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Structure and light-regulated expression of the *gsa* gene encoding the chlorophyll biosynthetic enzyme, glutamate 1-semialdehyde aminotransferase, in *Chlamydomonas reinhardtii*

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Abstract

The *gsa* gene, which encodes glutamate 1-semialdehyde (GSA) aminotransferase (GSAT), an enzyme in the chlorophyll and heme biosynthetic pathway, has been cloned from *Chlamydomonas reinhardtii* by complementation of an *Escherichia coli hemL* mutant. The deduced *C. reinhardtii* GSAT amino acid sequence has a high degree of similarity to GSAT sequences from barley, tobacco, soybean and various prokaryotic sources. *In vitro* enzyme activity assays from *E. coli* transformed with the *C. reinhardtii* GSAT cDNA showed that higher levels of GSAT activity are associated with the expression of the cDNA insert. Analysis of changes in mRNA levels in light:dark synchronized *C. reinhardtii* cultures was done by northern blotting. The level of GSAT mRNA nearly doubled during the first 0.5 h in the light and increased over 26-fold after 2 h in the light. This increase is comparable to previously reported increases in GSAT activity in dark-grown cultures transferred to the light, and is the first report of induction by light of a gene encoding an ALA biosynthetic enzyme in plant or algal cells. The accumulation of GSAT mRNA follows the pattern of chlorophyll accumulation and the pattern of chlorophyll *a*/*b*-binding protein (*cabII-1*) mRNA accumulation in these cells, suggesting that the two genes may be regulated by light through a common mechanism. Additional evidence that the GSAT mRNA may be transcriptionally regulated by light is found in the genomic sequence of the *gsa* gene. Two areas that are similar to sequences involved in the light regulation of genes from other organisms are located upstream of the GSAT-encoding region, and a third was detected internal to the coding region.

Introduction

δ-Aminolevulinic acid (ALA) is the first universal committed precursor in the tetrapyrrole biosynthetic pathway that produces chlorophylls, hemes, and bilins. In chloroplasts, as well as in cyanobacteria and most other bacteria, ALA is formed from glutamate in a three-step process, referred to

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers U03632 and U03633.

as the five-carbon pathway. Initially, glutamate is ligated to $tRNA^{Glu}$. It is then reduced to glutamate 1-semialdehyde (GSA) by glutamyl-tRNA reductase and the GSA is converted to ALA by GSA aminotransferase (GSAT) [1]. In plants, all three enzymes of the five-carbon pathway are nuclear-encoded, although the required glutamyl-tRNA is encoded in the chloroplast genome.

GSAT has been purified from barley and *Synechococcus* strain PCC 6301 [14] and from *Chlamydomonas reinhardtii* [23]. The enzyme isolated from these organisms is a 43–46 kDa subunit protein that requires a pyridoxal phosphate (PALP) cofactor for activity. The bacterial gene encoding GSA aminotransferase, designated *hemL* because of the heme requiring phenotype of *Escherichia coli* GSAT mutants, is also known as *Gsa* in barley and soybean, and has been cloned from various prokaryotic and eukaryotic species [12, 13, 20, 35].

Light is known to stimulate the synthesis of ALA and chlorophyll in plants [2, 18], and illumination increases the *in vitro* activities of ALA synthesizing enzymes from cells transferred from the dark [43]. In *Euglena gracilis*, light increases the cellular level of plastid $tRNA^{Glu}$ as well as the enzymes of the five-carbon pathway [29]. However, in *C. reinhardtii*, the transcription of the chloroplast glutamyl-tRNA gene *trnE*1 is not affected by light [22] and the steady-state level of barley plastid glutamyl-tRNA is unchanged in light or dark [3]. Therefore, light appears to stimulate ALA-forming activity by affecting the nuclear-encoded ALA biosynthetic enzymes in *C. reinhardtii*, rather than the plastid-encoded $tRNA^{Glu}$. A light-stimulated increase in GSAT activity has been measured directly in extracts of *C. reinhardtii* [28], *E. gracilis* [29], and plastids of greening barley seedlings [26]. Of the three enzymes in the five-carbon pathway, GSAT was more strongly induced than the others in *E. gracilis* and *C. reinhardtii* cells transferred from dark growth conditions to the light. However, it is not known if the increases in GSAT activity in *C. reinhardtii* and *E. gracilis* reflect regulation at the protein or mRNA level. The inducing effect of light on GSAT activity in these protists appears to be very different from the effect of light on GSAT gene expression during greening of etiolated plant seedlings. In etiolated soybean leaves, the high level of GSAT mRNA was only slightly increased after 24 h of light, and no change in GSAT enzyme activity was observed [35]. In barley, the increase of GSAT activity induced by light was not correlated with transcript abundance. Barley GSAT mRNA levels were high in etiolated seedlings, remained constant after transfer of seedlings from the dark to the light, and decreased during the later phases of greening [12]. Thus, the increase in GSAT activity in greening barley leaves must be regulated by a translational or post-translation event. In contrast, the activity of porphobilinogen deaminase, a light-induced enzyme that catalyzes a later step of chlorophyll biosynthesis, appears to be regulated at the level of mRNA abundance in greening etiolated pea leaves [45].

To more thoroughly investigate the effects of light on the expression of the genes involved in ALA synthesis, we have cloned a cDNA and a genomic copy of the GSAT-encoding gene from *C. reinhardtii*, which we have designated *gsa* to correspond with the plant nomenclature, and examined GSAT mRNA levels in light/dark synchronous cultures. We report here the first evidence of light-induction of an ALA biosynthetic enzyme at the level of mRNA accumulation in *C. reinhardtii*.

