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The chloroplast β-subunit allows assembly of the *Escherichia coli* F_0 portion of the energy transducing adenosine triphosphatase

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The effect of the expression of the chloroplast F_1-ATPase β-subunit in two *Escherichia coli* β-subunit mutant st RA ND was investigated. The amount of chloroplast β-subunit formed in *E. coli* was increased by introduci ng 'Shine-Dalgarno' sequence upstream from the translation start site. The chloroplast β-subunit was meml bound but was unable to functionally replace the mutant β-subunit in a strain carrying the *uncD409* ε However, in an *E. coli* mutant strain unable to form the β- and ε-subunits the presence of the chloro β-subunit enabled the assembly of a functional proton pore.

Introduction

The F_0F_1-ATPase enzyme complex catalyses the terminal step in oxidative phosphorylation or photophosphorylation and is located in mitochondrial, chloroplast and bacterial membranes. The structure is highly conserved and the complex can be readily dissociated into two portions, the water-soluble F_1-ATPase and the membrane-bound F_0 portion which forms a proton pore (see Ref. 1). In both chloroplasts and *E. coli* the F_1 complex is composed of five subunits α, β, γ, δ and ε with the stoichiometry of α_3, β_3, γ_1, δ_1 and ε_1. The F_0 complex of *E. coli* (ECF_0) contains three polypeptide species (*a*, *b* and *c*) in the ratio of a_1, b_2, c_{6-12} while chloroplast F_0 (CP_0) appears to contain four polypeptide species (I, II, III and IV) of unknown stoichiometry [2]. In *E. coli* the F_0F_1-ATPase subunits are encoded in a single operon located at about 83 min on the *E. coli* chromosome with the genes in the order *unc* IBEFHAGDC (see Ref. 3). The *uncI* gene product does not appear to be part of the F_0F_1-ATPase complex and the remaining genes encode the subunits *a*, *c*, *b*, δ, α, γ, β and ε, respectively. In spinach chloroplasts six of the nine subunits of the F_0F_1-ATPase are encoded by genes in the chloroplast genome. Th chloroplast genes are arranged in two operons b each case the order is similar to that of the *E. co* genes. Thus, one operon carries the genes codin the CF_0IV, CF_0III, CF_0I and the CF_1 α-subunits and the other carries the genes for the CF_1 β- anc ε-subunits [7,8]. Table I summarises the equiv polypeptides and genes for the F_0F_1-ATPases c *coli* and chloroplasts.

The similarities in the structures of the F_0F_1-AT complexes from *E. coli* and from chloroplasts, plu similarities in gene arrangement, prompted us to i tigate whether *unc* mutants of *E. coli* could be plemented by the appropriate gene from the sp chloroplast. A modified CF_0I subunit has been s to substitute for the *b*-subunit in an appropriate tant strain of *E. coli* [9]. In the present pape describe work on the expression of the chlorc gene encoding the CF_1 β-subunit in *E. coli* an effects in various *E. coli unc* mutant strains.

Materials and Methods

Enzymes and chemicals. All chemicals and enz used were of the highest quality available. Restri endonucleases, T4-polynucleotide kinase and T4 l ligase were obtained from Pharmacia (Austr [α-^{35}S]dATP and L-[^{35}S]methionine were obtained Amersham (Australia). Oligonucleotides were sy

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TABLE I

Subunits and corresponding genes of the E. coli and spinach chloroplast F_0 F_1-ATPases

Subunit		Molecular mass (kDa)		Gene			
E. coli	chloroplast	*E. coli*	chloroplast	*E. coli*		chloroplast	
				old	proposed [b]	old	proposed [b]
α	α	55	56	*uncA*	*atpA*	*atpA*	*atpA*
β	β	50	54	*uncD*	*atpB*	*atpB*	*atpB*
γ	γ	31	36	*uncG*	*atpC*	*atpC*	*atpC*
δ	δ	19	20	*uncH*	*atpD*	*atpD*	*atpD*
ε	ε	15	15	*uncC*	*atpE*	*atpE*	*atpE*
a	CF_0IV	30	25	*uncB*	*atpF*	*atpI*	*atpF*
b	CF_0I	17	19	*uncF*	*atpG*	*atpF*	*atpG*
–	CF_0II	–	16 [a]	–	–	*atpG*	*atpH*
c	CF_0III	8	8	*uncE*	*atp*1	*atpH*	*atpI*

[a] Molecular mass estimated after SDS-gel electrophoresis.

