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Calcium and Calmodulin Are Involved in Blue Light Induction of the *gsa* Gene for an Early Chlorophyll Biosynthetic Step in Chlamydomonas

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The *Chlamydomonas reinhardtii* nuclear gene *gsa*, which encodes the early chlorophyll biosynthetic enzyme glutamate 1-semialdehyde aminotransferase (GSAT), is specifically induced by blue light in cells synchronized in a 12-hr-light and 12-hr-dark regime. Light induction required the presence of a nitrogen source in the incubation medium. Maximal induction also required acetate. However, in the absence of acetate, partial induction occurred when Ca^{2+} was present in the medium at concentrations of ≥1 μM. The Ca^{2+} channel-blocking agents Nd^{3+} and nifedipine partially inhibited the external Ca^{2+}-supported induction of GSAT mRNA but did not inhibit acetate-supported induction. The calmodulin antagonists trifluoperazine and *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide inhibited both external Ca^{2+}-supported and acetate-supported induction. The Ca^{2+} ionophore A23187 caused a transient induction in the dark. These results suggest that Ca^{2+} and calmodulin are involved in the signal transduction pathway linking blue light perception to the induction of GSAT mRNA. The electron transport uncoupler carbonyl cyanide *m*-chlorophenylhydrazone inhibited acetate-supported induction of GSAT mRNA but did not inhibit external Ca^{2+}-supported induction. It is proposed that in the presence of acetate, an internal pool of Ca^{2+} can be mobilized as a second message, whereas in the absence of acetate, internal Ca^{2+} is not available but the requirement for Ca^{2+} can be partially met by an external Ca^{2+} source. The mobilization of internal Ca^{2+} may require energy derived from metabolism of acetate.

INTRODUCTION

Developmental responses to light are ubiquitous in plants, algae, and other organisms that depend on sunlight for photosynthetic growth. The responses to light depend on cellular photoreceptors and signal transduction systems that link light perception to downstream consequences, such as gene transcription.

Higher plants contain several photoreceptor systems that may play roles in light-dependent development of chloroplasts and the attainment of photosynthetic capability, including one or more red/far-red light photoreversible phytochrome-based systems, blue light photoreceptors that may be based on flavins (Ahmad and Cashmore, 1993) or carotenoids (Quiñones and Zeiger, 1994), light-dependent protochlorophyllide reductase, which may function as a developmental photoreceptor over and above its essential biosynthetic role in angiosperms, lacking a light-independent protochlorophyllide reduction system, and photosynthesis itself, which can provide developmental cues to the plant regarding the amount of photosynthetically useful light available (Beale and Appleman, 1971; Maxwell et al., 1995).

Chlamydomonas reinhardtii is a unicellular green alga that has been used as a model system for the study of plant-type photosynthesis and chloroplast structure and function. This organism is also an attractive model system for the study of light regulation of gene expression because it lacks phytochrome and phytochrome-based photoreversible responses while still retaining blue light–based and protochlorophyllide reductase–based photoresponses.

We have been using Chlamydomonas to study light regulation of early steps of chlorophyll biosynthesis. We reported previously that two Chlamydomonas nuclear genes encoding enzymes for early steps of chlorophyll and heme biosynthesis are induced by blue light (Matters and Beale, 1994, 1995a, 1995b). These genes are *gsa*, which encodes glutamate 1-semialdehyde aminotransferase (GSAT), and *alad*, which encodes δ-aminolevulinic acid (ALA) dehydratase (ALAD). In cells synchronized in a 12-hr-light and 12-hr-dark regime, both genes are maximally expressed at 2 hr into the light phase. Of the two genes, *gsa* exhibits the greater influence of light on its expression, and the GSAT mRNA level at 2 hr into the light phase is >25-fold higher than the level immediately before the beginning of the light phase. Also, GSAT mRNA induction is absolutely dependent on light in cells synchronized in a light-and-dark regime, whereas ALAD mRNA appears to be partially induced during the light phase, even in cells that are kept

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in the dark, suggesting the influence of a circadian or cell cycle effect.

In this report, we have determined some components of the signal transduction chain that links perception of blue light to the induction of GSAT mRNA in Chlamydomonas. The results indicate that Ca^{2+} and calmodulin are involved in the signal transduction chain. A preliminary account of portions of this work is provided in Matters et al. (1996).

RESULTS

Nutrient Requirements for Light Induction of Chlamydomonas GSAT mRNA

As described previously, GSAT mRNA is induced early in the light phase in cells synchronized in a 12-hr-light and 12-hr-dark regime and reaches a maximum at 2 hr (Figure 1, lanes 1 and 2). To determine which, if any, nutrients present in the Tris–acetate–phosphate (TAP) culture medium (Harris, 1989) are required for induction, cells were grown synchronously in TAP medium and transferred to various simpler media in the dark immediately before the onset of the last light phase. Cells were then harvested at 2 hr into the light phase, and the GSAT mRNA level was determined. The major nitrogen and carbon sources in TAP medium are 7.5 mM NH_3 and 17.5 mM acetate, respectively, and the final pH is 7.0 (Harris, 1989). In medium containing only 10 mM Pipes buffer, pH 7.0, 17.5 mM Na-acetate, and 7 mM NH_3, induction at 2 hr was approximately equal to that in TAP medium (Figure 1, lane 4). However, the omission of NH_3 from the medium completely abolished light induction,

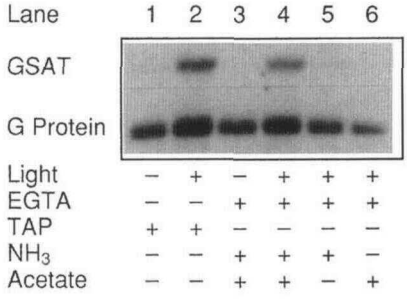

Figure 1. Dependence of Light Induction of Chlamydomonas GSAT mRNA on Acetate and NH_3.

