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Translation of mRNAs for subunits of chloroplast coupling factor 1 in spinach

(plastid genes/nuclear genes/protein precursor)

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ABSTRACT **The chloroplast coupling factor 1 consists of five nonidentical subunits, three of which (α, β, and ε subunits) have been shown in several laboratories to be synthesized within chloroplasts. The site of synthesis of the remaining two (γ and δ subunits) was investigated by analyzing products directed by spinach leaf RNAs in wheat germ and reticulocyte translation systems *in vitro*. It was found that poly(A)$^+$ RNA directs the synthesis of two distinct polypeptides, one of which is immunochemically related to the γ subunit but is 4,000 daltons larger. The other shares antigenic sites with the δ subunit but is 8,000 daltons larger. When wheat germ or reticulocyte translation systems were programmed with RNAs from purified chloroplasts, the only products related to CF_1 that we could detect were a putative precursor of β, 2,000 daltons larger than the mature subunit, and some smaller polypeptides, which appear to be incomplete translation products of β. From these results it appears likely that the γ and δ subunits are synthesized in the cytoplasm as larger precursors and that β is synthesized within the chloroplast as a precursor.**

It has been demonstrated in several laboratories that isolated intact chloroplasts, using light as a sole energy source, incorporate labeled amino acids into α, β, and ε subunits of chloroplast coupling factor 1 (CF_1) (1–5). Inhibitor studies of CF_1 biosynthesis suggested that the remaining subunits of CF_1, γ and δ, are synthesized in the cytoplasm and imported into the chloroplasts, where the five subunits are assembled into the CF_1 complex (6, 7). In contrast to this model, Nelson *et al.* (8) recently reported that, in addition to the α, β, and ε subunits, the γ subunit was synthesized within isolated chloroplasts.

Using antibodies specific to individual CF_1 subunits, Nelson *et al.* (8) also detected precursors of the δ subunit in the *in vitro* translation products of a reticulocyte lysate system programmed by total cell RNA from spinach leaves. A trace amount of radiolabeled polypeptide was immunoprecipitated by anti-γ, but it was not characterized further.

Using wheat germ and reticulocyte translation systems *in vitro*, we find that poly(A)$^+$ RNA from spinach leaves contains mRNAs for polypeptides that may be precursors of γ and δ subunits, whereas chloroplast RNA directs only the synthesis of polypeptides antigenically related to the β subunit.

MATERIALS AND METHODS

Extraction and Fractionation of Spinach Leaf RNA. Spinach leaves (*Spinacia oleracea* Linnaeus) were harvested from plants grown in the field, deribbed, washed with water, and used for RNA extraction and chloroplast preparation (9, 10). Total leaf RNA was extracted from 30 g of the leaves by homogenization with 150 ml of 50 mM Tris·HCl, pH 9.0/1% (wt/vol) NaDodSO$_4$ and an equal volume of 90% (vol/vol) phenol (11) in a Waring Blendor. The aqueous phase was collected after centrifugation of the homogenate at 1,500 × *g* for 15 min and extracted twice more with equal volumes of 90% phenol. RNA was precipitated by the addition of 2 vol of ethanol followed by incubation at −20°C for at least 2 hr. The precipitate was collected, partially dried, and dissolved in water, and the high molecular weight RNA was precipitated from the total leaf RNA by the addition of LiCl to 2 M. After standing at 0°C for several hours, the precipitate was collected, washed twice with 70% (vol/vol) ethanol to remove residual LiCl, and dissolved in water. The high molecular weight RNA was fractionated into poly(A)$^+$ RNA and poly(A)$^-$ RNA by oligo(dT)-cellulose column chromatography (12). The procedures for binding and elution of the RNAs were as described previously (13), except that the temperature shift was omitted. The fractionated RNA was precipitated and washed with 70% ethanol, dried under reduced pressure, and then dissolved in a minimal volume of water.

Chloroplast RNA was prepared from intact chloroplasts isolated and purified as described (9, 10). Intact chloroplasts were recovered from gradients of colloidal silica, washed twice with sorbitol/*N*-[tris(hydroxymethyl)methyl]glycine (Tricine) medium (0.33 M sorbitol/50 mM Tricine KOH, pH 8.4), and resuspended in 50 mM Tris·HCl, pH 9.0, containing 1% NaDodSO$_4$ and chlorophyll at 0.4 mg/ml. The suspension was shaken with equal volume of 90% phenol at room temperature for 10 min and processed further to obtain high molecular weight RNA as described for total leaf RNA. When chloroplast RNA is isolated in this way, contamination with cytoplasmic RNA is negligible (13).