Materials and methods

Cell culture

C. reinhardtii strain CC124 was obtained from the *Chlamydomonas* culture collection, (Duke University, Durham, NC) and was routinely maintained on TAP medium [17] at 25 °C under continuous lighting (32 μE m^{-2} s^{-1} supplied by equal numbers of red and cool-white fluorescent tubes). Cells were grown to mid-exponential phase (2–5 $\times$ 10^6 cells/ml) for RNA isolations or to stationary phase for DNA isolation in liquid TAP medium.

cDNA cloning

A cDNA library was obtained from J.P. Woessner (Washington University, St. Louis, MO). The library was constructed in λZAP II phagemid (Stratagene) with mRNA from vegetative-stage, light-grown cells of *C. reinhardtii* strain NO^- [42]. A sample (10^5 pfu) of the library was excised with helper phage VCSM13 into XL1-Blue *E. coli* cells, and recovered as pBluescript plasmids. Competent *hemL E. coli* cells (strain GE 1377) [20] were transformed with the excised plasmid mixtures and selected for ALA-independent growth on LB-ampicillin plates.

GSAT activity assay

E. coli cells were grown to mid-exponential phase in the presence or absence of 1 mM IPTG. Pelleted cells were washed once in extraction buffer (150 mM Tricine pH 7.9, 0.3 M glycerol, 20 mM $MgCl_2$, 5 mM dithiothreitol, 20 μM PALP, 0.004% phenylmethylsulfonyl fluoride), resuspended in 1.0 ml of buffer, and sonicated on ice 10 times for 15 s each separated by 30 s cooling periods. To remove endogenous ALA, sonicated extracts were passed over a Sephadex G-25 column equilibrated with assay buffer (50 mM Tricine pH 7.9, 1 M glycerol, 15 mM $MgCl_2$, 1 mM dithiothreitol, 20 μM PALP, 0.004% phenylmethylsulfonyl fluoride) and 1.5 ml samples were collected. GSAT activity assays were done in assay buffer containing 5 mM levulinic acid, 100 mM DL-GSA (synthesized by the method of Gough *et al.* [11]), and cell extract (0.13 to 0.15 mg total protein). To correct for nonenzymic conversion of GSA to ALA, control incubations with heat-denatured enzyme were also assayed and their values were subtracted from sample activities. Assays mixtures were incubated at 30 °C for 20 min, and ALA was purified by Dowex chromatography and quantitated as previously reported [43]. Protein concentration was determined by the dye-binding method of Bradford [4], using bovine serum albumin as a standard.

DNA sequencing

Double-strand sequencing of cDNA and genomic DNA clones was done using a Sequenase kit (U.S. Biochemical) according to the manufacturer's protocol. Sequence data were compiled with The DNA Inspector IIe (Textco, West Lebanon, NH) sequence analysis program.

RNA isolation and northern blot analysis

Total RNA was isolated from 10^9 to 10^{10} cells that were resuspended in extraction buffer (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 5 mM EDTA), treated with 40 μg/ml of Proteinase K (Fisher) for 20 minutes, and phenol:chloroform-extracted until the interface was clear. The aqueous layer was precipitated with ethanol, the pellet resuspended in H_2O, and the RNA was precipitated overnight at 4 °C with an equal volume of 4 M LiCl. The LiCl pellet was washed with ethanol, dried, and resuspended in H_2O. Poly(A)$^+$ RNA was selected by two passes over oligo(dT) cellulose columns (BRL).

For northern blots, 2 μg of poly(A)$^+$ RNA was denatured with glyoxal, separated on a 1% (w/v) agarose gel, and blotted onto a nylon membrane (Nytran, Schleicher and Schuell). Blots were UV-crosslinked and hybridized to nick-translated probes in a solution containing 50% formamide, 5× SSPE, 2× Denhardt's reagent, and 50 μg/ml of sonicated salmon testes DNA, at 45 °C. Final washes were done at 55 °C in 0.2× SSPE containing 0.1% sodium dodecyl sulfate (SDS). Densitometric scans of autoradiographs were taken with an LKB Ultroscan XL laser densitometer. Scans of autoradiographs from several different exposure times were taken to confirm that the densitometric data were obtained from exposures that were within the linear response range of the film.

Genomic Southern analysis and genomic library screening

Genomic DNA from *C. reinhardtii* CC124 (10^{10} cells) was extracted overnight at 4 °C in a solu-

tion containing 100 mM Tris-HCl pH 8.0, 0.4 M NaCl, 40 mM EDTA, 2% (w/v) SDS, and 200 μg/ml of Pronase E (Sigma). Cells were extracted once with phenol/chloroform, then once with chloroform, and the supernatant was precipitated with ethanol. Nucleic acids were resuspended in TE pH 8.0 and separated on CsCl gradients. The DNA band was extracted with butanol 4 times, dialyzed overnight against TE and precipitated with ethanol.

For genomic Southern analysis, 10 μg of DNA was digested overnight with various restriction enzymes, and the fragments were separated on 0.8% agarose gel in 1 × TBE. The gel was blotted onto a Nytran membrane and probed with the GSAT cDNA insert as described for the northern blot. For construction of a genomic library, DNA (1 μg) was partially digested with *Mbo* I to an average size of 20 kb, ligated to λEMBL3 *Bam* HI cut arms (Stratagene), packaged (Gigapack II Gold, Stratagene), and plated on XL1-Blue MRA P2. Library lifts were hybridized with the GSAT cDNA insert, and a single hybridizing plaque was identified. The genomic DNA insert (15 kb) was restriction mapped, the position of the *gsa* gene was identified by Southern analysis, and the relevant restriction fragments were subcloned and sequenced. The transcription start site was determined by primer extension [34] using a 21 bp oligonucleotide primer homologous to the 5′ end of the cDNA.

