Transcription and translation of the chloroplast atpB-gene and assembly of ATP synthase subunit β

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In vitro transcription and subsequent translation of the cloned *Chlamydomonas* chloroplast atpB gene was used to study assembly of ATP synthase. Translation in the presence of thylakoids resulted in association of the β subunit with the membrane. The in vitro synthesized polypeptide bound to the membrane copurified with CF_1 on sucrose gradients. This provides more evidence for the self-assembly of CF_1.

Chloroplast; Translation; atpB-gene; CF_1 assembly; ATP synthase

1. INTRODUCTION

Chloroplast ATP synthase (CF_0-CF_1) couples proton translocation across the thylakoid membrane to synthesis or hydrolysis of ATP. The extrinsic part of this complex, CF_1, can be extracted from the membrane and shows Ca^{2+}- and Mg^{2+}-dependent ATPase activity [1]. Enzymatic properties of CF_1, the closely related mitochondrial and bacterial F1 complexes have been extensively studied [2,3]. A heterodimer α_3/β_3 has been shown to bind nucleotides and have ATPase activity. This trimer contains one active site, while the other binding sites have regulatory functions [2,4]. Recently, Mg^{2+}-dependent ATPase activity has been found in isolated β-subunit of spinach CF_1 [5].

CF_1 is composed of 5 subunits, three of which are synthesized and encoded in the chloroplast [6]. The atpB gene from *Chlamydomonas* chloroplasts has been sequenced [7] and subunit β was isolated after overexpression in *E. coli* [8]. The subchloroplast site of atpB mRNA translation is a topic of some controversy. Polyribosomes and mRNA directing synthesis of the β-subunit have been found associated with membranes in spinach and *Chlamydomonas* [9,10]. Soluble and membrane-bound polyribosomes capable of β CF_1 translation were found in pea [11]. Recently, it has been proposed that the thylakoid-bound mRNAs for CF_1 proteins are electrostatically bound to thylakoids of *Vicia faba* [12]. Thus, there may not be functional association of translation and membrane association. Investigation of mutants deficient in photophosphorylation was used to study ATP synthase assembly. Alterations in the β-subunit prevented assembly of the entire complex [13]. However, isolated β-subunit could restore photophosphorylation in chromatophores of *R. rubrum* [14]. Therefore, the β-subunit may have a central role in the activity and assembly of CF_1.

In this paper we describe the in vitro transcription/translation of the cloned atpB gene. The in vitro translated β-subunit associated with thylakoids and copurified with CF_1. This may provide more evidence for the self-assembly model of CF_1.

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2. MATERIALS AND METHODS

The 2.6-kb *Hin*dIII-*Kpn*I fragment of plasmid pB7 (a gift from Dr N. Gillham), containing the *Chlamydomonas* chloroplast atpB gene, was directionally cloned into pT7-2. The fragment containing the atpB gene was isolated from gels of the plasmid cut with *Kpn*I, blunt ends were made using the Klenow fragment of pol I (BRL) plus deoxynucleotides, phenol extracted, precipitated and then digested with *Hin*dIII to give a ~2.5 kbp fragment with a blunt end and a *Hin*dIII compatible end. The plasmid (pT7-2) was digested with *Sma*I and then with *Hin*dIII to yield a linear vector with a blunt end and a compatible *Hin*dIII overhang. Each DNA fragment was then isolated from agarose gels by a standard procedure [15]. The vector was treated with bacterial alkaline phosphatase (BRL), phenol extracted, precipitated with ethanol, and resuspended in water. Ligation of the plasmid to the insert was done in two stages. The first stage was at low T4 ligase (BRL) concentrations at room temperature for 4 h to ligate the *Hin*dIII ends. The reaction was then diluted 10-fold and incubated at 5 × concentration of T4 ligase at 15°C overnight. The entire reaction was then ethanol precipitated after the addition of carrier tRNA. The resulting plasmid was then used to transform competent cells (DH5α, BRL) [15]. Transformants were selected on solid media containing 50 μg/ml ampicillin. The resulting clones were analyzed by electrophoresis for the presence of the correct plasmid by restriction endonuclease analysis of alkaline minipreps [7,15].