Cell-Free Translation Systems. Wheat germ extract was prepared by the procedure of Marcu and Dudock (14). The conditions for the translation reaction were optimized by using poly(A)$^+$ RNA from spinach leaves as mRNA, and were as follows: 20 mM Hepes–KOH at pH 7.5, 70 mM KCl, 1.5 mM $MgCl_2$, 0.6 mM spermidine, 1 mM ATP, 8 mM creatine phosphate, creatine kinase (Sigma) at 5 μg/ml, 20 μM GTP, 1 mM dithiothreitol, 19 amino acids (excluding methionine or leucine) each at 50 μM, [^{35}S]methionine (700 Ci/mmol, Amersham) at 0.7 mCi/ml or [^{3}H]leucine (140 Ci/mmol, Amersham) at 0.5 mCi/ml, and poly(A)$^+$ RNA at 70 μg/ml or poly(A)$^-$ RNA or chloroplast RNA each at 0.3 μg/ml (1 Ci = 3.7 × 10^{10} becquerels). The translation reaction was carried out at 30°C for 1 hr and stopped by the addition of RNase A at 10 μg/ml. The mixture was then incubated at 30°C for 30 min. The incorporation of radioactive amino acids into hot trichloroacetic acid-insoluble materials was determined for the aliquots of the reaction mixture as described (15). Reticulocyte lysate was purchased from Amersham and used under the conditions directed by the sup-

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Abbreviation: CF_1, coupling factor 1.

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plier. The concentrations of RNAs and radiolabeled amino acids were the same as for wheat germ system.

Isolation of CF_1 and Its Subunits. CF_1 was isolated from washed thylakoid membranes of spinach leaves by the procedures of Binder *et al.* (16). The sucrose density gradient centrifugation in the purification process was replaced with preparative electrophoresis on a 4% polyacrylamide gel, using a Hoefer model SE501 apparatus under the conditions suggested by the manufacturer. CF_1 subunits were separated by $NaDodSO_4$/polyacrylamide gel electrophoresis as described (1) and extracted separately from the stained gel as described (4). The extracts were concentrated to a minimal volume by using a Millipore immersible concentrator (excluding molecular weight >10,000). The purified subunits were found by analysis with $NaDodSO_4$/polyacrylamide gel electrophoresis to contain traces of degraded polypeptides of the subunits, but no cross-contamination among the five species (data not shown).

Immunochemical Procedures. Antiserum to CF_1 was raised in a rabbit against the purified CF_1 protein, which was dissociated in 2% $NaDodSO_4$/25 mM Tris·HCl, pH 7.5/0.14 M NaCl, at 1 mg/ml prior to mixing with Freund's complete adjuvant. Antisera specific to α, β, γ, and ε subunits were a generous gift from A. T. Jagendorf of Cornell University. IgG was precipitated by the addition of an equal volume of saturated ammonium sulfate solution, dialyzed against saline (25 mM Tris·HCl, pH 7.5/0.14 M NaCl), and used as antibody without further purification. Antibody to CF_1 was shown to possess specificity to each of the five subunits by immunoreplicas (17) of thylakoid membrane proteins separated on $NaDodSO_4$/polyacrylamide gels. Because the antibodies specific to individual subunits contained trace activities toward other subunits, they were used after preincubation with excess amounts of purified subunits of the contaminating species at 30°C for 2 hr, so that they precipitate only the designated subunits.

The cell-free translation products were dissociated by the addition of $NaDodSO_4$ at 2% (vol/vol) and incubated at 80°C for 3 min. The mixture was made 3% (wt/vol) with Triton X-100, diluted with Triton/saline (25 mM Tris·HCl, pH 7.5/0.14 M NaCl/0.1% Triton X-100) and then incubated with 50 μg of IgG at 30°C for 1 hr. To the immunoreaction mixture was added 20 μl (packed volume) of staphylococcal protein A-Sepharose CL-4B (Pharmacia) and the incubation was continued for 1 hr with occasional stirring. The Sepharose particles were transferred to a small plastic column and washed successively with 5 ml of Triton/saline, 1 ml of saline, and 1 ml of water. The antigen–antibody complex bound to protein A was eluted with 60 μl of sample buffer for $NaDodSO_4$/polyacrylamide gel electrophoresis (18) and dissociated by incubation at 80°C for 3 min.