Results

Identification of a C. reinhardtii *GSAT cDNA clone*

A near-full length cDNA clone encoding *C. reinhardtii* GSAT was isolated by complementation of an *E. coli hemL* mutant with a vegetative phase *C. reinhardtii* cDNA expression library. Three independent *E. coli hemL* transformants that showed ALA-independent growth were identified and each contained a pBluescript plasmid with a 2.0 kb insert. Two of the clones were identical in length, while the third was slightly shorter at the 5′ end of the insert. To confirm the identity of these cDNAs, one of the longer putative *C. reinhardtii* GSAT clones was transformed into *E. coli* DH5α, and extracts of cells transformed with the putative GSAT cDNA, or with the pBluescript vector only, were tested for *in vitro* GSAT activity. Cells transformed with vector only had low GSAT activity, while cells transformed with the *C. reinhardtii* cDNA had 4 to 5 times more GSAT activity (Table 1). The level of GSAT activity in cells transformed with the *C. reinhardtii* cDNA was not dependent on IPTG, perhaps because the high copy number of the plasmid surpassed the number of available *lac* repressor molecules. We did not attempt to determine GSAT activity in extracts of *E. coli hemL* cells because the exogenous ALA used to the support growth of these cells would interfere with the enzyme assay. It was previously reported that GSAT purified from *E. coli hemL* cells had less than 2% of the wild-type activity [19]. The ability of the *C. reinhardtii* cDNA clone to functionally complement an *E. coli hemL* mutant and the presence of increased GSAT activity in *E. coli* cells that express the *C. reinhardtii* cDNA insert indicates that it encodes a functional *C. reinhardtii* GSAT.

Sequence analysis of the C. reinhardtii *GSAT cDNA*

The *C. reinhardtii* GSAT cDNA was restriction-mapped and sequenced. The cDNA has an open

Table 1. GSAT activity of extracts of *E. coli* cells transformed with *C. reinhardtii* GSAT cDNA. GSAT enzyme activity was analyzed in *E. coli* DH5α cells transformed with either pBluescript vector alone or with vector containing a putative *C. reinhardtii* GSAT cDNA. *In vitro* GSAT activity assays were done with extracts of mid-exponential phase cells grown in the presence or absence of 1 mM IPTG as indicated.

Plasmid	ALA formation (net nmol per mg protein)
No insert–IPTG	4.45
No insert + IPTG	3.44
C. reinhardtii GSAT cDNA insert–IPTG	19.21
C. reinhardtii GSAT cDNA insert + IPTG	21.09

reading frame that encodes a 463 amino acid protein (Fig. 1). The 2002 bp insert contains 19 bp of 5′ untranslated DNA, the entire protein-coding region for GSAT including a putative chloroplast transit peptide of 30 residues (Fig. 1, underlined), and 591 bp of 3′-untranslated DNA. Codon usage, which is known to be selective in *C. reinhardtii*, was similar to that of other nuclear-encoded *C. reinhardtii* genes [31]. The 5′-most Met residue appears to be the initiation Met, as deduced by its position in a favorable translation initiation context [31]. This assignment was verified by examination of the *gsa* genomic clone (described below), where an in-frame stop codon was found just upstream of the 5′ end of the cDNA. The amino acid sequence of the presumed GSAT chloroplast transit peptide, although similar in size and base composition to other *C. reinhardtii* chloroplast transit peptides [8], has little sequence similarity to the barley GSAT transit peptide, and no discernible sequence similarity to the transit peptides deduced from *C. reinhardtii* Rubisco small subunit genes [10] or the OEE2 gene [30]. The beginning of the mature protein amino acid sequence was deduced from the beginning of the region of high sequence similarity to other GSAT proteins and by the preceding VRA sequence which is present at the end of other *C. reinhardtii* transit peptides [8]. The deduced *C. reinhardtii* mature GSAT protein contains 433 amino acids and has a molecular weight of 45 879 Da. This molecular weight is in close agreement with the reported subunit molecular weight for the purified *C. reinhardtii* GSAT, 43 000 Da [23] and for the barley GSAT, 46 000 Da [14]. The long 3′ untranslated region contains the presumed *C. reinhardtii* polyadenylation signal, TGTAA, 19 bp upstream of the polyadenylation site [40]. No significant open reading frames or similarity to known DNA sequences were found in the 3′-untranslated region.

The sequences of nine other GSAT proteins, deduced from the cloned genes of both prokaryotic and eukaryotic organisms, have been reported. These include *Synechococcus* sp. PCC 6301 [13], barley [12], soybean [35], *Bacillus subtilis* [16], *Salmonella typhimurium* [7], *E. coli* [20], *Propionibacterium freudenreichii* [32], and *Xanthomonas campestris* [33]. The sequence of tobacco GSAT was obtained from GenBank. Alignment of the deduced *C. reinhardtii* GSAT protein sequence with that of barley is shown in Fig. 2. In a comparison of all 10 GSAT sequences, there are 118 (27.2%) absolutely conserved amino acids (Fig. 2, upper-case letters), and an additional 59 (13.6%) residues that are identical in 9 of the 10 sequences (Fig. 2, lower-case letters). The sequence of the *C. reinhardtii* GSAT was most similar to *Synechococcus* sp. PCC 6301 GSAT, with 72.3% sequence identity, and the similarity to the soybean (70.4% identity), barley (70.2% identity), and tobacco (69.6% identity) GSAT proteins was also high. One area of sequence similarity (Thr-269 to Gly-278 of the mature *C. reinhardtii* protein) includes a conserved lysine residue (Lys-273) which previously has been shown to bind the PALP cofactor required for enzyme activity [23]. Other regions of similar amino acid sequence include Tyr-60 and Ser-122/Gly-123/Thr-124, which are residues common to most members of the aspartate aminotransferase family. The highly conserved sequence of 10 amino acids from Tyr-301 to Pro-310 is of as yet unknown function.

