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The *CRY1* Gene in *Chlamydomonas reinhardtii*: Structure and Use as a Dominant Selectable Marker for Nuclear Transformation

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We have cloned and sequenced the *CRY1* gene, encoding ribosomal protein S14 in *Chlamydomonas reinhardtii*, and found that it is highly similar to S14/rp59 proteins from other organisms, including mammals, *Drosophila melanogaster*, and *Saccharomyces cerevisiae*. We isolated a mutant strain resistant to the eukaryotic translational inhibitors cryptopleurine and emetine in which the resistance was due to a missense mutation (*CRY1-1*) in the *CRY1* gene; resistance was dominant in heterozygous stable diploids. Cotransformation experiments using the *CRY1-1* gene and the gene for nitrate reductase (*NIT1*) produced a low level of resistance to cryptopleurine and emetine. Resistance levels were increased when the *CRY1-1* gene was placed under the control of a constitutive promoter from the ribulose bisphosphate carboxylase/oxygenase small subunit 2 (*RBCS2*) gene. We also found that the 5′ untranslated region of the *CRY1* gene was required for expression of the *CRY1-1* transgene. Direct selection of emetine-resistant transformants was possible when transformed cells were first induced to differentiate into gametes by nitrogen starvation and then allowed to dedifferentiate back to vegetative cells before emetine selection was applied. With this transformation protocol, the *RBCS2/CRY1-1* dominant selectable marker gene is a powerful tool for many molecular genetic applications in *C. reinhardtii*.

Chlamydomonas reinhardtii has been an excellent model organism for many areas of research, particularly for the study of flagellar structure and function (25), chloroplast function (1), and cell-cell interactions during mating (9). The development of reliable nuclear transformation procedures (18) opened the *Chlamydomonas* field to techniques such as insertional mutagenesis (47) and homologous recombination (46). One limitation, however, has been a shortage of selectable marker genes. Heterologous genes are very poorly expressed when transformed into *C. reinhardtii*. A few reports of selection using heterologous genes have appeared (11, 13, 38), although no heterologous gene has allowed efficient recovery of transformants. Several *C. reinhardtii* genes corresponding to auxotrophic mutants, including the genes for nitrate reductase (*NIT1*) (7, 19), argininosuccinate lyase (*ARG7*) (4), and oxygen-evolving enhancer protein 1 (*OEE1*) (29), have been cloned and characterized for use as efficient transformation markers.

Dominant *C. reinhardtii* mutations that confer resistance to herbicides and other drugs are promising candidates for providing selectable marker genes. We identified a mutation in the ribosomal protein S14 gene of *C. reinhardtii* that produced resistance to the eukaryotic translation inhibitors cryptopleurine and emetine, providing an easily clonable selectable marker gene. Mutations in the ribosomal protein S14/rp59 genes of *Cricetulus griseus* (Chinese hamster ovary cells) and *Saccharomyces cerevisiae* are responsible for resistance to emetine and cryptopleurine, respectively (31, 37), and these and other S14 genes have been sequenced and found to be highly conserved (Fig. 1).

We isolated cDNA and genomic clones of the *Chlamydomonas* gene for ribosomal protein S14 (*CRY1*) by using a degenerate oligonucleotide mixture and an S14 cDNA from maize as hybridization probes (22). The deduced amino acid sequence of the *C. reinhardtii* S14 protein showed a high degree of similarity to sequences of the previously characterized rp59/S14 proteins. We determined the locations of the two introns of the S14 gene by comparing cDNA and genomic clones and determined the transcription start site by primer extension. We isolated a mutant strain, c18c, which was resistant to cryptopleurine and emetine and determined that the c18c lesion was a single missense mutation that changed the carboxyl-terminal amino acid of the S14 ribosomal protein from leucine to proline. The cloned mutant gene, *CRY1-1*, conferred cryptopleurine and emetine resistance upon transformation into wild-type cells. The level of resistance could be substantially increased by putting *CRY1-1* expression under the control of the constitutive promoter of the ribulose bisphosphate carboxylase/oxygenase small subunit 2 (*RBCS2*) gene. The increased expression of the *CRY1-1* gene afforded by the *RBCS2* promoter allowed selection of transformants directly on emetine.

MATERIALS AND METHODS

***Chlamydomonas* strains, media, and plasmids.** The *C. reinhardtii* strains used were 137c (*mt*$^+$), A54-e18 (*ac17 nit1-Δ1 sr1 mt*$^+$) from R. Schnell, and J9 (*cw15 nit1-305 mt*$^-$). Cells were grown and maintained on M (minimal), R (acetate), and SGII media (12, 39, 43). Nitrate media (MNO_3, RNO_3, and $SGIINO_3$) were prepared by replacing NH_4NO_3 with 2 mM KNO_3 (7). SGII without a nitrogen source (SGII-N) was prepared by omitting NH_4NO_3 and doubling the concentration of K_2HPO_4. Solid media were prepared by using 1% agar (JRH Biosciences) after extensive washing with water. Cryptopleurine (Chemasea Manufacturing Pty. Ltd., Sydney, Australia) was dissolved in ethanol at a concentration of 1 g/liter and stored at −20°C. The molar concentration was calculated on the basis of a cryptopleurine molar extinction coefficient (A_{258}) of 57,540. Emetine dihydrochloride (Sigma) was pre-

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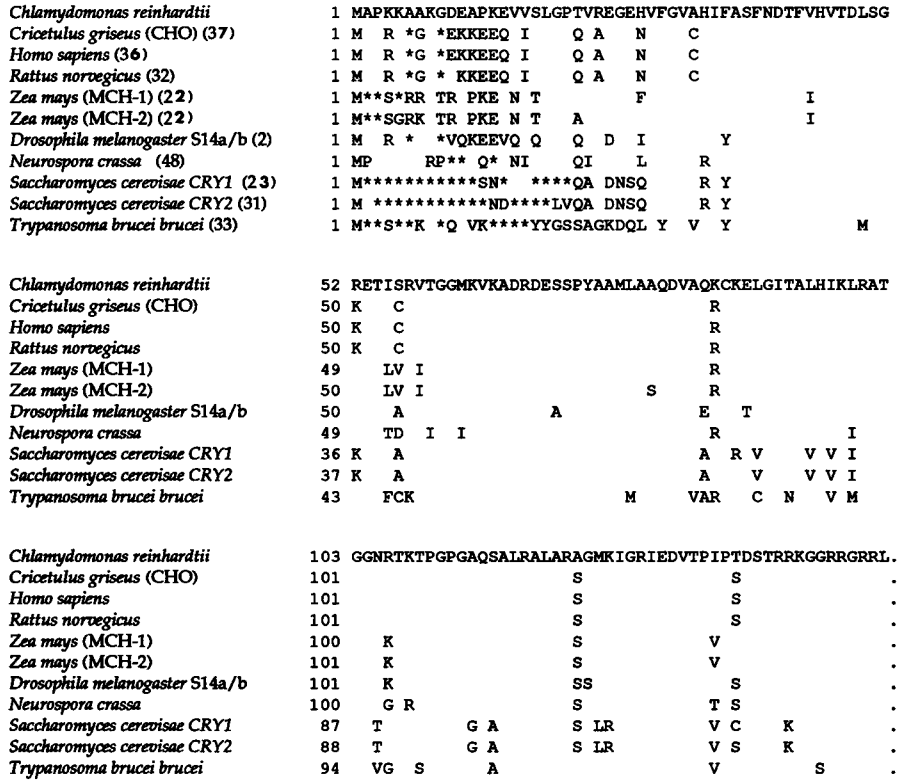

FIG. 1. Comparison of eukaryotic ribosomal protein S14 homologs. The predicted S14 amino acid sequences from several species were aligned by using the GENALIGN program (IntelliGenetics, Inc.), using the Needleman-Wunsch algorithm. Spaces inserted to preserve the alignment are designated with asterisks. Blank spaces represent amino acids identical to those of the *C. reinhardtii* wild-type *CRY1* sequence, and periods indicate the positions of the termination codons.

pared fresh before use as a stock solution of 50 g/liter in ethanol. The drugs were added to agar media cooled to 55°C after autoclaving. Plates containing the drugs were made the day before use and stored in the dark. After cells were spread on drug plates, the plates were placed under light, and growth of colonies was scored after 5 to 7 days.

