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Expression of Chimeric Genes by the Light-Regulated *cabII*-1 Promoter in *Chlamydomonas reinhardtii*: A *cabII*-1/*nit1* Gene Functions as a Dominant Selectable Marker in a *nit1*⁻ *nit2*⁻ Strain

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In *Chlamydomonas reinhardtii*, expression of the *cabII*-1 gene increases dramatically in response to light (*cabII*-1 encodes one of the light-harvesting chlorophyll *a*/*b*-binding proteins of photosystem II). We have used a region upstream of the *cabII*-1 gene in translational fusions to the bacterial *uidA* gene (encodes β-glucuronidase) and transcriptional fusions to the *Chlamydomonas* nitrate reductase gene (*nit1*). *Chlamydomonas* transformants carrying intact copies of the chimeric *uidA* gene do not express β-glucuronidase at the level of enzyme activity or mRNA accumulation. Methylation in the *cabII*-1 promoter region of the introduced gene is extensive in these strains, suggesting that newly introduced foreign genes may be recognized and silenced by a cellular mechanism that is correlated with increased methylation. Transformants that express the chimeric *cabII*-1/*nit1* gene have been recovered. In contrast to the endogenous *nit1* gene, the chimeric *cabII*-1/*nit1* gene is expressed in ammonium-containing medium. Moreover, *nit1* mRNA accumulation is dramatically stimulated by light, with a time course that is indistinguishable from that of the endogenous *cabII*-1 gene. The *cabII*-1/*nit1* gene has been used to select transformants in a *nit1*⁻ *nit2*⁻ *Chlamydomonas* strain (CC400G) and should be useful for transformation of the large number of mutants in the Ebersold-Levine lineage, which carry the same mutations.

In many photosynthetic organisms, the expression of nuclear genes that encode chloroplast proteins increases dramatically in response to light. The most intensively studied light-responsive genes have been nuclear genes that encode abundant chloroplast proteins, in particular the *rbcS* genes, which encode the small subunit of ribulose-bisphosphate carboxylase, a stromal enzyme, and the *cab* genes, which encode the light-harvesting chlorophyll *a*/*b*-binding proteins of photosystem II, integral thylakoid membrane proteins. In a number of flowering plants, it has been demonstrated that light activates expression of these genes by increasing the rate of transcription initiation (37, 60, 61). Mutants with abnormal photomorphogenic responses have been isolated in *Arabidopsis thaliana* and crop plants. These show either constitutive expression of genes that are normally silent in the dark (5, 6, 14) or phytochrome deficiencies that lead to irregularities in light-induced gene expression (1, 7, 55).

Chlamydomonas reinhardtii is a single-celled eukaryotic alga with many favorable attributes for genetic and molecular biological studies. In contrast to flowering plants, *Chlamydomonas* cells can grow in the dark using acetate as a carbon and energy source. It is therefore possible to isolate photosynthetic mutations in *Chlamydomonas* cells that would be lethal in higher plants. The expression of a number of genes, including one of the *rbcS* genes (23), at least one *cab* gene (32, 56), the carbonic anhydrase gene (16), and several heat shock genes (25, 29), is regulated by light in *C. reinhardtii*.

In *C. reinhardtii* there are six or seven *cab* genes that encode a family of closely related light-harvesting chlorophyll *a*/*b*-binding proteins (8, 28). The expression of one highly expressed member of this gene family, *cabII*-1, increases dramatically in response to light in cells that have been grown phototrophically under a 12-h dark–12-h light diurnal cycle (31, 32). Nuclear run-on transcription assays suggest that the light-induced increase in *cabII*-1 mRNA is due to an increase in transcription initiation (34).

We are interested in elucidating the mechanism by which the light-induced increase in *cabII*-1 gene expression occurs. We are taking two approaches toward this goal: (i) to locate and characterize *cis*-acting DNA sequences that mediate light-activated transcription in vivo and (ii) to develop a genetic selection for mutants that are unable to express the *cabII*-1 gene in response to light. Both of these approaches take advantage of recently developed nuclear transformation technology in which *Chlamydomonas* genes have been used to complement auxotrophic or photosynthetic mutations (11, 21, 35, 44, 57).

Selectable markers commonly used for transformation in other organisms, such as kanamycin resistance, do not work in *Chlamydomonas* cells. The difficulty in expressing foreign genes has been attributed to extremely biased codon usage in highly expressed *Chlamydomonas* genes (26, 44). Codon choice can significantly affect the level of expression of introduced genes in other organisms (17, 40), but there is as yet no experimental evidence that this is the cause for the failure to express foreign genes in *Chlamydomonas* cells. Indeed, *Chlamydomonas* genes expressed at low levels may exhibit a less biased codon choice (12).

In this paper, we describe attempts to express chimeric genes containing the bacterial *uidA* gene (encodes β-glucuronidase [GUS] [30]) or the *Chlamydomonas nit1* gene (encodes nitrate reductase [NR]). Transformants carrying intact copies of a translational fusion between the *cabII*-1

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promoter and *uidA* do not express GUS enzymatic activity nor GUS mRNA. However, transformants carrying a transcriptional fusion of the *cabII*-1 upstream region to the *Chlamydomonas nit1* gene can be used to transform a *nit1*$^-$ recipient to *nit*$^+$, and expression of the chimeric gene appears to be regulated by the *cabII*-1 promoter. The use of this construct as a dominant selectable marker for transforming a *nit1*$^-$ *nit2*$^-$ *Chlamydomonas* mutant is described.

MATERIALS AND METHODS

***Chlamydomonas* strains.** *nit1*-305 is a stable NR mutation (reversion, $\sim10^{-8}$) originally isolated in wild-type strain 6145c (58). A cell wall-deficient strain carrying this mutation was constructed by P. Lefebvre (15). A *nit*$^+$ revertant of the *nit1*-305 strain was isolated and used as the *nit*$^+$ control in several experiments.

CC400G (*cw*15 [32]) is derived from the Ebersold-Levine lineage of laboratory strains, which contain mutations in both *nit1* and *nit2* (26).

Construction of plasmid vectors. (i) *cabII*-1/GUS translational fusions. A 1.8-kb *Pst*I fragment, containing 1.7 kb of the *cabII*-1 upstream region, the 40-bp 5′ untranslated region, and 12 codons of the transit peptide, was ligated into the *Pst*I site of pBSGUS, a promoterless GUS construct that is flanked at the 3′ end by the polyadenylation site from the *Agrobacterium tumefaciens* nopaline synthetase gene (NOS [46]). This construct is called 1.8-kb *cabII*-1/GUS/NOS and is shown in Fig. 1A. To produce 1.8-kb *cabII*-1/GUS/*cab*, the NOS terminator was removed by digestion with *Sst*I, and the ends were trimmed with T4 DNA polymerase. A 0.7-kb *Pst*I fragment from the 3′ end of the *cabII*-1 gene was trimmed with T4 DNA polymerase and ligated into this site. The 4-kb *cabII*-1/GUS/*cab* construct was created by ligating a 3-kb *Kpn*I-*Nde*I fragment from the upstream region of *cabII*-1 into 1.8-kb *cabII*-1/GUS/*cab* that had been digested with *Kpn*I and *Nde*I.