Cells synchronized in a light-and-dark regime at the beginning of the light phase were incubated for 2 hr in the light (+) or dark (–), as indicated, in TAP culture medium or in medium containing 10 mM Pipes, pH 7.0, plus the indicated additions of NH_3 (7 mM), potassium acetate (17.5 mM), and EGTA (1 mM). Total RNA was then extracted, electrophoresed on a 1% (w/v) agarose gel, blotted onto a nylon membrane, and hybridized with probes specific for GSAT mRNA and for a constitutively expressed G protein β subunit–like mRNA that served as a standard to control for unequal gel loading.

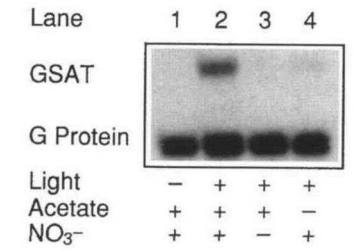

Figure 2. Dependence of Light Induction of Chlamydomonas GSAT mRNA on Acetate and NO_3^-.

The experiment was performed as described in the legend to Figure 1, except that strain CC1690 cells, which can use NO_3^- as a nitrogen source, were used instead of strain CC124 cells, and NO_3^- was used instead of NH_3 as the nitrogen source in the growth medium and in the incubations. RNA gel blots were produced as described in the legend to Figure 1.

and omission of acetate almost completely abolished induction (Figure 1, lanes 5 and 6).

To determine whether the sole role of NH_3 in supporting light induction of GSAT mRNA is to act as a nitrogen source or whether there might be a specific requirement for a reduced nitrogen source, perhaps to influence the intracellular redox state, an experiment was performed in which NO_3^- was substituted for NH_3. For this experiment, CC1690, a strain capable of utilizing NO_3^- as a nitrogen source, was used in place of CC124, which is deficient in nitrate reductase (Harris, 1989). CC1690 cells were grown under a 12-hr-light and 12-hr-dark regime in modified TAP medium in which 7.5 mM KNO_3^- was substituted for the normal nitrogen source, 7.5 mM NH_4Cl. The CC1690 cells synchronized in a light-and-dark regime exhibited light induction of GSAT mRNA only when the incubation medium contained NO_3^- (Figure 2). The small amount of induction that occurred in the absence of acetate was somewhat more than that which occurred in CC124 cells in the absence of acetate, suggesting that compared with CC124 cells, CC1690 cells are less dependent on acetate for induction. We conclude that the role of NH_3 or NO_3^- is to act as a nitrogen source and that a nitrogen source is required for light induction of GSAT mRNA.

External Ca^{2+} Supports Light Induction of GSAT mRNA in the Absence of Acetate

In contrast to the absolute dependence on an external nitrogen source for light induction of GSAT mRNA, the requirement for acetate could be supplanted by Ca^{2+} (Figure 3). Acetate-independent induction was dependent on the Ca^{2+} concentration, with slight induction occurring at 10^{-7} M Ca^{2+}, significant induction at 10^{-6} M, and maximal induction at 10^{-4} M Ca^{2+}. Higher Ca^{2+} concentrations did not result in greater induction (data not shown). At 10^{-4} M Ca^{2+}, the light-induced level of GSAT mRNA was approximately two-thirds of the level

induced by light in the presence of acetate. In these experiments, the Ca^{2+} concentration was controlled by the use of Ca^{2+}–EGTA buffers that were made according to the equations described by Blinks et al. (1982). EGTA itself had only a slight effect on light induction of GSAT mRNA in the presence of acetate, confirming that external Ca^{2+} is not required for induction in the presence of acetate (Figure 3, lanes 1 and 2).

Ca^{2+} Channel Blockers Inhibit External Ca^{2+}-Supported Light Induction of GSAT mRNA