The *CRY1-1* mutant strain was isolated after UV irradiation of strain 137c, followed by selection on cryptopleurine. The cells were grown to a density of 1.25×10^7 cells per ml in R medium and concentrated 25-fold in M medium. The cells were stirred in a petri dish while being exposed to UV light (General Electric G8T5 germicidal lamp) at a distance of 18 cm for 200 s; 5 to 10% of the cells survived the mutagenesis. The cells were immediately aliquoted into tubes of M medium and placed in the dark for 18 h to minimize light-activated repair. The cells were then placed under light for 3 days and spread onto plates containing 0.4 μM cryptopleurine. Upon retesting, strain c18c was highly resistant to both cryptopleurine and emetine (Table 1) and was selected for further study. Diploid construction and restriction fragment length polymorphism mapping were performed as described previously (17, 35).

Plasmids pMN24 and pMN56 contain the complete *C. reinhardtii* gene for nitrate reductase (*NIT1*) (7); the 9-kb *Xba*I-*Eco*RI fragment of pMN24 was subcloned into pUC119 to create pMN56. p1.03 is an *RBCS2* subclone generously provided by M. Goldschmidt-Clermont (University of Geneva, Geneva, Switzerland) (8). All plasmids were maintained in *Escherichia coli* K-12 DH5α.

Isolation and sequencing of *CRY1* cDNA and genomic clones. A λ gt10 cDNA library made from *C. reinhardtii* mRNA (strain 137c), generously provided by J. S. Gantt (University of Minnesota), was screened by using an eightfold-degenerate oligonucleotide mixture of the sequence GG(C/T)GG(C/T)ATGAAGGT(C/G)AAGGC (corresponding to the amino acid sequence GGMKVKA). A mixture of two maize S14 cDNA clones, MCH-1 and MCH-2 (generously provided by J. C. Larkin, University of Minnesota), was used as a hybrid-

TABLE 1. Cryptopleurine and emetine resistance

Strain	*CRY1* genotype (plasmid)[a]	No. of transformants tested	Fold increase[b] in resistance to:	
			Cryptopleurine	Emetine
A54-e18	+		1	1
c18c	*CRY1-1*		10	5
Diploid	+/*CRY1-1*		7	2
J9	+(pCRY1-1)	12	1–3	ND
A54-e18	+(pJN1)	320	1	1
A54-e18	+(pJN4)	13	5–10	2.5–5
A54-e18	+(pJN4)[c]	60	10	5–10

[a] All transformant strains were obtained following selection on nitrate by using cotransformation methods 1 and 2 except as noted.

[b] Relative to the haploid parent strain, A54-e18; 1 is equivalent to 0.3 μM cryptopleurine or 80 μM emetine. ND, not determined.

[c] Obtained after direct selection on emetine by using transformation method 3.

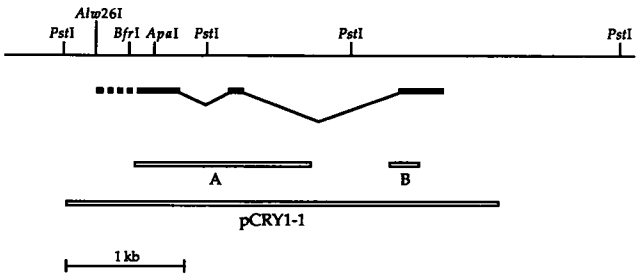

FIG. 2. *CRY1-1* gene structure. All three *Pst*I fragments were sequenced. Filled lines represent the cDNA, the broken line designates the position of the 5′ end of the mRNA as determined by primer extension, and thin lines represent introns. Probe A is a 1,458-bp *Bfr*I-*Sph*I fragment from pCRY1-1, and region B is the 246-bp PCR product sequenced to determine the *CRY1-1* mutation.

ization probe for the tertiary screen of positive plaques. Five positive clones were subcloned into M13mp18 and partially sequenced. Two of the five showed substantial similarity to other S14 genes, and one of these was subcloned into pUC119. Sequential deletion clones were prepared as described previously (14), and both strands were sequenced by using Sequenase 2.0 (United States Biochemical Corp.) as instructed by the manufacturer.

Approximately eight nuclear genomic equivalents (120,000 plaques) of an EMBL4 λ library, constructed by using strain 21gr DNA (49), were screened with the *CRY1* cDNA insert as described above. Eight λ clones were purified, and their restriction maps were compared. In all of the clones, three common *Pst*I fragments hybridized to the cDNA insert. These three fragments were subcloned into pUC119 and sequenced as described above.

Determination of the 5′ end of the *CRY1* transcript. Primer extension was used to determine the 5′ end of the transcript. An 18-mer complementary to a region 59 bp downstream of the AUG start codon (see Fig. 3) was end labeled with [γ-^{32}P]dATP and T4 polynucleotide kinase (Bethesda Research Laboratories). The primer extension reaction mixture contained 5 ng of oligomer, 1 μg of poly(A) RNA, 3 μl of 5× Moloney murine leukemia virus reverse transcriptase buffer, and water to 10 μl. The reaction mixture was heated to 80°C for 3 min and slowly cooled to 37°C. Next, 4 μl of nucleotide solution (2.5 mM each deoxynucleoside triphosphate [dNTP]) and 200 U of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) were added, and the reaction mixture was incubated at 37°C for 1 h. The reaction was stopped by adding an equal volume of sequencing stop mix (United States Biochemical Corp.), and the mixture was stored at −20°C. The reaction mixtures were denatured by heating to 75°C in the presence of formamide and size separated on a urea-polyacrylamide sequencing gel. A set of sequencing reaction mixtures of a *CRY1* subclone primed with the same oligonucleotide was loaded in adjacent lanes as a control.

Determination of the *CRY1-1* mutation. A 246-bp region containing the last 22 codons of the S14 gene (B in Fig. 2) was amplified from genomic DNA of strain c18c, using asymmetric PCR (30) with the primers 5′-GCTCTCACCCATGTGCG GCCTC-3′ and 5′-CCACCAAAACCACCACCGGCTG-3′. A 100-μl reaction volume contained 1 μg of DNA, 50 pmol of the first primer, 1 pmol of the second primer, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.001% gelatin, 2.5% dimethyl sulfoxide, 50 mM tetramethylammonium chloride (16), 20 μM each dNTP, and 2.5 U of *Taq* DNA polymerase. One hundred microliters of paraffin oil was overlaid onto the reaction mixture. The amplifications were performed in a Perkin-Elmer Cetus DNA Thermal Cycler 480, using the following program: 94°C for 5 min and then 94°C for 1.5 min, 65°C for 1 min, and 72°C for 2 min for 35 cycles and 72°C for 5 min. A 10-μl volume of each reaction mixture was run on an agarose gel to check amplification efficiency; the remaining product was precipitated with 2 M ammonium acetate–50% isopropanol and resuspended in 10 μl of TE (10 mM Tris-HCl [pH 8], 1 mM EDTA). Three microliters of the resuspended product and 2 pmol of the second primer were used in duplicate sequencing reactions using Sequenase 2.0.