Light regulation of the C. reinhardtii gsa *gene*

To determine whether the *C. reinhardtii* GSAT mRNA level is influenced by light, RNA was isolated from mid-exponential growth phase cells synchronized under a 12 h light/12 h dark regime. Cells harvested at 0.5, 1, and 2 h into the light phase had increasing amounts of a 2.2 kb GSAT mRNA (Fig. 3). However, by 4 h into the light phase, the GSAT mRNA level dropped significantly, and from 8 h through the 12 h time point, just prior to the start of the dark phase, GSAT mRNA was nearly undetectable. The northern blot was also probed with a G protein β subunit-like cDNA from *C. reinhardtii* (obtained from K. L. Kindle, Cornell University), which is known to be constitutively expressed in light/dark synchronized cultures [36, 37], to quantitate the relative

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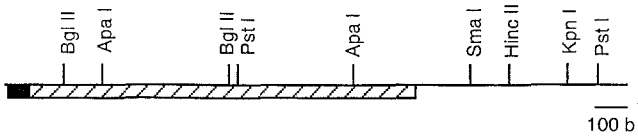

B

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                                                                GTTCAGTTTAGTAGCCAAT   19

ATGCAGATGCAGCTGAACGCCAAGACCGTGCAGGGCGCCTTCAAGGCGCAGCGCCCTCGCTCTGTCCGCGGCAAC   94
MetGlnMetGlnLeuAsnAlaLysThrValGlnGlyAlaPheLysAlaGlnArgProArgSerValArgGlyAsn   25

GTGGCGGTGCGCGCAGTGGCCGCTCCCCCTAAGCTGGTCACCAAGCGCTCCGAGGAGATCTTCAAGGAGGCTCAG  169
ValAlaValArgAlaValAlaAlaProProLysLeuValThrLysArgSerGluGluIlePheLysGluAlaGln   50

GAGCTGCTGCCCGGTGGCGTGAACTCGCCCGTGCGCGCTTTCCGCTCGGTTGGTGGCGGCCCCATCGTCTTCGAC  244
GluLeuLeuProGlyGlyValAsnSerProValArgAlaPheArgSerValGlyGlyGlyProIleValPheAsp   75

AGGGTCAAGGGTGCCTACTGCTGGGACGTCGATGGCAACAAGTACATCGACTACGTTGGCTCTTGGGGCCCTGCC  319
ArgValLysGlyAlaTyrCysTrpAspValAspGlyAsnLysTyrIleAspTyrValGlySerTrpGlyProAla  100

ATTTGCGGCCACGGCAACGACGAGGTCAACAACGCCCTGAAGGCGCAGATCGACAAGGGCACCTCGTTCGGTGCT  394
IleCysGlyHisGlyAsnAspGluValAsnAsnAlaLeuLysAlaGlnIleAspLysGlyThrSerPheGlyAla  125

CCCTGCGAGCTGGAGAACGTGCTGGCCAAGATGGTGATTGACCGCGTGCCCTCGGTGGAGATGGTGCGCTTCGTG  469
ProCysGluLeuGluAsnValLeuAlaLysMetValIleAspArgValProSerValGluMetValArgPheVal  150

TCCTCGGGCACTGAGGCGTGCCTGTCGGTGCTGCGCCTGATGCGCGCATACACCGGCCGCGAGAAGGTGCTGAAG  544
SerSerGlyThrGluAlaCysLeuSerValLeuArgLeuMetArgAlaTyrThrGlyArgGluLysValLeuLys  175

TTCACCGGCTGCTACCACGGCCACGCCGACTCCTTCCTGGTGAAGGCCGGCTCCGGTGTGATCACCCTGGGCCTG  619
PheThrGlyCysTyrHisGlyHisAlaAspSerPheLeuValLysAlaGlySerGlyValIleThrLeuGlyLeu  200

CCCGACTCGCCCGGTGTGCCCAAGAGCACCGCCGCCGCCACCCTGACCGCCACCTACAACAACCTGGACTCCGTG  694
ProAspSerProGlyValProLysSerThrAlaAlaAlaThrLeuThrAlaThrTyrAsnAsnLeuAspSerVal  225

CGCGAGCTGTTCGCCGCCAACAAGGGCGAGATTGCCGGTGTGATCCTGGAGCCCGTGGTCGGCAACAGCGGCTTC  769
ArgGluLeuPheAlaAlaAsnLysGlyGluIleAlaGlyValIleLeuGluProValValGlyAsnSerGlyPhe  250

ATTGTGCCCACCAAGGAGTTCCTGCAGGGCCTGCGCGAGATCTGCACGGCTGAGGGCGCCGTGCTGTGCTTCGAT  844
IleValProThrLysGluPheLeuGlnGlyLeuArgGluIleCysThrAlaGluGlyAlaValLeuCysPheAsp  275

GAGGTCATGACCGGCTTCCGCATTGCCAAGGGCTGCGCCCAGGAGCACTTCGGTATCACCCCCGACCTGACCACC  919
GluValMetThrGlyPheArgIleAlaLysGlyCysAlaGlnGluHisPheGlyIleThrProAspLeuThrThr  300