Many Ca^{2+}-requiring processes are inhibited by compounds that inhibit Ca^{2+} transport through specific channels at the cytoplasmic membrane or at the limiting membranes of internal cellular compartments. Two widely used Ca^{2+} channel–blocking agents, the substituted dihydropyridine nifedipine (Janis and Triggle, 1983; Conrad and Hepler, 1988; Reiss and Beale, 1995) and Nd^{3+} (Tew, 1977; Lansman, 1990; Reiss and Beale, 1995), were tested for their effects on light induction of GSAT mRNA. These two compounds are effective in plants as well as animals; they block different classes of Ca^{2+} channels and have different effectiveness for different Ca^{2+}-requiring processes. For acetate-independent light induction of GSAT mRNA in the presence of a suboptimal Ca^{2+} concentration (1 μM), nifedipine (10 μM) and Nd^{3+} (10 nM) inhibited GSAT mRNA induction by ~15 and 33%, respectively, and the combination of the two compounds inhibited the induction by ~50% (Figure 4, lanes 5 to 8). In contrast, the two inhibitors, added together, had no effect on the light induction that occurs in the presence of acetate and that does not require external Ca^{2+} (Figure 4, lanes 2 and 4). In this set of experiments, the concentrations of Ca^{2+} and Nd^{3+} were controlled by use of Ca^{2+}–Nd^{3+}–EGTA buffers, which were made up by a modification of the equations described by Blinks et al. (1982), using the Nd^{3+}–EGTA association constants given by Martell and Smith (1974). Nifedipine was added from a 10 mM stock solution in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide was 0.1% (v/v), and all samples contained this amount of dimethyl sulfoxide, which by itself had no effect on GSAT mRNA induction.

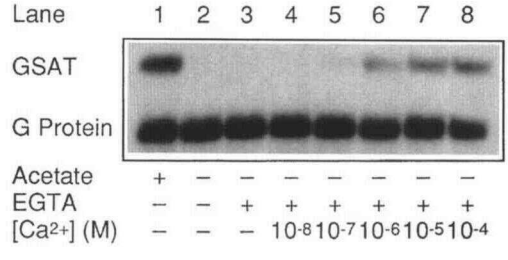

Figure 3. Ca^{2+} Requirement for Light Induction of Chlamydomonas GSAT mRNA in Acetate-Free Medium.

Cells synchronized in a light-and-dark regime at the beginning of the light phase were incubated for 2 hr in the light in medium containing 10 mM Pipes, pH 7.0, 7 mM NH_3, and either potassium acetate (17.5 mM) or EGTA (1 mM) plus sufficient $CaCl_2$ to give the indicated free Ca^{2+} concentration. RNA gel blots were produced as described in the legend to Figure 1.

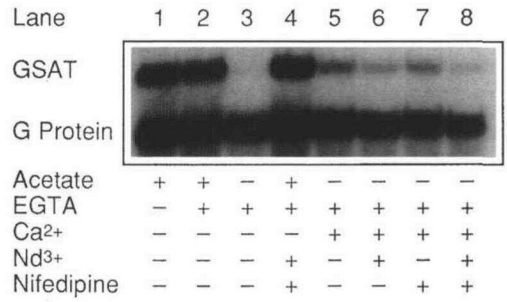

Figure 4. Effects of EGTA and Ca^{2+} Channel Blockers on Acetate-Dependent and Ca^{2+}-Dependent Light Induction of Chlamydomonas GSAT mRNA.

Cells synchronized in a light-and-dark regime at the beginning of the light phase were incubated for 2 hr in the light in medium containing 10 mM Pipes, pH 7.0, 7 mM NH_3, plus, as indicated, potassium acetate (17.5 mM), EGTA (1 mM), nifedipine (10 μM), sufficient $CaCl_2$ to give a free Ca^{2+} concentration of 1 μM, and sufficient $NdCl_3$ to give a free Nd^{3+} concentration of 10 nM. RNA gel blots were produced as described in the legend to Figure 1.

Ca^{2+} Ionophore A23187 Causes Transient Induction of GSAT mRNA in the Dark

It was reported previously that cells synchronized in a 12-hr-light and 12-hr-dark regime that were kept in the dark after the end of a dark phase do not show induction of GSAT mRNA (Matters and Beale, 1994, 1995b). However, when the cells were exposed in the dark to the Ca^{2+} ionophore A23187 in Ca^{2+}-containing medium, there was a transient induction of GSAT mRNA (Figure 5). Maximal induction occurred at ~15 min after the administration of A23187, and the degree of induction was ~15% of that occurring after 2 hr in the light.

Mg^{2+} Supports Weak Light Induction of GSAT mRNA in the Absence of Acetate and Ca^{2+}

During the course of these experiments, it was found that a small amount of light induction of GSAT mRNA occurred in the absence of acetate and Ca^{2+} when Mg^{2+} was present in the medium (Figure 6, lanes 1 to 7). Mg^{2+} supported this induction only at concentrations at or above 1 mM, in contrast to Ca^{2+}, which supported acetate-independent induction at micromolar concentrations. Because EGTA-buffered solutions were used in these experiments, the possibility that the observed effect of Mg^{2+} was caused by contamination of the Mg^{2+} solutions with Ca^{2+} was excluded. An alternative explanation is that external Mg^{2+} acts by facilitating the release of

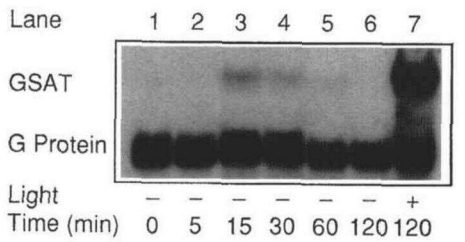

Figure 5. Transient Induction of Chlamydomonas GSAT mRNA in the Dark in A23187-Treated Cells.

