B, ClustalW alignment of phosphatase domains of *Chlamydomonas* RDP1, 2, 3 with CDC25 from different organisms. Conserved residues are shaded; asterisks indicate position of catalytic site (HCXSSSRGP) and conserved Asp residue. At, *Arabidopsis*; Cr, *Chlamydomonas reinhardtii*; Dm, *Drosophila melanogaster*; Mm, *Mus musculus*; Os, *Oryza sativa*; Ot, *Ostreococcus tauri*; Pc, *Pneumocystis carinii*; Sc, budding yeast; Sp, fission yeast. See Supplemental Table II for GenBank accession numbers.

et al., 2003). A parallel annotation project on the green microalga *O. tauri* has also been carried out independently (H. Moreau, personal communication). The genome of a more primitive acidophilic red alga, *Cyanidioschyzon merolae*, has recently been sequenced (Matsuzaki et al., 2004), but to our knowledge the *C. merolae* cell cycle genes have not yet been annotated. Below we compare the cell cycle regulators from *Chlamydomonas* with those in higher plants and metazoans and discuss the implications of our findings for understanding eukaryotic cell cycles.

A Single-Celled Plant?

Although there are some differences between *Chlamydomonas* and higher plants, the most striking

features are the similarities. These include (1) similarity in both sequence and expression pattern of the major cell cycle CDKs, CDKA and CDKB, the former being constitutively expressed and the latter up-regulated during G2/M; (2) the presence of and similarity between the other CDK family members (C–E); (3) the presence of all three major classes of cyclin genes (A, B, and D) that are also found in metazoans but are much more diverged in the fungi; and (4) the retention of the RB-E2F pathway, also present in metazoans but lost from the fungi.

There are several classes of regulators that we expected to find but that were either missing or ambiguous. These include cyclin H, CDC25, and CKI proteins. H-type cyclins bind CDKD to form a CAK complex (Fisher and Morgan, 1994; Makela et al., 1994;

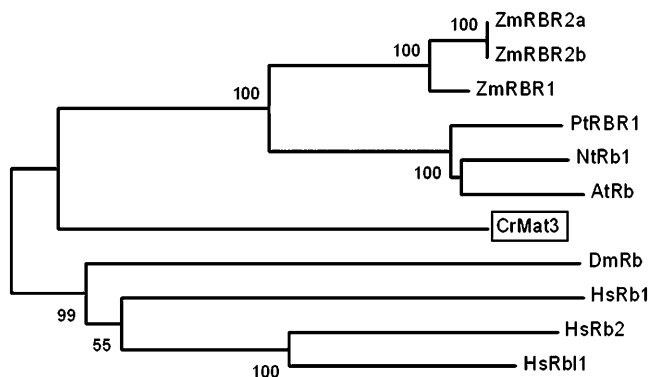


Figure 11. Neighbor-joining tree of RB and RBR proteins. Bootstrap values of 50% or higher are shown for each clade. At, *Arabidopsis*; Cr, *Chlamydomonas reinhardtii*; Dm, *Drosophila melanogaster*; Hs, *Homo sapiens*; Mm, *Mus musculus*; Nt, *Nicotiana tabacum*; Pt, *Populus tremula* × *Populus tremuloides*; Zm, *Zea mays*. See Supplemental Table II for GenBank accession numbers.

Yamaguchi et al., 2000). The presence of a CDKD ortholog in *Chlamydomonas* and the conservation of a CAK phosphorylation site in CDKA suggest that there is a cyclin H-like activity that interacts with CDKD1. The gene encoding this cyclin H-like activity could be missing from this genome sequence, diverged beyond recognition, or one of the other cyclin genes. One possibility for a cyclin H substitute is the C-type cyclin CYCC1, which belongs to a closely related sister clade. A second possibility is that a novel cyclin, such as CYCM1, has taken over the role of cyclin H. Direct testing will ultimately be required to identify the composition of the predicted CAK activity in *Chlamydomonas*.