The purified plasmid containing the atpB gene was linearized with restriction endonuclease *Eco*RI, phenol extracted, precipitated and used to produce RNA in vitro with T7 RNA polymerase as described by the supplier (BRL). After digestion with RNase free DNase (Pharmacia), the in vitro transcribed RNA was phenol-extracted, precipitated by addition of 2 vols of ethanol and analyzed by elec-

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trophoresis to determine concentration and homogeneity of each preparation [15].

Chloroplast membranes were prepared from synchronous cultures of *Chlamydomonas* and chlorophyll determined as previously published [9]. Reticulocyte lysate translation of the in vitro transcribed RNA (25 μg/ml) in the presence or absence of thylakoids (300 μg/ml chlorophyll) was done according to the supplier (Promega) at 30°C with the Mg^{2+} and K^+ concentrations adjusted to 1.5 and 120 mM and addition of [^{35}S]methionine (~1000 Ci/mmol, Amersham). Incorporation was measured by liquid scintillation of hot TCA-insoluble radioactivity on filter paper discs of duplicate 1 μl samples from each reaction. Post-translational thylakoid association of β CF_1 were done as follows: reactions were prepared with and without thylakoids, after 1 h incubation at 30°C cycloheximide (10 μg/ml) was added to all reactions, thylakoids were added to those translations not containing membranes and incubation continued for an additional hour.

The membranes were recovered from translation reactions by centrifugation and washed in R buffer (120 mM KAc, 1.2 mM $MgAc_2$, 25 mM Hepes/KOH, pH 7.8). They were solubilized either for electrophoretic analysis or in 1% Triton, 10 mM EDTA, 50 mM Tris, pH 7.6, and centrifuged at 12000 × *g* for 5 min. The soluble material was mixed with solubilized cold thylakoids (0.5 mg chlorophyll) as carrier and used to isolate CF_1 by sucrose gradient centrifugation as published [9]. Gradient fractions (0.3 ml) were collected with an ISCO gradient fractionator and protein precipitated with addition of 1.5 ml acetone on ice for 1 h. The precipitated proteins were collected by centrifugation at 12000 × *g*, the pellets dried under vacuum and solubilized in electrophoresis sample buffer. Both agarose and acrylamide gel electrophoresis was done as described [15,16].

A parallel gradient of solubilized membranes was fractionated. Nitrocellulose filters containing electrophoretically separated proteins (10 μl of each sample) were probed with an antibody raised against the α/β subunits of CF_1 [9]. Bound antibodies were detected by protein A conjugated alkaline phosphatase (Sigma no. P9650) according to the protocol of Promega.

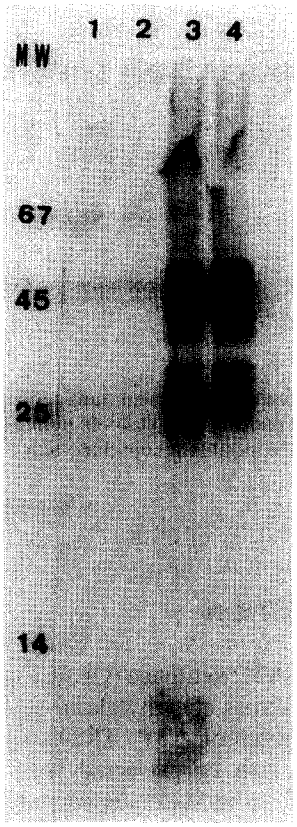

Fig. 1. Co- and post-translational association of atpB gene product. Equal amounts of recovered membranes were analyzed after post- and co-translational insertion in the absence (lanes 1, 2, respectively), or presence of RNA (lanes 3 – post-translation, 4 – co-translation).

