The EMBO Journal vol.8 no.10 pp.2777 – 2783, 1989

Phytochrome activation of two nuclear genes requires cytoplasmic protein synthesis

Eric Lam, Pamela J.Green[1], Mary Wong[2] and Nam-Hai Chua

Laboratory of Plant Molecular Biology, The Rockefeller University, 1230 York Avenue, New York, NY 10021-6399, USA

[1]Present address: MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, MI 48824, USA

[2]Present address: Ciba-Geigy, Pharmaceutical Division, V131, 556 Morris Avenue, Summit, NJ 07901, USA

Communicated by P.Bennoun

We have investigated the effects of protein synthesis inhibitors on light-induced expression of two plant nuclear genes, *Cab* and *rbcS*, in wheat, pea and transgenic tobacco. Light activation of these two genes is very sensitive to cycloheximide, an inhibitor of cytoplasmic protein synthesis but not to chloramphenicol, an inhibitor of organellar protein synthesis. Studies with chimeric gene constructs in transgenic tobacco seedlings show that cycloheximide exerts its effect at the transcriptional level. As a control, we show that the expression of the cauliflower mosaic virus (CaMV) 35S promoter is enhanced by cycloheximide treatment, irrespective of the coding sequence used. Escape-time analyses with green wheat seedlings show that the cycloheximide block for *Cab* gene expression is after the primary signal transduction step linked to phytochrome photoconversion. Our results suggest that phytochrome activation of *Cab* and *rbcS* is mediated by a labile protein factor(s) synthesized on cytoplasmic ribosomes.

Key words: cycloheximide/gene regulation/signal transduction/light-responsive genes

Introduction

Light is a critical environmental regulator of plant development. Among the various known photoreceptors involved in photomorphogenesis, phytochrome is by far the best characterized. A classical assay for the involvement of this receptor is the red light induction of the far-red light reversal of the observed phenomena. Phytochrome can regulate nuclear genes in a positive or negative manner, depending on the gene in question. Two of the best characterized plant genes are known to be under positive regulation by phytochrome. These are the nuclear genes *Cab* and *rbcS*, which encode the chlorophyll *a*/*b*-binding protein and the small subunit of ribulose-1,5- bisphosphate carboxylase, respectively (Tobin and Silverthorne, 1985; Kuhlemeier *et al.*, 1987).

A major advance in the characterization of the molecular mechanism of the light responsiveness of *Cab* and *rbcS* is the definition of the *cis*-acting DNA elements required for their expression in transgenic tobacco (Morelli *et al.*, 1985; Timko *et al.*, 1985; Fluhr *et al.*, 1986a; Simpson *et al.*, 1986; Nagy *et al.*, 1987; Kuhlemeier *et al.*, 1988). These studies have shown that 5′ sequences from these genes can confer light responsiveness on chimeric gene constructs driven by constitutive promoter elements (Fluhr *et al.*, 1986a; Simpson *et al.*, 1986; Nagy *et al.*, 1987; Poulsen *et al.*, 1988). A nuclear protein factor, GT-1, that recognizes light-responsive elements upstream of pea *rbcS* has been identified (Green *et al.*, 1987, 1988; Kuhlemeier *et al.*, 1988, 1989). Another factor that binds to a conserved G-box in the tomato *rbcS* promoter has also been described recently (Giuliano *et al.*, 1988). Thus, the molecular characterization of the *cis*-acting element for light–dark regulation and the analysis of *trans*-acting factors which interact with these elements are well underway. In contrast, the intermediate step(s) between the signal reception by phytochrome and the activation of *Cab* and *rbcS* transcription is completely undefined. Although phytochrome has been reported to activate transcription in isolated nuclei (Ernst and Oesterhelt,

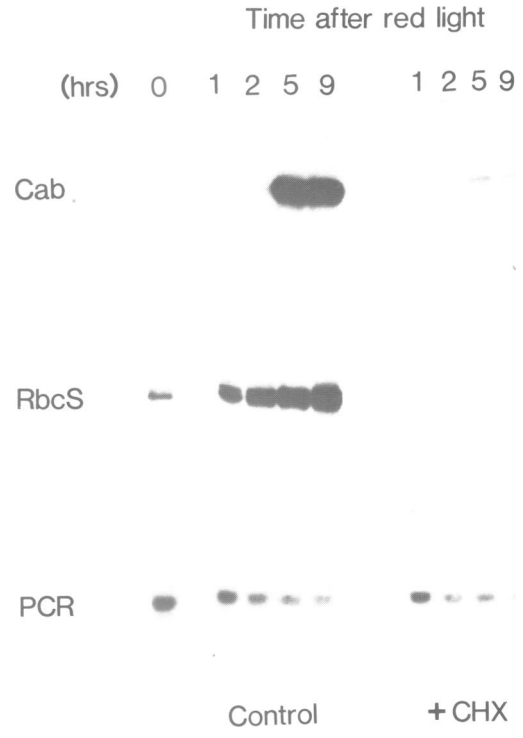

Fig. 1. Effects of cycloheximide treatment on phytochrome-regulated gene expression in etiolated wheat. Etiolated wheat seedlings were cut and immersed in 20 mM HEPES–KOH (pH 7.0) buffer with or without 0.3 mM CHX as described in Materials and methods. All manipulations were done in a dark room under a dim, green safe-light. After cutting, the plants were incubated in the buffer for 2 h in the dark before treatment with 5 min of red light. Plants were then returned to the dark boxes and samples were collected at 0, 1, 2, 5 and 9 h after the red flash, and immediately frozen in liquid nitrogen. RNAs (10 μg per lane) from these samples were analyzed, either by 5′ S1 nuclease protection assays (*Cab* and *rbcS*) or by Northern blot hybridization (protochlorophyllide reductase, PCR) as described in Materials and methods.