ATGGGCAAGGTCATTGGTGGCGGCATGCCTGTGGGCGCCTACGGCGGCAAGAAGGAGATCATGAAGATGGTCGCC  994
MetGlyLysValIleGlyGlyGlyMetProValGlyAlaTyrGlyGlyLysLysGluIleMetLysMetValAla  325

CCCGCCGGCCCCATGTACCAGGCCGGCACCCTTTCGGGCAACCCCATGGCCATGACTGCCGGCATCAAGACGCTG 1069
ProAlaGlyProMetTyrGlnAlaGlyThrLeuSerGlyAsnProMetAlaMetThrAlaGlyIleLysThrLeu  350

GAGATCCTGGGCCGCCCCGGCGCCTACGAGCACCTGGAGAAGGTGACCAAGCGCCTGATCGACGGCATCATGGCC 1144
GluIleLeuGlyArgProGlyAlaTyrGluHisLeuGluLysValThrLysArgLeuIleAspGlyIleMetAla  375

GCCGCCAAGGAGCACAGCCACGAGATCACCGGCGGCAACATCAGCGGCATGTTTGGCTTCTTCTTCTGCAAGGGC 1219
AlaAlaLysGluHisSerHisGluIleThrGlyGlyAsnIleSerGlyMetPheGlyPhePhePheCysLysGly  400

CCTGTGACCTGCTTCGAGGACGCCCTGGCGGCCGACACTGCCAAGTTCGCGCGCTTCCACCGCGGCATGCTGGAG 1294
ProValThrCysPheGluAspAlaLeuAlaAlaAspThrAlaLysPheAlaArgPheHisArgGlyMetLeuGlu  425

GAGGGCGTCTACCTGGCTCCCTCGCAGTTCGAGGCCGGCTTCACCTCTCTGGCCCACTCCGAGGCGGACGTGGAT 1369
GluGlyValTyrLeuAlaProSerGlnPheGluAlaGlyPheThrSerLeuAlaHisSerGluAlaAspValAsp  450

GCCACGATCGCCGCCGCTCGCCGCGTGTTCGCCCGCATC                                      1408
AlaThrIleAlaAlaAlaArgArgValPheAlaArgIle                                       463

TAAGCGGCGCGTGCACAGCTTGCAGCAAATTTATCGTGGGTGGGTAGGATGATGGAGCAGCAGACCCGGATGCTA 1483
GTGGCGCGCGGCAGCCTCGGTTGAAGGCAACCCTTGCGCGAGTGTCCGCTGCGTGAGTGTCCTTGCGGCTCGACC 1558
CGGGATTCGGGAGGCGTGCTTGCGTGATGCAGGCGGAGCTGGTTCTTCCGTGTGTATGCGTCGAGGCATGCATGG 1633
TATGCGGAAGAAGTTCAGACGTTGACAGCAAACTCTGTGCAGAGCGCTCAATACTGGGCCTATGACGATGACCTG 1708
ACCCATAATTGTTTATGACTAACCTGACTTCATGAGTGATGATATAGTGTTGCGTGTGTTGGTGAGCTTGACAGA 1783
GCTTTGACCTCTTGACACCGAGTTTAGAGTGCGGGTGCGCCCTGGGCGGGTACCGAAGCAAGTGCTGCTGACAGT 1858
GCTGCGCCAGGCGCAAGAGCTGAGAAGCTAGGAGAAACGAAGAGCAGGATGATGGCGGCGTCTACGGCTGCAGAT 1933
GTAGGGATGTAGGCTATGCAAGGGGGCTCTGATTGCACCCTCAGAAAGTGTGTAAGATTACAACCTGGT       2002
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Chlamy    1 -VAAPPKLVT-KRSEEIFKEAQELLPGGVNSPVRAFRSVGGGPIVFDRVKGA 50
Barley      A·SIDE·AY·VQK·····NA·K··M··········K····Q·····S···S
Conserved                S      A  l PGGV SPVRAf  Vgg P       g

Chlamy   51 YCWDVDGNKYIDYVGSWGPAICGHGNDEVNNALKAQIDKGTSFGAPCELE  100
Barley      HM·····E···········I··AD·K··A··IETLK·····A··
Conserved      D D   YiDyV sWGP   GH       a       g SFGAP   E

Chlamy  101 NVLAKMVIDRVPSVEMVRFVSSGTEACLSVLRLMRAYTGREKVLKFTGCY  150
Barley      ····Q···SA···I·····N·····MGA···V··F·····I···E···
Conserved      A  v   vp    VR v SGTEA     Rl R  TgR    Kf GCY

Chlamy  151 HGHADSFLVKAGSGVITLGLPDSPGVPKSTAAATLTATYNNLDSVRELFA  200
Barley      ·············A···········GATVG····P··DA·A·KK··E
Conserved   HGH D  LvkAGSG  tLG P SPGvP      T t  yn        F

Chlamy  201 ANKGEIAGVILEPVVGNSGFIVPTKEFLQGLREICTAEGAVLCFDEVMTG  250
Barley      D·····A·F·····A···P·QPA··NA···VTKQD··L·V·····P
Conserved         A    Epv gN     P   f   lr        lL  DEVmTg

Chlamy  251 FRIAKGCAQEHFGITPDLTTMGKVIGGGMPVGAYGGKKEIMKMVAPAGPM  300
Barley      ··L·Y·····Y·····V··L··I····L·····R·D··E·····
Conserved   FR     aQ   G  PD t  GK IGGG PvgA GG    M   AP GP