(ii) *cabII*-1/*nit1* transcriptional fusions. pSE*cabII*-1 contains a *Sma*I-*Eco*RI fragment that includes the entire *cabII*-1 coding region and 2.5 kb of upstream sequence (35) (Fig. 1B). pMN24 is a genomic clone from *C. reinhardtii* that includes the entire *nit1* gene (21). A 2-kb *Hin*dIII fragment, which begins approximately 40 bp upstream from the *nit1* translation start (38), was isolated from pMN24. The ends were filled with Klenow fragment of DNA polymerase I, and the fragment was ligated into pSE*cabII*-1 at the unique *Hpa*I site at the *cabII*-1 transcription start site, which lies 40 bp upstream of the initiating ATG (28). This intermediate plasmid was cut with *Hpa*I and *Eco*RI to remove the *cabII*-1 gene and 3′ end. It was then ligated to a 7.8-kb *Hpa*I-*Eco*RI fragment from pMN24, which contains part of the *nit1* coding region, the putative polyadenylation site, and the 3′-flanking region. The resulting construct, 2.5-kb *cabII*-1/*nit1*, is a transcriptional fusion between 2.5 kb of *cabII*-1 upstream sequences and the *nit1* gene and 3′-flanking region.

The *Kpn*I-*Eco*RI fragment containing *cabII*-1 was subcloned from a λEMBL4 clone containing ~15 kb of the *cabII*-1 region into pBS-SKII$^-$ (Stratagene). In this clone, a unique *Nde*I site is located approximately 1 kb upstream from the *cabII*-1 coding sequence. The subclone was digested with *Nde*I and *Eco*RI to remove 1 kb of the *cabII*-1 upstream region and the entire *cabII*-1 coding region. This region was replaced by the corresponding fragment from 2.5-kb *cabII*-1/*nit1*. This clone, called 4-kb *cabII*-1/*nit1*, contains 4 kb of *cabII*-1 upstream region transcriptionally fused to the *nit1* gene and flanking sequence.

To make 8.5-kb *cabII*-1/*nit1*, the *Kpn*I-*Nde*I fragment of 4-kb *cabII*-1/*nit1* was replaced by a 7.3-kb *Eco*RI-*Nde*I fragment from the *cabII*-1 upstream region, as follows. The 4-kb *cabII*-1/*nit1* was digested with *Kpn*I, trimmed with T4 DNA polymerase, and then cut with *Nde*I. A plasmid containing 8.5 kb of *cabII*-1 upstream sequence was cut with *Eco*RI, trimmed with T4 DNA polymerase, and then cut with *Nde*I; a 7.3-kb *cabII*-1 upstream genomic fragment was isolated. The fragments were ligated to generate 8.5-kb *cabII*-1/*nit1*.

Transformation. Rapidly dividing cultures of cell wall-deficient *Chlamydomonas* strains were transformed by agitation in the presence of 0.5-mm-diameter glass beads (33). Plasmid DNA was linearized at a site in the polylinker by appropriate restriction digests. Two micrograms of selectable DNA (*nit1* or *cabII*-1/*nit1*) and, where appropriate, 4 μg of the unselected cotransforming DNA were used to transform $\sim6 \times 10^7$ cells, which were spread on a single plate for selection (Sager-Granick medium II [SG II] in which KNO_3 replaced NH_4NO_3 [35]).

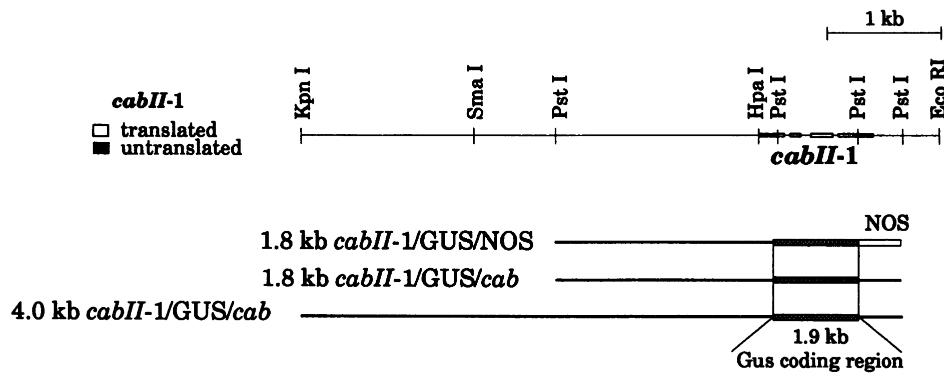
Analysis of nucleic acids. (i) Isolation of DNA and RNA. For DNA isolation, cells were grown to late log phase in nonselective liquid medium (SG II plus NH_4NO_3) and DNA was isolated as described elsewhere (35).

For RNA analysis, cells were first grown in liquid HSA medium (26) and then inoculated at low density ($\sim5 \times 10^4$ cells per ml) into M medium (KNO_3 as the sole nitrogen source [53]) or HS medium (contains NH_4Cl [26]). Cells were grown in a New Brunswick Scientific psychrotherm at 25°C, with shaking at a rate of 120 rpm, under a 12-h dark–12-h light illumination regimen. A mixture of CO_2 (5%) and air (95%) was continuously blown over the cultures. RNA was isolated as described previously (32) from mid-log-phase cultures at the end of the dark period or after various periods of illumination.

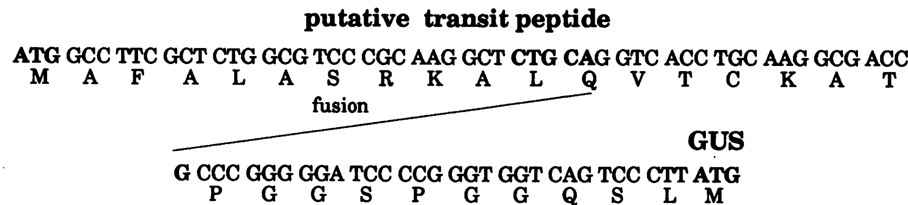
(ii) Nucleic acid blots. Nucleic acids were separated electrophoretically and then transferred to a nylon membrane (NYTRAN; Schleicher & Schuell) by capillary blotting or with a Stratagene PosiBlot apparatus. Radioactive DNA probes were prepared by using the Klenow fragment of DNA polymerase to extend annealed random hexamer oligonucleotides (18). RNA probes were prepared by using SP6 RNA polymerase as described by Stratagene. Hybridization with DNA probes was performed as described elsewhere (9) in a Robbins Scientific hybridization incubator. Hybridizations with RNA probes were performed in 40% formamide (41). Blots were exposed to X-ray film (Kodak XAR5) at −70°C with a single intensifying screen. In cases in which the signal was very weak, blots were exposed to Phosphor Imager screens (Molecular Dynamics).

(iii) Quantitation of gene expression. Lanes on formaldehyde gels were loaded with equal amounts of RNA as determined by measuring the A_{260}. After electrophoresis and transfer to nylon membranes, RNA was hybridized with probes specific for the introduced chimeric gene(s). Then the blots were stripped, and the RNA was hybridized with the endogenous *cabII*-1 gene and/or *Aedes aegypti* actin gene. A 1.5-kb *Bam*HI-*Eco*RI fragment from 2.5-kb *cabII*-1/*nit1*, which includes the putative polyadenylation site, was used as a hybridization probe for *nit1* transcripts. pHS16 (56), which contains sequences from the *cabII*-1 3′ untranslated region, was used to detect *cabII*-1 mRNA; this probe does not hybridize with the other *cabII* genes. pA19 is a clone of the coding region of the *A. aegypti* actin gene (32). RNA probes prepared from this template hybridize with rRNA as well as actin mRNA and were used to correct samples for

A. *cabII*-1/GUS



nucleotide and amino acid sequence at *cabII*-1/GUS fusion junction:



B. *cabII*-1/*nit1*

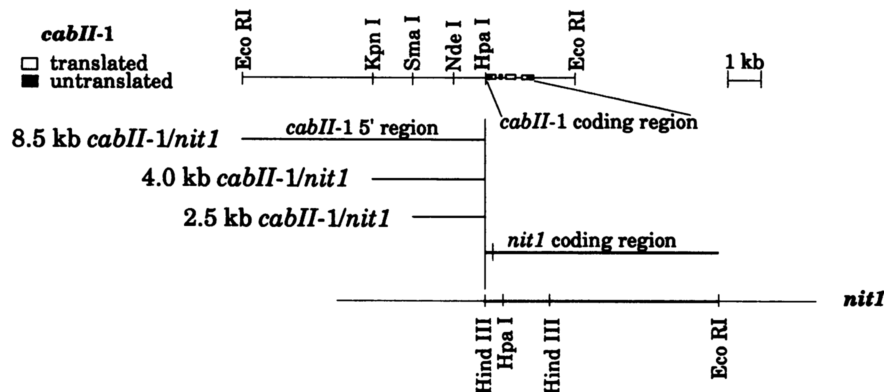


FIG. 1. Description of *cabII*-1 chimeric plasmids. (A) The upstream region of the *cabII*-1 gene (1.8 or 4.0 kb) was translationally fused to the coding region of GUS by using a *Pst*I site in exon I of *cabII*-1 for the fusion. The nucleotide and amino acid sequences of the fusion junction are shown (28, 30). (B) The *cabII*-1 upstream region (8.5, 4.0, or 2.5 kb) was fused to *nit1* by using a *Hpa*I site located at the transcription initiation site of *cabII*-1 and a *Hin*dIII site located 40 bp upstream of the putative initiation codon of *nit1*.

equal loading. Phosphor Imager screens were exposed to membranes and scanned, and hybridization with each probe was quantified by using Imagequant software. Background values were usually determined on a randomly selected area of the filter. In cases in which background hybridization was associated with the lane, this background was subtracted from the value in the band. For normalization, values obtained with the *cabII*-1/*nit1* probe were divided by those obtained with actin (rRNA).

GUS assays. (i) Histochemical staining. To test for GUS expression, *Chlamydomonas* cells were resuspended in GUS-staining solution (10 mg of X-Gluc [5-bromo-4-chloro-3-indolyl-β-D-glucuronide] was dissolved in 200 μl of dimethyl sulfoxide and then added to 20.0 ml of 0.01 M EDTA–0.1 M $NaPO_4$ [pH 7.0]–0.5 mM K-ferrocyanide [45]). Triton X-100 was added to the GUS-staining solution at concentrations ranging from 0 to 0.006% (vol/vol). Cells were incubated at room temperature for several days,

washed in 70% ethanol to remove chloroplast pigments, and observed microscopically for blue pigment deposition.

(ii) Enzyme assays. *Chlamydomonas* cells were grown to mid-log phase in SG II plus NH_4NO_3, harvested, and resuspended in freeze-thaw extraction buffer (50 mM Na_2HPO_4-NaH_2PO_4 [pH 7.0], 0.01 M 2-mercaptoethanol, 0.01 M Na_2EDTA [26]). Cells were frozen on dry ice and thawed at room temperature three times. Particulate residue was removed by centrifugation in an Eppendorf Microfuge for 10 min. GUS assays were performed with methylumbelliferyl β-D-glucuronide as the substrate (30).

RESULTS

Transformants carrying the *cabII*-1/GUS fusion gene do not express GUS activity nor accumulate GUS mRNA. The coding region of GUS was translationally fused to either 1.8 or 4 kb of the *cabII*-1 upstream region as shown in Fig. 1A. These *cabII*-1/GUS chimeric genes are expected to direct the synthesis of a transcript with approximately 40 bases of the *cabII*-1 5′ untranslated leader, 35 bases encoding ~12 amino acids of the *cabII*-1 transit peptide, and the entire GUS coding region. The GUS coding region is flanked at its 3′ end by either a NOS 3′ end fragment from *A. tumefaciens* (in 1.8-kb *cab*/GUS/NOS) or a 700-bp *cabII*-1 3′ fragment encoding approximately 450 bases of the 3′ untranslated region of *cabII*-1 mRNA followed by a *Chlamydomonas* polyadenylation site (in 1.8-kb *cabII*-1/GUS/*cab* and 4-kb *cabII*-1/GUS/*cab*).

These plasmids were introduced into a cell wall-deficient *Chlamydomonas* NR structural gene mutant (the *nit1*-305 *cw*15 strain, hereafter called the *nit1*-305 strain) by a cotransformation procedure in which cells were agitated with *nit1* DNA, *cabII*-1/GUS DNA, and glass beads. Nit$^+$ colonies were selected on agar plates with nitrate as the sole source of nitrogen.

Transformants in which DNA blot analysis indicated that the entire chimeric gene had integrated intact were tested for GUS activity by histochemical staining and a highly sensitive fluorescent assay. Enzyme activity above background was not detected in any of 18 tested transformants, including 4 1.8-kb *cabII*-1/GUS/NOS transformants, 10 1.8-kb *cabII*-1/GUS/*cab* transformants, and 4 4.0-kb *cabII*-1/GUS/*cab* transformants. (The 4.0-kb *cabII*-1/GUS transformants were shown to contain at least 3.1 kb of the *cabII*-1 upstream region.)

RNA was isolated from transformants that had been grown in a 12-h dark–12-h light illumination regime, 6 h after the onset of illumination, when *cabII*-1 mRNA abundance is high. When RNA blots were hybridized with a GUS probe, a broad region of hybridization was seen in lanes containing RNA prepared from transformants containing *cabII*-1/GUS DNA but not in strains lacking *cabII*-1/GUS DNA (not shown). This heterodisperse hybridization was seen in samples from both dark- and light-exposed cells and in transformants containing the *cabII*-1 promoter in the reverse orientation. In no case was a discrete GUS transcript detected, suggesting that transcripts are not synthesized from the *cabII*-1 promoter and/or that the chimeric transcript is unstable.

A *cabII*-1/*nit1* chimeric gene can be used to select Nit$^+$ transformants. A series of transcriptional fusions between the *cabII*-1 upstream region and *nit1* was constructed; 2.5, 4.0, or 8.5 kb of the *cabII*-1 upstream region was fused at the *cabII*-1 transcription initiation site to the *nit1* gene (Fig. 1B). The putative *nit1* translation initiation codon is located approximately 40 bp downstream from the *Hin*dIII site used for the fusion (38). The *cabII*-1/*nit1* chimeric genes were transformed into the *nit1*-305 strain, and a significant number of Nit$^+$ colonies were recovered. However, the transformation efficiency was only 5 to 10% of that with the wild-type *nit1* gene (see Table 1). Transformation with a promoterless construct yielded few Nit$^+$ colonies, only slightly more than control transformations with no added DNA (reversion rate, ~10^{-8}).

TABLE 1. Recovery of *nit1*-305 (*nit1*$^-$ *nit2*$^+$) and CC400G (*nit1*$^-$ *nit2*$^-$) transformants with *nit1* and *cabII*-1/*nit1* DNA

Expt[a] and DNA	*nit1*-305 result		CC400G result[b]	
	No. of plates	No. of transformants/ plate	No. of plates	No. of transformants/ plate
I				
No DNA	11	<1 (1/11 plates)	3	0
Promoterless *nit1*	7	<1 (6/7 plates)	ND	ND
2.5-kb *cabII*-1/*nit1*	13	4	5	>10
pMN24 (*nit1*)	11	>100	5	0
II				
4.0-kb *cabII*-1/*nit1*	3	10	ND	ND
pMN24 (*nit1*)	2	>150	ND	ND
III				
8.5-kb *cabII*-1/*nit1*	4	28	ND	ND
pMN24 (*nit1*)	2	>300	ND	ND

[a] I, II, and III represent three independent experiments.
[b] ND, not done.