There are three genes in *Chlamydomonas*—*RDP1*, *RDP2*, and *RDP3*—that encode RDPs related to CDC25 but lack the N-terminal extension typical for metazoan CDC25s. It has been argued for higher plants that the presence of a *WEE1*-encoded activity means that an opposing CDC25-like activity must exist (Vandepoele et al., 2002), and the same can be argued for *Chlamydomonas*. The CDC25-like protein described in *Arabidopsis* is orthologous to the RDPs and has been shown to have phosphatase activity that can activate CDKs purified from plant extracts (Landrieu et al., 2004). The RDPs, therefore, are the best candidates for CDC25 in *Chlamydomonas*. A more definitive conclusion about CDC25 function in *Chlamydomonas* and the role of the RDP proteins can be drawn after biochemical and genetic data become available.

The only conserved activity that appears to be missing from *Chlamydomonas* is the CKIs. This is not surprising given the low similarity between different members of this family (Wang et al., 1997). Since CKIs are a widespread group, they are probably also present in *Chlamydomonas*, but their identification will require either more sensitive sequence search methods or direct biochemical assays.

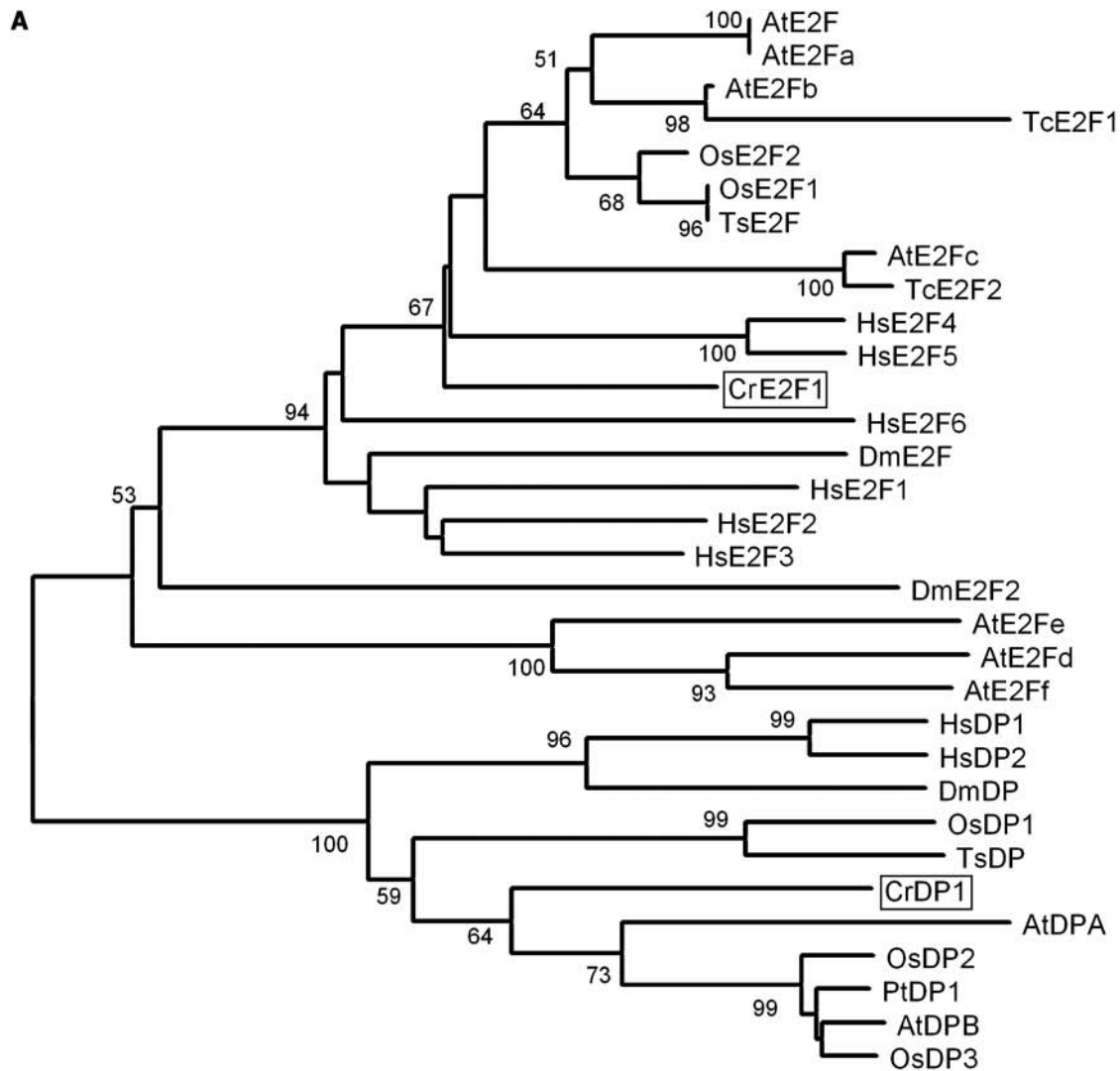
There is one major difference between *Chlamydomonas* and higher plants that is especially noteworthy.