Cloning of the *CRY1-1* allele and the *RBCS2* promoter. Plasmid pCRY1-1 was constructed by using PCR to amplify the 3.6-kb genomic region containing the entire *CRY1-1* transcription unit and 250 bp of upstream sequence (Fig. 2), using the primers 5′-GCTCTAGAACCTTGTCCAGCACGCGC-3′ and 5′-AAGA GCTCGTGACCTGCACCTACGCC-3′ (40). The underlined bases were changed from the endogenous sequence to make the restriction sites *Xba*I and *Sac*I, respectively, to facilitate cloning. Reactions were the same as described above except that 0.2 μg of DNA, 10 pmol of each primer, and 200 μM each dNTP were used and trimethylammonium chloride was omitted. Amplifications were performed with a program of 94°C for 5 min and then 35 cycles of 94°C for 2 min, 60°C for 3 min, and 72°C for 5 min, which was followed by 72°C for 5 min. The amplification products were separated on low-melting-temperature agarose, and the 3.6-kb product was excised from the gel and isolated. The fragment was digested with *Xba*I and *Sac*I and then cloned into pUC119 digested with *Xba*I and *Sac*I.

Plasmid pRS2-1 was constructed by amplifying a 1,073-bp region of the *RBCS2* promoter from plasmid p1.03 (8) with the primers 5′-CACCGTGCATTGCTGCCTTAG-3′ and 5′-CG GCGGCCACTTTAAGATGTT-3′. The second primer has a C in position 10, which abolishes translational initiation within the *RBCS2* sequence by changing the ATG start codon for *RBCS2* to GTG. The reactions were carried out in a Perkin-Elmer Cetus GeneAmp PCR System 9600 thermal cycler with a program of 94°C for 2 min and then 35 cycles of 94°C for 1 min, 57°C for 1 min, and 72°C for 4 min. Reagents in the reactions were 0.1 ng of p1.03 plasmid DNA, 10 pmol of each primer, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2 mM $MgCl_2$, 0.001% gelatin, 2.5% dimethyl sulfoxide, 100 μM each dNTP, and 2.5 U of *Taq* DNA polymerase. The PCR product was cloned into pUC119 digested with *Sma*I and dT tailed with *Taq* DNA polymerase (28).

Construction of chimeric genes. pJN1 was constructed by inserting the 3.0-kb *Bfr*I-*Eco*RI fragment of pCRY1-1 into the *Hin*cII site of pRS2-1 (see Fig. 5). pJN4 was constructed by ligating the 474-bp *Alw*26I-*Apa*I fragment of pCRY1-1 to the 7.2-kb *Apa*I-*Bam*HI fragment of pJN1. Techniques for isolation of DNA fragments from low-melting-temperature agarose, ligation of DNA fragments, and transformation of *E. coli* were performed as described by Sambrook et al. (41).

Transformation of *C. reinhardtii*. Transformation protocols were modified from those of Kindle (18) and Kozminski et al. (21).

(i) Method 1. Strain J9 was grown to high density (~10^7 cells per ml) in 250 ml of SGII with bubbling. Cells were harvested by centrifugation at 1,700 × *g*, washed with $SGIINO_3$, and resuspended to a density of 10^8 cells per ml with $SGIINO_3$. Then 0.3 ml of cells (3×10^7) was added to a 15-ml conical tube containing 0.3 g of sterile glass beads (710- to 1,180-μm diameter; Sigma). Supercoiled plasmids pMN24 (2 μg) and pCRY1-1 (2-5 μg) were added to the cells, and then polyeth-

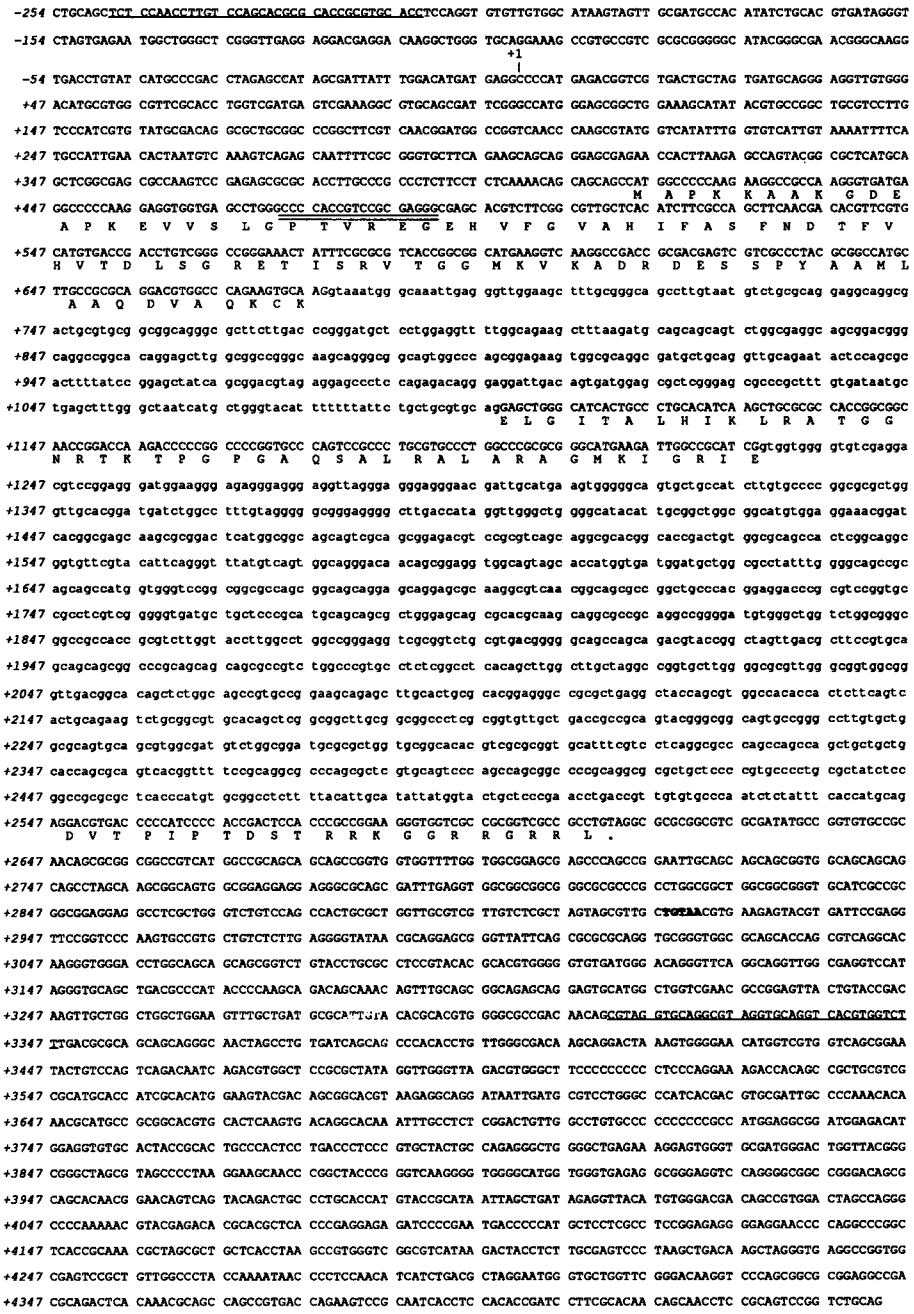

FIG. 3. Nucleotide sequence of the *CRY1* gene. Single-letter amino acid abbreviations are given under the coding regions, and the intron sequences are in lowercase letters. +1 designates the start of transcription, and the boldface TGTAA is the putative polyadenylation signal. The PCR primers used for cloning the *CRY1-1* allele are singly underlined, and the oligomer used for primer extension is doubly underlined.