3. RESULTS

Analysis of the recombinant plasmid by agarose gel electrophoresis after restriction endonuclease digestion showed the orientation of the atpB gene relative to the T7 promoter. The plasmid was then used for in vitro transcription and the recovered RNA was analyzed by denaturing agarose gel electrophoresis. The M_r of the RNA produced is similar to that determined by Northern blot analysis of cellular RNA (data not shown) [17].

Translation of the atpB mRNA in the reticulocyte extract showed synthesis of polypeptides with a maximum M_r of about 52 kDa. Translation was neither significantly stimulated nor inhibited in the presence of photosynthetic membranes. Analysis of recovered, washed membranes showed that the β-subunit was associated with thylakoids (Figs 1, 3). Approximately 10% of the in vitro translated β-subunit was recovered with membranes as calculated from radioactivity incorporated into membranes. We have investigated post-translational association of the atpB gene product with membranes. Translation in the absence of membranes and subsequent addition of thylakoids to reactions was compared to translation in the presence of membranes. In each case the membranes were recovered and washed before electrophoretic analysis (Fig. 1). The β-subunit of CF_1 was associated with recovered membranes under both conditions. The amount of β in membranes was 1.3-fold greater co-translationally as estimated from cpm and electrophoretic analysis (Fig. 1).

We wanted to determine if the membrane-associated β was part of the CF_1 complex or bound to thylakoids in a non-specific manner. Membranes were recovered from translation reactions, solubilized in detergent and analyzed as described in section 2. The position of CF_1 polypeptides in the gradient fractions was determined by Coomassie stain and Western blots using a well-characterized antibody (Fig. 2) [9]. Some radioactive β-subunit as well as lower molecular weight products of the translation were found at the top of the gradient, while radioactivity in the gradient was coincident with the position of CF_1 (Figs 2, 3).

4. DISCUSSION

We have measured translation/assembly of the CF_1 β-subunit in a heterologous system. The translation of chloroplast mRNAs in the reticulocyte lysate results in a product pattern comparable to that of in organello and in vivo pulse labelling [16]. In spite of the

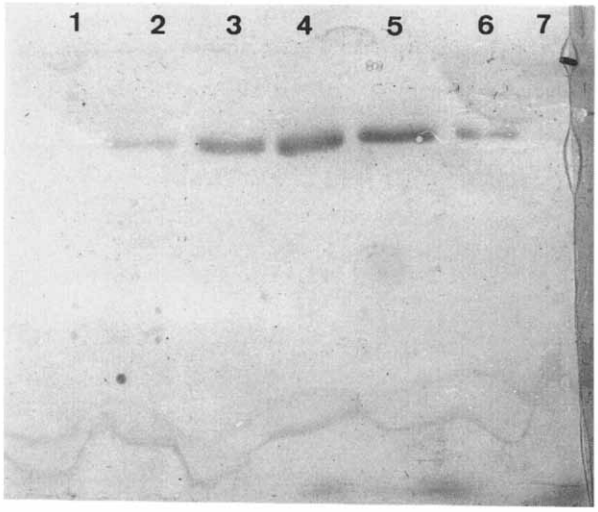

Fig. 2. The position of CF_1 in gradients of Triton-solubilized thylakoids was established by Western blot analysis (10 μl samples from fractions 1–7). The blot was probed with an antibody to the α/β-subunits of CF_1. This antibody was previously shown to be specific for the two subunits [9]. The highest amount of cross-reacting material occurred in fractions 4 and 5.

heterologous nature of the translation conditions it was possible to insert functional D1 protein into photoinhibited thylakoids and restore PS II function [18]. The in vitro translation pattern in the reticulocyte extract is similar to that of atpB expression in *E. coli* [8]. In each system many premature termination products are observed.