Whereas many of the key cell cycle regulators have undergone multiple duplications in higher plants (Mironov et al., 1999; Joubes et al., 2000; Vandepoele et al., 2002), most of the *Chlamydomonas* cell cycle regulatory genes are single copy. The genome of *C. reinhardtii* (approximately 100 Mb, approximately 20,000 predicted genes) is comparable in size to that of *Arabidopsis* (approximately 120 Mb, approximately 26,000–29,000 predicted genes) and is about 10-fold larger than the genome of *O. tauri* (approximately 10–12 Mb). Therefore, differences in genome size or gene number cannot explain the differing degrees of cell cycle gene duplication in green algae versus land plants. It is tempting to speculate that the cell cycle gene duplications in higher plants are coupled to increased developmental complexity, but it remains to be determined whether the correlation of copy number with developmental complexity holds for plants other than angiosperms. In any case, its lack of apparent genetic redundancy and simple unicellular life cycle are advantageous properties that position *Chlamydomonas* as a good model for the eukaryotic cell cycle.

The most notable exception to the single-copy rule is the D-cyclin family that has three members in *Chlamydomonas*. The three D-type cyclins in *Chlamydomonas* are not closely related to any of the seven *Arabidopsis* D-cyclin subfamilies, suggesting that *CYCD1*, *2*, and *3* are the result of independent duplication in *Chlamydomonas* (Fig. 5). It is not clear whether this duplication of the D-cyclin family is unique to *Chlamydomonas* since there is only a single cyclin D in *O. tauri* (H. Moreau, personal communication). It has been suggested that the proliferation of D-cyclins in plants is related to an elaboration of G1 control mechanisms (Meijer and Murray, 2000), and the same may be true in *Chlamydomonas*. It remains to be determined whether the *Chlamydomonas* D-cyclins have separate, overlapping, or redundant roles in cell cycle regulation.

Chlamydomonas-Specific Genes

It is interesting that, besides the higher plant repertoire of CDKs and cyclins, *Chlamydomonas* encodes four novel CDKs (G1, G2, H1, and I1), two novel cyclins (CYCAB1 and CYCM1), and a novel E2F-related protein (E2FR1). These novel classes are missing from *O. tauri* (H. Moreau, personal communication), meaning either that they were present early in the green algal/land plant ancestor and lost from some lineages, or that they evolved later in a subgroup of algae that includes *Chlamydomonas*. The fact that *O. tauri* is missing these genes may suggest that they carry out a specialized function in *Chlamydomonas*, but it is equally possible that *Ostreococcus*, which has a very small genome, might be the more derived species due to gene loss. More information on the cell division mechanism used by *Ostreococcus* and a broader sampling of genomes from green eukaryotes might shed light on these differences.