Cells synchronized in a light-and-dark regime at the beginning of the light phase were incubated in the light for 2 hr or in the dark for the indicated time in a medium containing the Ca^{2+} ionophore A23187. The composition of the medium for all incubations was 10 mM Pipes, pH 7.0, 17.5 mM acetate, 7 mM NH_3, 1 mM EGTA, sufficient $CaCl_2$ to give a free Ca^{2+} concentration of 10 μM, and 3 μM A23187. RNA gel blots were produced as described in the legend to Figure 1.

Ca^{2+} from some compartment that is inaccessible to EGTA rather than by directly supporting induction in a Ca^{2+}-independent manner. This hypothesis is consistent with the ability of calmodulin-specific reagents trifluoperazine (TFP) and *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) to block Mg^{2+}-supported induction of GSAT mRNA (see below). One possible location of a Ca^{2+} binding, EGTA-inaccessible compartment is the external surface of the cell membrane, which, if sufficiently negatively charged, might bind Ca^{2+} and repel EGTA. However, the cell wall itself was excluded as a possible source of a hypothetical EGTA-inaccessible Ca^{2+} pool because weak Mg^{2+}-supported light induction of GSAT mRNA was observed even in Chlamydomonas strain cw-15, which lacks a cell wall (data not shown). The ability of Mg^{2+} to partially support another Ca^{2+}-dependent response, flagellar shortening, in Chlamydomonas was previously reported and explained in terms of an Mg^{2+}-facilitated exchange of bound Ca^{2+} (Quader et al., 1978). The ability of Mg^{2+} to support weak light induction of GSAT mRNA in the absence of external Ca^{2+} and acetate was not investigated further.

Calmodulin Antagonists Inhibit Light Induction of GSAT mRNA

One way in which Ca^{2+} is involved in signal transduction leading to gene expression is through interaction with the ubiquitous Ca^{2+} binding protein calmodulin. The Ca^{2+}–calmodulin complex is capable of activating various protein kinases and phosphatases that in turn affect downstream events. In Chlamydomonas, calmodulin occurs in the cell body and flagella (Gitelman and Witman, 1980). TFP is a specific calmodulin antagonist that blocks its interaction with target effector proteins (Vandonselaar et al., 1994). TFP blocked the light induction of GSAT mRNA in a concentration-dependent manner (Figure 7). TFP was effective in blocking external Ca^{2+}-independent induction in the presence of acetate as well as external Ca^{2+}-supported induction in the absence of acetate. Although at lower concentrations the drug was somewhat more effective in blocking Ca^{2+}-supported induction than external Ca^{2+}-independent induction, at concentrations above 5 μM, both types of induction were completely blocked. Even the weak light induction of GSAT mRNA supported by Mg^{2+} in the absence of acetate and Ca^{2+} was blocked by TFP (Figure 6, lanes 8 and 9). This result supports the conclusion that Mg^{2+} acts by facilitating the release of Ca^{2+} from a compartment that is inaccessible to EGTA rather than by directly supporting GSAT mRNA induction in a Ca^{2+}-independent manner. In these experiments, the cell population density was carefully controlled at levels below 1.5×10^6 cells per mL for all incubations to prevent spurious requirements for higher TFP concentrations that can occur at higher cell densities (Detmers and Condeelis, 1986).

Another compound that inhibits calmodulin action by blocking its interaction with target effector proteins is W-7 (Detmers and Condeelis, 1986; Bloodgood and Salomonsky, 1990; Cheshire and Keller, 1991). Like TFP, W-7 blocked external Ca^{2+}-independent light induction of GSAT mRNA in the presence of acetate as well as external Ca^{2+}-supported induction in the absence of acetate (Figure 8). The W-7 analog *N*-(6-aminohexyl)-1-naphthalenesulfonamide (W-5), which is a much weaker inhibitor of calmodulin action than W-7, was ineffective at concentrations at which W-7 was effective. These results indicate that the inhibitory effect of W-7 is due specifically to its action on calmodulin.

Possible Roles for Acetate in Supporting Light Induction of GSAT mRNA

Acetate at high concentration or at low pH causes Chlamydomonas cells to shed their flagella (Hartzell et al., 1993). This action of acetate is a function of the concentration of the protonated form of the acid and is mediated through acidification of the cytoplasm. The cytoplasmic acidification was proposed

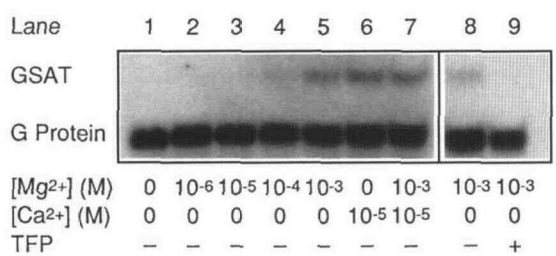

Figure 6. Weak Induction of GSAT mRNA by Light in Cells Incubated in Medium Containing Mg^{2+}.

Cells synchronized in a light-and-dark regime at the beginning of the light phase were incubated for 2 hr in the light in medium containing 10 mM Pipes, pH 7.0, 7 mM NH_3, 1 mM EGTA, and, where indicated, sufficient $CaCl_2$ and $MgCl_2$ to give the indicated free concentrations of Ca^{2+} and Mg^{2+}, respectively, and 6 μM TFP. RNA gel blots were produced as described in the legend to Figure 1.