Chlamy  301 YQAGTLSGNPMAMTAGIKTLEILGRPGAYEHLEKVTKRLIDGIMAAAKEH  350
Barley      ·········L·····H··KR·ME··T··Y·D···GE·VR··LDVGAKT
Conserved   YQAGTLSGNP Am AG   L     p     l      l  g

Chlamy  351 SHEITGGNISGMFGFFFCKGPVTCFEDALAADTAKFARFHRGMLEEGVYL  400
Barley      G··MC··H·R·····AG···HN·D··KKS·····G·····G·····
Conserved   g          mfg ff    V         D   F rf   ml  G  l

Chlamy  401 APSQFEAGFTSLAHSEADVDATIAAARRVFARI                  433
Barley      ·············TTQ·IEK·VE··EK·LRW·
Conserved   aPS fEa F S Ah   d   t  aa
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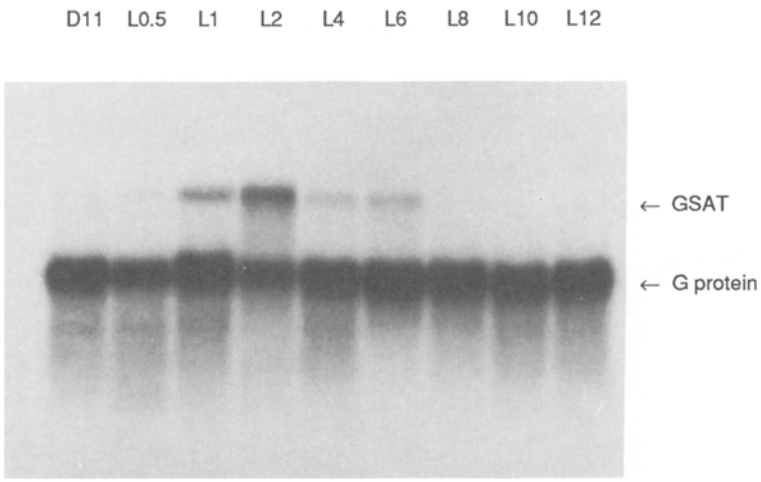
Fig. 2. Comparison of the deduced protein sequence of the *C. reinhardtii* (Chlamy) and barley GSAT proteins. Dots indicate sequence identity. Conserved residues are shown in the third line, with upper case letters indicating complete identity (10 out of 10 species) and lower case letters nearly invariant residues (9 out of 10 species).

RNA loading for each time point. When normalized to the amount of G protein subunit mRNA per lane, the GSAT mRNA increased over 1.8-fold within the first 0.5 h of light, and over 26-fold after 2 h of light (Fig. 3).

Genomic gsa *sequence analysis*

The results of a genomic Southern blot probed with the GSAT cDNA indicated that there is a single copy of the *gsa* gene in *C. reinhardtii* (data not shown). The GSAT cDNA was used to select a genomic DNA clone from a *C. reinhardtii* CC124 library. The genomic DNA clone contains the complete coding region, covering approximately 3.5 kb, and an additional 11.5 kb of 5′ upstream DNA, but less than 200 bp of 3′ DNA (Fig. 4). Approximately 700 bp of genomic DNA 5′ to the end of the cDNA was sequenced. The transcription start site was determined to be 23 bp beyond the end of the cDNA, and defined a 5′ untranslated region 37 bp in length. The putative TATA-like box present between bases −33 and −38 (TTAATT), followed by a G-C-rich region, appears to be typical of *C. reinhardtii* nuclear genes [36].

Fig. 1. A. Restriction map of the *C. reinhardtii* cDNA clone encoding GSAT. The area of the open reading frame is shown as the hatched box and the putative chloroplast transit peptide is shown as the solid box. B. DNA sequence and deduced amino acid sequence of the *C. reinhardtii* cDNA clone encoding GSAT. The putative chloroplast transit peptide (residues 1 to 30) and the presumed *C. reinhardtii* polyadenylation signal (TGTAA, bases 1984 to 1988) are underlined.



	Relative band density									Normalized induction ratio	
	D11	L0.5	L1	L2	L4	L6	L8	L10	L12	L0.5/D11	L2/D11
GSAT	0.197	0.239	1.481	3.749	0.668	0.794	0.000	0.000	0.000		
G protein	2.511	1.634	3.235	1.515	2.404	3.060	2.686	1.900	2.800		
GSAT/G protein	0.034	0.062	0.208	0.903	0.118	0.120	–	–	–	1.82	26.47

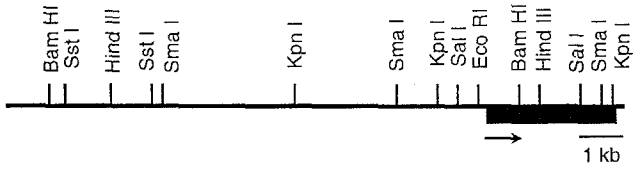
Fig. 3. Northern blot analysis of poly(A)$^+$ RNA isolated from 12 h light/12 h dark synchronized *C. reinhardtii* cultures at 11 h into the dark phase (D11), and 0.5, 1, 2, 4, 6, 8, 10, and 12 h into the light phase (lanes L0.5, L1, L2, L4, L6, L8, L10, and L12). Blots were probed with cDNA inserts for GSAT and for the constitutively expressed G protein β-like subunit [36] from *C. reinhardtii*. The autoradiograph was scanned and the density of the GSAT band (2.2 kb) normalized against the G protein band (1.2 kb) for each time point. Densitometric data for GSAT mRNA were obtained from a 40 h exposure (photograph shown) and the data for G protein mRNA were taken from a 16 h exposure (not shown). The normalized increase in the amount of GSAT mRNA in the first 0.5 and 2 h of light (normalized induction ratio) is shown at the right.