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis and Fluorography. Portions of the eluate from the protein A-Sepharose column were subjected to electrophoresis on a slab gel of 12.5% polyacrylamide containing 0.1% $NaDodSO_4$ with the discontinuous buffer system of Laemmli (18). After electrophoresis, the gel was stained with Coomassie brilliant blue R, destained, and then processed for fluorography using EN^{3}HANCE (New England Nuclear) under the conditions suggested by the manufacturer. The dried gel was exposed to a medical x-ray film (Fuji RX, Fuji Photo Film, Tokyo) at −80°C.

RESULTS

In principle one should be able to demonstrate the products of cytoplasmic protein synthesis by analyzing the products of cell-free translation of the mRNAs contained in the cytoplasm, but it is not generally feasible to obtain cytosol from green plant tissues that is free from chloroplast stroma. We prepared, therefore, total cellular RNA from spinach leaves and fractionated it into poly(A)$^+$ RNA, which is nearly pure cytoplasmic mRNA, and poly(A)$^-$ RNA, which is composed largely of rRNAs, but also contains some cytoplasmic or organellar mRNAs. We also prepared RNA from chloroplasts, which can be isolated free from cytoplasm (13). We then compared the cell-free translation products of poly(A)$^+$, poly(A)$^-$, and chloroplast RNAs.

The three classes of RNA stimulated the incorporation of [^{3}H]leucine into hot-trichloroacetic-insoluble materials with different efficiencies in the wheat germ system (Fig. 1). Poly(A)$^+$ RNA was the most active of the three, and the incorporation increased depending on its concentration up to 70 μg/ml. Poly(A)$^-$ RNA showed modest stimulation up to 0.7 mg/ml. Chloroplast RNA was the least efficient source of mRNA for the wheat germ system.

The translation products in the wheat germ system in the presence of [^{35}S]methionine were analyzed for polypeptides immunochemically related to CF_1 protein by using antibodies to CF_1 that included specificities to each of the five subunits. As shown in Fig. 2, all of the poly(A)$^+$ RNA preparations from different sizes of spinach leaves contained mRNAs for two polypeptides of molecular weights 42,000 and 30,000. Chloroplast RNA directed the synthesis of two major labeled polypeptides of molecular weight 53,000 and 41,000 when [^{35}S]methionine was used as a labeled amino acid. The molecular weight 41,000 product is distinctly separable from the molecular weight 42,000 product directed by poly(A)$^+$ RNA. The analysis of the translation products from poly(A)$^-$ RNA showed this RNA to contain the same mRNAs as those in poly(A)$^+$ RNA and chloroplast RNA. The molecular weight 41,000 and 42,000 products form a doublet band in the fluorogram.

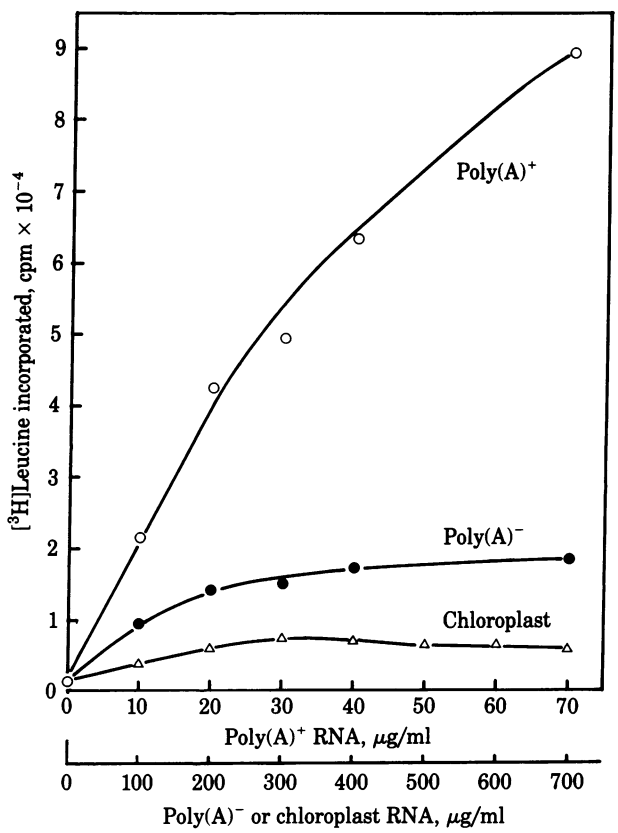

FIG. 1. Stimulation of translation by spinach leaf RNAs in the wheat germ system. Poly(A)$^+$ RNA, poly(A)$^-$ RNA, and chloroplast RNA were prepared from spinach leaves and translated at different concentrations in the wheat germ cell-free system in the presence of [^{3}H]leucine. After 1 hr of incubation, the reaction was terminated and radioactivity in hot-acid-insoluble materials was determined.