ylene glycol 8000 was added to 5%. The cells were vortexed at the highest setting for 45 s, immediately washed with 10 ml of $SGIINO_3$, pelleted, and spread onto MNO_3 plates. Transformed colonies were visible after 4 to 5 days and were picked into MNO_3 liquid medium after 7 to 10 days. Single colonies of the transformants were isolated and spotted onto medium containing cryptopleurine or emetine to score resistance.

(ii) Method 2. Strain A54-e18 was grown to high cell density as described above. The cells were concentrated 100-fold into $SGIINO_3$ and shaken slowly under bright light for 4 h (46). The cells were diluted to 1.7×10^8 cells per ml, and 0.3 ml (5×10^7 cells) was added to a tube containing 0.3 g of glass beads. Then 2 μg of each plasmid (pMN56 and pJN1 or pJN4, each linearized with *Eco*RI) was added, polyethylene glycol was added to 5%, and the cells were transformed as described above. Efficient transformation of this strain did not require autolysin treatment, even though the strain has a cell wall. One possible explanation is that during the 4 h in $SGIINO_3$, cells released sufficient autolysin to remove cell walls. After the $SGIINO_3$ wash, the cells were spread onto RNO_3 plates, and

the transformants were picked into RNO_3 medium and scored for resistance as described above.

(iii) Method 3. Strain A54-e18 was grown to high density and transformed with 1 μg of *Eco*RI-linearized pJN4 as in method 2, with the following changes: after the wash with 10 ml of $SGIINO_3$, the cells were resuspended in 10 ml of SGII and slowly shaken under bright light for 3.5 h; the cells were then washed once with SGII-N, transferred to a flask containing 100 ml of SGII-N, and bubbled under bright light for 4 days. The cells were harvested, resuspended in 40 ml of SGII, slowly shaken under bright light for 8 h, and spread onto R plates containing emetine. Different lots of emetine were found to have different effective killing concentrations for *C. reinhardtii*, so the level of emetine used was determined experimentally for each lot by testing the untransformed strain on a range of emetine concentrations. Test transformations were performed on a range of emetine concentrations starting at the lowest concentration at which the untransformed cells died. The optimal emetine concentration used for selection of transformants was the lowest concentration that resulted in few or no colonies in a control transformation without DNA.

Analysis of transformants. *C. reinhardtii* genomic DNA was isolated as described previously (43). Restriction enzyme digestion of the genomic DNA was followed by agarose gel electrophoresis and transfer of the fractionated DNA to Magna NT membranes (Micron Separations Inc., Westborough, Mass.) as instructed by the manufacturer. Hybridization probe A (Fig. 2) was isolated in low-melting-temperature agarose and labeled by using the Genius 2 nonradioactive DNA labeling kit (Boehringer Mannheim Biochemicals).

Nucleotide sequence accession number. The *Chlamydomonas CRY1* gene has been assigned GenBank accession number U06937.

RESULTS

Sequence of the *CRY1* cDNA and gene. We isolated and sequenced a cDNA clone containing the coding region of the *C. reinhardtii CRY1* gene. This clone was identified by using a hybridization probe consisting of a degenerate oligonucleotide mixture corresponding to a seven-amino-acid sequence found in the *S. cerevisiae* rp59 protein and the *C. griseus* and *Zea mays* S14 proteins. We took into account the codon bias seen in most *Chlamydomonas* nuclear genes when designing the oligonucleotide mixture (45). The sequence of the *CRY1* transcript contained a large open reading frame predicting a protein of 153 amino acids that was similar to the S14 proteins of other species. Only nine nucleotides upstream of the putative AUG start codon were present in the cDNA clone. The *Chlamydomonas* S14 protein is the longest yet described of the S14 homologs, with most of the identity being in the carboxyl-terminal two-thirds of the protein (Fig. 1).

Overlapping genomic clones containing the *CRY1* gene were isolated from a genomic λ library. Three common *Pst*I fragments from these clones that hybridized with the *CRY1* cDNA were sequenced, revealing two introns of 420 and 1,318 nucleotides within the *CRY1* coding region (Fig. 2 and 3). The splice junctions of both introns agree with the consensus junctions from other *Chlamydomonas* genes (24). The start of the *CRY1* transcript was determined by primer extension to be 414 nucleotides upstream of the AUG start codon. This 5′ untranslated region (UTR) is the longest to date from *Chlamydomonas* species and contains 11 AUG codons upstream of the AUG that initiates the S14 protein translation. One of these is in the same sequence context, but not the same frame, as the initiator AUG. The 11 upstream AUG codons start or are within five different upstream open reading frames that either are out of frame with or terminate before the S14 coding region.

TABLE 2. Restriction fragment length polymorphism mapping data

Markers in cross[a]	PD:NPD:TT[b]	Map distance (m.u.)
CRY1 × pCF6-2	10:0:1	4.5
CRY1 × *pf2*	6:0:7	26.9
pCF6-2 × *pf2*	8:0:4	16.6

[a] Tetrads from a *C. reinhardtii* × *C. smithii* cross were scored for segregation of the phenotypic marker *pf2* and molecular markers pCF6-2 and *CRY1*.
[b] PD, parental ditype; NPD, nonparental ditype; TT, tetratype.

Characterization of a *Chlamydomonas* mutant resistant to cryptopleurine and emetine. A mutant (c18c) was isolated on cryptopleurine medium after UV mutagenesis. The strain showed a high level of resistance to both cryptopleurine and emetine (Table 1). Resistance segregated as a single Mendelian trait in backcrosses to wild type. Stable heterozygous diploids constructed from c18c and a wild-type strain showed an intermediate level of resistance to cryptopleurine and emetine between the two parent strains (Table 1). The codominance of the c18c allele (*CRY1-1*) was surprising because all of the *S. cerevisiae* and *C. griseus* resistance alleles were recessive. This result also suggested that transformation of *CRY1-1* into a wild-type strain might produce levels of drug resistance sufficient for selection of transformants.

The *CRY1* locus was mapped to linkage group XI, as the most centromere-distal marker on the right arm of the linkage group. It is 4.5 map units (m.u.) from molecular marker pCF6-2 and 26.9 m.u. from the *pf2* locus (Table 2). This localization on the molecular map is consistent with genetic linkage of the *CRY1-1* mutation to *pf2* (23 parental ditype:0 nonparental ditype:10 tetratype; 15.1 m.u.).