The 5′ upstream region was searched for similarity to sequence motifs found upstream of light-regulated genes. One area in the *gsa* 5′ upstream region (bases −174 to −159) has similarity to the consensus G-box sequence found adjacent to many light-regulated higher plant genes such as *rbcS* and *cab* [9, 15]. A second region (bases −111 to −100) was very similar to the parsley chalcone synthase Box II, which is involved in UV-light induction [38, 44]. A third region with sequence similarity to the rice phytochrome gene *phyA* GT motif [5], and to the pea *rbcS-3A* GT-1 boxes [9] was found not in the upstream area, but at the first exon-intron border within the coding region (bases +167 to +175).

Discussion

A near-full length cDNA encoding the chlorophyll biosynthetic pathway enzyme GSAT was selected from a vegetative-stage, light-grown *C. reinhardtii* expression library. Selection of the cDNA was based on its ability to complement a GSAT-deficient, ALA-requiring *hemL* mutant strain of *E. coli*. The clone was verified as a GSAT-encoding cDNA by experiments that showed a 4- to 5-fold increase in *in vitro* GSAT activity in *E. coli* cells expressing the cDNA insert, and by significant sequence similarity of the protein deduced from the cDNA open reading frame to other GSAT proteins. We have desig-

A



B

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                                                                  CAGCTGCTGTCGCTTGATCTCCGGGTGTTTCCATCGCTCAATGTTAACAGTTCTGCTGCTGCTAAT -601
GTTGCTGCAGCCAGAATATTGACCAAGACAGCGGTAGACCGGCGAGTTCCAAAGCCGCGCTGTGTGCTTATGTAT -526
TGTTATCAGCTTGCTAACATTGATGTAATAGATAACGGCACTCGATAGTCAACAACGGCTTTTTACTTCGCTAGA -451
AACAATCGAAACTTACGAAAGCATGCTCGCAAGTCCTAGCCCGTGTGTCATTTTAGCCGACACTACAGTATCTGC -376
TGTCAAAGCATAAAATCTTTGGTTCTAAGTTTGCGCGCCACATTTGCAAGTGCAACACCCGTCCAAGCGACTTCT -301
GCCACGCTGCACCTTTCCTAGATCTTTTGATGCCAAGCAATGTTTTAGAGCCGAATTTTGTGTCTGGTCATGAAT -226
TCAAGCTGTGATTCAGGCTGTGAGTGGTCCGCTTGCAGCTCGCTCAGGGAAGCATGCATGTGGACGTCGCACTGA -151
AAGAATGATGAAAACGTCTCATAGGCATCTCATGGGTCTTTCCACCTGGCCCGAATCGAGGGTTTCCAGGCGGAA  -76
TGAAACTATGCCCCATCGCGACCTTCTGGATCTGCACTTAATTCTCGCGCAGGCGCTGTGGTCTTTGCATTTCTA   -1
CGCTATCTGGGCATAACAGTTCAGTTTAGTAGCCAATATGCAGATGCAGCTGAACGCCAAGACCGTGCAGGGCGC   75
CTTCAAGGCGCAGCGCCCTCGCTCTGTCCGCGGCAACGTGGCGGTGCGCGCAGTGGCCGCTCCCCCTAAGCTGGT  150
CACCAAGCGCTCCGAGGAGgtaaattgacacatgagatcctagtacttgagcgacttctcggggtctggatgtgc  225
cgatgggcacctttttcgggtggtcaggcacagtaatcacccaaatccgggccttcgcagATCTTCAAGGAGGCT  300
CAGGAGCTGCTGCCCGGTGGCGTGAACTCGCCCGTGCGCGCTTTCCGCTCGGTTGGTGGCGGCCCCATCGTCTTC  375
GACAGGGTCAAGGGTGCCTACTGCTGGGACGTCGATGGCAACAAGTACATCGACTACGTTGGCTCTTGGGGCCC   449
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C

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Chlamydomonas gsa          -174 GCATGCATGTGGACGT -159
Consensus rbcS G Box [15]       T·T·A··C····CAY·

Chlamydomonas gsa          -111 TTCCACCTGGCC -100
Parsley CHS Box II [44]         ·····G·····

Chlamydomonas gsa          +167 GAGgtaaat +175
Rice phyA GT motif [5]          ·C·····T·
Pea rbcS-3A GT-1 Box II [9]     ·T···T···
Pea rbcS-3A GT-1 Box II* [9]    ·······TA
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Fig. 4. A. Restriction map of a *C. reinhardtii* genomic *gsa* clone. The genomic region hybridizing to the GSAT cDNA is shown as the black box and the direction of transcription is indicated by the arrow. B. Partial DNA sequence of the *C. reinhardtii* genomic *gsa* clone. Sequence corresponding to the cDNA is in bold upper case and the first intron is in lower case. The presumed translational initiation site is double-underlined, and a TATA-like box is underlined with dashes. Three areas of sequence similarity to promoters of various light-regulated genes are underscored with asterisks. C. Comparison of the areas of sequence similarity to promoters of various light-regulated genes. Dots indicate sequence identity.

nated the *C. reinhardtii* gene encoding this GSAT cDNA as *gsa*.

