Journal of Cellular Biochemistry 25:1–13 (1984)
Biosynthesis of the Photosynthetic Apparatus:
Molecular Biology, Development and Regulation 211–223

Differential Regulation of the Accumulation of the Light-Harvesting Chlorophyll a/b Complex and Ribulose Bisphosphate Carboxylase/Oxygenase in Greening Pea Leaves

John Bennett, Gareth I. Jenkins, and Martin R. Hartley

Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, United Kingdom

The photoregulation of chloroplast development in pea leaves has been studied by reference to three polypeptides and their mRNAs. The polypeptides were the large subunit (LSU) and the small subunit (SSU) of ribulose 1,5-bisphosphate carboxylase/oxygenase (RUBISCO), and the light-harvesting chlorophyll a/b protein (LHCP). The polypeptides were assayed by a sensitive radioimmune assay, and the mRNAs were assayed by hybridization to cloned DNA probes. LSU, LSU mRNA, and LHCP mRNA were detectable in etiolated seedlings but LHCP, SSU, and SSU mRNA were at or below the limit of detection. During the first 48 hr of de-etiolation under continuous white light, the mRNAs for LSU, SSU, and LHCP increased in concentration per apical bud by about 40-fold, at least 200-fold, and about 25-fold, respectively, while the total RNA content per apical bud increased only 3.5-fold. In the same period, the LSU, SSU, and LHCP contents per bud increased at least 60-, 100-, and 200-fold, respectively. The LHCP increased steadily in concentration during de-etiolation, whereas the accumulation LSU, SSU, and SSU mRNA showed a 24-hr lag. The accumulation of SSU, SSU mRNA, and LHCP mRNA showed classical red/far-red reversibility, indicating the involvement of phytochrome in the regulatory mechanism. LSU and LSU mRNA were induced equally well by red and far-red light. The LHCP failed to accumulate except under continuous illumination. These results indicate that the accumulation of SSU is controlled largely through the steady-state level of its mRNA, which is in turn almost totally dependent on light as an inducer and on phytochrome as one of the photoreceptors. The accumulation of LSU is largely but not totally determined by the level of its mRNA, which appears to be under strong photoregulation, which has yet to be shown to involve phytochrome. Phytochrome is involved in the regulation of LHCP mRNA levels but substantial levels of the mRNA also occur in the dark. LHCP accumulation is not primarily governed by the levels of LHCP mRNA but by posttranslational stabilization in which chlorophyll synthesis plays a necessary but not sufficient role.

Key words: mRNA levels, regulation of biosynthesis, light-harvesting chlorophyll a/b complex, chloroplast, ribulose bisphosphate carboxylase, oxygenase

Abbreviations used: cDNA, complementary DNA; LHCP, light-harvesting chlorophyll a/b protein; LSU and SSU, large and small subunits of ribulose bisphosphate carboxylase/oxygenase (RUBISCO); P_r and P_{fr}, red and far-red absorbing forms of phytochrome; SSC, 0.15 M NaCl-0.015 M Na_3 citrate.

John Bennett is now at Biology Department, Building 463, Brookhaven National Laboratory, Upton, NY 11973.

Received August 22, 1983; revised and accepted December 20, 1983.

During leaf formation in higher plants, chloroplasts develop from small, undifferentiated organelles known as proplastids or eoplasts [1] by a process involving the synthesis of nucleic acids, proteins, and lipids [2] and characterized at the ultrastructural level by the elaboration of a complex system of internal photosynthetic membranes [1,2]. In most flowering plants, but not in all [3], this process is light-dependent [4] and much current research is devoted to identifying the biosynthetic events which are under photocontrol. A commonly adopted approach is to study the changes in plastid composition and structure which occur when dark-grown seedlings are transferred to various light regimes. The plastids which form in dark-grown plants are known as etioplasts, and the transformation of etioplasts into apparently normal chloroplasts may be followed by transfer of plants into continuous white light or into day/night cycles. Alternatively, when the roles of light in this transformation are to be dissected, other less physiologically normal light regimes may be employed, such as intermittent illumination (eg, 2 min of white light every 100-120 min) or continuous illumination with light of various colours, or one or a few pulses of light followed by long intervals of darkness. The result of the use of these various light regimes has been to establish the existence of at least three distinct photoreceptors which affect different aspects of chloroplast development to different extents. The three photoreceptors are (1) phytochrome, which is a chromoprotein existing in two forms (P_r and P_{fr}) that are interconvertible and absorb principally red and far-red light, respectively [5], (2) protochlorophyllide, which is a chlorophyll precursor and absorbs principally in the blue and orange regions of the spectrum [6], and (3) a chemically undefined blue-light photoreceptor [7].