Table I. Effects of inhibitors on the incorporation of [^{35}S]methionine into TCA precipitate of green wheat seedlings

Sample	Relative incorporation (%)	Inhibition of total protein synthesis (%)	Inhibition of cytoplasmic synthesis (%)
control	100	0	0
+CAP	35	65	0
+CAP, CHX	2	98	94
+CAP, ANI	20	80	40
+CAP, EME	45	55	0

The amount of [^{35}S]methionine incorporation into TCA precipitate in the control sample was taken as a measure of total protein synthesis. Addition of CAP reduced [^{35}S]methionine incorporation by 65%. The CAP-insensitive [^{35}S]methionine incorporation (35% of total) was assumed to be due to cytoplasmic protein synthesis. CAP, chloramphenicol (0.15 mM); CHX, cycloheximide (0.3 mM); ANI, anisomycin (0.3 mM) and EME, emetine (0.3 mM).

1984), the *in vivo* relevance of this observation is unknown at present.

In this paper we address the question of whether *de novo* protein synthesis is required for the signal transduction between Pfr (the far-red absorbing form of phytochrome) and the expression of *Cab* and *rbcS*. Our results indicate that cytoplasmic protein synthesis is needed for the light activation of *Cab* and *rbcS* whereas the de-activation of NADPH−protochlorophyllide reductase gene (*PCR*) expression by phytochrome does not require protein synthesis. In addition, using transgenic tobacco plants with chimeric gene constructs, we show that cycloheximide (CHX) inhibits light-responsive transcription mediated by the *Cab* and *rbcS* 5′ upstream regions, irrespective of the coding sequence.

Results

Effects of protein synthesis inhibitors on gene expression in wheat

We examined the effects of cycloheximide treatment on the expression of three light-responsive genes in etiolated wheat. The *Cab* and *rbcS* families respond positively to red light treatment while the gene for PCR is negatively regulated by red light (Batschauer and Apel, 1984). Figure 1 shows that red light induces an increase in the *Cab* and *rbcS* mRNA levels in etiolated wheat seedlings while CHX pre-treatment abolishes most of the light-activated accumulation of these two mRNAs. In contrast, the red light induced decrease of the *PCR* mRNA is not affected by cycloheximide treatment. Without red light, the level of *PCR* mRNA remains essentially constant in the dark with or without cycloheximide (data not shown). Thus, the phytochrome-induced increase in *Cab* and *rbcS* mRNA is inhibited by CHX while the red light induced decrease of *PCR* gene expression is not. It should be pointed out that the data for *Cab* in Figure 1 was obtained with a probe specific for the wheat *Cab*-1 transcript. Northern blot analyses with the same RNAs using the *Cab* coding sequence as a probe shows essentially identical results (data not shown). Thus, the *Cab* family as a whole behaves similarly under our conditions.

The effects of various protein synthesis inhibitors on light activation of *Cab*-1 in etiolated wheat have also been compared (data not shown). Chloramphenicol (CAP), a 70S ribosome inhibitor, has no significant effect while the 80S ribosome inhibitor, CHX, prevents the accumulation of *Cab-1* mRNA when added either alone (Figure 1) or together with CAP (data not shown). In contrast, two other 80S ribosome inhibitors, anisomycin and emetine, have no significant effects (data not shown). To see whether the apparent specificity of the CHX effect is due to the inability of the other 80S ribosome inhibitors to efficiently inhibit protein synthesis in wheat, we have quantitated the effects of the various inhibitors on the incorporation of [^{35}S]methionine into proteins by green wheat seedlings.

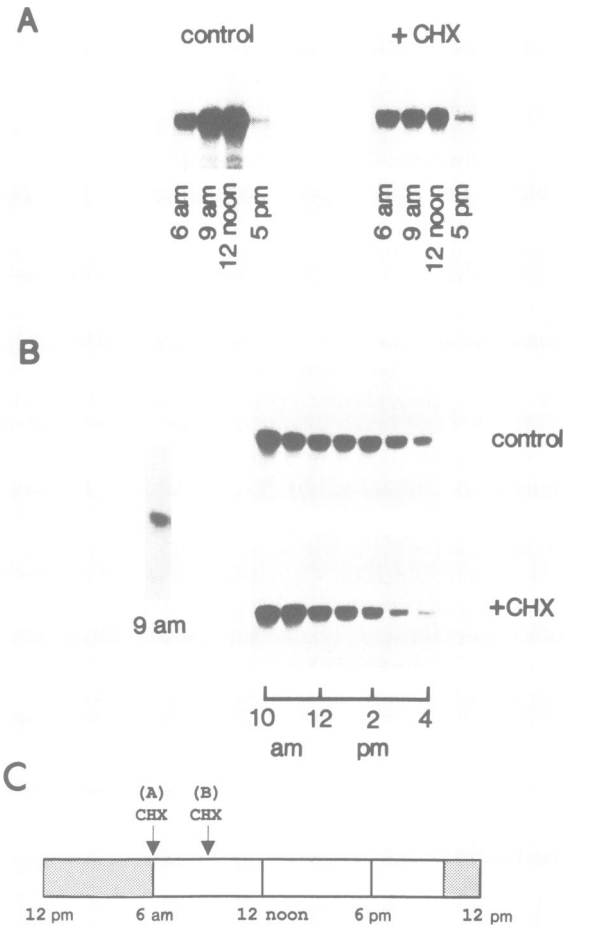

Fig. 2. The escape time for cycloheximide inhibition of *Cab* expression is between 6 am and 9 am. CHX (300 μM) was added to green wheat seedlings either at 6 am (**A**) or at 9 am, (**B**). Control samples contained only 0.1% (v/v) DMSO. Samples were collected for 5′ S1 nuclease protection assay using a wheat *Cab-1* probe (Nagy *et al.*, 1987). Each lane contained 10 μg RNA. Panel C shows a schematic diagram depicting the light:dark cycle in which the experiment was carried out. The shaded portions represent dark periods of the cycle. Arrows indicate the times when the seedlings were treated with CHX for the experiments in (A) and (B).