Many of the large collection of *Chlamydomonas* mutations have been isolated in a *nit1*$^-$ *nit2*$^-$ genetic background; *nit2* is a positive regulatory gene whose function is required for expression of the wild-type *nit1* gene (19). If the chimeric *cabII*-1/*nit1* gene is expressed under the control of the *cabII*-1 promoter, it may be possible to express the gene in a *nit2*$^-$ background. Therefore, we transformed strain CC400G (*nit1*$^-$ *nit2*$^-$ *cw*15) with the 2.5-kb *cabII*-1/*nit1* construct. Nit$^+$ colonies were recovered after 4 weeks by using 2.5-kb *cabII*-1/*nit1* DNA but not by using the wild-type *nit1* gene (pMN24; Table 1). Interestingly, although the *cabII*-1/*nit1* transformants took much longer to appear in the *nit2*$^-$ background than in *nit2*$^+$ cells, once they had been recovered, they grew as well as the wild type even under selective conditions.

To establish that the Nit$^+$ colonies were transformants that had integrated the chimeric *cabII*-1/*nit1* gene, genomic DNA was isolated, digested with *Sma*I, and hybridized with a 2.5-kb *Sma*I-*Hpa*I probe from the *cabII*-1 upstream region. DNA blots from CC400G cells transformed with 2.5-kb *cabII*-1/*nit1* DNA are shown in Fig. 2. In each transformant, the probe hybridized with a 5.7-kb endogenous *cabII*-1 fragment and also with additional bands. Integrated 2.5-kb *cabII*-1/*nit1* DNA with an intact 5′ end should give rise to a 4.5-kb *Sma*I fragment that hybridizes with the probe. Transformants A, E, F, and K have integrated a single intact copy of the *cabII*-1/*nit1* gene; transformants C and D show stronger hybridization and presumably have at least two intact copies. In some cases (transformants A and C), additional copies of *cabII*-1/*nit1* have been integrated, but they must have been truncated at the 5′ end, since the size of the *Sma*I fragments that hybridize with the probe differs from 4.5 kb. In transformants with a single integrated

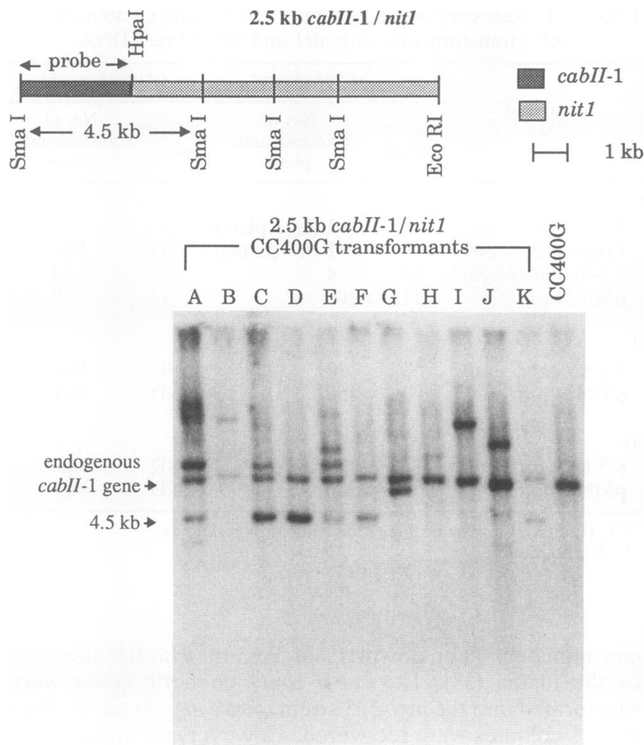


FIG. 2. Integrated copies of *cabII-1/nit1* in CC400G transformants. The 2.5-kb *cabII-1/nit1* DNA was linearized with *Eco*RI and transformed into *Chlamydomonas* strain CC400G by agitating with glass beads (33). Genomic DNA from Nit$^+$ transformants was digested with *Sma*I, electrophoresed in an agarose gel, and transferred to a nylon membrane which was incubated with the 2.5-kb *Sma*I-*Hpa*I *cabII-1* upstream fragment shown in the figure. Intact integration should generate a 4.5-kb *Sma*I fragment that hybridizes with the probe.

cabII-1/nit1 gene, we assume that the coding region and 3′ end are intact because the *nit1* defect has been complemented. However, in transformants carrying more than one copy of the *cabII-1/nit1* gene, we cannot be sure that all of the genes contain an intact 3′ end and are functional. Nevertheless, this does not appear to be a problem since the results of gene expression studies have been similar whether the transformants contain a single intact integrated copy of the chimeric gene or more than one copy (see below).

***cabII-1/nit1* gene expression is induced by light and not repressed by ammonium.** To determine whether the accumulation of *nit1* mRNA is regulated by the *cabII-1* promoter in transformants carrying the chimeric gene, we analyzed *nit1* mRNA accumulation during a 12-h dark–12-h light illumination regime. The time course of light-induced *cabII-1* mRNA accumulation is similar in the *nit1-305* strain to that previously reported for strain CC400G (32) except that the dark level of *cabII-1* mRNA is much lower in the *nit1-305* strain; consequently, the light induction is significantly greater in the *nit1-305* strain (~20-fold in the *nit1-305* strain compared to three- to fourfold in CC400G). An RNA blot that compares the level of expression of the *cabII-1/nit1* gene in the dark and light in transformants from CC400G and the *nit1-305* strain is shown in Fig. 3. The cells were grown in medium containing ammonium, conditions under which the endogenous *nit1* gene is repressed (see *nit1-305* and CC400G in Fig. 3A). In all transformants, *nit1* mRNA accumulates in the presence of ammonium, indicating that the *cabII-1/nit1* gene is expressed under these conditions. Furthermore, there is a very significant increase in *nit1* mRNA abundance in response to light. The magnitude of the light-induced increase for both *nit1* mRNA and *cabII-1* mRNA is shown in Fig. 3B. CC400G transformants accumulated twice as much *nit1* mRNA as *nit1-305* transformants, but the light induction is more pronounced in *nit1-305* than in CC400G transformants.

The expression of *cabII-1/nit1* is slightly increased by distal upstream DNA but is not proportional to gene copy number. Although *cabII-1/nit1* gene expression responds to light like the endogenous *cabII-1* gene qualitatively, *nit1* mRNA accumulates to a much lower level than *cabII-1* mRNA. Differences in probe length and specific activity preclude precise quantitation, but from the time required to get comparable exposures of RNA blots, we estimate that there is less than 1/10 as much *nit1* mRNA as *cabII-1* mRNA in *cabII-1/nit1* transformants. To see whether sequences further upstream might boost the overall level of expression, we examined *nit1* mRNA accumulation in Nit$^+$ colonies resulting from transformation with *cabII-1/nit1* genes with 2.5, 4.0, or 8.5 kb of upstream sequence (Fig. 4). These particular transformants were chosen because they expressed *nit1* mRNA well in preliminary experiments and most contained an intact upstream region (see the Fig. 4 legend). In all transformants, *nit1* mRNA accumulation from the chimeric gene was markedly stimulated by light. For 8.5-kb *cabII-1/nit1* transformants, the amount of expression in the light was only about threefold higher than in transformants containing 2.5-kb *cabII-1/nit1*. The abundance of *nit1* mRNA in 2.5-kb *cabII-1/nit1* transformants induced by light for 6 h was similar to the level in Nit$^+$ cells induced by transfer from ammonium- to nitrate-containing medium for 2 h in continuous light (Fig. 4).