B

CrE2F1	11	TGGCRYDSS	LGMLTKRFL	NLINTA DGI	LDLN QA	AE	TLK	VQ	RRRIYDIT	ITNVLE	EGVGLTE	KKSKNN	IRWKA
OsE2F1	97	VGTCRYDSL	SGLLTKRFL	NLLKQAPDCI	LDLN	.	NAAET	LEVQ	RRRIYDIT	ITNVLE	EGIGLIE	KTTLKN	IRWKG
TsE2F	129	AGTCRYDSL	SGLLTKRFL	NLLKQAE	LDLN	.	NAAET	LEVQ	RRRIYDIT	ITNVLE	EGIGLIE	KTTLKN	IRWKG
OsE2F2	66	VGGCRYDSL	SGLLTKRFL	NLLKGAPGGI	VDLN	.	NAAET	LEVQ	RRRIYDIT	ITNVLE	EGIGLIE	KTTLKN	IRWKG
AtE2F	191	SFMSRVTS	.LLTKRFV	NLIKQAKDGM	LDLN	.	KAAET	LEVQ	RRRIYDIT	ITNVLE	EGIDLIE	KPFKNR	ILWKG
AtE2Fa	147	SGSCRYDSL	SGLLTKRFV	NLIKQAKDGM	LDLN	.	KAAET	LEVQ	RRRIYDIT	ITNVLE	EGIDLIE	KPFKNR	ILWKG
AtE2Fb	112	AGTCRYDSL	SGLLTKRFL	NLIKQAE	LDLN	.	KAAET	LEVQ	RRRIYDIT	ITNVLE	EGIGLIE	KTTLKN	IRWKG
AtE2Fc	137	SINCRYDSL	SGLLTKRFV	KLIQEAEDGT	LDLN	.	YCAVV	LEVQ	RRRIYDIT	ITNVLE	EGIGLIE	KTTLKN	IRWKA
TcE2F2	131	N.NCRYDSL	SGLLTKRFV	NLIREAEDGS	LDLN	.	YCAVV	LEVQ	RRRIYDIT	ITNVLE	EGIGLIE	KTTLKN	IRWKA
CrDP1	84	KGRTTYNE	VADELVGFMS	KMEAMNKG	QYDEKN	IRRRVYDA	INVL	MAMDI	IQEKK
HsDP1	118	KGRTTYNE	VADELVAEFS	AADNHILPNE	S	.	.	.	AYDQKN	IRRRVYDAL	NVLMAM	NIISKE	KK
HsDP2	78	KGRTTYNE	VADELVSEET	NSNNHLAADS	Q	.	.	.	AYDQKN	IRRRVYDAL	NVLMAM	NIISKE	KK
DmDP	166	KGRTTYNE	VADDLVSEEM	KNN...AYD	N	.	.	.	NCQDKN	IRRRVYDAL	NVLMAM	NIISKE	KK
AtDPB	107	KGRTTYNE	VADELVAEFA	LPNNDGTS	PD	.	.	.	QQYDEKN	IRRRVYDAL	NVLMAM	DIISKE	KK
PtDP1	107	KGRTTYNE	VADELVAEFA	DPNSVSTPD	Q	.	.	.	QQYDEKN	IRRRVYDAL	NVLMAM	DIISKE	KK
OsDP2	100	KGRTTYNE	VADELVAEFA	DPNNFASPD	PD	NPN	.	.	TPQFDEKN	IRRRVYDAL	NVLMAM	DIISKE	KK
OsDP3	125	KGRTTYNE	VADELVAEFA	DPNNSILPPD	PD	NPN	.	.	AQQYDEKN	IRRRVYDAL	NVLMAM	DIISKE	KK
OsDP1	105	KGRTTYNE	VADEIFAE	LK SITQNG	LEFDEKN	IRRRVYDA	FNVLT	AI	IRVIA
TsDP	73	KGRTTYNE	VADEIYSELK	SMAHIG	QYFDEKN	IRRRVYDA	FNVLT	AI	IRVIA
CrE2FR1	38	RSTTTIYAD	VADALCAASS	AGPAS	ARDMKS	IRRRVYDAL	NVLM	SAGL	IERV

Figure 12. A, Neighbor-joining tree of E2F and DP families. Bootstrap values of 50% or higher are shown for each clade. B, ClustalW alignment of DNA-binding domains of *Chlamydomonas* E2F/DP with E2F/DP from different organisms. Conserved residues are shaded; asterisks indicate position of DNA-binding motif. At, *Arabidopsis*; Cr, *Chlamydomonas reinhardtii*; Dm, *Drosophila melanogaster*; Hs, *Homo sapiens*; Os, *Oryza sativa*; Pt, *Populus tremula* × *Populus tremuloides*; Tc, *Thlaspi caerulescens*; Ts, *Triticum* sp. See Supplemental Table II for GenBank accession numbers.