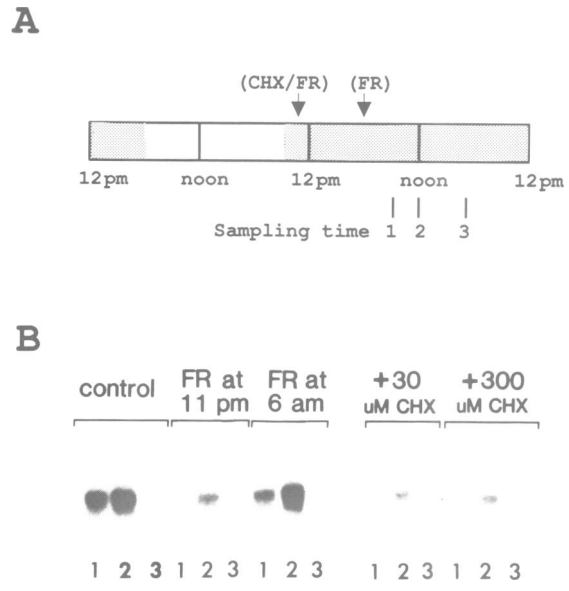

Fig. 3. The escape time for far-red reversibility of *Cab*-1 expression is between 11 pm and 6 am. (**A**) Wheat seedlings grown under 16 h light:8 h dark cycle were cut at 11 pm and transferred immediately to dark boxes. Seedlings were treated with control buffer or the same buffer containing CHX (30 or 300 μM) at 11 pm. One set of control buffer-treated samples were illuminated with 15 min of far-red (FR) light at 11 pm while another set was illuminated 7 h later at 6 am of the next day. Samples were then collected from each set of samples in the dark at 9 am (1), 12 noon (2) and 5 pm (3) for RNA preparation. Shaded portions of the schematic diagram represent dark period (**B**) RNA samples (10 μg per lane) were analyzed by 5′ S1 nuclease protection assay according to Nagy *et al.* (1987) for quantitation of *Cab-1* transcript. Lanes 1, 2 and 3 contained samples collected at 9 am, 12 noon and 5 pm, respectively (cf. A).

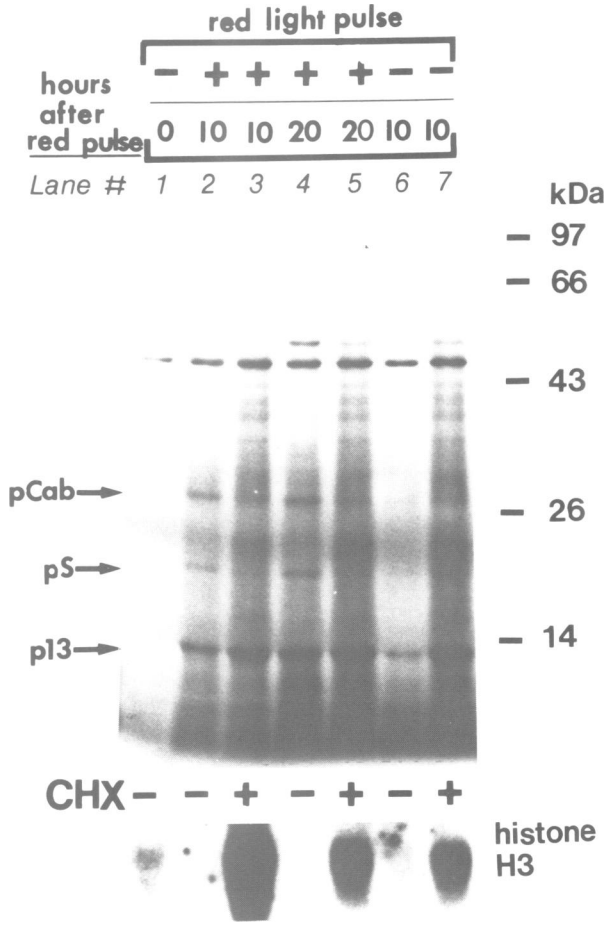

Fig. 4. Effects of cycloheximide on translatable mRNA levels in etiolated wheat seedlings. Etiolated wheat seedlings were cut at 11 pm and incubated with either control buffer or buffer with 0.3 mM CHX for 2 h as indicated at the bottom of the fluorogram. When indicated, the samples were treated with 5 min of red light and then returned to the dark for 10 or 20 h before harvesting. Total RNA was isolated by the guanidinium thiocyanate method as described in Materials and methods. RNA (5 μg) from each sample were used for *in vitro* translation assays with rabbit recticulocyte lysate (Amersham) and [^{35}S]methionine was added as label. **Lane 1**, translation products of RNAs from the etiolated seedlings at 11 pm; **Lanes 2** and **3**, samples taken 10 h after treatment with red light; **Lanes 4** and **5**, samples taken 20 h after treatment with red light; **Lanes 6** and **7**, samples were kept in the dark for 10 h with no light treatment after the cutting. The positions of phosphorylase b (97 kd), bovine serum albumin (66 kd), ovalbumin (43 kd), chymotrypsinogen (26 kd) and lysozyme (14 kd) are indicated. The lower panel shows a Northern blot analysis of the same RNA samples using the coding region of the wheat histone H3 gene as a probe (Tabata *et al.*, 1984). Each lane contains 5 μg of RNA. Ethidium bromide staining of the agarose gel before transfer to nitrocellulose revealed a similar pattern and intensity of fluorescence in the different samples (data not shown).

Table I shows that CAP treatment inhibits 65% of the total protein synthesis in wheat. The remaining protein synthesis can be inhibited effectively (>90%) by further treatment with CHX. Anisomycin only inhibits ~50% of the CAP-insensitive activity while emetine is completely ineffective. Thus, the inability of anisomycin and emetine to attenuate phytochrome activation of *Cab* can be correlated with their poor inhibitory action on protein synthesis in wheat.