A number of 2.5-kb *cabII-1/nit1* transformants were examined in detail to determine the number of intact and truncated chimeric genes. DNA blots were prepared from size-fractionated *Sma*I-digested genomic DNA and hybridized with a series of probes from the *cabII-1* upstream region (not shown). In general, there does not appear to be a positive correlation between the amount of gene expression and the number of integrated gene copies. Several CC400G transformants contain a single copy of the introduced gene yet express the gene at a high level. Two CC400G transformants contain multiple copies of the *cabII-1/nit1* gene, but compared with expression in transformants with a single integrated copy, expression of *cabII-1/nit1* is poor.

The time course of *cabII-1/nit1* light induction is identical to that of *cabII-1*. The *cabII-1/nit1* gene is expressed in the presence of ammonium, and expression increases in response to light. In flowering plants, genes encoding NR are induced by light in dark-adapted plants and regulated by a circadian rhythm in plants grown under alternating dark and light periods (4, 13, 47, 52). Therefore, we examined *nit1* mRNA accumulation in response to light during the light-dark diurnal cycle in *Chlamydomonas* cells. With a Nit$^+$ strain grown in nitrate-containing M medium, *nit1* mRNA was detectable in the dark but increased significantly after 6 h of light (data not shown). Because *nit1* mRNA accumulation increases in response to light in nitrate-containing medium, it is important to establish that light-induced expression from the *cabII-1/nit1* fusion gene is due to the *cabII-1* promoter rather than to sequences within the *nit1* gene. We therefore compared the time course of light induction of the chimeric *cabII-1/nit1* gene in a *nit1-305*

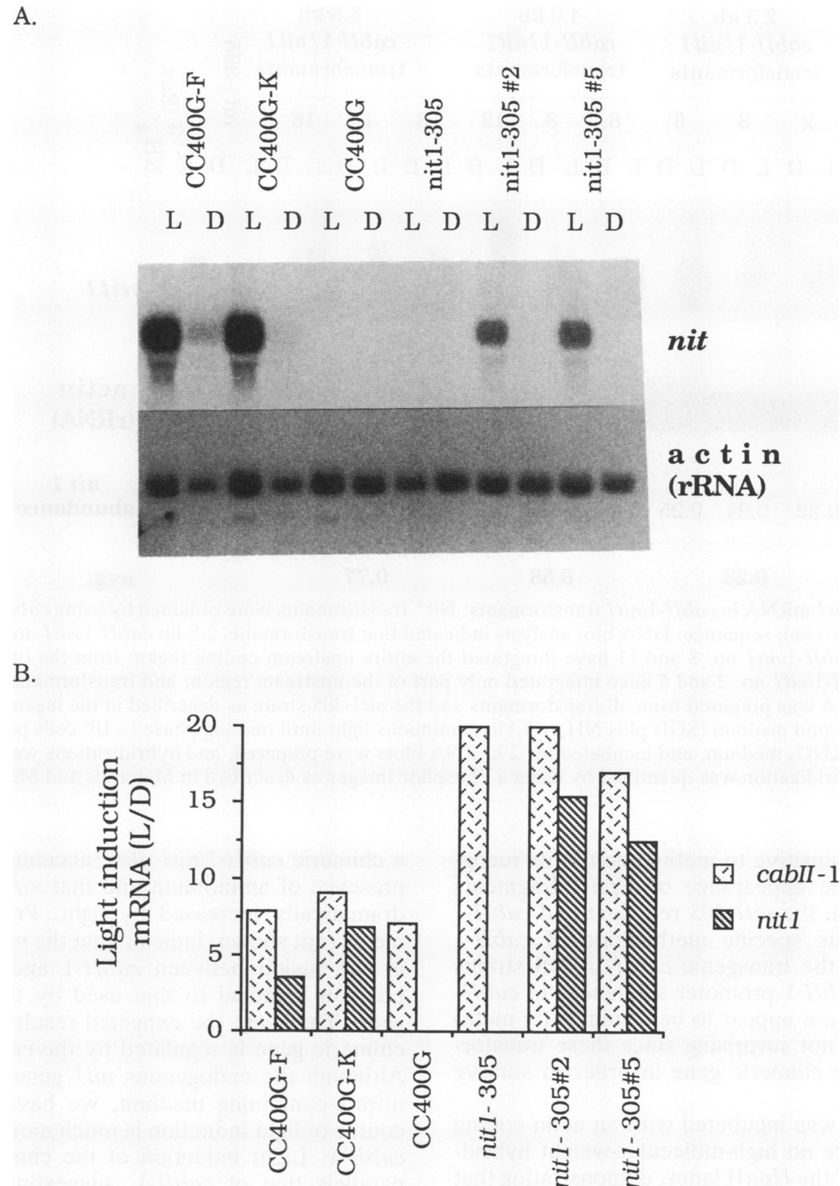


FIG. 3. Expression of 2.5-kb *cabII*-1/*nit1* genes in two *Chlamydomonas* strains. Untransformed and transformed isolates of two strains were grown in HS medium under a 12-h dark–12-h light illumination regime. RNA was isolated from cells at the end of the dark cycle (D) or after 6 h of illumination (L). RNA was separated in formaldehyde gels and transferred to nylon filters, which were incubated with a 1.5-kb *Bam*HI-*Eco*RI *nit1* 3' end probe or *A. aegypti* actin probe. (A) Autoradiogram from RNA blot; (B) quantitation. Data are expressed as light induction, defined as the mRNA abundance in the light divided by mRNA abundance in the dark.

transformant with that of the endogenous *cabII*-1 and *nit1* genes, in either HS medium (contains ammonium) or M medium (contains nitrate). In a 4.0-kb *cabII*-1/*nit1* transformant grown in HS medium, the time course of light-induced *nit1* mRNA accumulation from *cabII*-1/*nit1* was similar to that for the *cabII*-1 gene (Fig. 5A); the endogenous *nit1* gene is repressed by ammonium in this medium. In M medium, accumulation of *nit1* mRNA reached a maximum within 1 h of the onset of illumination, declined, and then increased again in parallel with *cabII*-1. When the Nit$^+$ revertant strain was grown in M medium, *nit1* mRNA abundance reached a maximum after 1 h of illumination, declined to about 50% of the maximal level after 2 h, and then remained constant (Fig. 5B). Thus, *nit1* mRNA accumulation in the 4.0-kb *cabII*-1/*nit1* transformant appears to result from combined expression of the endogenous *nit1* gene and the introduced *cabII*-1/*nit1* gene(s) in M medium.

Chimeric genes that are not expressed show increased methylation in the promoter region. The inability to detect GUS mRNA in *Chlamydomonas* transformants that carried full-length copies of the *cabII*-1/GUS gene could be due to transcript instability or low (or negligible) transcription. Since increased methylation has been correlated with the failure to express introduced genes in fungi and higher plants (24, 42, 49, 50, 59, 62), genomic DNA from a number of *cabII*-1/GUS and *cabII*-1/*nit1* transformants was analyzed by digestion with the isoschizomer *Hpa*II or *Msp*I and hybridized with a 2.5-kb *Sma*I-*Hpa*I *cabII*-1 upstream