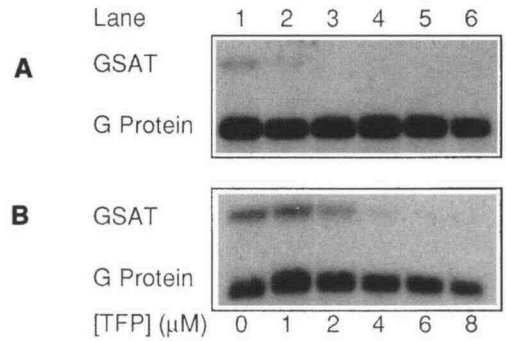

Figure 7. Effects of TFP on Light Induction of Chlamydomonas GSAT mRNA.

(A) Induction was supported by external Ca^{2+} in acetate-free medium. **(B)** Induction was supported by acetate in the absence of external Ca^{2+}.

Cells synchronized in a light-and-dark regime at the beginning of the light phase were incubated for 2 hr in the light in medium containing 10 mM Pipes, pH 7.0, 7 mM NH_3, 1 mM EGTA, the indicated concentration of TFP, and either sufficient $CaCl_2$ to give a free Ca^{2+} concentration of 10 μM **(A)** or 17.5 mM acetate **(B)**. RNA gel blots were produced as described in the legend to Figure 1.

to activate phospholipase C, leading to inositol trisphosphate production and activation of an inositol trisphosphate–gated Ca^{2+} channel and an increase in cytosolic Ca^{2+}, which triggers flagellar excision (Hartzell et al., 1993). Other permeant weak acids could be substituted for acetate in triggering flagellar excision, including benzoic acid, which at 15 mM was approximately as effective as 50 mM acetate (Hartzell et al., 1993). Although acetate does not cause cytoplasmic acidification at the concentration (17.5 mM) and pH (7.0) at which it supports light induction of GSAT mRNA (Hartzell et al., 1993), we nevertheless examined whether benzoate could also support this induction. Under our incubation conditions, 15 mM benzoate did not induce flagellar excision (data not shown), and it did not support light induction of GSAT mRNA, although it inhibited the induction somewhat in the presence of 17.5 mM acetate (Figure 9). It thus appears that the mechanism by which acetate supports light induction of GSAT mRNA is not through cytoplasmic acidification.

Another possible role for acetate is raising the intracellular ATP concentration or energy charge, thereby facilitating the release of Ca^{2+} from intracellular stores in response to the light signal. Evidence favoring this role for acetate was provided by the observation that acetate-supported light induction of GSAT mRNA is inhibited by carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), an uncoupler of respiratory ATP synthesis (Figure 10). It is of interest that CCCP did not inhibit external Ca^{2+}-supported light induction of GSAT mRNA. The specific effect of CCCP in inhibiting acetate-supported but not external Ca^{2+}-supported induction suggests that acetate may act by providing an energy source needed by the cells to enable them to utilize internal Ca^{2+} as a second message in the signal transduction pathway.

DISCUSSION

The results of this study clearly demonstrate the involvement of Ca^{2+} and calmodulin in light-regulated expression of GSAT mRNA in Chlamydomonas cells synchronized in a light-and-dark regime. Key findings leading to this conclusion include the requirement for external Ca^{2+} in the absence of acetate, inhibition of both Ca^{2+}- and acetate-supported induction by the calmodulin antagonists TFP and W-7, and transient induction in the dark by the Ca^{2+} ionophore A23187. Recently, Ca^{2+} but not calmodulin has been implicated as a component of a signal transduction pathway linking the perception of blue light and expression of a specific gene in Arabidopsis (Christie and Jenkins, 1996).

Chlamydomonas is a useful experimental organism in which to study the effects of blue light, because it lacks phytochrome and does not require light for chlorophyll biosynthesis; therefore, the possibility of complications in data interpretation caused by the interaction of multiple photoreceptor systems can be minimized. We previously showed that in cells synchronized in a light-and-dark regime, GSAT mRNA levels are increased by light, that the increase is due mainly to increased synthesis and not to decreased turnover of the mRNA, and that blue light is effective whereas red light is not (Matters and Beale, 1994, 1995b). The promoter region of the Chlamydomonas *gsa* gene contains sequences that are similar to those identified as conferring light regulation of several plant genes (Matters and Beale, 1994). We have used these observations as the basis for continuing studies of the mechanism of blue light–regulated gene expression.

To determine which, if any, components of the culture medium are required for light induction of GSAT mRNA, cells synchronized in a light-and-dark regime were transferred at

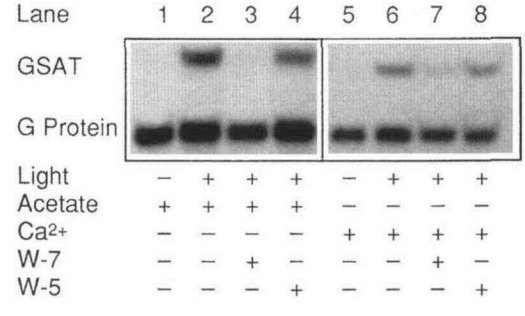

Figure 8. Effects of W-7 and W-5 on Light Induction of Chlamydomonas GSAT mRNA.