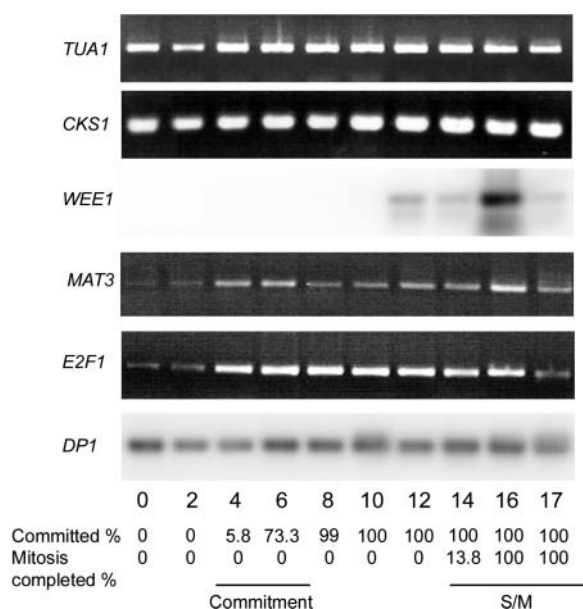


Figure 13. Expression of *Chlamydomonas* *CKS1*, *WEE1*, *MAT3*, *E2F1*, and *DP1*. RT-PCR products were detected by Southern blotting (*WEE1*, *DP1*) or ethidium bromide staining (*CKS1*, *MAT3*, *E2F1*). Fraction of cells that had passed Commitment and completed cell division when the sample was prepared is indicated at the bottom. α -Tubulin transcript (*TUA1*) detected by ethidium bromide staining serves as an internal control.

Although *CDKH1* belongs to the plant/animal *CDKC/CDK9* family, it appears to be an outlier in the *CDKC/CDK9* clade. The fact that *Chlamydomonas* encodes another protein that appears to be a bona fide *CDKC* ortholog suggests that *CDKH1* may have a novel, specialized function as compared to the other *CDKC/CDK9* proteins.

CYCAB1 is intriguing. Even though it lacks the canonical signature motifs found in either A- or B-type cyclins (Table I), it is clearly related to the A- and B-type cyclin families that play a conserved role in eukaryotic cell cycles (Fig. 5). Importantly, *CYCAB1* has a putative destruction box, suggesting that its proteolysis may be linked to cell cycle progression. Moreover, the cell cycle-regulated message abundance of *CYCAB1* suggests a role for *CYCAB1* in regulating S phase and/or mitosis.

E2FR1 is clearly related to the *E2F/DP* family of transcription factors, but has diverged considerably and does not cluster with any known *E2F* or *DP* family members in phylogenetic trees (data not shown). Most other *E2F*-containing organisms have at least one repressor *E2F*-related gene, so an interesting possibility is that *E2FR1* acts as a novel type of repressor *E2F* in *Chlamydomonas*. However, it remains to be determined whether *E2FR1* interacts with the *RB-E2F* pathway in *Chlamydomonas*.

Implications for the *Chlamydomonas* Cell Cycle

The finding of potential *Chlamydomonas*-specific cell cycle regulators raises the question of whether

these novel proteins contribute to aspects of cell division that are unique to *Chlamydomonas*. Many chlorophyte algae utilize a multiple fission cell cycle similar to that of *C. reinhardtii*. Although multiple fission appears unusual, it incorporates cell cycle modifications that are quite common in animals and plants. Egg cells grow in the absence of cell division to achieve their characteristic large size, and the early embryos of some species undergo a series of rapid S/M cycles in the absence of growth (Saucedo and Edgar, 2002). Therefore, it is possible that *Chlamydomonas* and other algae evolved multiple fission by altering the regulation of core conserved cell cycle genes and not by utilization of novel genes. In any case, it will require more direct tests of gene function to resolve this question.

There are at least two major modifications required to generate a *Chlamydomonas*-like multiple fission cell cycle. The first modification involves suppression of S-phase entry after passage through Commitment. This suppression generates an extended G1 period during which cells can grow to many times their original size before initiating the S/M part of the cell cycle. The second modification allows for mother cells to undergo rapid successive rounds of S phase and mitosis with either greatly reduced or absent G1 and G2 intervals. Exit from these rapid S/M cycles is connected to cell size through the *RB-E2F* pathway (Umen and Goodenough, 2001), but may also involve other types of regulation. The *Chlamydomonas*-specific *CYCAB1* gene is expressed during S/M phase and is conceivably involved in regulating entry into or exit from S phase or mitosis.