To better understand the mechanism of CHX inhibition of *Cab-1* expression, we have compared the effect of CHX at different times during *Cab-1* activation. This is most easily done with green wheat seedlings that have been grown under a constant 16 h light:8 h dark cycle. Under these conditions, *Cab-1* expression is regulated by two factors. The steady state *Cab-1* mRNA level exhibits circadian rhythmicity; it is high during the day and low at night. This oscillation continues for at least one cycle, when the plants are transferred to continuous darkness (Nagy *et al.*, 1988). Secondly, far-red light treatment given at the end of the day has been shown to attenuate the *Cab-1* mRNA level in the next cycle in the dark, i.e. the subjective light period, thus demonstrating phytochrome involvement. Both regulatory mechanisms apparently operate at the level of transcription since these features of gene expression can be observed in transgenic tobacco with chimeric gene constructs driven by the *Cab-1* 5′ upstream sequence (Nagy *et al.*, 1988). We grew wheat seedlings under a 16 h light (6 am to 10 pm) and 8 h dark (10 pm to 6 am) cycle. Under this condition, the expression of *Cab-1* in wheat is repressed sometime between 1 pm and 4 pm and is reactivated after 6 am in the next morning. Taking advantage of this cyclic character of *Cab-1* expression, we treated green wheat seedlings at various times in the morning and examined the effects of CHX before and after *Cab-1* was activated. Figure 2 shows that CHX addition at 6 am is still effective in the arrest of *Cab*-1 activation. Thus, the peak of *Cab-1* mRNA accumulation at ~12 noon is abolished and the mRNA level is essentially arrested at the 6 am level. However, addition of CHX at 9 am, when the *Cab-1* mRNA increase is already well underway, has no dramatic effect on *Cab-1* mRNA level for up to 4 h after the treatment. We observed reproducibly lower *Cab-1* mRNA levels at the later time points, which

may reflect a more complete turn-off of transcription in the CHX-treated samples during the down phase of the circadian rhythm regulated cycle. Another possibility is that CHX treatment accelerates the turn-over rate of *Cab-1* mRNA. We will address the latter point with chimeric constructs in transgenic tobacco seedlings.

To establish the temporal relationship of the CHX-sensitive step of *Cab-1* activation and phytochrome activation, we have determined the time when *Cab-1* expression becomes insensitive to far-red light. Figure 3 shows that the *Cab-1* mRNA level continues to oscillate even when the wheat seedlings were shifted to continuous darkness from a 16 h light:8 h dark cycle. During the subjective light period the *Cab-1* mRNA level increases and reaches a maximum at about 12 noon (lane 2). Exposure of the seedlings to far-red illumination at 11 pm decreases *Cab-1* expression during the subjective light period. However, far-red light is ineffective when given at 6 am (Figure 3B). thus, the CHX block of *Cab-1* activation, which occurs readily at 6 am, is temporally beyond the primary event of signal transmission between Pfr and its transducer. Figure 3 also compares the effects of CHX addition to those of far-red light treatment. The inhibition of *Cab-1* expression is apparently saturated at 30 μM CHX since addition of 300 μM CHX does not give further inhibition. Determination of [^{35}S]methionine incorporation shows that the level of protein synthesis inhibition is also saturated at 30 μM CHX (data not shown).

We have also examined the effects of CHX treatment on the general profile of translatable mRNAs in order to gain a more global view of the status of mRNA levels before and after the treatment. Figure 4 shows the *in vitro* translation products of total RNA from etiolated wheat seedlings after various treatments. Three translation products with approximate masses of 35−40 kd are induced by CHX treatment irrespective of light (Figure 4, lanes 3, 5 and 7). A translation product of 13 kd (p13) is induced by red light treatment of etiolated seedlings (Figure 4, lanes 2 and 4). Interestingly, this transcript is also induced to comparable levels by treatment with CHX in the dark (Figure 4, lane 7). Two prominent translation products with relative molecular masses of 30 and 18 kd, which likely correspond to the precursors for the *Cab-1* and *rbcS* gene products, respectively (p*Cab* and pS in Figure 4), are induced by red light and the induction is inhibited by CHX treatment (Figure 4, lanes 2−5). As a control, we carried out Northern blot analysis with the same RNA samples using the wheat histone H3 gene coding region as a probe (Tabata *et al.*, 1984). Figure 4 shows that CHX causes a significant increase in the steady state level of the histone H3 mRNA (Figure 4). This result is in agreement with previous studies on HeLa cells by Sive *et al.* (1984), which show that inhibition of protein synthesis causes activation of histone H4 and H3 transcription as well as stabilization of these mRNAs. Our observations suggest that treatment with CHX does not cause a general inhibition of transcription. Since the accumulation of a light inducible mRNA (p13) is not inhibited by CHX treatment, the inhibition of the light activation of *Cab* and *rbcS* can be considered to be specific.

To see whether CHX acts at the transcriptional level and thus depends only on the promoter of the gene in question, we examined the effect of CHX on the expression of either a wheat *Cab-1* coding sequence with its own promoter (from −1029 to +1100) or the *Cab-1* coding region (+31 to +1100) fused to the constitutive CaMV 35S promoter (−941 to +8) in transgenic tobacco seedlings. Figure 5 shows that the addition of CHX prevents light-induced accumulation of the *Cab-1* transcript in etiolated seedlings. In addition, CHX treatments also inhibits *Cab-1* expression in transgenic tobacco seedlings grown under 16 h light:8 h dark cycle. In contrast, with the 35S promoter, CHX addition increased the *Cab-1* transcript level by ~2- to 3-fold. We also found that CHX treatment inhibits light induction of a chimeric gene construct with the *Cab-1* 5′ upstream region (−1029 to +31) driving the expression of the chloramphenicol acetyltransferase (CAT) coding region (data not shown).