Cells synchronized in a light-and-dark regime at the beginning of the light phase were incubated for 2 hr in the light in medium containing 10 mM Pipes, pH 7.0, 7 mM NH_3, and 1 mM EGTA, plus W-7, W-5, and acetate (17.5 mM) or Ca^{2+} (10 μM), as indicated. In the incubations containing acetate (lanes 1 to 4), the W-7 or W-5 concentration was 50 μM, and in the incubations containing Ca^{2+} (lanes 5 to 8), the W-7 or W-5 concentration was 20 μM. RNA gel blots were produced as described in the legend to Figure 1.

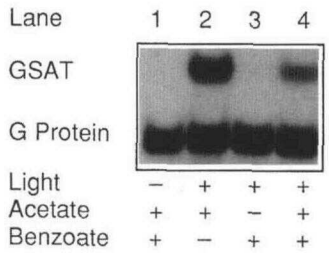

Figure 9. Effects of Benzoate on Light Induction of GSAT mRNA.

Cells synchronized in a light-and-dark regime at the beginning of the light phase were incubated for 2 hr in the dark or light, as indicated, in medium containing 10 mM Pipes, pH 7.0, 7 mM NH_3, and 1 mM EGTA, plus the indicated additions of 17.5 mM acetate and 15 mM benzoate. RNA gel blots were produced as described in the legend to Figure 1.

the end of a dark phase to buffered solutions containing one or more components of the TAP culture medium, and the GSAT mRNA level was determined after the cells were exposed to light for 2 hr. Preliminary experiments indicated that both a nitrogen source and acetate are required for GSAT mRNA induction by light. Full induction of GSAT mRNA occurred in cells exposed to light in the presence of 7 mM NH_3 (or NO_3^- in a strain that can utilize NO_3^- as a nitrogen source) and 17.5 mM acetate, the concentrations at which these components are present in TAP medium, whereas little or no induction occurred if either of these components was omitted. A dependence on nitrogen for the expression of several Chlamydomonas nuclear genes that are involved in chloroplast development has been noted, and it was suggested that this nitrogen requirement may be the basis for chlorosis caused by nitrogen deficiency (B.U. Bruns and G.W. Schmidt, personal communication). The nitrogen requirement for GSAT mRNA induction was not explored further in this study.

In contrast to the complete absence of induction in medium lacking a nitrogen source, a small amount of induction occurred in the absence of acetate. Moreover, the requirement for acetate was substantially relieved if the cells were provided with low concentrations of Ca^{2+} in the incubation medium. Partial induction occurred at Ca^{2+} concentrations as low as 1 μM, and higher levels of induction occurred at increasing Ca^{2+} concentrations, up to a maximum of ~60% of the acetate-supported induction at 100 μM Ca^{2+}. The external free Ca^{2+} concentration required for GSAT mRNA induction in the absence of acetate is comparable to the concentration required for antibody-induced glycoprotein movements within the flagellar membrane (Bloodgood and Salomonsky, 1990) and for deflagellation of detergent-permeabilized cells (Sanders and Salisbury, 1989) and cells treated with sodium benzoate at pH 6 (Quarmby and Hartzell, 1995).

The observation that external Ca^{2+} is required for light induction of GSAT mRNA only in the absence of acetate has several possible explanations. At high concentrations, or at low pH, acetate can enter the cells in the unionized form and cause intracellular acidification. This acidification has been proposed to activate a signal transduction chain leading to an increase in cellular Ca^{2+} concentration that triggers flagellar excision (Hartzell et al., 1993). However, this explanation was rejected because the acetate concentration (17.5 mM) and external pH (7.0) used in the experiments in this study are the same as those of the TAP culture medium. Under these conditions, acetate does not cause deflagellation (Hartzell et al., 1993). Moreover, benzoate, a permeant weak acid that is effective in triggering flagellar excision at low pH, did not induce deflagellation or support GSAT mRNA induction in the absence of acetate under our incubation conditions.

Another possible explanation for the observation that external Ca^{2+} is required for light induction of GSAT mRNA only in the absence of acetate is that the signal transduction pathway bifurcates into two independent branches, one requiring Ca^{2+} and the other requiring acetate instead of Ca^{2+}. However, this interpretation was rejected because low concentrations of TFP and W-7, specific inhibitors of Ca^{2+}–calmodulin interactions with effector molecules (Detmers and Condeelis, 1986; Bloodgood and Salomonsky, 1990; Cheshire and Keller, 1991; Vandonselaar et al., 1994), inhibited both external Ca^{2+}-supported (acetate-independent) and external Ca^{2+}-independent (acetate-supported) induction. The results with TFP and W-7 thus indicate that Ca^{2+}-activated calmodulin is involved in the induction even under conditions in which external Ca^{2+} is not required.