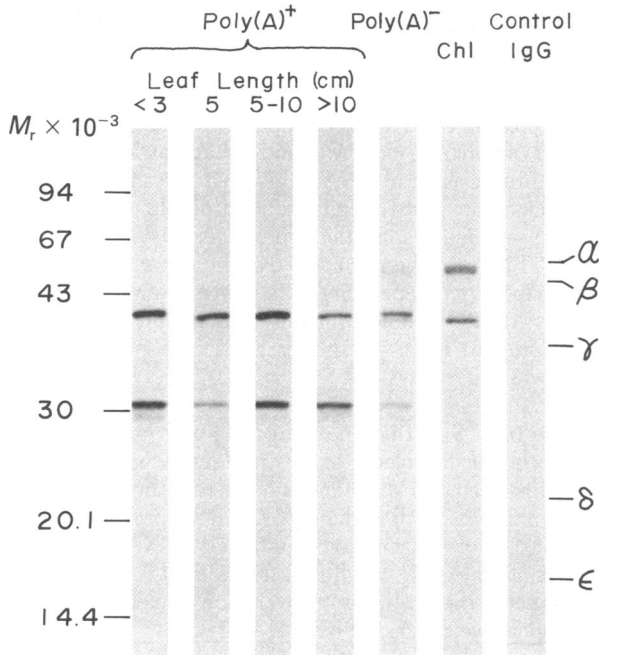

FIG. 2. NaDodSO$_4$/polyacrylamide gel electrophoresis of cell-free translation products antigenically related to CF$_1$ protein. Poly(A)$^+$ RNA was prepared from spinach leaves of different lengths. The RNAs were translated in the wheat germ system in the presence of [^{35}S]methionine, and the products reacting with antibody to CF$_1$ were analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis followed by fluorography. Chl, chloroplast RNA. Lane 7 shows the result of a similar experiment with control antibody.

All of the antigenic polypeptides found in the translation products in the wheat germ system were also detected by the reticulocyte lysate system. The products from chloroplast RNA in the latter system contained more minor polypeptides of molecular weights ranging between 20,000 and 40,000 (Fig. 3).

To identify the relationships of the observed translation products to individual subunits, antibody to CF$_1$ was incubated at 30°C for 2 hr with 1–10 μg of isolated subunits to block IgG molecules specific to the respective subunits. Antibodies directed against isolated subunits were also used.

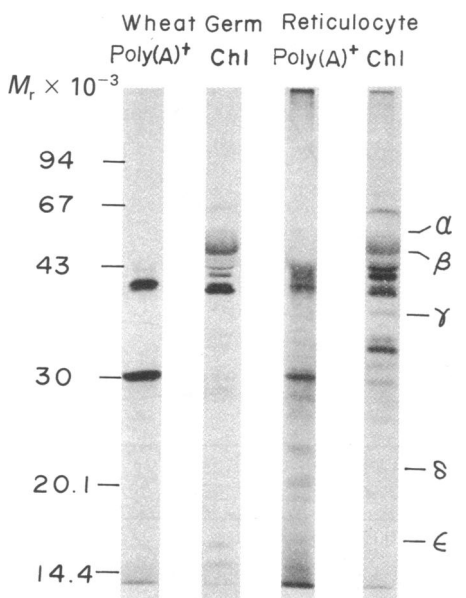

FIG. 3. Comparison of translation products of the wheat germ and reticulocyte systems. Experiments similar to those in Fig. 2 were performed with [^{35}S]methionine as the labeled amino acid. Translation products of poly(A)$^+$ RNA and chloroplast RNA were analyzed by NaDodSO$_4$ gel electrophoresis and fluorography.

Fig. 4 shows an analysis of the *in-vitro* translation products of chloroplast RNA with [^{35}S]methionine. When the individual subunits are employed to compete with translation products for the binding sites of anti-CF$_1$, only the β subunits produce significant qualitative changes, and it eliminates all of the translation products. Conversely, precipitation with anti-β enhances the collection of all the products bound by anti-CF$_1$. No significant amount of products is collected by anti-γ or anti-ε.