Our previous results indicated that α/β-subunits of CF_1 were translated by rough thylakoids. We postulated that chloroplast translated subunits of CF_1 are synthesized and may exist in a transient manner unassembled in the membrane [9]. In this publication we show that β CF_1 produced from in vitro transcription/translation of the atpB gene is capable of assembly into CF_1, either by exchange with existing CF_1 or by assemby of new CF_1 from pools of the other subunits present in the membrane, as postulated in our previous work.

Both atpA and atpB genes show strict coordinate regulation in abundance, transcription and translation in the *Chlamydomonas* cell cycle. Their expression during the light period of the cell cycle may mainly be regulated by mRNA abundance [9]. This would indicate that coordinate synthesis of the two polypeptides is required for ATP synthase assembly.

Point mutations in the atpB gene result in cells deficient for CF_1 assembly. The mutated atpB gene was transcribed, translated, but not accumulated on the membrane. The authors concluded that subunit β is necessary for CF_1 assembly and binding to the membrane [13]. However, these results also permit the conclusion that the mutated beta subunit prevents CF_1 assembly and therefore induces high turnover of the unassembled subunits in vivo.

It has been demonstrated that the β-subunit coupled to a transit sequence is imported into isolated chloroplasts but not assembled in CF_1 [20]. Therefore, translation should take place within the chloroplast for proper assembly. It is not clear, at this time, whether translation must be associated with thylakoids [9–11]. Our results show that translation of atpB mRNA was unaffected by the presence or absence of membranes. A small increase in cotranslational association to membranes occurred compared to post-translational association. We do not know whether the post-translationally membrane-associated β-subunit is complexed in CF_1.

Recent reports have demonstrated chloroplast transformation using the atpB gene to restore autotrophic growth in deletion mutants of

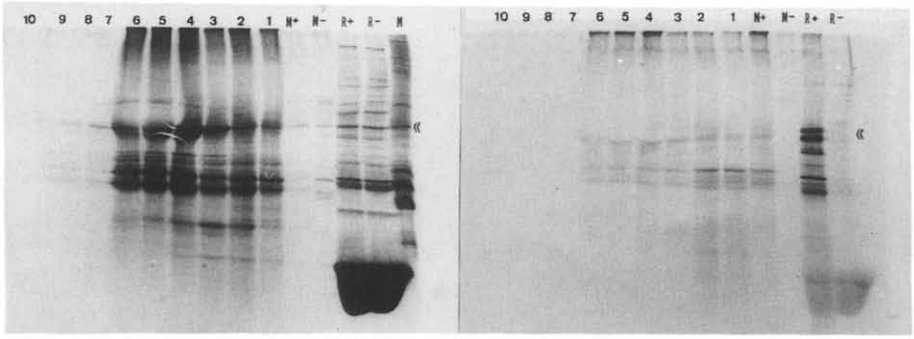

Fig. 3. Translation reactions in the presence of membranes and RNA were solubilized and fractionated by centrifugation prior to electrophoresis. The left panel is the stained, dried gel indicating the position of the α/β-subunits of CF_1 in the gradient. The right panel is the X-ray film of these fractions. The gradient fractions of all proteins precipitated with acetone are numbered 1 (top) through 10 (bottom); Triton-solubilized membranes recovered from the translation reactions in the presence (M+) or absence (M−) of RNA prior to addition of cold membranes for sucrose gradient centrifugation resulting in a two-fold dilution of original radioactivity in membranes (for M+), which was the starting material for the gradient; total translation mixtures in the presence (R+) and absence (R−) of RNA; isolated unlabelled thylakoids (M) used as a reference standard for the position of the α/β-subunits of CF_1 («). The difference in radioactivity of R+ and M+ lanes was the result of M+ sample dilution giving 5% of total radioactivity seen in lane R+. There is an apparent increase in specific radioactivity associated with β CF_1 in fraction 4 of gradient (compared with M+).

Chlamydomonas [21]. In vitro assembly of mutated atpB genes in addition to chloroplast transformation can now be used to investigate the molecular basis for assembly and function of this polypeptide in CF_1.

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