In view of the foregoing, we favor the hypothesis that Ca^{2+} and calmodulin are essential components in the signal transduction chain for blue light induction of GSAT mRNA. We propose that an internal Ca^{2+} pool can be mobilized for induction when acetate is present in the medium and that external Ca^{2+} can partially substitute for the internal pool, which cannot be mobilized by blue light when acetate is not supplied to the cells. Acetate may act by raising the intracellular ATP level or energy charge, a possible requirement for mobilization of the intracellular Ca^{2+} pool. This hypothesis is supported by the ability of CCCP, an uncoupler of electron

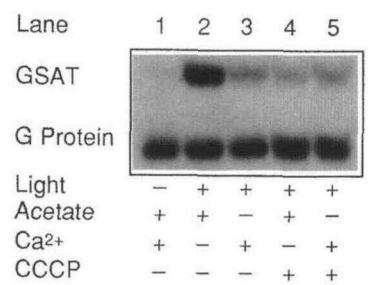

Figure 10. Effects of CCCP on Light Induction of GSAT mRNA.

Cells synchronized in a light-and-dark regime at the beginning of the light phase were incubated for 2 hr in the dark or light, as indicated, in medium containing 10 mM Pipes, pH 7.0, 7 mM NH_3, and 1 mM EGTA, plus the indicated additions of 17.5 mM acetate, sufficient $CaCl_2$ to give a free Ca^{2+} concentration of 10 μM, and 1 μM CCCP. RNA gel blots were produced as described in the legend to Figure 1.

transport, to inhibit acetate-supported but not external Ca^{2+}-supported induction.

External Ca^{2+} enters cells through one or more classes of Ca^{2+} channels, the permeability of which can be modulated by various factors, including ATP hydrolysis, protein phosphorylation, phospholipids and their hydrolysis products, and the membrane potential (Janis and Triggle, 1983; Tsien et al., 1987). Transport of Ca^{2+} through different classes of Ca^{2+} channels is inhibited to varying extents by a variety of blocking agents. Two Ca^{2+} channel-blocking agents, nifedipine and Nd^{3+}, were tested for their ability to block light induction of GSAT mRNA in the presence or absence of external Ca^{2+}. These agents were chosen because they were previously shown to inhibit differentially the enhancement by phytochrome and cytokinin of light-induced chlorophyll accumulation in etiolated cucumber cotyledons (Reiss and Beale, 1995). Each agent partially inhibited external Ca^{2+}-dependent induction of GSAT mRNA in the absence of acetate, and Nd^{3+} was somewhat more effective than nifedipine at the concentrations used. The inhibitory effects of the two agents were approximately additive. In contrast, the blocking agents did not inhibit the external Ca^{2+}-independent induction in the presence of acetate. These results reinforce the conclusion that in the presence of acetate, external Ca^{2+} is not required for light induction of GSAT mRNA. The absence of inhibition in the presence of acetate also supports the conclusion that the blocking agents do not affect the ability of the cells to respond to light, except by partially blocking the influx of external Ca^{2+} that is required for induction of GSAT mRNA in the absence of acetate.

It is notable that the Ca^{2+} ionophore A23187 caused a transient induction of GSAT mRNA even in the dark. This positive effect confirms the conclusions reached from the results with external Ca^{2+}, Ca^{2+} channel blockers, and the calmodulin antagonists TFP and W-7 and provides independent evidence for the involvement of Ca^{2+} and calmodulin in the signal transduction pathway linking blue light perception with gene induction in Chlamydomonas.

It is likely that Ca^{2+} and calmodulin exert their effects on the GSAT mRNA level by increasing the transcription rate of the gene rather than by lowering the stability of the message. The half-life of the message was previously determined to be short: <30 min in the light and 40 min in cells placed in the dark after 2 hr of light induction (Matters and Beale, 1995b). An effect of the absence of Ca^{2+} to shorten the life of the GSAT mRNA would have to be specific (because the reference G protein mRNA is unaffected), mediated through calmodulin (because the increase is blocked by calmodulin inhibitors), and be so great as to account for the complete absence of message when calmodulin action is inhibited by TFP or W-7. In view of the unlikelihood of this scenario, we favor the simpler model in which Ca^{2+}-activated calmodulin is required for transcription of the *gsa* gene. The latter model has been adopted to explain the involvement of Ca^{2+} and calmodulin in regulating the transcription of many other genes, including the phytochrome-mediated light induction of some genes in higher plants (see below).

Calcium has long been known to be involved in many cellular responses in Chlamydomonas, including photoaccumulation (Stavis and Hirschberg, 1973; Stavis, 1974), photophobic responses (Schmidt and Eckert, 1976), phototaxis (Nultsch, 1979), chemotaxis (Ermilova et al., 1995), and various flagellar responses (Hyams and Borisy, 1975, 1978; Lefebvre et al., 1978; Quader et al., 1978). Ca^{2+} has also been shown to be involved in the regulation of chloroplast development in plants. For example, in excised etiolated cucumber cotyledons, external Ca^{2+} is required for greening and for the enhancement of greening by preexposure to phytochrome and cytokinin (Reiss and Beale, 1995). Calmodulin antagonists inhibit phytochrome-mediated induction of the genes that encode the major light-harvesting chlorophyll binding protein in soybean cell suspension cultures (Lam et al., 1989). Moreover, ionomycin, a Ca^{2+} ionophore, induces transient low-level expression of the genes in the dark (Lam et al., 1989). Microinjection experiments have demonstrated that Ca^{2+}-activated calmodulin is a component of a signal transduction chain linking the perception of light by phytochrome to the induction of some but not all phytochrome-regulated genes in leaf cells of a phytochrome-deficient tomato strain (Neuhaus et al., 1993; Bowler et al., 1994).