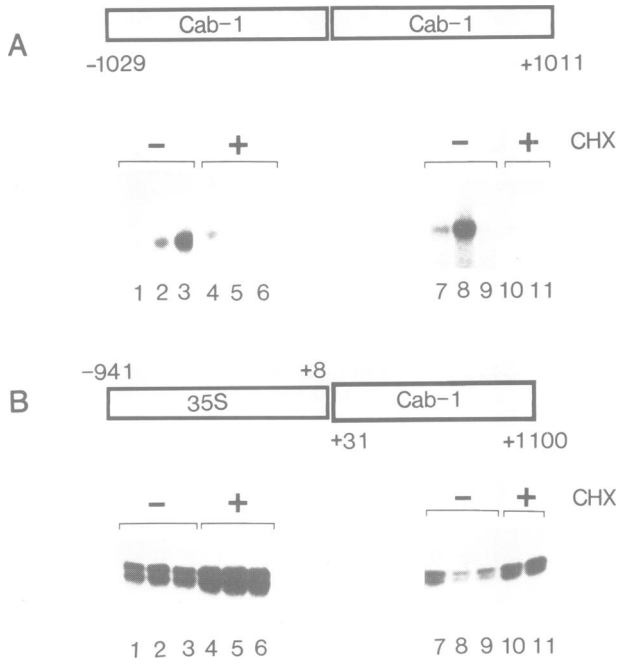

Fig. 5. Effects of cycloheximide on the wheat *Cab-1* expression in transgenic tobacco. Transgenic tobacco seedlings were germinated on MS plates for about 10 days either in the dark (lanes 1−6), or in the light (lanes 7−11). The plates were then treated with either control buffer or with cycloheximide (300 μM) as indicated. For etiolated seedlings (lanes 1−6), the plates were incubated with buffer in the dark for 1 h and transferred to continuous light. Samples were then collected in the dark at the beginning of the experiment (lanes 1 and 4), 10 h (lanes 2 and 5) and 20 h (lanes 3 and 6) after exposure to continuous light. For green seedlings, lane 7 shows samples collected at 11:30 pm in the dark, at which time either the control buffer or the cycloheximide-containing buffer as added. Samples were then collected at 11 am (lanes 8 and 10) and 7 pm (lanes 9 and 11) on the following day. RNAs were prepared from the samples and analyzed by 5′ S1 nuclease protection as described in Materials and methods. For each sample, 10 μg RNA was used for analysis. In (**A**) the transgenic seedlings contained the wheat *Cab-1* gene with 1029 bp of 5′ upstream sequence. In (**B**) the transgenic seedlings contained a chimeric construct with the *Cab-1* coding region driven by the CaMV 35S promoter. Details of both constructs were given in Nagy *et al.* (1987).

Effects of protein synthesis inhibitors on rbcS gene expression in pea and chimeric gene constructs in transgenic tobacco seedlings

To assess the generality of our observations, we have examined the effects of the protein synthesis inhibitors on light-induced *rbcS* expression in pea seedlings. The *rbcS* transcripts from pea seedlings, after exposure to continuous white light for 4 and 16 h, were analyzed by 3′ S1 nuclease analysis. We have shown previously that three of the *rbcS*

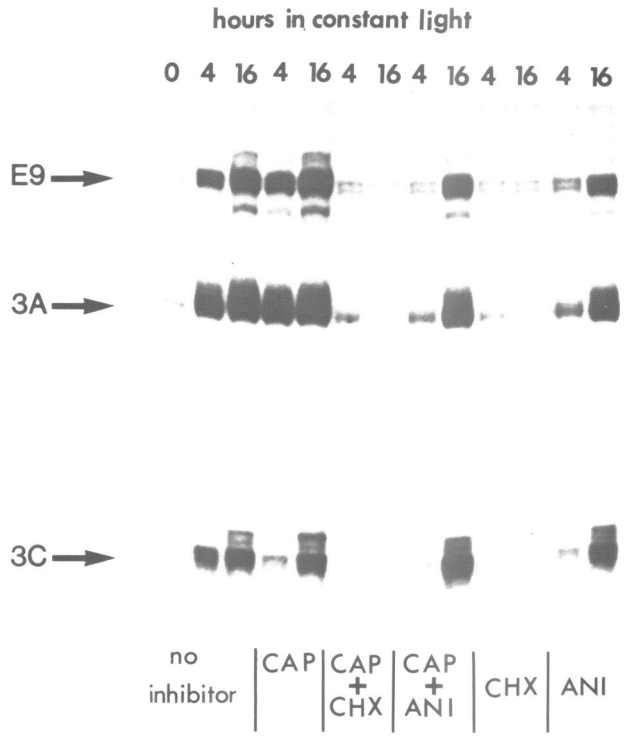

Fig. 6. Effects of protein synthesis inhibitors on light-induced *rbcS* gene expression in seedlings. Eight-day old green pea seedlings grown in a greenhouse were transferred to the dark for 2 days. The plants were then cut in the dark and immediately immersed in buffers with the indicated inhibitors. After incubation in the dark for 2 h, the plants were illuminated with continuous white fluorescent light to activate *rbcS* transcription. The first lane from the left (0 h) shows the sample collected at the beginning of the light period. Samples were then collected at 4 and 16 h after exposure to continuous white light. RNA samples (10 μg per lane) were analyzed by S1 nuclease protection with an *rbcS-E9* 3′ end probe (Fluhr *et al.*, 1986). The positions of the signal from the different members (*E9*, *3A* and *3C*) of the pea *rbcS* family (Fluhr *et al.*, 1986) are indicated on the left. The inhibitors used are given at the bottom of the lanes. CAP, chloramphenicol (0.15 mM); CHX, cycloheximide (0.3 mM) and ANI, anisomycin (0.3 mM).