An especially intriguing aspect of chloroplast development is that at the level of macromolecular synthesis three subcellular compartments are involved: the nucleus, the cytoplasm and the plastids themselves [8]. This may be illustrated by reference to the two most abundant proteins of the chloroplast: (1) the soluble, CO_2 fixing-enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (RUBISCO) whose large subunit (LSU) is encoded in chloroplast DNA and synthesized on chloroplast ribosomes, and whose small subunit (SSU) is encoded in nuclear DNA, synthesized on cytoplasmic ribosomes in precursor form and transported into chloroplasts [8], and (2) the membrane-bound light-harvesting chlorophyll a/b complex, whose pigment components are synthesized in the chloroplast and whose apoproteins (LHCP) resemble the SSU in sites of coding and synthesis [8,9]. Phytochrome is known to regulate the total activity of RUBISCO in various species including pea (Pisum sativum) [10] and white mustard (Sinapis alba) [11]. More detailed studies reveal that in duckweed (Lemna gibba) the level of SSU mRNA in the cytoplasm is controlled by phytochrome [12, 13]. Phytochrome was implicated by the classical red/far-red photoreversibility test [5]. Because P_r (the form of phytochrome which accumulates in dark-grown plants) is converted into P_{fr} by a single brief exposure to red light and P_{fr} is converted back to P_r by far-red light, any physiological response dependent on the production of P_{fr} will, in principle, be elicited by a pulse of red light, but not by a pulse of far-red light. Furthermore far-red light, if administered sufficiently quickly after red light, will prevent the expression of the response to red light. These effects are indeed observed in L gibba for SSU mRNA levels [12]. However, in S alba the level of LSU mRNA in the chloroplast does not satisfy the red/far-red photoreversibility test; far-red light is at least as potent as red light in inducing this mRNA [14]. It may be that this response is saturated by the small amount of P_{fr} formed by far-red light alone. The levels of LHCP mRNA have, however, been shown to satisfy the reversibility

test in barley (Hordeum vulgare) [15,16] and L gibba [12,13]. In contrast, chlorophyll synthesis in these and most other flowering plants (Angiosperms) is only in part controlled by phytochrome. Phytochrome appears to regulate the total capacity of the chlorophyll biosynthetic pathway [11], whereas the extent to which the total capacity is utilized is determined by excitation of protochlorophyllide [17]. The activity of the plastid enzyme protochlorophyllide reductase (PCR) is directly light-dependent. The ternary complex of NADPH-enzyme-protochlorophyllide is converted to NADP-enzyme-chlorophyllide only after the absorption of a photon by the bound protochlorophyllide molecule [17]. As a result, continuous synthesis of chlorophyllide and of chlorophyll requires essentially continuous illumination. Thus, for both RUBISCO and the light-harvesting chlorophyll a/b complex there is reason to believe that the different components are synthesized under different photoregulation.

We decided to investigate the problem of the coordination of synthetic events during chloroplast development by comparing the photoregulation of LSU, SSU, LHCP, and their mRNAs in a single species of plant (P sativum) exposed to standard light regimes. The three polypeptides have been assayed by a sensitive and specific radioimmune assay and the mRNAs quantified by DNA-RNA hybridization using cloned DNA probes. We have asked four questions: (1) Does phytochrome regulate the steady-state level of the three mRNAs? (2) Can the level of each protein be explained in terms of the level of its mRNA? (3) Is the level of LSU coordinated with that of SSU? (4) Is the level of LHCP coordinated with those of chlorophyll a and chlorophyll b? The results provide for the first time a comparison between two major chloroplast proteins with respect to the levels at which light regulates their accumulation through events inside and outside the organelle. This study is also unusual in its emphasis on quantitation of both protein levels and mRNA levels during chloroplast development.

MATERIALS AND METHODS

Growth of Plants

Seeds of Pisum sativum L cv Feltham First were sown in moist potting fibre and grown in darkness in a controlled environment cabinet at 20°C. After 8 d some plants were transferred to continuous white light for up to 48 hr; illumination was provided at 20°C by warm white fluorescent tubes (photon fluence rate of photosynthetically active radiation = 100 μmol $m^{-2}sec^{-1}$). Other plants were exposed to either red light (662-nm interference filter, 15 min at a photon fluence rate of 15 μmol $m^{-2}sec^{-1}$), or far-red light (735-nm interference filter, 15 min at a photon fluence rate of 8 μmol $m^{-2}sec^{-1}$), or red light followed by far-red light, and then returned to darkness for 48 hr. After the indicated times, samples were harvested and placed directly into liquid N_2 and stored at −80°C until required. The tissue harvested from dark-grown plants or briefly illuminated plants consisted of the apical bud which was excised from the epicotyl at the plumular hook. Light-grown plants were cut across the epicotyl just below the node bearing the first-formed leaf.

Extraction and Quantitation of Nucleic Acids

For the preparation of RNA for northern hybridization, and dot hybridization, total nucleic acid was extracted from frozen tissue by the phenol/detergent method of Parish and Kirby [18] with the modifications described in [19], except that the organic

phase for the second extraction consisted of phenol: chloroform (1:1, v/v). Following ethanol precipitation the pellet was washed twice in 70% ethanol, 50 mM NaCl, and dissolved in sterile 50 mM Mes-NaOH, pH 7.0, 2 mM magnesium acetate at 2 mg nucleic acid/ml and incubated on ice for 30 min with 15 μg/ml RNAase-free DNAase I (Worthington). The mixture was extracted with phenol and then with phenol:chloroform. The aqueous phase was removed and one-tenth volume of 2 M sodium acetate was added, followed by ethanol precipitation. The washed pellet was dried in vacuo, dissolved in sterile water at about 3 mg RNA/ml, and stored at −90°C.