Results of the corresponding experiments with [^{3}H]leucine are presented in Fig. 5. Again, neither γ nor δ subunits compete significantly for anti-CF$_1$ and, alone among the antisera to specific subunits, only anti-β collects detectable amounts of translation products.

The principal translation product crossreacting with β migrates as a polypeptide of molecular weight 53,000 (Figs. 4 and 5), which is about 2,000 larger than the mature subunit. The two other principal products correspond to molecular weights of 41,000 and 14,000. We suspect these are incomplete translation products. The observation that the molecular weight 14,000 product is labeled only with [^{3}H]leucine may indicate the absence of methionine from the amino-terminal sequence of β.

The identities of the mRNAs in the poly(A)$^+$ fraction were similarly determined by competition by individual subunits and by binding with antibody to γ. Fig. 6 also shows that only γ subunit competed with the molecular weight 42,000 polypeptide, and that only δ subunit competed with the molecular weight 30,000 polypeptide. These observations indicate that the molecular weight 42,000 and 30,000 polypeptides translated from poly(A)$^+$ RNA share antigenic sites with γ and δ subunits,

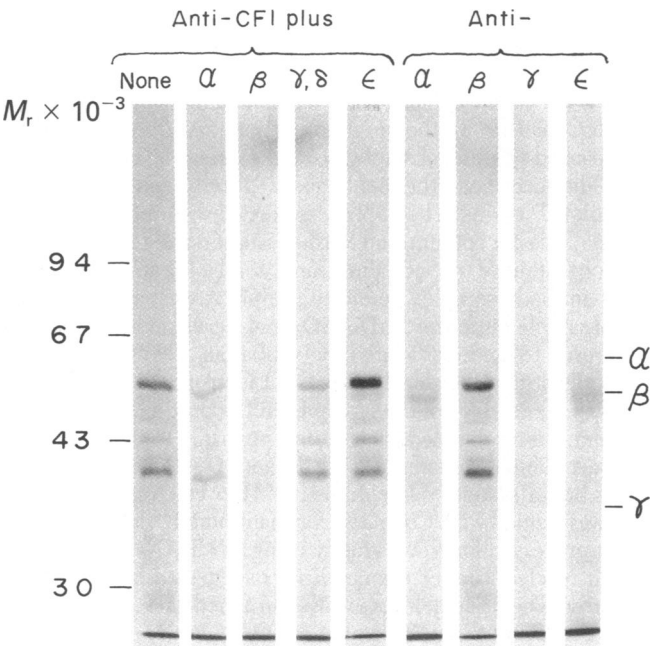

FIG. 4. Immunochemical identification of the polypeptides translated from chloroplast RNA in the presence of [^{35}S]methionine. Translation products from chloroplast RNA labeled with [^{35}S]methionine in the wheat germ system were allowed to react with antibody to CF$_1$ that had been incubated with saline or various subunits of CF$_1$ (lanes 1–5) or with antisera to various subunits (lanes 6–9). The immunoprecipitates were analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis followed by fluorography.

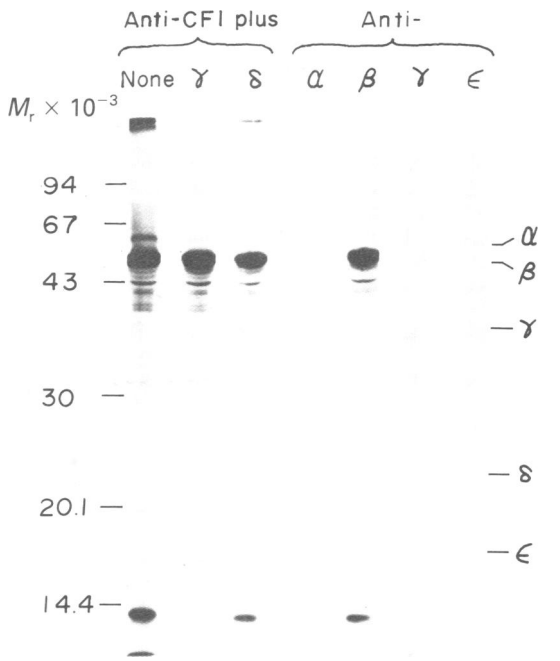

FIG. 5. Immunochemical identification of the polypeptides translated from chloroplast RNA in the presence of [^{3}H]leucine. Translation products from chloroplast RNA labeled with [^{3}H]leucine in the wheat germ system were allowed to react with antibody to CF_1 that had been incubated with saline, γ, or δ subunit (lanes 1–3) or with antibodies to the several subunits (lanes 4–7). The immunoprecipitates were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis followed by fluorography.