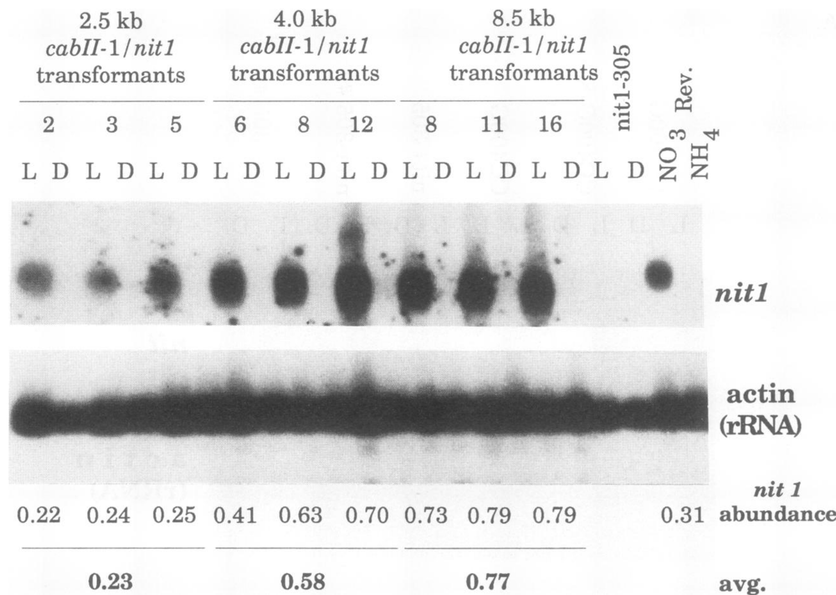


FIG. 4. Expression of *nit1* mRNA in *cabII-1/nit1* transformants. Nit^+ transformants were obtained by using *cabII-1/nit1* plasmids with 2.5, 4.0, or 8.5 kb of *cabII-1* upstream sequence. DNA blot analysis indicated that transformants 2.5-kb *cabII-1/nit1* no. 3; 4.0-kb *cabII-1/nit1* no. 6, 8, and 12; and 8.5-kb *cabII-1/nit1* no. 8 and 11 have integrated the entire upstream coding region from the original transforming DNA. Transformants 2.5-kb *cabII-1/nit1* no. 2 and 5 have integrated only part of the upstream region, and transformant 8.5-kb *cabII-1/nit1* no. 16 has not been analyzed. RNA was prepared from all transformants and the *nit1*-305 strain as described in the legend to Fig. 3. Nit^+ revertant cells (rev.) were grown in liquid medium (SGII plus NH_4NO_3) in continuous light until mid-log phase (~10^6 cells per ml), harvested, washed, resuspended in SGII plus KNO_3 medium, and incubated for 2 h. RNA blots were prepared, and hybridizations were performed as described in the legend to Fig. 3. Hybridization was quantified by using a Phosphor Imager as described in Materials and Methods; units are arbitrary.

probe. (*Hpa*II is more sensitive to methylation in its recognition site, CCGG.) The appearance of *Hpa*II fragments larger than those seen in the *nit1*-305 recipient and *cabII*-1 plasmid control suggests specific methylation of *cabII*-1 promoter sequences in the transgenic *cabII*-1/GUS strains (Fig. 6). In contrast, *cabII*-1 promoter sequences in *cabII-1/nit1* transformants do not appear to be significantly methylated. This is perhaps not surprising since these transformants must express the chimeric gene in order to survive selection.

When the same filter was incubated with an actin coding region probe, there were no high-molecular-weight hybridizing fragments in any of the *Hpa*II lanes, demonstrating that the genomic DNA was digested to completion in all samples. With a *cab* coding region probe, a small number of high-molecular-weight *Hpa*II fragments were labeled in genomic DNA isolated from *cab*/GUS transformants (three for *cab*/GUS-A and one for *cab*/GUS-M), suggesting that the endogenous *cab* coding sequences are slightly methylated in these transformants (data not shown). However, the number of *Hpa*II-insensitive sites in the *cab* coding regions is much lower than in the *cabII*-1 promoter(s), suggesting that the methylation is fairly specific for the introduced DNA. Interestingly, in transformants carrying the entire *cabII*-1 gene marked by a small insertion of bacteriophage λ DNA, a *cab* coding region probe hybridized extensively with high-molecular-weight *Hpa*II fragments, suggesting significant methylation of the coding region in these transformants (data not shown).

DISCUSSION

Regulation of *cabII*-1 and *nit1* gene expression. We have demonstrated that *Chlamydomonas* transformants carrying a chimeric *cabII-1/nit1* gene accumulate *nit1* mRNA in the presence of ammonium and that *nit1* mRNA abundance is dramatically increased by light. Primer extension experiments (not shown) indicate that the *nit1* transcript is initiated at the fusion between *cabII*-1 and *nit1* sequences, at a position identical to that used by the endogenous *cabII*-1 gene. These are the expected results if transcription of the chimeric gene is regulated by the *cabII*-1 upstream region. Although the endogenous *nit1* gene is induced by light in nitrate-containing medium, we have shown that the time course of light induction is much more rapid for *nit1* than for *cabII*-1. Light induction of the chimeric *cabII-1/nit1* gene parallels that of *cabII*-1, suggesting that the *cabII*-1 upstream region regulates expression of the chimeric gene. Thus, light-induced expression of both the endogenous *cabII*-1 and chimeric *cabII-1/nit1* genes is due to increased transcription mediated by DNA sequences in the *cabII*-1 upstream region.

We have demonstrated that *nit1* mRNA accumulation increases transiently at the beginning of the light period in cells exposed to a 12-h dark–12-h light illumination regimen (Fig. 5). In higher plants, the accumulation of NR mRNA is also regulated in response to light during a diurnal cycle. In *Arabidopsis* strains and tobacco, NR mRNA begins to increase before the end of the dark period, is maximal by the onset of illumination or within a few hours, and then declines through the remainder of the light period (4, 13). In maize plants grown under alternating periods of light and dark, NR mRNA increases during the first hour of light and then decreases, with a time course very similar to that observed in *Chlamydomonas* cells (39). In *Chlamydomonas* cells, this regulation clearly occurs at the level of transcription, since it is altered by replacement of the *nit1* upstream region with that of *cabII*-1. Since expression of the chimeric *cabII-1/nit1*