The nucleic acid content of buds was determined on replicate samples. Ten frozen pea buds were powdered in a mortar and pestle at liquid N_2 temperature and total nucleic acid was quantitatively extracted from the powder as described [19]. The final washed nucleic acid pellet was dissolved in electrophoresis buffer (E buffer, [20]) and the total nucleic acid content was estimated from the A_{260}, assuming that 1 mg of nucleic acid/ml has a $A^{1\ cm}_{260}$ of 20. A 30-μg sample was fractionated by electrophoresis in a 2.4% polyacrylamide gel [20], and the gel was scanned at 260 nm with a Gilford linear transporter. The relative amounts of DNA and RNA (ie, rRNA and tRNA) were determined by measurement of the areas of the respective peaks. These areas were expressed as a percentage of the total area and related to the mass of nucleic acid applied to the gel.

Agarose-formamide gel electrophoresis of RNA and transfer to nitrocellulose sheets. Samples (20 μg) of total RNA in 40 μl of 60% formamide, 0.1 × E buffer were incubated at 60°C for 5 min. The samples were cooled on ice, 5 μl of 50% glycerol containing bromophenol blue was added, and the nucleic acids were fractionated by electrophoresis in horizontal 1.5% agarose gels (220 × 150 × 3 mm) containing 50% formamide, 0.1 × E buffer, as described [in 21]. Electrophoresis was carried out at 8 V/cm for 5 hr. The gel was stained in 300 ml of 2 μg/ml ethidium bromide for 30 min, destained in water for 30 min, and photographed under uv light. The gel was then soaked in 300 ml of 10% (v/v) formaldehyde for 60 min, rinsed with water, and soaked in 300 ml of 20 × SSC for 30 min. RNA was transferred to nitrocellulose filters (BA85, Schleicher and Schull) with 10 × SSC, essentially as described by Southern [22].

Dot Hybridization

RNA samples were applied directly to nitrocellulose sheets as discrete spots using a manifold (Bethesda Research Laboratories Inc, Gaithersburg, Maryland, USA) which allowed a slight vacuum to be applied to the underside of the sheets. Before use, the sheets were placed in sterile distilled water for 10 min and then in 20 × SSC for 30 min. Routinely, 0.8 μg RNA was applied per spot in 10–100 μl 15 × SSC and each sample well was flushed with 80 μl of 15 × SSC to ensure that all the RNA entered the nitrocellulose. The sheets were dried and baked and incubated with ^{32}P-labelled hybridization probes as described below. Following hybridization and washing the sheets were cut up and the radioactivity hybridized to each spot was determined by scintillation counting. The cpm bound to control spots of 15 × SSC were subtracted from each sample. The radioactivity bound to spots of Xenopus laevis total RNA was not significantly different from that in the above control. For each hybridization probe employed, the relationship between log cpm hybridized and log μg RNA applied was essentially linear over the range 0.01–5 μg total RNA for a sample extracted from pea apical buds which had been de-etiolated for 48 hr.

Preparation and Nick Translation of Cloned DNA Probes

The clones from which hybridization probes specific for the mRNAs of LSU, SSU, and LHCP were prepared were the following: for LSU mRNA, pZmC37 [23]; for SSU mRNA, pSSU 160 [24]; and for LHCP mRNA, pFab31 (S.M. Smith, personal communication). The pFab31 clone was constructed by ligation of Bam H1 linkers to double-stranded cDNA made from Pisum sativum leaf poly(A)$^+$ RNA and inserted into the Bam H1 site of pBR322. The clone was shown to encode the DNA sequence for the larger of the two major LHCP precursors by hybrid-release translation. Supercoiled plasmid DNAs were prepared as described by Clewell [25]. The cDNA insert in pSSU 160 was excised by digestion with Hind III, as described [26]; that in pFab37 was excised by digestion with Bam H1, using 1 unit of enzyme/μg DNA in 20 mM TRIS-HCl, pH 7.0, 100 mM NaCl, 7 mM $MgCl_2$, 2 mM 2-mercaptoethanol for 2 hr at 37°C. The digestion products were brought to 12.5 mM EDTA, heated at 60°C for 5 min, and then fractionated at 5°C by centrifugation in 12 ml 5–25% sucrose density gradients containing 50 mM sodium acetate-acetic acid, pH 6.0, 1 mM EDTA, 2 μg/ml ethidium bromide for 15 hr at 180,000g in a 6 × 14 ml swing out rotor (MSE Ltd). DNA bands were detected in uv light and the upper band (insert) was removed by puncturing the side of the tube with a needle. The DNA was ethanol precipitated, and the pellet was washed twice in 70% ethanol, 50 mM NaCl, dried in vacuo, and dissolved in sterile water. Plasmid pZmC37 (20 μg) was digested with 40 units of Pst 1 in 50 mM TRIS-HCl, pH 8.0, 10 mM $MgCl_2$, 50 mM NaCl for 3 hr at 34°C. Following digestion the DNA was precipitated with spermine [27] and fractionated in a 1.4% low-melting agarose gel (Sea Plaque, Miles). A segment of the gel containing a 580-bp fragment derived from within the LSU structural gene [23] was excised, and the DNA recovered as described in Langridge et al [28].