The deduced protein sequence of the *C. reinhardtii* GSAT is most similar to that of cyanobacterial and higher-plant GSATs, although there are several areas of sequence similarity to eubacterial GSATs. The cDNA contains the entire protein-coding region, including a putative chloroplast transit peptide. Despite the presence of the transit peptide-encoding sequence and 19 bp of 5′-untranslated DNA, the cDNA clone expressed in *E. coli* has GSAT activity *in vivo* and *in vitro*. The significance of the long 3′-untranslated region in the GSAT cDNA is unclear, although the presence of long 3′-untranslated regions is not uncommon among *C. reinhardtii* cDNAs. Unlike the *C. reinhardtii* chloroplast *atpB* gene, in which inverted repeats with the potential for stem-loop formation in the 3′-untranslated region influence mRNA accumulation [41], no apparent stem-loop forming sequences are present in this region of the GSAT cDNA.

Expression of the *gsa* gene in light/dark synchronized *C. reinhardtii* cells appears to be induced by light. The GSAT mRNA level was low just prior to the beginning of the light phase, it increased slowly at the beginning of the light phase, reached a high point at 2 h in the light, and then decreased throughout the remainder of the light phase. Unlike in barley, where the GSAT mRNA level in etiolated seedlings transferred to the light decreases, or in soybean leaves, where the GSAT mRNA level increases only slightly in the light, the *C. reinhardtii* GSAT mRNA level increases 26-fold from the end of the dark phase to 2 h into the light phase. The increase in GSAT mRNA mirrors the appearance of chlorophyll in these cells. After an initial lag of 3 to 4 h during the beginning of the light phase, total chlorophyll content increases steadily during the next 6 h and levels off at 9 to 10 h into the light phase [25]. The degree to which GSAT mRNA increases in the light is similar to the degree of induction of GSAT activity when dark-adapted *C. reinhardtii* cultures are transferred to the light: after 6 h of light exposure, GSAT activity increased 16-fold [28]. Therefore, the light-induced increase in GSAT activity can be accounted for by the increase in GSAT mRNA. The expression of the *C. reinhardtii cabII-1* gene, which encodes a member of the chlorophyll *a*/*b*-binding protein family, also shows a light-induced pattern of mRNA appearance that is not cell-cycle related [25, 39], and the increase in *cabII-1* mRNA is known to be caused by transcriptional activation of the gene [24]. The pattern of *cabII-1* expression in light/dark cultures is similar to the pattern of GSAT mRNA accumulation, and the regulation of expression of the two genes may be related. Differences between the effect of light on GSAT mRNA levels between light/dark *C. reinhardtii* cultures and greening angiosperm leaves may reflect physiological differences between etiolated tissues and the light/dark cycled cells used in this study. Etiolated tissues are poised for greening, and to fulfill the need for rapid ALA synthesis they may require high levels of GSAT mRNA to be present before the light signal is perceived. In contrast, light/dark synchronized cultures, which divide once during the dark phase, synthesize chlorophyll for new cells but may not require an extremely rapid phase of chlorophyll accumulation. The *C. reinhardtii* cultures may more closely resemble natural diurnal variations in chlorophyll synthesis in photosynthetic tissues that contain dividing cells.

To facilitate further exploration of how light affects GSAT mRNA levels, a genomic copy of the *C. reinhardtii gsa* gene was isolated and partially sequenced. The genomic Southern hybridization analysis indicates that there is a single *gsa* gene in *C. reinhardtii*, and a single genomic clone was selected from the genomic library. Two sequences with similarity to the promoter regions associated with light-induced transcriptional regulation of higher plant genes were identified in the *gsa* upstream region. One area has a particularly strong sequence similarity to the Box II region of the UV-light induced parsley chalcone synthase promoter, with 9 out of 10 identical bases. Although transcription of many higher plant *cab* genes is light-regulated through phytochrome signal transduction systems, in *C. reinhardtii*, *cabII-1* mRNA is induced by blue light

and is independent of photosynthesis or chlorophyll biosynthesis [25, 27]. This indicates the presence of a *C. reinhardtii* blue light receptor system. In addition, no physiological evidence for phytochrome or for red light effects have been found in *C. reinhardtii*. Thus, the similarity of the *gsa* upstream region to a UV-light responsive promoter suggests that light in the blue to UV range may be important in regulating *gsa* expression. A third region of similarity to light-regulated promoter sequences was found not in the upstream area, but at the first exon-intron junction. Although the significance of this region and its position in the *gsa* gene is not yet known, light-responsive elements regulating expression of the pea ferredoxin (*Fed-1*) gene are located within the transcribed region of the gene [6].

It is possible that *gsa* expression is an important control point for chlorophyll biosynthesis in *C. reinhardtii*. It has been shown previously that chloroplast $tRNA^{Glu}$ transcription is unaffected by light in *C. reinhardtii* [22]. The level of glutamyl-tRNA synthetase, which is active in protein synthesis as well as ALA formation, does not appear to increase during greening, and the level of glutamyl-tRNA reductase increases only slightly [28]. In addition, in the presence of charged $tRNA^{Glu}$, *C. reinhardtii* glutamyl-tRNA synthetase and glutamyl-tRNA reductase, the first two enzymes in the ALA biosynthetic pathway, can form a stable complex which does not include GSAT [21]. The complex may help to channel glutamyl-tRNA into tetrapyrrole biosynthesis, while level of GSAT, which varies significantly in response to light, can serve as the up-regulation point when increased chlorophyll synthesis is required. The fact that, of the three ALA biosynthetic enzymes, GSAT is the most responsive to light modulation in both *C. reinhardtii* [28] and *E. gracilis* [29], supports a regulatory role for this enzyme under normal diurnal (i.e., not etiolation) developmental conditions. The regulatory mechanism may differ in etiolated plant tissues, where, for example, the GSAT mRNA level remains relatively constant during greening in soybean [35] and barley [12].

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