The site of the *CRY1-1* mutation was determined by sequencing the third exon of the *CRY1-1* gene (Fig. 2). All of the resistance mutations in the rp59 genes of *S. cerevisiae* and the S14 gene of *C. griseus* are found in this highly conserved carboxyl-terminal region (Fig. 4) (31, 37). Comparison of the sequences of the S14 gene from the *CRY1-1* mutant and the corresponding sequence from a wild-type strain showed a single base pair transition (T-A to C-G) at position 2610 that changes the carboxyl-terminal amino acid of the protein from leucine to proline (Fig. 4).

Transformation of the *CRY1-1* gene into cells conferred cryptopleurine and emetine resistance. The ability of the *CRY1-1* allele of the S14 gene to confer resistance to cryptopleurine and emetine was tested by cotransforming the *CRY1-1* gene (plasmid pCRY1-1; Fig. 5) into cells along with the *NIT1*

C. reinhardtii CRY1	DSTRRKGGRRGRRL.
C. reinhardtii CRY1-1	P.
C. griseus wild-type (37)	.
C. griseus Em^r-2 (37)	C .
C. griseus Em^r-2-3 (37)	CH .
S. cerevisiae CRY1, CRY2 (23, 31)	K .
S. cerevisiae cry1 (31)	K S .
S. cerevisiae cry2 (31)	K .

FIG. 4. Alignment of the wild-type and mutant S14/rp59 proteins from *C. reinhardtii*, *C. griseus*, and *S. cerevisiae*. Shown are the carboxyl-terminal 14 amino acids of each protein. Blank spaces represent amino acids identical to those of the *C. reinhardtii* wild-type *CRY1* sequence, and periods indicate positions of the termination codons. Em^r, emetine resistant.

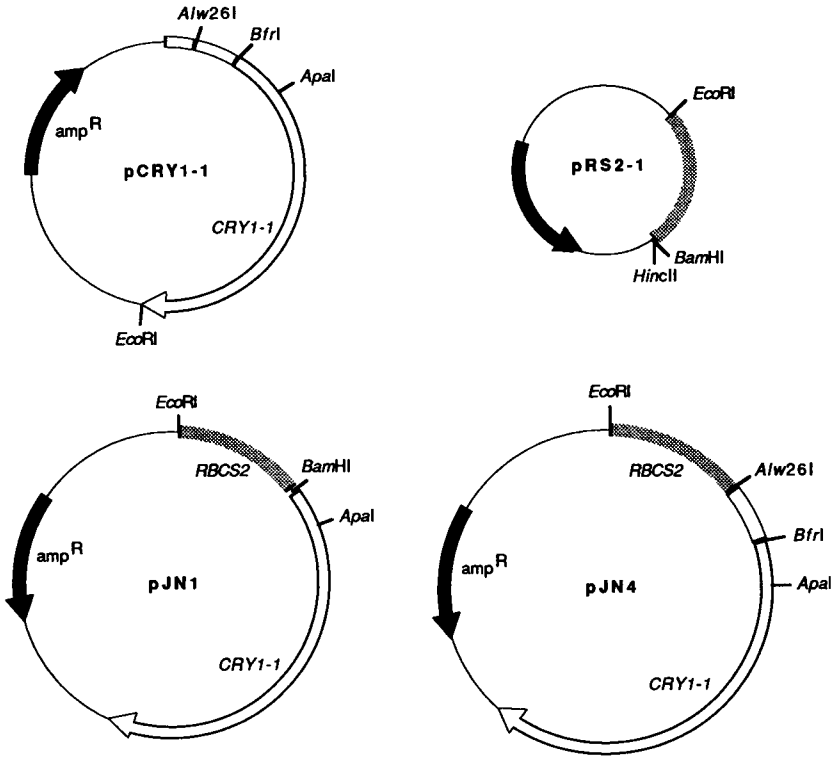

FIG. 5. Plasmids used in transformation experiments. pCRY1-1 contained the 3.6-kb PCR product from genomic DNA of the *CRY1-1* mutant strain. pRS2-1 contained the 1.1-kb *RBCS2* promoter. pJN1 was constructed by inserting the *Bfr*I-*Eco*RI piece of pCRY1-1 into the *Hin*cII site of pRS2-1. In pJN4, the *Bam*HI-*Apa*I fragment of pJN1 was replaced by the *Alw*26I-*Apa*I fragment of pCRY1-1.

gene (in pMN24) as the selectable marker. The *CRY1-1* allele used for these experiments encompassed the entire transcribed region, plus 250 bp of upstream sequence and 425 bp downstream of the putative polyadenylation signal (TGTAA) (Fig. 3) (49). In cotransformations of plasmid pCRY1-1 with pMN24 (transformation method 1; Materials and Methods), 6% of the Nit^+ transformants were more resistant to cryptopleurine than the untransformed strain, but the resistance obtained was not as high as that observed for the heterozygous diploid (Table 1). All of the resistant transformants, as well as some of the sensitive transformants, contained at least one copy of pCRY1-1 in genomic DNA blot analysis (not shown), indicating that one copy of the transgene was sufficient to confer the level of resistance observed in the transformants. Copies of pCRY1-1 in the sensitive transformants were presumably nonfunctional as a result of rearrangement or mutation of the plasmid sequence upon integration into the *C. reinhardtii* genome or silencing of pCRY1-1 expression after integration.

The levels of resistance in these cotransformants were not as high as expected, given the codominant nature of the *CRY1-1* allele in the heterozygous diploid (Table 1). In an attempt to increase expression of the *CRY1-1* transgene and therefore increase drug resistance in the transformants, a promoter known to produce high, constitutive levels of expression, the promoter of the *C. reinhardtii RBCS2* gene (8), was fused to the *CRY1-1* coding region (Fig. 5). This constitutive promoter was used previously to express nonacetylatable α-tubulin to a high level in *C. reinhardtii* (21). The chimeric gene on plasmid pJN1 retained 94 bp of the 414-bp *CRY1-1* 5′ UTR between the *RBCS2* promoter and the *CRY1-1* coding region. None of the 320 Nit^+ strains from cotransformation of pJN1 and pMN56 (transformation method 2) were resistant to cryptopleurine or emetine. However, 8 of 16 transformants assayed by genomic DNA blot analysis contained at least one copy of pJN1 (not shown).

One possible explanation for the inability of pJN1 to confer resistance was that the 320 bp of the *CRY1-1* 5′ UTR which were not included in pJN1 were necessary for expression of the S14 protein. To test this possibility, a new chimeric construct was created in plasmid pJN4, in which the complete 414-bp 5′ UTR and coding region of the *CRY1-1* gene were included downstream of the *RBCS2* promoter (Fig. 5). Cotransformation with this plasmid and *NIT1* (transformation method 2) resulted in a significant number of drug-resistant cotransformants; 7% of the Nit^+ strains were highly resistant to cryptopleurine and emetine (Table 1). Analysis of genomic DNA isolated from 11 resistant and 3 sensitive transformants (Fig. 6) showed that all resistant strains had at least one copy of pJN4 and that one copy was sufficient for resistance in several transformants. As observed previously with pCRY1-1, one of the sensitive transformants (Fig. 6, lane 13) has a nonfunctional copy of pJN4 as a result of either silencing after integration or deletion and rearrangement of plasmid sequences by the recipient cell. Two of the resistant cotransformants with single copies of pJN4 were crossed to wild-type *CRY1* strains to check whether the integrated plasmids were stable through meiosis. Tetrad analysis showed 2:2 segregation of resistance, and the resistant progeny were as resistant as the parent transformants, indicating stable integration and expression of the transgene (data not shown).