The DNA probes were radiolabelled to a specific activity of about 6×10^7 cpm/μg by nick translation [29] using either α-^{32}P dCTP or α-^{32}P dGTP (Amersham International, 3000 Ci/mmol). DNA was recovered by spermine precipitation [27].

Nucleic Acid Hybridization

Nitrocellulose filters bearing immobilized RNA blotted from gels, or applied as dots, were prehybridized in sealed polythene bags overnight at 42°C in hybridization buffer containing 50% (v/v) formamide, 3 × SSC, 50 mM HEPES-NaOH, pH 7.0, 0.5 mg/ml yeast RNA, 10 μg/ml sheared salmon sperm DNA, 10 μg/ml poly(U) supplemented with Denhart reagents [30]. Hybridization with cDNA probes ($3–8 \times 10^6$ cpm per filter) was performed in hybridization buffer containing additionally 10% (w/v) dextran sulphate [31] for 48 hr at 42°C. Following hybridization, filters were washed twice in 1 × SSC, 0.5% SDS at room temperature for 15 min, once in 0.1× SSC at room temperature for 15 min, twice in 0.1 × SSC at 60°C for 30 min (55°C for LSU probe), and once in 0.1 × SSC at room temperature. After washing, filters were air-dried and autoradiographed for 1–7 d at −80°C using Kodak X-Omat RP film and a Dupont Cronex "Lightening Plus" intensifying screen.

Radioimmune Assay for LSU, SSU, and LHCP

Up to ten apical buds were frozen in liquid nitrogen and stored at −80°C until required. The buds were powdered in a mortar and pestle under liquid N_2 and allowed

to thaw in the presence of 10 ml of buffer (100 mM Tricine-NaOH, pH 8.0, 10 mM dithiothreitol, 1% SDS). The powder was homogenized in the buffer and frozen again with liquid N_2. On thawing the extract was heated to 70°C for 4 min, and centrifuged at 3,000 g for 5 min. The supernatant was retained for the determination of LSU, SSU, and LHCP contents. Samples were made 10% in glycerol and 0.01% in bromophenol blue, 10–50 μl aliquots were subjected to SDS-polyacrylamide gel electrophoresis through 10% polyacrylamide slab gels, and proteins were transferred electrophoretically to nitrocellulose sheets as described in [32]. LSU, SSU, and LHCP were detected by a radioimmune assay using antibodies raised against pea RUBISCO [33], Lemna gibba SSU [12] and pea LHCP [34]. The antibody-antigen complexes were detected with ^{125}I-labelled protein A, as described [32]. Autoradiograms were scanned to provide a quantitative estimate of the amount of each antigen in each sample.

RESULTS

When 8-d-old, etiolated spa pea seedlings are exposed to continuous white light for 48 hr, chlorophyll synthesis is initially slow but gradually accelerates (Table I). The lag phase in chlorophyll synthesis is longer in pea than in many other plants [2, 4,11], because etiolation arrests leaf development at an especially early stage in peas. Consequently, de-etiolation in white light is accompanied not only by the tranformation of etioplasts into chloroplasts but also by major leaf expansion [10]. Total nucleic acid content increases markedly in the period 24–48 hr after the onset of illumination (Table I).

The absence of chlorophyll from etiolated peas is due to the light dependence of protochlorophyllide reductase [17] and raises questions about the levels of chlorophyll-binding proteins (such as LHCP) in the absence of chlorophyll. Can the LHCP accumulate in etiolated peas? And if it cannot accumulate, at what point(s) in the pathway of LHCP biosynthesis is light required? In an earlier study of the LHCP content of pea leaves [34], the LHCP was separated from other proteins by SDS-polyacrylamide gel electrophoresis and the LHCP content was estimated by quantitative microdensitometry of stained gels. While that method is adequate when LHCP is abundant, it is insufficiently sensitive and discriminating when LHCP is in low concentration. We have therefore turned to a radioimmuneassay in which total leaf proteins are extracted in SDS, fractionated by gel electrophoresis, transferred to nitrocellulose sheets, and then challenged with specific antibodies and ^{125}I-protein A. Autoradiography reveals the location and amount of antigen-antibody-protein A complex. Figure 1 A,B shows the method as applied to the detection of LHCP in etiolated and de-etiolated plants; each track contained total protein from one thirtieth of a pea bud. LHCP was very readily detectable in plants exposed to light for 48 hr but was below the limit of detection in etiolated plants. Since the extract of de-etiolated plants can be diluted about 200-fold before the signal ceases to be detectable above the background on the x-ray film, it may be concluded that the LHCP content of pea leaves increases at least 200-fold during the first 48 hr of de-etiolation. Since the assay in Figure 1A,B refers to total leaf protein, it eliminates the possibility that LHCP accumulates in some form other than the mature (24–26 kDa), thylakoid-bound polypeptide (eg, a soluble 30–32-kDa precursor).

The absence of LHCP from etiolated plants is not due to a lack of translatable LHCP mRNA. An earlier report from this laboratory [35] showed that LHCP mRNA

TABLE I. Amounts of Chlorophylls a and b and Total RNA in Apical Buds of Pisum sativum During De-Etiolation*

Time of de-etiolation (hr)	Chlorophyll a (μg/bud)	Chlorophyll b (μg/bud)	RNA (μg/bud)
0	0	0	266
3	2.5	0	ND[a]
6	5.1	1.4	ND
12	12	3.1	256
24	30	10	408
48	111	39	908

*Plants were grown in darkness for 8 d and then transferred into continuous white light for up to 48 hr. Amounts of chlorophylls a and b were calculated from absorbance measurements at 663 and 645 nm [44]. RNA was determined as described in Materials and Methods.
[a]ND, not determined.