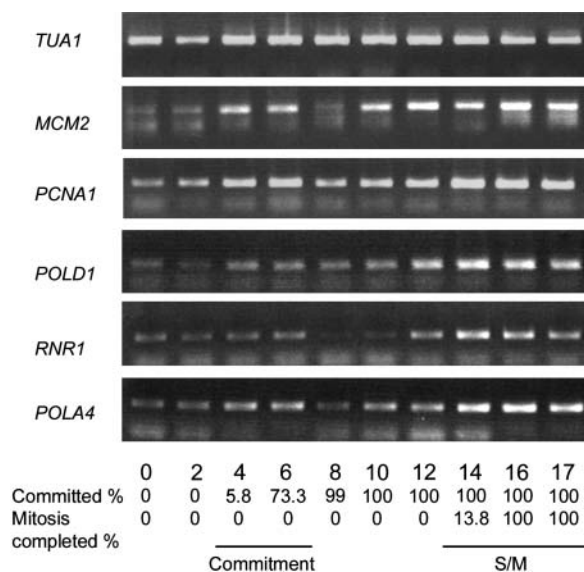


Figure 14. Expression of S-phase genes *MCM2*, *PCNA1*, *POLD1*, *RNR1*, and *POLA4*. RT-PCR products were detected by ethidium bromide staining. Fraction of cells that had passed Commitment and completed cell division when the sample was prepared is indicated at the bottom. α -Tubulin transcript (*TUA1*) detected by ethidium bromide staining serves as an internal control.

It is intriguing that expression of a subset of *Chlamydomonas* genes (e.g. *CYCA1*, *CYCB1*, *CDKB1*, *RNR1*, *POLA4*), most of which are likely to be RB-E2F pathway targets, is up-regulated prior to Commitment and down-regulated just after passage through Commitment. These same genes are up-regulated a second time during S/M phase, which is when they are likely to function. Although we cannot rule out a function for these genes during Commitment, to our knowledge there is no evidence for DNA synthesis or increased DNA repair in G1 cells as they pass Commitment. Instead, we favor an alternative explanation for the transient up-regulation of these genes at Commitment. In a conventional cell cycle, passage through the equivalent of Commitment entails the immediate preparation for S phase and subsequent mitosis, and therefore necessitates the expression of S/M-phase genes. It is possible that the transient increase in S/M-phase genes that we see in *Chlamydomonas* cells as they pass Commitment is an atavistic signature of the conventional cell cycle from which multiple fission evolved. When a G1 delay period arose in algae as a modification required for multiple fission, the expression of S/M-phase genes at Commitment became either unnecessary or detrimental, and their expression was suppressed. However, the suppression was incomplete, so we still observe a temporary derepression of S/M-phase genes during Commitment.

Passage through Commitment in *Chlamydomonas* is under the control of the RB-E2F pathway (Umen and Goodenough, 2001), and loss of MAT3 (RBR) presumably leads to deregulated E2F activity. However, a normal post-Commitment G1 interval was observed in *mat3* deletion strains, indicating that the post-Commitment S/M-phase transcriptional suppression mechanism still operates in these cells. These genetic data, taken together with the expression profiles of prospective RB-E2F transcriptional targets, suggest the existence of a G1 transcriptional control mechanism that is activated after Commitment and that suppresses expression of some RB-E2F pathway genes independently of MAT3. The mechanism by which this RB-independent suppression is achieved will be an interesting area for future investigation.

CONCLUSIONS AND PERSPECTIVES

A simplistic analogy based on the divergence between yeasts and animals would have predicted a large divergence between single-celled algae and higher plants. Contrary to this prediction, the majority of cell cycle regulatory genes in higher plants have orthologs in *Chlamydomonas*, and the overall conservation between *Chlamydomonas* and metazoan cell cycle regulators is substantial. The sequence similarities that we have observed suggest that, outside the fungal kingdom, there are greater underlying similarities between the cell cycle regulators of unicellular and multicellular eukaryotes than has previously been

appreciated. Importantly, cell cycle regulatory genes in *Chlamydomonas* have not undergone the extensive duplications seen in higher plants and vertebrates. Its relatively small number of cell cycle genes, combined with its extensive molecular genetic toolkit (for review, see Grossman et al., 2003), make *Chlamydomonas* an attractive model for eukaryotic cell cycles.