genes in pea (E9, 3A and 3C) have homologous 3′ non-coding sequences. Thus, using a single 3′ probe, we can detect simultaneously all three *rbcS* transcripts since they have different lengths of 3′ non-translated sequences that will hybridize to the probe (Fluhr *et al.*, 1986b). Figure 6 shows that CAP treatment does not have any effect on either the extent or the rate of accumulation of the transcripts for *rbcS-3A or rbcS-E9*. CHX, in the presence or absence of CAP, inhibits the light induction of these *rbcS* transcripts almost completely. Interestingly, the dark level of the *rbcS-E9* and *rbcS-3A* transcripts is not affected by CHX treatment. Emetine is without any effect (data not shown) while anisomycin inhibits the rate of accumulation of the *rbcS* transcripts after light treatment. Thus, after 4 h in constant light, pre-treatment with anisomycin significantly inhibits accumulation of the *rbcS* mRNAs compared to that of control while after an additional 12 h, the *rbcS* mRNAs accumulate to nearly control levels. The protein synthesis inhibitors anisomycin and emetine have been shown to be effective in the activation of auxin-inducible mRNA accumulation in etiolated pea seedlings (Theologis *et al.*, 1985). However, both inhibitors are not as effective as CHX in their ability to inhibit cytoplasmic protein synthesis and to induce the

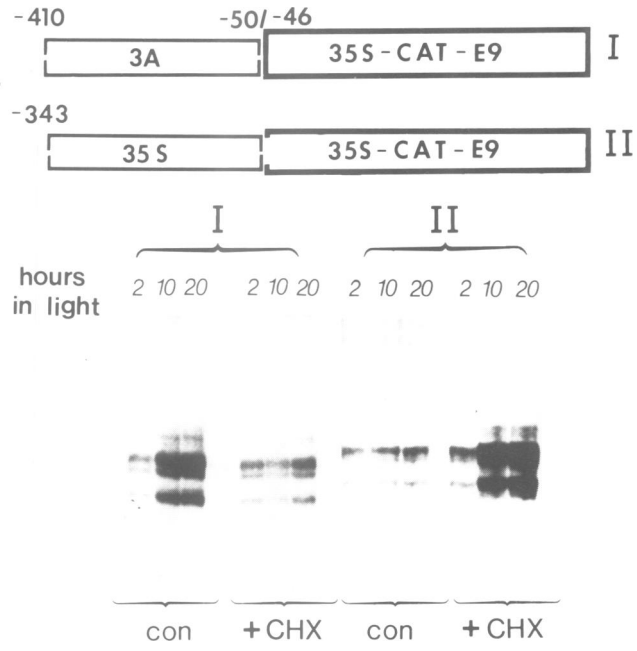

Fig. 7. Effects of cycloheximide on mRNA levels in transgenic tobacco: dependence on the enhancer element. Transgenic tobacco seedlings containing either construct I or II were compared with respect to the effects of cycloheximide treatment. Both constructs I and II contain the CaMV 35S TATA box (from −46 to +8) fused to the coding region of the bacterial CAT followed by the *rbcS-E9* polyadenylation sequence (Fluhr *et al.*, 1986). In construct I, an *rbcS-3A* enhancer (from −410 to −50) was used to drive the transcription unit whereas in construct II, a CaMV 35S enhancer (from −343 to −46) was used. Details of these constucts have been described (Aoyagi *et al.*, 1988). Samples were collected at 2, 10 and 20 h after the light treatment, as indicated. RNAs (10 μg per lane) were analyzed by 3′ S1 nuclease protection assay using an *rbcS-E9* 3′ probe (Fluhr *et al.*, 1986). Con, control, CHX, with 0.3 mM cycloheximide.

auxin regulated transcripts even when added at >10-fold higher concentrations (Theologis *et al.*, 1985).

We have previously shown that the 5′ upstream fragment (−410 to −50) of the pea *rbcS-3A* gene can act as an enhancer-like element to drive light-responsive transcription from a heterologous TATA box (Aoyagi *et al.*, 1988). Therefore, we examined the effects of CHX on the CAT transcript level in transgenic plants containing the *rbcS-3A*/35S TATA/CAT chimeric construct (Figure 7, construct I). Figure 7 confirms previous findings that the CAT mRNA level is low in dark seedlings but can be elevated ~10-fold upon exposure to light. Adding CHX abolishes the light-induction but has little effect on the basal expression level. In contrast, when the CaMV 35S promoter (−343 to +8) is used instead, CHX treatment leads to an increase of the steady state level of the CAT mRNA (Figure 7, construct II). This CHX-induced increase is independent of light (data not shown). From these data we conclude that CHX treatment specifically inhibits light activation of the *rbcS* enhancer while increasing the level of transcripts expressed by the 35S promoter.

Discussion

The signal transduction pathway between phytochrome activation and gene expression is a subject of considerable interest. Calcium flux has been implicated in a study

involving phytochrome regulation of *Onoclea* spore germination (Wayne and Hepler, 1984), However, the relevance of ionic currents to control of gene expression has not been defined. Phytochrome-responsive genes are known to have drastically different fluence requirements for their expression (Kaufman *et al.*, 1984). Thus, it is probable that after the initial step of signal conversion between phytochrome and its immediate transducer, a branch point occurs which subsequently gives rise to a diverse set of secondary messengers. The results of this paper suggest that the phytochrome stimulated transcription of *Cab* and *rbcS* in higher plants requires a labile protein factor which is involved in the signal transduction pathway. Comparison of the escape time for cycloheximide inhibition with that for phytochrome photoreversion suggests that this labile protein factor acts after the initial signal transducing step leading to *Cab-1* transcription. From our work with transgenic seedlings containing chimeric gene constructs we conclude that CHX inhibits transcription of *Cab* and *rbcS* with little effect on their transcript stability. It is interesting that the regulation of *PCR* by phytochrome is not significantly affected by CHX (Figure 1). Moreover, Lissemore and Quail (1988) have recently reported that the down-regulation by phytochrome of the oat phytochrome gene is also not affected by inhibition of cytoplasmic protein synthesis. Therefore, at least one of the secondary messenger(s) for the down regulation of gene expression by phytochrome must be distinct from those involved in the activation of the *Cab* and *rbcS*. Taken together, these data suggest that phytochrome regulation of gene expression is likely to involve a diverse set of regulatory pathways at the molecular level.