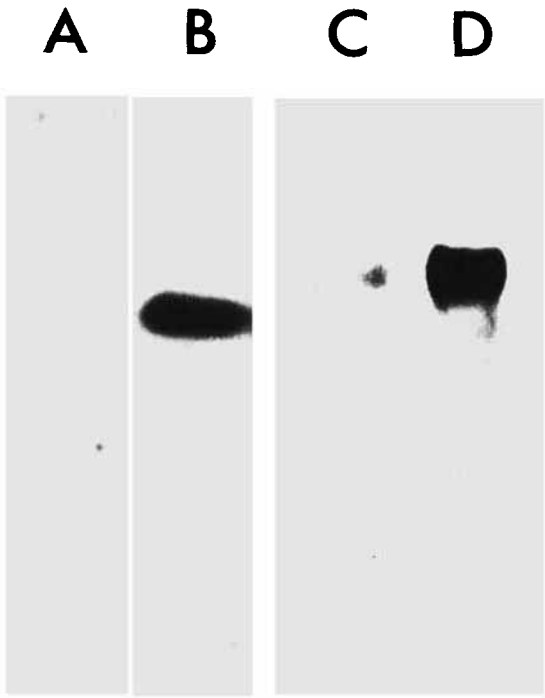

Fig. 1. Detection of LHCP polypeptide and LHCP mRNA in etiolated and de-etiolated seedlings of Pisum sativum. Apical buds were harvested either from seedlings grown in darkness for 8 d or from seedlings grown in darkness for 8 d and then transferred to continuous white light for 48 hr. The LHCP polypeptide was detected by radioimmunoassay using a specific antibody and ^{125}I-protein A following SDS gel electrophoresis of total protein extracts and transfer to nitrocellulose sheets (see Materials and Methods). Each gel track contained protein from one-thirtieth of an apical bud. For the detection of LHCP mRNA, total RNA extracts were fractionated by agarose gel electrophoresis, transferred to nitrocellulose, and then hybridized to a ^{32}P-labelled cloned cDNA probe (see Materials and Methods). Each gel track contained 20 μg total RNA. The autoradiograms show, A) LHCP polypeptide, dark-grown plants; B) LHCP polypeptide, illuminated plants; C) LHCP mRNA, dark-grown plants; D) LHCP mRNA, illuminated plants.

is readily detectable in the polyadenylated fraction extracted from etiolated peas. That assay involved in vitro translation of polyadenylated mRNA, followed by immunoprecipitation to detect the 30- and 32- kDa LHCP precursors. The availability of a cloned cDNA probe for the mRNA encoding the 32-kDa precursor has enabled us to improve the assay and to detect not only LHCP mRNA that is both polyadenylated and translatable but also LHCP mRNA that may be untranslatable or not polyadenylated. In this new assay, total leaf RNA is fractionated by agarose gel electrophoresis, transferred to nitrocellulose sheets by blotting, and then challenged with denatured, ^{32}P-labelled cloned cDNA. Figure 1 C,D shows the hybridization patterns obtained for RNA extracted from etiolated and de-etiolated pea leaves; each track was loaded with 20 μg of total RNA. A single band of hybridization is visible for each sample, with approximately ten times more hybrid in the case of the leaves that had been de-etiolated for 48 hr than in the case of etiolated plants. This result confirms the earlier observation [35] that etiolated peas contain LHCP mRNA and contrasts with the reported absence of LHCP mRNA from etiolated barley leaves [15,36].

The mRNA assay illustrated in Figure 1 C,D is not sufficiently convenient and accurate for the quantitative analysis of multiple samples. A better method, made feasible by the evident specificity of the cDNA as a hybridization probe, is simply to deposit total RNA directly on to nitrocellulose in the form of replicate dots and then to probe the dot blot with ^{32}P-labelled cDNA clone. The amount of hybridization can be measured by scintillation counting. This method is highly reproducible and is applicable over at least a 200-fold range of mRNA concentrations [37].

We have used the radioimmune assay to determine the levels of LHCP, LSU, and SSU during de-etiolation. We have also employed the dot blot assay to quantitate the levels of the corresponding mRNAs. Table II presents these results, expressed as a percentage of the protein or mRNA content of leaves that had been de-etiolated for 48 hr. The protein and mRNA levels have been calculated per bud. This presented no problem in the case of the protein measurements, because total protein coresponding to a known fraction of a pea bud was loaded onto each track, as in Figure 1 A,B. However, in the case of the mRNA assays, it was necessary to convert from ^{32}P counts hybridized per unit total RNA to ^{32}P counts hybridized per bud. To achieve this conversion it was necessary to determine the total RNA content per bud (Table I).