Subsequent sequence analysis of the pRS2-1 construct revealed that only 597 bp of the intended 1,073-bp PCR product (bases −905 to −309 [8]) had been cloned into pUC119, and in

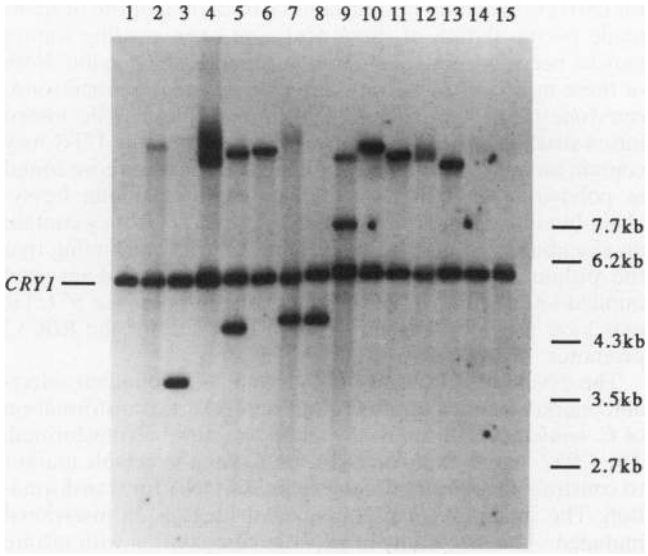

FIG. 6. Analysis of Nit$^+$ strains after cotransformation with pJN4 and pMN56. A DNA blot of genomic DNA digested with *Kpn*I was hybridized with probe A (Fig. 2). Lanes: 1, untransformed strain A54-e18; 2 to 12, Nit$^+$ transformant strains resistant to cryptopleurine and emetine; 13 to 15, Nit$^+$ transformant strains sensitive to the inhibitors. *CRY1* designates the 5.6-kb *Kpn*I fragment of the endogenous *CRY1* gene. Size markers are shown at the right.

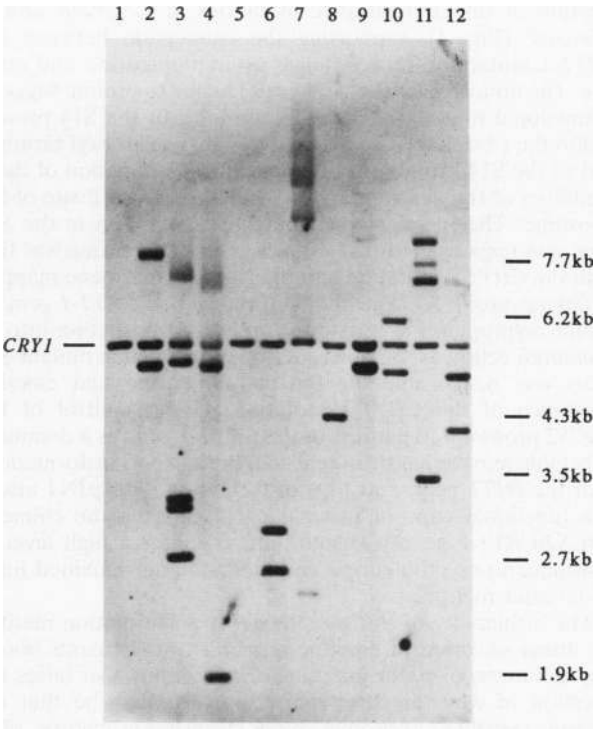

FIG. 7. Analysis of emetine-resistant strains after transformation with pJN4. Genomic DNA was digested with *Kpn*I, and a blot of the DNA was hybridized with probe A (Fig. 2). Lanes: 1, untransformed strain A54-e18; 2 to 12, resistant transformant strains. Size markers are shown at the right.

the reverse orientation. However, this inverted promoter sequence, in the context of pJN4, allowed sufficient expression to confer high levels of resistance to cryptopleurine and emetine.

Although pJN4 produced high levels of resistance when introduced in cotransformation experiments, we were unable to select drug-resistant cells directly by using standard transformation protocols. This observation is not surprising if the cells were unable to assemble ribosomes containing the mutant S14 protein before they were subjected to selection on emetine. To overcome this problem, we delayed putting the cells under selection after transformation. In addition, cells were allowed to differentiate into gametes after transformation but before selection was applied. During the differentiation of vegetative cells into gametes induced by nitrogen starvation, *Chlamydomonas* cells degrade up to 90% of their ribosomal protein (44). RNA blots from differentiating and dedifferentiating cells probed with the *CRY1* cDNA clone showed that the level of the *CRY1* transcript slowly decreased about twofold over 24 h after the removal of nitrogen, and the transcript level increased about twofold within 1 h after the readdition of ammonium to the medium (42). Therefore, we predicted that cells induced to differentiate into gametes after transformation and then allowed to dedifferentiate would assemble new ribosomes containing the mutant S14 ribosomal protein. Using this gamete step transformation procedure (transformation method 3), resistant transformants were obtained at a high efficiency. Between 100 and 300 emetine-resistant colonies per transformation were obtained by inducing differentiation to gametes following transformation, keeping the cells as gametes for 4 days (gametes remain viable for weeks), and then allowing cells to dedifferentiate for 8 h before plating the cells on emetine-containing medium. This same efficiency was observed in standard transformation of strain A54-e18 with pMN56, followed by selection on RNO_3 (transformation method 2). Figure 7 shows that 10 of 11 resistant strains contained at least one copy of pJN4. The strain in lane 5, which had no copies of pJN4, was weakly resistant to emetine in subsequent tests. This colony probably arose spontaneously as a result of a mutation conferring low-level emetine resistance. The remaining transformants were resistant to high levels of cryptopleurine and emetine, comparable to those of the original *CRY1-1* mutant (Table 1).

DISCUSSION

The protein encoded by the *Chlamydomonas CRY1* gene is highly similar to S14/rp59 proteins in other organisms, showing 80 to 100% identity in the carboxy-terminal three-fourths of the protein. This result is not surprising given the high degree of conservation in structure and function of ribosomal proteins in general.

Chlamydomonas strains resistant to the protein synthesis inhibitors emetine and cryptopleurine have been isolated and characterized. Previous experiments have demonstrated that cryptopleurine and emetine both bind to the S14 ribosomal protein (3, 10). One of these strains, carrying *CRY1-1*, was selected for further characterization on the basis of its high level of resistance to both cryptopleurine and emetine (Table 1). The results of this characterization provided three reasons to believe that a mutation in the gene encoding ribosomal protein S14 was responsible for conferring the resistance.

Nucleotide sequencing of a portion of the S14 coding region in the *CRY1-1* strain revealed a T-to-C transition in the codon preceding the translation stop signal, which changes the carboxyl-terminal leucine to proline. The location of the mutation at the carboxyl terminus of the protein coincides with the

location of similar resistance mutations in *C. griseus* and *S. cerevisiae* (Fig. 4), supporting the connection between the *CRY1-1* mutation and resistance to cryptopleurine and emetine. The nonconservative change of leucine to proline suggests a functional role of the carboxyl terminus of the S14 protein within the ribosome. The amino acids at the carboxyl-terminal end of the S14 protein may lie near the site of action of these inhibitors of translocation, possibly near the A or P site of the ribosome. The second indication that a mutation in the S14 gene was responsible for resistance to cryptopleurine was that both the *CRY1-1* mutation and the cloned *CRY1* gene mapped to linkage group XI. The ability of the cloned *CRY1-1* gene to confer cryptopleurine resistance upon transformation into *C. reinhardtii* cells was the third confirmation that the mutant S14 locus was responsible for the cryptopleurine and emetine resistance of the *CRY1-1* mutation. Under control of the *RBCS2* promoter, the utility of the *CRY1-1* gene as a dominant selectable marker has been realized. Both in cotransformations with the *NIT1* gene and in transformation with pJN4 alone, one functional copy of plasmid pJN4 carrying the chimeric *RBCS2/CRY1-1* gene was sufficient to confer a high level of resistance to cryptopleurine and emetine and remained functional after meiosis.