MATERIALS AND METHODS

Tree Construction and Phylogenetic Analysis

Protein sequences were aligned by ClustalW using default parameters (Thompson et al., 1994) and then optimized manually. The resulting alignments were used to construct phylogenetic trees in Mega 2.1 (Kumar et al., 2001). Unrooted trees were produced by the neighbor-joining method using p-distance for distance matrix and 1,000 replicas for bootstrapping. Bootstrap values are presented as a percentage; values lower than 50% are not shown on the trees.

Culture Conditions and Cell Cycle Analysis

Chlamydomonas reinhardtii cultures of wild-type strain 21gr (CC-1690; *Chlamydomonas* Genetics Center, Duke University, Durham, NC) were synchronized by growth in Erlenmeyer flasks in inorganic high-salt medium (HSM) aerated with 0.5% CO₂ in air, alternating 14/10-h light/dark cycles, at 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 24°C. Cell density was maintained between 10⁵ cells/mL and 10⁶ cells/mL by dilution into fresh media at the beginning of each light phase. Passage through Commitment was evaluated at hourly intervals by spreading a 1-mL aliquot of culture on an HSM plate, decanting excess liquid, and then incubating the plate in the dark to prevent further growth. After 12- to 16-h dark incubation, the fraction of committed cells (those that produced 2, 4, 8, or more daughters) was determined by microscopic examination. Cell volumes were measured with a Coulter counter (MULTISIZER 3; Beckman Coulter, Miami) in samples fixed with glutaraldehyde (0.2% final concentration). The percentage of cells that completed cell division was determined in fixed samples that were observed microscopically.

RNA and DNA Preparation and Southern Analysis

Cell pellets containing 2×10^7 cells were harvested at each time point, fast frozen in liquid nitrogen, and stored at -70°C. RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions for one set of samples or according to a previously described protocol for a second set of samples (Verwoerd et al., 1989).

For isolation of genomic DNA, 1×10^7 cells were harvested and resuspended in 10 mM Tris, pH 8.0, 10 mM EDTA, 150 mM NaCl, lysed by adding SDS and sarcosyl to final concentrations of 2% (w/v) and pronase to final concentration 2.5 mg/mL, and incubated 40 min at 37°C. DNA was extracted with phenol/chloroform and precipitated with 2 volumes of ethanol 2 h at -20°C. The DNA pellet was washed with 70% ethanol and resuspended in 10 mM Tris, pH 7.4, 1 mM EDTA. Approximately 3 μg of DNA was restriction digested overnight in the recommended buffer supplemented with 2 mM spermidine and 0.1 mg/mL RNase A. Southern blotting and hybridization were carried out as described previously (Church and Gilbert, 1984) using Hybond-XL membranes (Amersham Pharmacia Biotech, Uppsala) and a UV cross-linker (UV STRATALINKER 1800; Stratagene, La Jolla, CA) to attach DNA to membranes. The stringency was reduced by lowering the wash temperature from 65°C to room temperature.

cDNA Synthesis and RT-PCR

Five micrograms of total RNA were treated with RQ1 RNase-free DNase (Promega, Madison, WI), according to the manufacturer's instructions. DNase-free RNA was reverse transcribed using the ThermoScript RT-PCR system (Invitrogen), according to the manufacturer's instructions, in a total volume of 40 μL using oligo(dT) primers. One microliter of cDNA was used for each RT-PCR reaction. PCR fragments were amplified by ExTaq DNA polymerase (TAKARA, Shiga, Japan), according to the manufacturer's instructions, in a final volume 20 μL in the presence of 1 μM primers and 2% DMSO. Primers and conditions used for RT-PCR are listed in Supplemental Table III. Each PCR