Table II shows that, although LHCP is below the limit of detection in etiolated peas, it becomes detectable by 6 hr of illumination (when chlorophyll b also becomes detectable). Thereafter, LHCP accumulates steadily. As mentioned above, the LHCP increases in concentration per bud at least 200-fold over 48 hr of de-etiolation. In contrast the mRNA for LHCP is readily detectable in dark-grown plants, and increases only about 25-fold per bud in 48 hr of de-etiolation.

LSU and its mRNA are both detectable in dark-grown plants, as studies on protein synthesis in isolated etioplasts would indicate [38]. However, whereas the mRNA level increases markedly in the first 12 hr of illumination, the LSU itself remains at a low level for about 24 hr and only then accumulates rapidly. It is at this 24–48-hr stage that the SSU and its mRNA also begin to increase rapidly in concentration. In dark-grown plants SSU and its mRNA are at or below the limit of detection, and both increase only slightly during the first 24 hr of de-etiolation. Thus these three mRNAs show different photoregulation.

The role of phytochrome in the light-dependent accumulation of LHCP, LSU, and SSU was investigated by exposing dark-grown plants to pulses of red light

TABLE II. Accumulation of LSU, SSU and LHCP Polypeptides and Their mRNAs During De-Etiolation of Pisum sativum Apical Buds*

	(% of 48 hr de-etiolated value)					
Time of de-etiolation (hr)	LSU	LSU mRNA	SSU	SSU mRNA	LHCP	LHCP mRNA
0	1.5	2.6	≤1.0[a]	0.5	≤0.5[a]	4.1
6	1.7	ND[b]	≤1.0[a]	ND	0.6	ND
12	3.8	7.1	1.1	1.8	3.0	3.7
24	8.2	20.6	4.6	5.6	15.0	9.9
48	100	100	100	100	100	100

*Plants were grown in darkness for 8 d and then transferred into continuous white light for up to 48 hr, as described in Materials and Methods. Amounts of the polypeptides and mRNAs were determined by radioimmune assay and dot hybridization, respectively. Values are expressed as a percentage of those obtained for 48 hr de-etiolated seedlings.
[a]At or below the limit of detection.
[b]ND, not determined.

TABLE III. Phytochrome Control of mRNA and Protein Levels for LSU, SSU, and LHCP in Pisum sativum Apical Buds*

	(% of 48-hr white light value)					
Light regime	LSU	LSU mRNA	SSU	SSU mRNA	LHCP	LHCP mRNA
48-hr white light	100	100	100	100	100	100
48-hr dark	1.2	1.6	≤1.0[a]	0.3	≤0.5[a]	2.9
15-min red, 48-hr dark	6.5	15.4	15.0	2.0	≤0.5	11.8
15-min far-red, 48-hr dark	6.0	16.2	3.1	0.8	≤0.5	6.2
15-min red, 15-min far-red, 48-hr dark	5.1	16.9	3.9	0.7	≤0.5	5.5

*Plants were grown in darkness for 8 d and then illuminated as shown (see Materials and Methods). Amounts of the polypeptides and mRNAs were determined as described in the legend of Table II.

(662 nm) and far-red light (735 nm) and determining the levels of these proteins and their mRNAs after a further 48 hr in darkness (Table III). The levels of SSU and its mRNA are clearly under phytochrome control since they satisfy the classical red/far-red photoreversibility test: the inductive effect of a 15-min pulse of red light can be largely abolished by immediate exposure to a 15-min pulse of far-red light. However, a single 15-min pulse of red light followed by 48 hr of darkness is nowhere near as inductive as continuous illumination, suggesting that the maximal response requires either repeated phytochrome activation or the activation of some other photoreceptor.

LHCP mRNA levels are also regulated by phytochrome, though a substantial level of the mRNA is present in dark-grown plants (Table III). On the other hand, the level of LHCP itself is not explicable simply in terms of phytochrome-mediated regulation of its mRNA content. Accumulation of LHCP occurs only in the case of the continuously illuminated plants.

The situation with respect to LSU and its mRNA is rather different. The results in Table III show that in peas, red light and far-red light are approximately equally inductive of both LSU and LSU mRNA. In other words, the classical red/far-red photoreversibility test is not satisfied. Although this result could be interpreted to indicate that phytochrome is not the photoreceptor for the light-dependent increase in LSU and its mRNA, it is possible that phytochrome is involved but is activated even by the far-red light used in this experiment (see Discussion).

DISCUSSION

We began by asking four questions. The first question was: Does phytochrome regulate the steady-state level of the mRNAs for LSU, SSU, and LHCP? The data in Table III indicate that phytochrome certainly mediates the steady-state level of SSU and LHCP mRNAs in P sativum in that the red/far-red photoreversibility test is at least substantially satisfied in both cases. However, the fact that the level of SSU mRNA achieved following a single pulse of red light is less than 10% of that obtained under continuous illumination suggests that additional controls may be involved. In this respect we need to study in detail the time course of SSU mRNA accumulation following a single pulse of red light to determine whether the mRNA is subject to breakdown in the 48-hr period between illumination and harvest.