The availability of the gamete step transformation method for direct selection of emetine-resistant transformants boosts the usefulness of pJN4 for transformation but also raises the question of why this step is necessary. It may be that the emetine-sensitive ribosomes block translation before ribosomes containing the emetine-resistant S14 protein can be assembled. Also, by inducing gamete differentiation after transformation, it was possible to keep the cells from dividing while they recovered from the transformation procedure and to ensure that resistant ribosomes were assembled before selection was applied.

An apparent side effect of the gamete step transformation method was an increase in the number of copies of pJN4 inserted during transformation. The average copy number in resistant cotransformants in Fig. 6, without regard to functionality of the different copies, was 1.5, whereas the average was 2.5 for the resistant transformants in Fig. 7. This increase also occurred in gamete step transformations with the *NIT1* gene, perhaps because during the extra time before selection is applied the gametes are able to integrate extra copies of the plasmid that they have taken up. Another effect of the gamete step transformation with *NIT1* is a 100-fold increase in transformation efficiency; however, the dramatic increase in copy number discourages the use of the gamete step transformation method for applications such as insertional mutagenesis.

It was quite unexpected to find that plasmid pJN4 produced efficient transformation to emetine resistance, whereas pJN1, which is identical to pJN4 except for the absence of 320 bp of the *CRY1-1* 5′ UTR, produced no resistant colonies by cotransformation. The 320-bp region of the *CRY1-1* 5′ UTR missing from pJN1 contains 10 AUG codons, 1 of which is in the same strong translational context as the AUG that initiates the S14 protein translation (20, 42). The putative initiator codon is the only AUG in frame with the predicted S14 amino acid sequence. One other AUG codon in the 94-bp region of the *CRY1-1* 5′ UTR included in pJN1 is neither in frame with the predicted S14 amino acid sequence nor in a strong translational context (20). These 11 upstream AUG codons start or lie within five open reading frames that either are out of frame with or terminate before the S14 coding region (42). The presence of these upstream AUG codons suggests that the scanning model of initiation of translation is not used on the S14 mRNA (20). The upstream AUG codons may be required for correct regulation of translational initiation, or the proteins made by translation of these upstream open reading frames may be necessary for reinitiation at the S14 AUG codon. Both of these mechanisms of translation have been described for *S. cerevisiae* (15). Alternatively, the entire UTR may be folded into a structure that is recognized by ribosomes, the UTR may contain an internal ribosome binding site, such as those found in poliovirus RNA and the human immunoglobulin heavy-chain-binding protein RNA (26, 33), or the UTR may contain an essential mRNA stability element. Our recent finding that the promoter region of pJN4 was incomplete and inverted implied an alternative, technical explanation that the 5′ UTR may have provided a transcriptional start site for the *RBCS2* promoter.

The development of the *CRY1-1* gene as a dominant selectable marker offers a number of advantages for transformation of *C. reinhardtii*. Virtually any strain can now be transformed. The *CRY1-1* gene could be incorporated as a selectable marker to construct libraries of *Chlamydomonas* DNA for transformation. The small size of pJN4 makes it ideal as an insertional mutagen. Also, the utility of pJN4 in combination with nitrate reductase allows more sophisticated gene disruption strategies to be developed. For example, it is possible to select against expression of nitrate reductase by using chlorate toxicity; cells with active nitrate reductase are sensitive to killing by chlorate (6). Thus, a positive-negative selection for targeted gene disruptions (27) in *C. reinhardtii* may now be developed.

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REFERENCES

1. **Boynton, J. E., and N. W. Gillham.** 1993. Chloroplast transformation in *Chlamydomonas*. Methods Enzymol. **217**:510–536.
2. **Brown, S. J., D. D. Rhoads, M. J. Stewart, B. Van Slyke, I.-T. Chen, T. K. Johnson, R. E. Denell, and D. J. Roufa.** 1988. Ribosomal protein S14 is encoded by a pair of highly conserved, adjacent genes on the X chromosome of *Drosophila melanogaster*. Mol. Cell. Biol. **8**:4313–4321.
3. **Bucher, K., and L. Skogerson.** 1976. Cryptopleurine—an inhibitor of translocation. Biochemistry **15**:4755–4759.
4. **Debuchy, R., S. Purton, and J-D. Rochaix.** 1989. The argininosuccinate lyase gene of *Chlamydomonas reinhardtii*: an important tool for nuclear transformation and for correlating the genetic and molecular maps of the *ARG7* locus. EMBO J. **8**:2803–2809.
5. **Diaz, J.-J., D. D. Rhoads, and D. J. Roufa.** 1990. Genetic analysis of a vital mammalian housekeeping locus using CHO cells that express a transfected mutant allele. Somatic Cell Mol. Genet. **16**:517–528.
6. **Fernández, E., and J. Cárdenas.** 1989. Genetic and regulatory aspects of nitrate assimilation in algae, p. 101–124. *In* J. L. Wray and J. R. Kinghorn (ed.), Molecular and genetic aspects of nitrate assimilation. Oxford University Press, Oxford.
7. **Fernández, E., R. Schnell, L. P. W. Ranum, S. C. Hussey, C. D. Silflow, and P. A. Lefebvre.** 1989. Isolation and characterization of the nitrate reductase structural gene of *Chlamydomonas reinhardtii*. Proc. Natl. Acad. Sci. USA **86**:6449–6453.
8. **Goldschmidt-Clermont, M., and M. Rahire.** 1986. Sequence, evolution and differential expression of the two genes encoding variant small subunits of ribulose bisphosphate carboxylase/oxygenase in *Chlamydomonas reinhardtii*. J. Mol. Biol. **191**:421–432.
9. **Goodenough, U. W., B. Shames, L. Small, T. Saito, R. C. Crain, M. A. Sanders, and J. L. Salisbury.** 1993. The role of calcium in the *Chlamydomonas reinhardtii* mating reaction. J. Cell Biol. **121**:365–374.