In mammalian systems, the effects of cytoplasmic protein synthesis inhibitors on gene expression have been well documented. The stabilization of certain mRNAs such as the histone H3 and H4 mRNAs, has been reported (Sive *et al.*, 1984). Activation of nuclear gene transcription by protein synthesis inhibitor has also been shown by nuclear run-on assays (Sive *et al.*, 1984; Gonzalez and Nebert, 1985). This activation is promoter-specific as well as cell-type specific in some cases (Gonzalez and Nebert, 1985; Linial *et al.*, 1985; Altus *et al.*, 1987) and the possibility of labile negative factor has been raised. There are also several mammalian genes whose expression is blocked by cytoplasmic protein synthesis inhibitors. A detailed study by Linial *et al.* (1985) has shown that the activation of c-*myc* gene expression by the LTR sequences of the avian leukosis virus is dependent on cytoplasmic protein synthesis. Interestingly, this inhibition of transcription is cell type dependent and is associated with a concomitant disappearance of a DNase I hypersensitive site in the LTR. More recently, NFAT-1, a factor which binds to the T cell activation-specific enhancer of interleukin-2, has been shown to be induced *de novo* by activation of T cells with phytohaemaglutinin (PHA) and phorbol myristate acetate (PMA) (Shaw *et al.*, 1988). The appearance of NFAT-1, as measured by DNase I footprinting, is inhibited by cytoplasmic protein synthesis inhibitors. Constitutive c-*myc* and induced c-*fos* gene expressions in these same cells are not affected by protein synthesis inhibition whereas interleukin-2 gene activation is completely abolished. This study provides direct evidence for the model where *de novo* synthesis of *trans*-acting factor is the final step of the signal transduction process leading to gene activation.

Our present work raises the possibility that a positive transcription factor for the activation of *Cab* and *rbcS* is synthesized *de novo* subsequent to the activation of phytochrome. Such a factor is predicted to be distinct from GT-1, a protein factor which binds the light-responsive elements of the *rbcS-3A* promoter and is present in roughly the same amounts in light-grown and dark-adapted pea plants (Green *et al.*, 1987). At this moment, however, it is equally probable that the labile nature of one or some of the intermediate messengers is the cause of the sensitivity of the light activation process to protein synthesis inhibitors. Continued synthesis of these protein factors may be needed to maintain the signal transduction pathway leading from phytochrome photoreception to the activation of *Cab* and *rbcS*. Alternatively, the inhibition of cytoplasmic protein synthesis may have triggered a protein or DNA modification enzyme that specifically inactivates positive factors which ultimately lead to *Cab* and *rbcS* expression.

Our results provide evidence that transcription of the CaMV 35S promoter is stimulated by CHX treatment. Construct I and II in Figure 7 are identical except that the sequences from −410 to −50 of the *rbcS-3A* in construct I was replaced by the enhancer region (−343 to −46) of the 35S promoter in construct II. As shown in Figure 7, treatment with CHX does not affect the dark level of the CAT transcript expressed by the *rbcS-3A* 5′ upstream region, but the light activation is inhibited. In contrast, the level of CAT mRNA clearly increased significantly after CHX treatment when transcription is driven by the 35S enhancer. These data thus suggest that the increase in CAT mRNA upon CHX addition is due to the activation of transcription driven by the 35S enhancer. One simple model assumes the presence of labile negative factors which bind to the 35S enhancer. Alternatively, the inhibition of protein synthesis may activate a positive transcription factor for the 35S enhancer. Such transcription factors can appear as a result of modifying pre-existing factors and thus does not require *de novo* protein synthesis.

Materials and methods

Plant materials and treatment with protein synthesis inhibitors

Wheat and pea were grown on vermiculite for 7−8 days in a greenhouse under 16 h light (from 6 am to 10 pm): 8 h dark (from 10 pm to 6 am) cycles. Etiolated wheat plants were grown in dark boxes in a dark room at a constant temperature of ~26°C. Green pea plants were dark adapted by shifting the plants into dark boxes from a greenhouse. For treatment with inhibitors, wheat or pea plants were cut with a razor blade under water and immediately immersed in 40 ml of 20 mM HEPES−KOH buffer, pH 7.0, in a 150 ml beaker. Protein synthesis inhibitors were dissolved in DMSO and an identical amount of this solvent was added to the control buffer samples. The final concentration of DMSO was 0.1% (v/v). Transgenic tobacco seeds were surface-sterilized and germinated on 1% agar plates (100 × 20 mm) with Murashige and Skoog (MS) medium. The plates were incubated at 26°C under 16 h light:8 h dark cycles for 10−12 days. To obtain etiolated tobacco seedlings, the plates were covered with aluminum foil and incubated in dark boxes as for the etiolated wheat. For inhibitor studies, 10 ml of 20 mM HEPES−KOH buffer with either 0.1% DMSO alone or with cycloheximide were added to the Petri dishes.

RNA isolation and anlaysis for transcript levels

For S1 nuclease protection and Northern blot analyses RNA was isolated from frozen tissues as described previously (Kuhlemeier *et al.*, 1988). The probes and conditions for S1 nuclease protection assay were as reported (Fluhr *et al.*, 1986b; Nagy *et al.*, 1987, 1988; Keith and Chua, 1986). For Northern blot analysis, the RNA samples were denatured in a formaldehyde−formamide buffer and then separated in 1% agarose gel with

formaldehyde. After blotting to nitrocellulose, specific transcripts were visualized by hybridization to nick-translated DNA probes and subsequent autoradiography. For *in vitro* translation studies, RNA was isolated using guanidinium thiocyanate as a denaturant (Nagy *et al.*, 1988). Usually, 5 μg of total RNA was incubated in a reticulocyte lysate translation mix (Amersham) containing [^{35}S]methionine at 30°C. After 1 h, two volumes of saturated ammonium sulfate were added to precipitate the translation products. The pellet was resuspended in 10 mM Tris–HCl, 1 mM EDTA (pH 7.5) and then mixed with two volumes of solubilization buffer containing 2 M urea, 1% SDS and 20 mM Tris–HCl (pH 7.5). The mixture was boiled and then separated on a 10–15% Laemmli gel. After electrophoresis, the gel was fixed in methanol:acetic acid and treated with Enlightening (New England Nuclear) before fluorography.