Another possibility is that phytochrome is the sole photoreceptor governing SSU mRNA levels, but it must be activated several times during the 48-hr period to achieve maximal SSU mRNA accumulation. This is also under investigation. Thirdly, another photoreceptor may be involved, or photosynthesis may contribute indirectly to SSU mRNA accumulation. The presence of LHCP mRNA in dark-grown peas could be interpreted to show that phytochrome activation is not essential for a certain level of mRNA accumulation. However, since P sativum is known to contain multiple genes for LHCP ([39], J.R. Bedbrook and S.M. Smith, personal communication) it is possible that the LHCP genes expressed in dark-grown peas are distinct from those genes that are regulated by phytochrome. It should be possible in the near future to distinguish between a "leaky control" on several LHCP genes and a tight phytochrome-dependent control on specific LHCP genes.

The involvement of phytochrome in the expression of SSU and LHCP genes is in accord with published results for other plant species [12,15]. Furthermore, our inability to demonstrate red/far-red photoreversibility in the case of LSU mRNA is also in accord with the literature [14]. It is unlikely that this difference in photoregulation between LSU mRNA on the one hand, and SSU and LHCP mRNAs on the other, is due simply to the location of LSU genes in the chloroplast and SSU and LHCP genes in the nucleus, because in S alba the level of another chloroplast mRNA (that coding for the 32-kD thylakoid herbicide binding protein) shows red/far-red photoreversibility [14]. Nor does failure to demonstrate photoreversibility disprove the involvement of phytochrome in controlling LSU mRNA levels: The low levels of P_{fr} produced by 735-nm light may be adequate to elicit a change in LSU mRNA production or stability. Further research on this point is required.

The second question posed in the Introduction was: Can the levels of LSU, SSU, and LHCP be explained in terms of the level of their mRNAs? In the case of SSU, the data on de-etiolation and on red/far-red reversibility indicate that there is a very tight correlation between the level of the protein and its mRNA suggesting that

the major and perhaps only control acting on SSU accumulation is a control on mRNA levels which may in turn be due to the effects of phytochrome on transcription. In the case of LSU, there is a good but not perfect correlation between protein level and mRNA level. Both mRNA and protein are induced equally by red and far-red light pulses, and both accumulate rapidly during the latter stages of de-etiolation (24–48 hr). However, during the early stages of de-etiolation (0–12 hr), there is a suggestion that there is more LSU mRNA than might be expected from the level of LSU itself. This could indicate that a translational or post-translational control is operative. Three possibilities suggest themselves: (1) a general translation control due to the low level of ribosomes found in etioplasts and in young etiochloroplasts [2,40]; (2) a specific translation control mediated by, for example, the SSU; or (3) a specific posttranslational control involving LSU breakdown in the absence of SSU. These last two possibilities will be considered again below.

In the case of LHCP, there is a poor correlation between protein and mRNA levels. As discussed in some detail elsewhere [9,37] the level of LHCP is determined not only by the level of LHCP mRNA but also by a posttranslational mechanism for which chlorophyll a and chlorophyll b synthesis is necessary but not sufficient. This leads us to our last two questions: Is the level of LSU coordinated with the level of SSU, and is the level of LHCP coordinated with the levels of chlorophylls a and b? Although tight coordination has been reported for LSU and SSU during the cell cycle Chlamydomonas reinhardtii [41] the coordination appears to be less stringent in soybean (Glycine max), where LSU synthesis can continue for several hours in the absence of SSU synthesis [42]. The fact that far-red light induces LSU to a much higher extent than SSU (Table III) might be taken as evidence that the coordination is also loose in pea. Further work is required to distinguish between the three mechanisms of coordination listed above. As far as LHCP is concerned, it is known that at least some of the members of the LHCP family of barley require chlorophyll b synthesis for stability [43]. However, even LHCP associated with chlorophyll a and chlorophyll b is unstable in darkness at an early stage of chloroplast development [9]. A photon fluence rate of at least 5 μmol $m^{-2}sec^{-1}$ of white light is required to stabilize LHCP in pea [37]. It has been suggested that LHCP is stabilized only when it has been incorporated into grana [9,34].

Thompson et al [45] have studied phytochrome control of RNA levels in developing pea and mung bean leaves. Their data for pea are in many respects comparable to ours. For example, they find that while the mRNAs for both LSU and LHCP increase in concentration on illumination of dark-grown peas, the relative increase for SSU mRNA is subtantially greater. In addition, they agree that the light-dependent increase in LHCP mRNA content can be ascribed in large measure to a phytochrome-mediated effect, whereas the contribution of phytochrome to the light-dependent increase in SSU mRNA content is comparatively minor. The only apparent disparity between the two sets of data lies in the response of LSU mRNA to red and far-red light. They illuminate dark-grown pea seedlings with 3 min of red light (with or without 8 min of far-red light) on the fourth, fifth, and sixth d after sowing. Under these light conditions, the level of LSU mRNA shows red/far-red photoreversibility. This suggests that the 15-min pulse of red light used in our studies was too long to permit photoreversibility in the case of LSU mRNA, even though it was not too long to permit photoreversibility in the cases of the SSU and LHCP mRNAs, reinforcing our conclusion that LSU and SSU genes are under different photoregulation.