10. **Grollman, A. P.** 1966. Structural basis for inhibition of protein synthesis by emetine and cycloheximide based on an analogy between ipecac alkaloids and glutarimide antibiotics. Proc. Natl. Acad. Sci. USA **56:**1867–1874.
11. **Hall, L. M., K. B. Taylor, and D. D. Jones.** 1993. Expression of a foreign gene in *Chlamydomonas reinhardtii*. Gene **124:**75–81.
12. **Harris, E. H.** 1989. The *Chlamydomonas* sourcebook: a comprehensive guide to biology and laboratory use. Academic Press, Inc., San Diego, Calif.
13. **Hasnain, S. E., E. K. Manavathu, and W.-C. Leung.** 1985. DNA-mediated transformation of *Chlamydomonas reinhardi* cells: use of aminoglycoside 3′-phosphotransferase as a selectable marker. Mol. Cell. Biol. **5:**3647–3650.
14. **Henikoff, S.** 1984. Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene **28:**351–359.
15. **Hinnebusch, A. G.** 1988. Novel mechanisms of translational control in *Saccharomyces cerevisiae*. Trends Genet. **4:**169–174.
16. **Hung, T., K. Mak, and K. Fong.** 1990. A specificity enhancer for polymerase chain reaction. Nucleic Acids Res. **18:**4953.
17. **James, S. W., C. D. Silflow, M. D. Thompson, L. P. W. Ranum, and P. A. Lefebvre.** 1989. Extragenic suppression and synthetic lethality among *Chlamydomonas reinhardtii* mutants resistant to anti-microtubule drugs. Genetics **122:**567–577.
18. **Kindle, K. L.** 1990. High-frequency nuclear transformation of *Chlamydomonas reinhardtii*. Proc. Natl. Acad. Sci. USA **87:**1228–1232.
19. **Kindle, K. L., R. A. Schnell, E. Fernández, and P. A. Lefebvre.** 1989. Stable nuclear transformation of *Chlamydomonas* using the *Chlamydomonas* gene for nitrate reductase. J. Cell Biol. **109:**2589–2601.
20. **Kozak, M.** 1986. Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell **44:**283–292.
21. **Kozminski, K. G., D. R. Diener, and J. L. Rosenbaum.** 1993. High-level expression of nonacetylatable α-tubulin in *Chlamydomonas reinhardtii*. Cell Motil. Cytoskel. **25:**158–170.
22. **Larkin, J. C., J. P. Hunsperger, D. Culley, I. Rubenstein, and C. D. Silflow.** 1989. The organization and expression of a maize ribosomal protein gene family. Genes Dev. **3:**500–509.
23. **Larkin, J. C., J. R. Thompson, and J. L. Woolford, Jr.** 1987. Structure and expression of the *Saccharomyces cerevisiae CRY1* gene: a highly conserved ribosomal protein gene. Mol. Cell. Biol. **7:**1764–1775.
24. **Lee, V. D., M. Stapleton, and B. Huang.** 1991. Genomic structure of *Chlamydomonas* caltractin: evidence for intron insertion suggests a probable genealogy for the EF-hand superfamily of proteins. J. Mol. Biol. **221:**175–191.
25. **Lefebvre, P. A., and J. L. Rosenbaum.** 1986. Regulation of the synthesis and assembly of ciliary and flagellar proteins during regeneration. Annu. Rev. Cell Biol. **2:**517–546.
26. **Macejak, D. G., and P. Sarnow.** 1991. Internal initiation of translation mediated by the 5′ leader of a cellular mRNA. Nature (London) **353:**90–94.
27. **Mansour, S. L., K. R. Thomas, and M. R. Capecchi.** 1988. Disruption of the proto-oncogene *int-2* in mouse embryo-derived stem cells: a general strategy for targeting mutations to non-selectable genes. Nature (London) **336:**348–352.
28. **Marchuk, D., M. Drumm, A. Saulino, and F. S. Collins.** 1991. Construction of T-vectors, a rapid and general system for direct cloning of unmodified PCR products. Nucleic Acids Res. **19:**1154.
29. **Mayfield, S. P., and K. L. Kindle.** 1990. Stable nuclear transformation of *Chlamydomonas reinhardtii* by using a *C. reinhardtii* gene as the selectable marker. Proc. Natl. Acad. Sci. USA **87:**2087–2091.
30. **McCabe, P. C.** 1990. Production of single-stranded DNA by asymmetric PCR, p. 76–83. *In* M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (ed.), PCR protocols: a guide to methods and applications. Academic Press, Inc., San Diego, Calif.
31. **Paulovich, A. G., J. R. Thompson, J. C. Larkin, Z. Li, and J. L. Woolford, Jr.** 1993. Molecular genetics of cryptopleurine resistance in *Saccharomyces cerevisiae*: expression of a ribosomal protein gene family. Genetics **135:**719–730.
32. **Paz, V., T.-L. Chang, A. Glück, and I. G. Wool.** 1989. The primary structure of rat ribosomal protein S14. Nucleic Acids Res. **17:**9484.
33. **Pelletier, J., and N. Sonenberg.** 1988. Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. Nature (London) **334:**320–325.
34. **Perelman, D., and J. C. Boothroyd.** 1990. Lack of introns in the ribosomal protein gene S14 of trypanosomes. Mol. Cell. Biol. **10:**3284–3288.
35. **Ranum, L. P. W., M. D. Thompson, J. A. Schloss, P. A. Lefebvre, and C. D. Silflow.** 1988. Mapping flagellar genes in *Chlamydomonas* using restriction fragment length polymorphisms. Genetics **120:**109–122.
36. **Rhoads, D. D., A. Dixit, and D. J. Roufa.** 1986. Primary structure of human ribosomal protein S14 and the gene that encodes it. Mol. Cell. Biol. **6:**2774–2783.
37. **Rhoads, D. D., and D. J. Roufa.** 1985. Emetine resistance of Chinese hamster cells: structures of wild-type and mutant ribosomal protein S14 mRNAs. Mol. Cell. Biol. **5:**1655–1659.
38. **Rochaix, J.-D., and J. van Dillewijn.** 1982. Transformation of the green alga *Chlamydomonas reinhardi* with yeast DNA. Nature (London) **36:**925–931.
39. **Sager, R., and S. Granick.** 1953. Nutritional studies with *Chlamydomonas reinhardtii*. Ann. N. Y. Acad. Sci. **466:**18–30.
40. **Saiki, R. K.** 1990. Amplification of genomic DNA, p. 13–20. *In* M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (ed.), PCR protocols: a guide to methods and applications. Academic Press, Inc., San Diego, Calif.
41. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
42. **Savereide, P. B.** 1991. The molecular basis of resistance to the protein synthesis inhibitor cryptopleurine in *Chlamydomonas reinhardtii*. Ph.D. thesis. University of Minnesota, St. Paul.
43. **Schnell, R. A., and P. A. Lefebvre.** 1993. Isolation of the *Chlamydomonas* regulatory gene *NIT2* by transposon tagging. Genetics **134:**737–747.
44. **Siersma, P., and K. S. Chiang.** 1971. Conservation and degradation of cytoplasmic and chloroplast ribosomes in *Chlamydomonas reinhardtii*. J. Mol. Biol. **58:**167–185.
45. **Silflow, C. D., and J. Youngblom.** 1986. *Chlamydomonas reinhardtii* tubulin gene structure. Ann. N. Y. Acad. Sci. **466:**18–30.
46. **Sodeinde, O. A., and K. L. Kindle.** 1993. Homologous recombination in the nuclear genome of *Chlamydomonas reinhardtii*. Proc. Natl. Acad. Sci. USA **90:**9199–9203.
47. **Tam, L.-W., and P. A. Lefebvre.** 1993. Cloning of flagellar genes in *Chlamydomonas reinhardtii* by DNA insertional mutagenesis. Genetics **135:**375–384.
48. **Tyler, B. M., and K. Harrison.** 1990. A *Neurospora crassa* ribosomal protein gene, homologous to yeast *CRY1*, contains sequences potentially coordinating its transcription with rRNA genes. Nucleic Acids Res. **18:**5759–5765.
49. **Youngblom, J., J. A. Schloss, and C. D. Silflow.** 1984. The two β-tubulin genes of *Chlamydomonas reinhardtii* code for identical proteins. Mol. Cell. Biol. **4:**2686–2696.