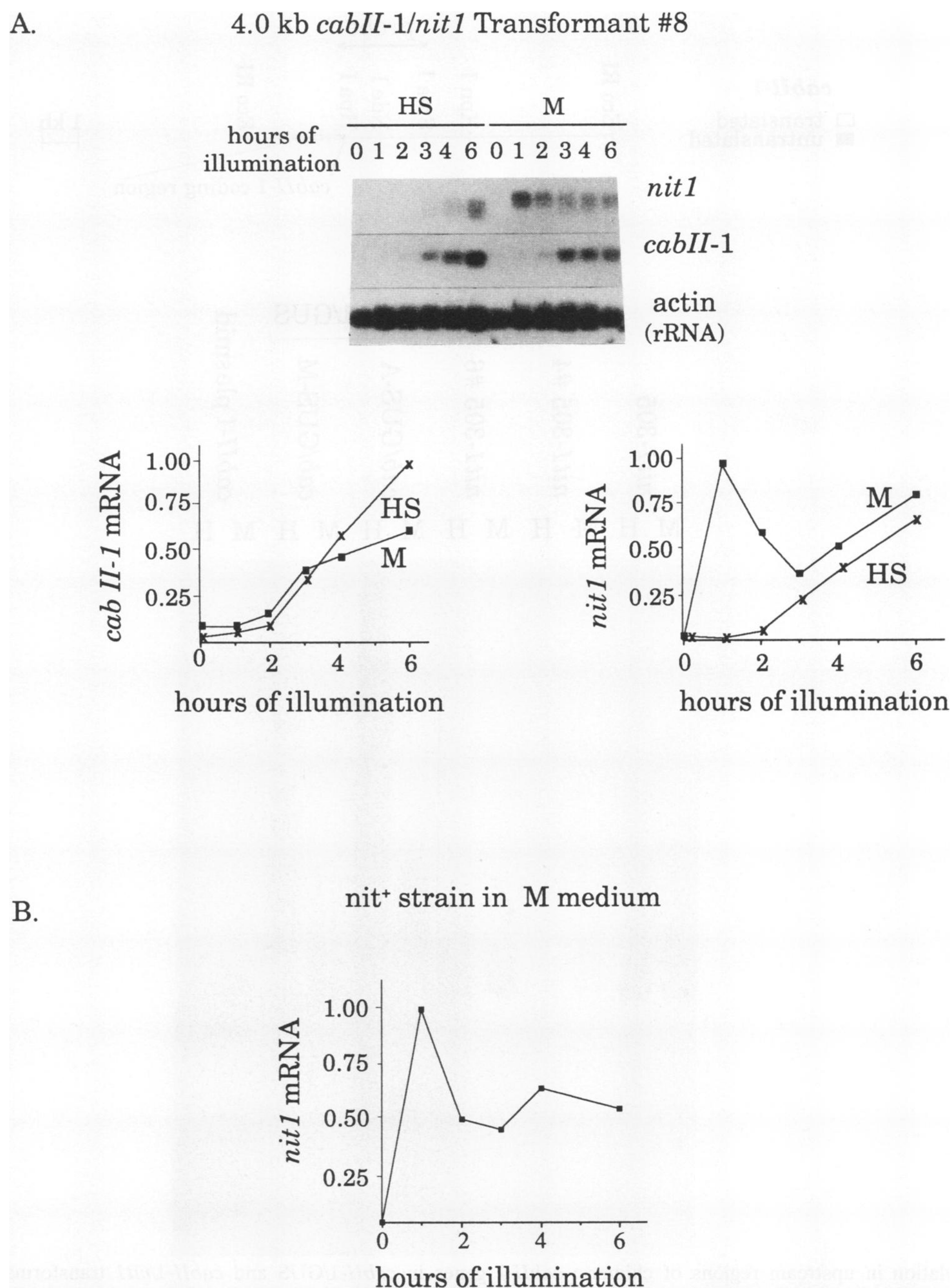


FIG. 5. Light induction of *cabII*-1/*nit1* and *nit1* in media containing nitrate or ammonium. A Nit$^+$ revertant strain derived from the *nit1*-305 strain and a 4.0-kb *cabII*-1/*nit1* transformant of the *nit1*-305 strain (no. 8) were grown in either M (nitrate) or HS (ammonium) medium under a 12-h dark–12-h light illumination regimen. RNA was harvested from cells at the end of the dark period and after various periods of illumination. After electrophoresis and transfer to a nylon membrane, the RNA was hybridized with the *nit1* 3'-end probe, the *cabII*-1 gene-specific probe, and the *A. aegypti* actin probe and normalized as described in Materials and Methods. (A) Autoradiograms and quantitation of *cabII*-1 and *nit1* mRNA accumulation in transformant 8; (B) *nit1* mRNA accumulation in the Nit$^+$ strain in M medium. In both panels, the maximum value for the time interval is arbitrarily defined as 1.0.

gene is not repressed by ammonium, ammonium repression of the endogenous *nit1* gene is probably also effected at the level of transcription. In related experiments, Vincentz and Caboche (63) have recently demonstrated that the cauliflower mosaic virus (CaMV) 35S promoter leads to constitutive light-independent expression of the *Nicotiana* 35S/NR gene in transgenic *Nicotiana plumbaginifolia* plants. In this case, expression of the NR gene no longer requires nitrate for induction (63).

A study of *nit1* mRNA accumulation and NR activity in transformants carrying the *cabII*-1/*nit1* gene should be useful for distinguishing transcriptional from posttranscriptional processes that regulate *nit1* gene expression (21). We have detected NR activity only in transformant and revertant cells

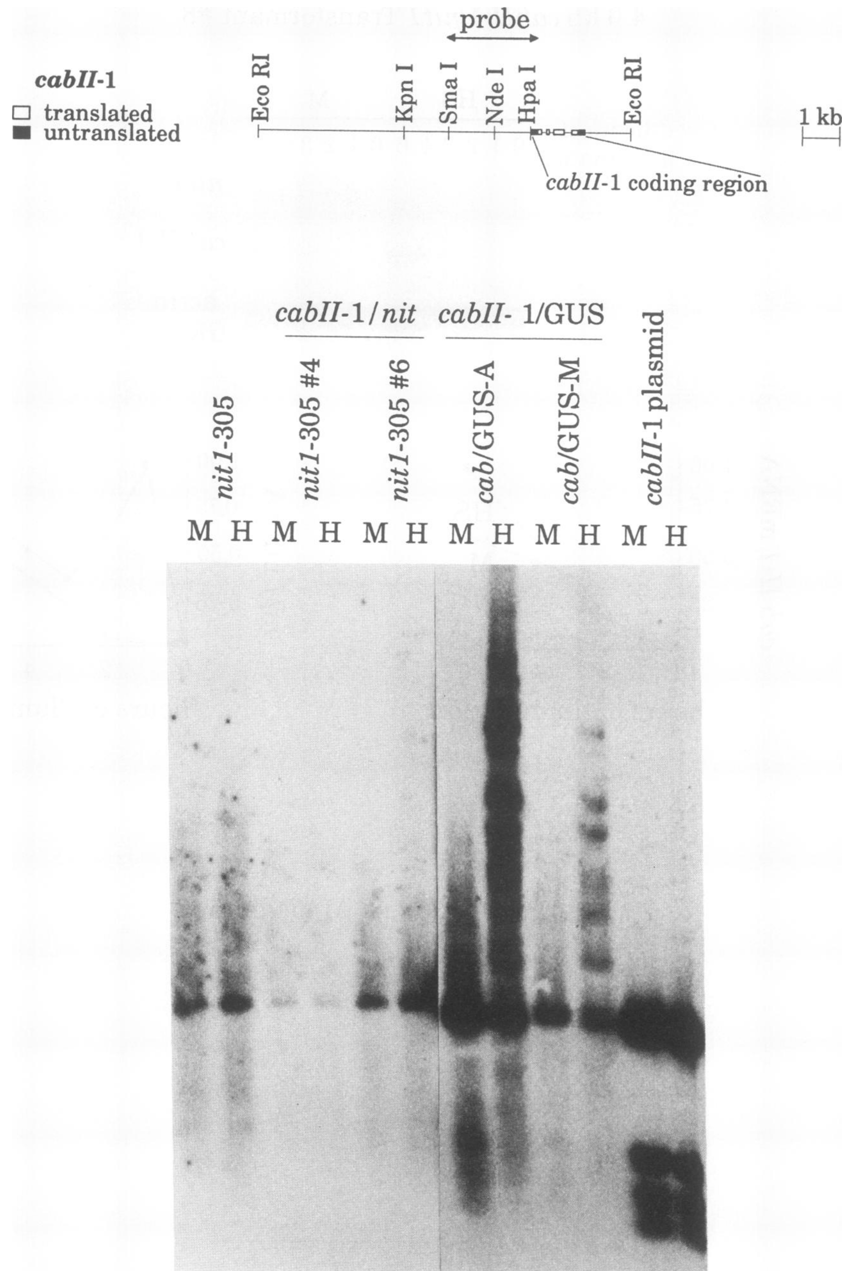


FIG. 6. Methylation in upstream regions of chimeric *cabII*-1 genes in *cabII*-1/GUS and *cabII*-1/*nit1* transformants. Genomic DNA, isolated from untransformed and transformed *nit1*-305 cells, was digested with *Hpa*II (H) or *Msp*I (M) and separated in an agarose gel. After transfer to a nylon membrane, the DNA was hybridized with a 2.5-kb *Sma*I-*Hpa*I *cabII*-1 upstream probe.

that were harvested during the light period (data not shown). This is consistent with the low level of *nit1* mRNA in the dark; however, other regulatory effects of light on enzyme activity have been documented. For example, light appears to be required for accumulation of NR protein in tobacco (13, 63) and is important under some conditions for activation of enzymatic activity via a flavin group in *Chlamydomonas* cells (2). It will be interesting to determine whether ammonium regulates synthesis and accumulation of NR protein posttranscriptionally. We have not detected NR enzyme activity in *cabII*-1/*nit1* transformants grown in the presence of ammonium, even though there is a substantial amount of *nit1* mRNA under these conditions (Fig. 3 to 5). However, ammonium can inhibit NR activity, and we have not measured NR protein abundance.