product was subcloned into pGEM-T easy vector (Promega). For each primer set, different cycle numbers were tested to ensure that the products were in the linear range for amplification. For the products that were detected by Southern blot (all except *CDKE*, *CYCD3*, *CKS1*, *MAT3*, *E2F1*, S-phase genes, and internal controls), the lowest number of cycles yielding a signal on an ethidium bromide-stained gel was determined, and five fewer cycles than this minimum were used for the Southern-blot gels. PCR products were separated in 1% agarose gels containing ethidium bromide and photographed. PCR fragments were subjected to Southern transfer under neutral conditions (Sambrook and Russell, 2001) onto Hybond XL membranes (Amersham Pharmacia Biotech), and transferred DNA was fixed by UV cross-linking (UV STRATALINKER 1800; Stratagene). Membranes were probed and washed as described (Church and Gilbert, 1984), and radioactive signals were visualized with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Preparation of CrCKS1 Beads and Affinity Purification of CDKs

A cDNA coding for the *C. reinhardtii* CKS1/suc1^{P13} homolog, *CKS1*, was amplified from oligo(dT)-primed cDNA by PCR with primers (5'-GGAATTCATGGCACAGAACAAACATTC-3', 5'-GCGCTCGAGTTACTGAACTGCAGAGCCT-3') and cloned into pGEM-T easy vector (Promega). The sequence was verified and the fragment was subcloned into the *EcoRI-XhoI* sites of pGEX-4T-1 (Amersham Pharmacia Biotech), yielding pGEX-CrCKS1. pGEX-CrCKS1 was transformed into BL21-CodonPlus(DE3)-RP cells (Stratagene). Cells were grown at 37°C until OD₆₀₀ 0.4, then transferred to 20°C for 30 min and induced with 0.5 mM isopropyl β-D-thiogalactoside for 2 h. The recombinant protein was purified using glutathione-Sepharose (Amersham Pharmacia Biotech), according to the manufacturer's instructions.

At intervals during synchronous growth, 2 × 10⁷ *Chlamydomonas* cells were harvested, fast frozen in liquid nitrogen, and stored at -70°C. Cell pellets were mixed with 300 μL of RIPA buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.1% SDS, 1% NP-40) containing 1× protease inhibitor cocktail (SIGMA P9599; Sigma, St. Louis), 1 mM Na₃VO₄, 1 mM benzimidazole, 10 mM NaF, and 150 μL zirconium beads (diameter 1.0 mm; Biospec, Bartlesville, OK) and vortexed 7 min at 4°C. Protein lysates were cleared by centrifugation 15 min at 14,000 rpm (EPPENDORF 5417 R). Twenty microliters of *Chlamydomonas* protein lysate were diluted 10-fold with RIPA buffer and incubated 1 h at 4°C with 20 μL of 50% CrCKS1 bead slurry. Unbound proteins were washed out by four consecutive washes with RIPA buffer and two washes with kinase buffer (20 mM HEPES, pH 7.5, 15 mM MgCl₂, 5 mM EGTA, 1 mM DTT; Brizuela et al., 1987; Draetta et al., 1987).

H1 Kinase Assay

Histone H1 kinase activity was assayed by a previously described method (Langan et al., 1989) with the following modifications. The kinase activity was assayed in a final volume of 10 μL in assay buffer (20 mM HEPES, pH 7.5; 15 mM MgCl₂, 5 mM EGTA, 1 mM DTT, 0.1% histone [SIGMA H4524], 0.1 mM ATP, and 0.185 MBq of [γ-³²P] ATP). Roscovitine was added where indicated to a final concentration 200 μM just prior to addition of radioactive ATP. The reaction was incubated at room temperature for 30 min and terminated by adding 5 μL of 5× SDS sample buffer (250 mM Tris-HCl, pH 6.8, 50% [w/v] glycerol, 10% SDS, 100 mM dithiothreitol, 0.5% [w/v] bromophenol blue), boiled for 2 min, and immediately cooled. Proteins were separated by SDS-PAGE in 12% gels (Laemmli, 1970). Phosphorylated histone bands were detected with a PhosphorImager (Molecular Dynamics).

Note Added in Proof

The data referred to here as H. Moreau, personal communication, are due to be published in *Molecular Biology and Evolution* as S. Robbens, B. Khadaroo, A. Camasses, E. Derelle, C. Ferraz, D. Inzé, Y. Van de Peer, and H. Moreau, Genome-wide analysis of core cell cycle genes in the unicellular green alga *Ostreococcus tauri*.

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