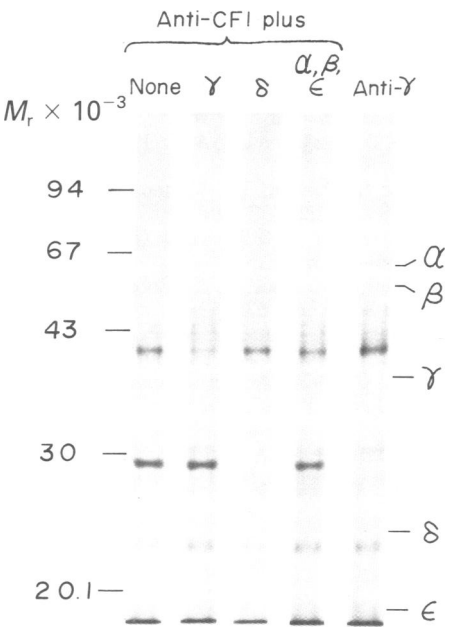

FIG. 6. Immunochemical identification of the polypeptides with molecular weights 42,000 and 30,000 translated from poly(A)$^+$ RNA. Translation products from poly(A)$^+$ RNA labeled with [^{35}S]methionine in the wheat germ system were allowed to react with antibody to CF_1 that had been incubated with saline or with various subunits (lanes 1–4) or with antibody to γ (lane 5). The immunoprecipitates were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis followed by fluorography.

respectively. Because their molecular weights are larger than those of the respective subunits by 4,000 and 8,000, these subunits are likely to be precursors of the mature subunits.

DISCUSSION

Our experiments, as well as the observations of most other investigators (2, 3, 5), have shown that isolated chloroplasts using light as an energy source synthesize the α, β, and ε subunits of CF_1. In the analysis of total thylakoid membrane proteins from the labeled chloroplasts by radioautography of $NaDodSO_4$/polyacrylamide gel electrophoresis, we were never able to detect a trace of incorporation of labeled amino acids into either the γ or δ subunits. Our present studies with cell-free translation of mRNAs clearly show that poly(A)$^+$ RNA from spinach leaves directs the synthesis of polypeptides immunochemically related to the γ and δ subunits, whereas RNA from purified chloroplasts does not. Our conclusions on the site of translation of the γ subunit therefore disagree with those of Nelson *et al.* (8).

There are two principal differences between the studies of Nelson *et al.* (8) and our studies. The Basel group employed crude chloroplast suspensions, a kind of preparation that is known to be contaminated with cytoplasmic particles (13), whereas our plastids were purified on gradients and contain no detectable contamination. Also, Nelson *et al.* employed exogenous ATP rather than light to drive protein synthesis. Neither of these differences, however, would seem to account for the discrepancies between our results.

Results substantially similar to ours have been obtained by Westoff *et al.* (19), except that they continue to claim that the γ subunit is a translation product of the spinach plastid.

We confirm the observation by Nelson *et al.* (8) that the δ subunit is synthesized as a polypeptide 8,000 daltons larger than the mature δ subunit. In addition we find that the γ subunit is synthesized as a precursor 4,000 daltons larger than mature γ. It seems likely that these polypeptides represent precursors of the mature subunits, analogous to the occurrence of a precursor to the small subunit of ribulosebisphosphate carboxylase (cf. ref. 20).

If either of the polypeptides of molecular weights 42,000 or 30,000 corresponds to mitochondrial F_1 ATPase, as suggested by Nelson *et al.* (8), one would expect to see comparable amounts of radioactivity associated with polypeptides migrating close to the α and β subunits, since all of the subunits of F_1 are reported to be synthesized in the cytoplasm (21). We expect that the chloroplast contains mRNAs for the α, β, and ε subunits, but only β was detected in the wheat germ and reticulocyte system. Westoff *et al.* (19), however, have obtained efficient translation of α and ε subunits hybrid-selected by restriction endonuclease fragments of chloroplast DNA.

We are tempted to speculate that the β subunit may be synthesized as a precursor, similar to findings for certain other chloroplast translation products: the molecular weight 32,000 protein (5, 22, 23), perhaps the large subunit of ribulosebisphosphate carboxylase (24), and cytochrome *b*-559 (4). Before a precursor role can be assigned to any of the CF_1 polypeptides detected *in vitro*, it is necessary to show that they function as intermediates in the formation of the mature protein.

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