How does phytochrome exert its effect on the levels of leaf mRNAs? Gallagher and Ellis [46] have shown, through the isolation of nuclei from dark-grown and illuminated peas, that the light-regulation of the expression of the SSU and LHCP genes occurs at the transcriptional level. Silverthorne and Tobin [47] have used the same approach to demonstrate that in Lemna gibba phytochrome controls the same genes at the transcriptional level. However, this is not the only way in which phytochrome controls mRNA levels. At the opposite extreme there is now abundant evidence that phytochrome can induce the rapid disappearance of certain mRNAs [45], including those for protochlorophyllide reductase [48] and phytochrome itself [49, 50]. These results point to an exceedingly complex involvement of phytochrome with gene expression in both the nucleus and the chloroplast.

ACKNOWLEDGMENTS

The authors gratefully acknowledge financial assistance from the Science and Engineering Research Council. We thank Dr. Steven Smith for the gift of cDNA clones and to Prof. Elaine Tobin for SSU antibody.

REFERENCES

1. Thomson WW, Whatley JM: Annu Rev Plant Physiol 31:375, 1980.
2. Kirk JTO, Tilney-Bassett RA: "The Plastids." North Holland: Elsevier, 1978, pp. 960.
3. Adamson H, Hiller RG: In Akoyunoglu G (ed) "Photosynthesis V: Chloroplast Development." Philadelphia: Balaban International Science Series, 1981, pp 213–221.
4. Boordman NK, Anderson JM, Goodchild DJ: Curr Top Bioenerget 8:35, 1978.
5. Pratt LJ: Annu Rev Plant Physiol 33:557, 1982.
6. Virgin H: Annu Rev Plant Physiol 32:451, 1981.
7. Senger H: Photochem Photobiol 35:911, 1982.
8. Ellis RJ: Annu Rev Plant Physiol 32:111, 1981.
9. Bennett J: Biochem J 212:1, 1983.
10. Graham D, Grieve AM, Smillie RM: Phytochemistry 10:2905, 1971.
11. Mohr H: Endeavour (New Series) 1:107, 1977.
12. Tobin EM: Plant Mol Biol 1:35, 1981.
13. Stiekema WJ, Wimpee CF, Silverthorne J, Tobin EM: Plant Physiol 72:717, 1983.
14. Link G: Planta 154:81, 1982.
15. Apel K: Eur J Biochem 97:183, 1979.
16. Gollmer I, Apel K: Eur J Biochem 133:309, 1983.
17. Griffiths WT: Biochem J 174:681, 1978.
18. Parish JH, Kirby KS: Biochim Biophys Acta 129:554, 1966.
19. Hartley MR, Ellis RJ: Biochem J 134:249, 1973.
20. Loening UE: Biochem J 113:131, 1969.
21. Covey SN, Lomonossoff GP, Hull R: Nucleic Acids Res 9:6735, 1981.
22. Southern EM: J Mol Biol 98:503, 1975.
23. Coen DM, Bedbrook JR, Bogorad L, Rich A: Proc Natl Acad Sci USA 74:5487, 1977.
24. Bedbrook JR, Smith SM, Ellis RJ: Nature 287:692, 1980.
25. Clewell DB: J Bacteriol 110:667, 1972.
26. Smith SM, Ellis RJ: J Mol Appl Gen 1:127, 1981.
27. Hoopes BC, McClure WR: Nucleic Acids Res 9:5493, 1981.
28. Langridge J, Langridge P, Berquist PI: Anal Biochem 103:264, 1980.
29. Rigby PWJ, Diekmann M, Rhodes C, Berg P: J Mol Biol 113:237, 1977.
30. Denhart DJ: Biochem Biophys Res Commun 23:641, 1966.
31. Wahl GM, Stem M, Stark GR: Proc Natl Acad Sci USA 76:3683, 1979.
32. Vaessen RTM, Kreike J, Groot GSP: FEBS Lett 124:193, 1981.

33. Highfield PE, Ellis RJ: Nature 271:420, 1978.
34. Bennett J: Eur J Biochem 118:61, 1981.
35. Cuming AC, Bennett J: Eur J Biochem 118:71, 1981.
36. Apel K, Kloppstech K: Eur J Biochem 85:581, 1978.
37. Jenkins GI, Hartley MR, Bennett J: Phil Trans R Soc Lond [Biol] 301:122, 1983.
38. Siddell SG, Ellis RJ: Biochem J 146:675, 1975.
39. Coruzzi G, Broglie R, Cashmore A, Chua NH: J Biol Chem 258:1399, 1983.
40. Ellis RJ, Hartley MR: Nature New Biol 233:193, 1971.
41. Iwanij V, Chua NH, Siekevitz P: J Cell Biol 64:572, 1975.
42. Barraclough R, Ellis RJ: Eur J Biochem 94:165, 1979.
43. Bellemare G, Bartlett S, Chua NH: J Biol Chem 257:7762, 1982.
44. Arnon DI: Plant Physiol 24:1, 1949.
45. Thompson WF, Everett M, Polans NO, Jorgensen RA, Palmer JD: Planta 158:487, 1983.
46. Gallagher TF, Ellis RJ: EMBO J 1:1493, 1982.
47. Silverthorne J, Tobin EM: Proc Natl Acad Sci USA 81:1112, 1984.
48. Apel K: Eur J Biochem 120:89, 1981.
49. Gottman K, Schäfer E: Photochem Photobiol 35:521, 1982.
50. Colbert JT, Hershey HP, Quail PH: Proc Natl Acad Sci USA 80:2248, 1983.