These earlier results clearly indicate the involvement of Ca^{2+} and calmodulin in phytochrome-mediated light-regulated gene expression in plants. The results of this study extend the range of involvement of Ca^{2+} and calmodulin in photoresponses by demonstrating their participation in the signal transduction chain linking the perception of blue light with the induction of a specific gene in Chlamydomonas. We expect that continued study of this system will provide additional information about signal transduction in blue light–regulated gene expression.

METHODS

Cell Culture

Chlamydomonas reinhardtii wild-type strains CC1690 and CC124 and cell wall–deficient strain CC406 (cw-15) were obtained from the Chlamydomonas culture collection (Duke University, Durham, NC). Strains CC124 and CC406 were routinely maintained on Tris–acetate–phosphate (TAP) medium (Harris, 1989) at 25°C under synchronous light cycles (12 hr of light and 12 hr of dark). The light (52 $\mu E\ m^{-2}\ sec^{-1}$) was supplied by cool-white fluorescent tubes. Strain CC1690 was grown in the same manner as the other strains, except that the normal TAP medium nitrogen source (7.5 mM NH_4Cl) was replaced by 7.5 mM KNO_3. Cultures were harvested in the mid-exponential growth phase (1 to 2 $\times$ 10^6 cells per mL) at the end of a dark phase by centrifugation.

Incubations

Nifedipine, A23187, trifluoperazine (TFP), *N*-(6-aminohexyl)-1-naphthalenesulfonamide (W-5), and *N*-(6-aminohexyl)-5-chloro-1-naph-

thalenesulfonamide (W-7) were obtained from Sigma. $NdCl_3$ was obtained from Aldrich Chemical Co. (Milwaukee, WI). $CaCl_2$, $NdCl_3$, TFP, W-5, and W-7 were made up as stock solutions in H_2O. Nifedipine was made up as a 10 mM stock solution in dimethyl sulfoxide. A23187 was made up as a 1 mg/mL stock solution in dimethyl sulfoxide. Benzoic acid was made up as a 1 M stock solution in ethanol. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was made up as a 10 mM solution in ethanol. For incubations containing controlled concentrations of Ca^{2+}, harvested cells were washed twice with a solution consisting of 10 mM Pipes, pH 7.0, and 3 mM EGTA and distributed into EGTA-treated flasks containing 10 mM Pipes, pH 7.0, 1 mM EGTA, 7 mM NH_4Cl (or 7 mM KNO_3 where indicated), and an amount of $CaCl_2$ calculated to give the desired concentration of free Ca^{2+} at pH 7.0 in the presence of 1 mM EGTA, based on the equations of Blinks et al. (1982). For incubations containing added combinations of Ca^{2+}, Mg^{2+}, and Nd^{3+}, the medium contained 10 mM Pipes, pH 7.0, 1 mM EGTA, and 7 mM NH_4Cl, as well as the amounts of $CaCl_2$, $MgCl_2$, and $NdCl_3$ necessary to give the desired free concentrations that were determined from the equations of Blinks et al. (1982), modified for use with Nd^{3+} by using the Nd^{3+}–EGTA association constants given by Martell and Smith (1974). Cultures were treated with the reagents for 10 min in the dark and then exposed to light for 2 hr before harvesting for RNA analysis.

RNA Isolation and Gel 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 Chemical Co., Pittsburgh, PA) for 20 min, and phenol–chloroform extracted until the interface was clear. The aqueous layer was precipitated with ethanol, the pellet was 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.

The probe for GSAT mRNA was the full-length *gsa* cDNA (Matters and Beale, 1994). Blots were also probed with a G protein β subunit–like cDNA from Chlamydomonas (obtained from K.L. Kindle, Cornell University, Ithaca, NY), which is known to be constitutively expressed in cells synchronized in light-and-dark regimes (Schloss et al., 1984; Schloss, 1990), to quantitate relative RNA loading.

For gel blots, 20 μg of RNA was denatured with glyoxal, separated on a 1% (w/v) agarose gel, and blotted onto a nylon membrane (Nytran+; Schleicher & Schuell). Blots were UV cross-linked and hybridized with nick-translated probes in a solution containing 50% (w/v) formamide, 5 × SSPE (1 × SSPE is 0.15 M NaCl, 10 mM sodium phosphate, pH 7.4, 1 mM EDTA), 2 × Denhardt's solution (1 × Denhardt's solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA), and 50 μg/mL sonicated salmon testes DNA at 45°C. Final washes were done at 55°C in 0.2 × SSPE containing 0.1% (w/v) SDS. Autoradiographs were taken with Kodak X-OMAT AR film. For quantification, RNA gel blots were scanned with a Fujix BAS-1000 MacBAS bioimaging analyzer (Fuji Photo Film Co., Tokyo, Japan).

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