As discussed above, *nit1* mRNA accumulates to a very low level in *cabII*-1/*nit1* transformants compared to the *cabII*-1 transcript. Clearly, differences in transcript stability could account for differences in net accumulation. However, the low abundance of *nit1* mRNA makes a measurement of *nit1* mRNA half-life technically difficult. It is possible that high-level expression of *nit1* is lethal and that we have selected against integration events that would allow high-level expression of the *cabII*-1/*nit1* gene. However, none of the *cabII*-1 chimeric constructs have been expressed at a level approaching that of the endogenous *cabII*-1 gene,

including a series in which a small fragment of bacteriophage λ DNA was inserted into the *cabII*-1 3′ untranslated region (3, 35). These latter constructs would direct the synthesis of a protein indistinguishable from the wild-type *cabII*-1 gene product, so there should be no selection against high-level expression. Therefore, we suspect that there is a fundamental limitation in achieving high-level chimeric gene expression using the promoter in its present form.

We have analyzed *cabII-1/nit1* transformants with as much as 8.5 kb of *cabII*-1 upstream sequence integrated into the genome but have observed only a threefold increase in *cabII-1/nit1* gene expression relative to 2.5-kb *cabII-1/nit1* transformants. Furthermore, the amount of gene expression is not correlated with gene copy number; if anything, there is an inverse correlation. Copy number-independent expression of introduced *Chlamydomonas* genes has also been observed for *nit1* (35), plastocyanin (48), and the γ subunit of chloroplast ATPase (57); however, increased expression of the nucleus-encoded 29-kDa oxygen-evolving protein was observed in transformants carrying the gene in multiple copies (43). DNA sequence elements important for integration site-independent, copy number-dependent expression of globin genes have been defined in mammalian cells and can act over very long distances (>50 kb [10]). Such a region may be required for active expression of the chimeric *cabII*-1 gene and could be located still further upstream.

Despite the low level of expression relative to that of *cabII*-1, we have shown that chimeric *cabII-1/nit1* constructs can be used as selectable markers for transformation in both a $nit1^-$ and a $nit1^-$ $nit2^-$ strain. Therefore, it should be possible to transform the many mutants that have been isolated in the Ebersold-Levine lineage of *C. reinhardtii*. Indeed, we have used the 2.5-kb *cabII-1/nit1* construct as the selectable marker in cotransformation experiments with *Chlamydomonas* mutant ac-208, which lacks plastocyanin. Using a genomic clone encoding plastocyanin as the unselected DNA, we have recovered Nit^+ transformants that contain the plastocyanin gene and accumulate plastocyanin (48). Although *cabII-1/nit1* is expressed in the $nit2^-$ background (Fig. 3), transformants take significantly longer to form colonies in the $nit1^-$ $nit2^-$ strain than in a $nit1^-$ $nit2^+$ background, suggesting that *nit2* regulates genes in addition to *nit1* that are required for growth on nitrate. This is consistent with a recent report showing that *nit2* regulates nitrite uptake and NR (22). Interestingly, following their delayed appearance, *cabII-1/nit1* transformants grow well on selective medium; the reason for this apparent recovery from the requirement for *nit2* gene function remains an unresolved question but suggests a possible regulatory role for functional NR. Alternatively, the *nit2* allele may be leaky or revert during the extended selection period.

Failure to express foreign genes in *Chlamydomonas* cells. Despite many attempts, convincing evidence for foreign gene expression in the *Chlamydomonas* nuclear genome has not yet been reported. We have shown that *cabII*-1/GUS transformants containing upstream regions sufficient for chimeric *cabII-1/nit1* gene expression do not express GUS enzyme activity or mRNA in *Chlamydomonas* cells. The 2.5-kb *cabII*-1/GUS/NOS construct led to low-level GUS expression in transiently transformed tobacco tissue culture cells (data not shown), so the translational fusion is in frame and produces a functional enzyme.

We have presented evidence suggesting that the *cabII*-1 upstream region in *cabII*-1/GUS transformants is highly methylated whereas in *cabII-1/nit1* transformants, which express the *nit1* gene, there is little or no methylation of the *cabII*-1 promoter. We suggest that the lack of expression of the *cabII*-1/GUS gene may be due to integration into transcriptionally inactive regions of the genome or to a cellular silencing mechanism in which foreign DNA sequences are preferentially methylated. In *Schizophyllum commune*, a foreign gene encoding hygromycin phosphotransferase was inactive in most (≥90%) cotransformants initially selected with another marker (TRP1). Inactivity of the hygromycin phosphotransferase gene was correlated with increased methylation of the integrated DNA sequences (49). There have been a number of reports in which the introduction of a gene into higher plants has resulted in the silencing of the endogenous and/or introduced gene (27, 42, 50, 54, 62). In some cases, this silencing has been correlated with increased DNA methylation (27, 42). In *Chlamydomonas* cells neither completely foreign GUS genes, such as those fused with the maize alcohol dehydrogenase promoter, the CaMV 35S promoter, or the rice actin promoter (3), nor the chimeric *Chlamydomonas cabII*-1/GUS genes discussed in this paper were expressed.

In an attempt to reduce cytosine methylation and allow GUS gene expression, we have grown transformants in 5-azacytidine; however, over the concentration range used (3 μM to 1 mM), there was no apparent inhibition of methylation. Endogenous *cabII*-1 expression was measured in a number of transformants containing *cabII*-1 chimeric genes. Although the introduced chimeric genes were expressed at a low level or not at all, expression of the endogenous *cabII*-1 gene was only very slightly reduced; in the most extreme case expression was 70% of the wild-type level (data not shown), suggesting that cosuppression of the endogenous gene is not significant.

This silencing process may be circumvented in transformants expressing the *cabII-1/nit1* gene, because there is a strong selection for *nit1* activity. It is noteworthy that transformation efficiency with *cabII-1/nit1* is low compared to that with the wild-type *nit1* gene. This may reflect a large fraction of cases in which the expression of the introduced chimeric gene is suppressed. Expression of cotransformed wild-type *Chlamydomonas* genes appears to be efficient (e.g., radial spoke protein 3 [15], dynein [48], plastocyanin [51], and the γ subunit of chloroplast ATPase [57]). Moreover, high-level expression of epitope-marked α-tubulin has been achieved by using the *Chlamydomonas rbcS2* promoter (36); however, mRNA abundance relative to the endogenous *rbcS2* transcript was not measured.

Despite the apparently low activity of the *cabII*-1 promoter in transformants carrying the *cabII-1/nit1* gene, *nit1* mRNA accumulates to levels comparable to or exceeding that of *nit1* in induced wild-type strains. We hope that these transgenic strains will be useful for isolating regulatory mutations in the light induction pathway. Mutants deficient in NR have been selected in the presence of chlorate in flowering plants, and many NR-deficient *Chlamydomonas* strains are chlorate resistant (20, 58). We hope to be able to select *cabII-1/nit1* transformants that are unable to express the chimeric *cabII-1/nit1* gene and endogenous *cabII*-1 gene in response to light. This will give us a genetic handle for identifying genes that are involved in the signal transduction pathway or that may encode critical transcription factors.

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