Determination of protein synthesis inhibition

The method for determining effects of the various inhibitors on protein synthesis was similar to that reported by Theologis *et al.* (1985). After incubation for 1–2 h with the inhibitors or in control buffer, the plants were transferred to a 1.5 ml Eppendorf tube with 0.5 ml of the same buffer containing ~10 μCi of [^{35}S]methionine (Amersham). The plants were allowed to incorporate [^{35}S]methionine for ~2 h, rinsed with 20 mM HEPES–KOH (pH 7.0), and then frozen in liquid nitrogen and stored at −70°C. To determine the incorporation of [^{35}S]methionine into proteins, the plants were homogenized in 0.5 ml of 50 mM HEPES (pH 7.5) and the homogenate spun at 8000 r.p.m. for 15 min. Aliquots of 2 μl of the supernatant from each sample were used to determine total [^{35}S]methionine uptake. The percent of TCA precipitable counts of the total [^{35}S]methionine taken up by the plant material was determined by scintillation counting. The value for the control sample was set at 100%.

Acknowledgements

We wish to thank Brian Keith for the wheat *rbcS* S1 probe, Steve Kay for the oat PCR fragment, Ferenc Nagy for the wheat *Cab* probe and transgenic tobacco seeds, Cris Kuhlemeier for the pea *rbcS* S1 probe and transgenic tobacco seeds, and Masaki Iwabuchi for the wheat histone H3 probe. We also thank Drs C.Kuhlemeier, P.Benfey and P.Gilmartin for critical reading of the manuscript, Wendy Roine for typing, and Hugh Williams for photographic work. This work was supported by a grant from Monsanto. E.L. and P.G. were supported by NIH post-doctoral fellowships GM10805-01 and GM11797, respectively.

References

Aoyagi,K., Kuhlemeier,C. and Chua,N.H. (1988) *Mol. Gen. Genet.*, **213**, 179–185.

Altus,M.S., Pearson,D., Horiuchi,A. and Nagamine,Y. (1987) *Biochem. J.*, **242**, 387–392.

Batschauer,A. and Apel,K. (1984) *Eur. J. Biochem.*, **143**, 593–597.

Ernst,D. and Oesterhelt,D. (1984) *EMBO J.*, **3**, 3075–3078.

Fluhr,R., Kuhlemeier,C., Nagy,F. and Chua,N.-H. (1986a) *Science*, **232**, 1106–1112.

Fluhr,R., Moses,P., Morelli,G., Coruzzi,G. and Chua,N.-H. (1986b) *EMBO J.*, **5**, 2063–2071.

Giuliano,G., Pichersky,E., Malik,V.S., Timko,M.P., Scolnick,P.A. and Cashmore,A.R. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 7089–7093.

Gonzalez,F.J. and Nebert,D.W. (1985) *Nucleic Acids Res.*, **13**, 7269–7288.

Green,P.J., Kay,S.A. and Chua,N.-H. (1987) *EMBO J.*, **6**, 2543–2549.

Green,P.J., Yong,M.-H., Cuozzo,M., Kano-Murakami,Y., Silverstein and Chua,N.H. (1988) *EMBO J.*, **7**, 4035–4044.

Kaufman,L.S., Thompson,W.F. and Briggs,W.R. (1984) *Science*, **226**, 1447–1449.

Keith,B. and Chua,N.-H. (1986) *EMBO J.*, **5**, 2419–2425.

Kuhlemeier,C., Green,P.J. and Chua,N.-H. (1987) *Annu. Rev. Plant Physiol.*, **38**, 221–257.

Kuhlemeier,C., Fluhr,R. and Chua,N.-H. (1988) *Mol. Gen. Genet.*, **212**, 405–411.

Kuhlemeier,C., Strittmatter,G., Ward,K. and Chua,N.H. (1989) The Plant Cell, **1**, 471–478.

Linial,M., Gunderson,N. and Groudine,M. (1985) *Science*, **230**, 1126–1132.

Lissemore,J.L. and Quail,P.H. (1988) *Mol. Cell Biol.*, **8**, 4840–4850.

Nagy,F., Boutry,M., Hsu,M.-Y., Wong,M. and Chua,N.-H. (1987) *EMBO J.*, **6**, 2537–2542.

Nagy,F., Kay,S.A. and Chua,N.-H. (1988) *Genes Dev.*, **2**, 376–382.

Shaw,J.-P., Utz,P.J., Durand,D.B., Toole,J.J., Emmel,E.A. and Crabtree,G.R. (1988) *Science*, **241**, 202–205.

Simpson,J., Van Montagu,M. and Herrera-Estrella,L. (1986) *Science*, **233**, 34–38.

Sive,H.L., Heintz,N. and Roeder,R.G. (1984) *Mol. Cell Biol.*, **4**, 2723–2734.

Tabata,T., Fukasawa,M. and Iwabuchi,M. (1984) *Mol. Gen. Genet.*, **196**, 397–400.

Theologis,A., Huynh,T.V. and Davis,R.W. (1985) *J. Mol. Biol.*, **183**, 53–68.

Timko,M.P., Kausch,A.P., Castresana,C., Fassler,J., Herrera-Estrella,L., Van den Broeck,G., Van Montagu,M., Schell,J. and Cashmore,A.R. (1985) *Nature*, **318**, 579–582.

Tobin,E.M. and Silverthorne,J. (1985) *Annu. Rev. Plant Physiol.*, **36**, 569–593.

Wayne,R. and Hepler,P.K. (1984) *Planta*, **160**, 12–20.

Received on April, 14, 1989; revised on June 20, 1989