[b] Proposed nomenclature change. The latest edition of the *E. coli* genetic map [27] has changed the mnemonic '*unc*' to '*atp*'. This necessitates a change also in the gene letter to conform with the previously proposed chloroplast system. If the old lettering system was retained [27] then *atpB* would encode the *a*-subunit in *E. coli* but would encode the β-subunit in the chloroplast. However, in the present paper the old '*unc*' nomenclature is used.

sised by K. Newell, Division of Plant Industry, CSIRO, Canberra on an Applied Biosystems 381A DNA Synthesiser.

Bacterial strains and plasmids. All of the bacterial strains used were derived from *E. coli* K12 and are described, together with the plasmids used, in Table II.

Genetic techniques. The techniques used for genetic experiments were as outlined previously [10].

Preparation of plasmids. Plasmid DNA was prepared as described by Selker et al. [11]

DNA sequencing. Nucleotide sequences were determined by the dideoxy chain-terminating method of Sanger et al. [12] using [α-^{35}S]dATP.

Site-directed mutagenesis. The M13-double primer protocol of Zoller and Smith [13] was used.

Preparation of CF_0I cDNA. The method used was that described by Hudson et al. [6].

Media and growth of organisms. The mineral salts minimal medium used and additions were as described previously [14]. Cells for the preparation of membranes

TABLE II

Strains of E. coli and plasmids used

Bacterial strain or plasmid	Genotype	Remarks
AN2967	pUC9/*minA minB PhoR79::Tn10 ara azi tonA lacY rpsL xyl mtl thi*	
AN2947	pAN369/*tonA lacY rpsl xyl mtl thi*	
AN2965	pAN480/*tonA lacY rpsl xyl mtl thi*	
AN1008	*uncD436 argH purE entA recA nal* ^{R}	
AN2694	*uncD409 argH purE entA lon100 phoR79::Tn10*	
AN2809	pUC18/*uncD436 argH purE entA recA nal* ^{R}	
AN2811	pAN480/*uncD436 argH purE entA nal* ^{R}	
AN2822	pUC18/*uncD409 argH pyrE entA lon100 phoR79::Tn10*	
AN2823	pAN480/*uncD409 argH pyrE entA lon100 phoR79::Tn10*	
JM101	*Δlac-pro supE thi* F'(*tra D36 proAB lacIq lacZ ΔM15*)	From J. Messing
C192	*minA minB ara azi tonA lacY rpsL xyl mtl thi*	
K37	Hfr *supD*	
pAN369	*atpB atpE bla*	2.3 kb *XbaI* fragment from plasmid pSocB149 inserted into *Sma*-digested pUC9. Insert in correct orientation
pAN379	*atpB atpE bla*	As for pAN369 except insert in the opposite orientation
pAN480	*atpB405 atpE bla*	2.1 kb *Bam*H1 fragment from pAN501 inserted into vector pUC18
pAN500	*atpB atpE bla*	2.1 kb *Bam*H1 fragment from pAN379 carrying *atpB* and *atpE* genes inserted into M13 mp19
pAN501	*atpB405 atpE bla*	Derived from pAN500 by site-directed mutagenesis

were grown in 14-litre fermenters as described previously [16]. The mineral salts medium in the fermenters was supplemented with 5% (v/v) Luria broth [17].

Turbidities of cultures were measured with a Klett-Summerson colorimeter. Growth yields were measured as turbidities after growth had ceased in medium containing limiting (5 mM) glucose.

Protein expression in minicells. Plasmids carrying the appropriate inserts were used to transform the minicell-producing strain CE192. The minicells were purified by the method of Mertens and Reeve [18] and labelled with L-[^{35}S]methionine.

Preparation of membranes. Membranes were prepared as described previously [19]. The F_1-ATPase was removed from the membranes by dialysing against a buffer system (pH 7.0) that contained Tes (5 mM), glycerol (15% v/v), dithiothreitol (0.5 mM) and 6-aminohexanoic acid (40 mM).

Other methods. ATPase and atebrin fluorescence quenching activities were assayed as previously described [14,15]. Protein concentrations were determined using Folin's phenol reagent [20] with bovine serum albumin as standard.

Results

Sub-cloning of the genes encoding CF_1 β and CF_1 ε and their expression in E. coli

A DNA fragment containing the chloroplast genes *atpB* and *atpE* was isolated from an *Xba* digest of the plasmid pSocB149 [7]. This fragment was then inserted into plasmid PUC9 to give plasmid pAN369 which had the insert in the correct orientation for expression of the β- and ε-subunits from the *lac* promoter. Plasmid pAN379 had the insert in the opposite orientation. Formation of the CF_1 β- and ε-subunits from the plasmid pAN369 was tested in an *E. coli* minicell-producing strain. Incorporation of [^{35}S]methionine into the ε-subunit occurred but only a relatively small amount of incorporation occurred in the β-subunit (see Fig. 1, trace 3). In addition there were additional bands of radioactivity presumed to correspond to fragments

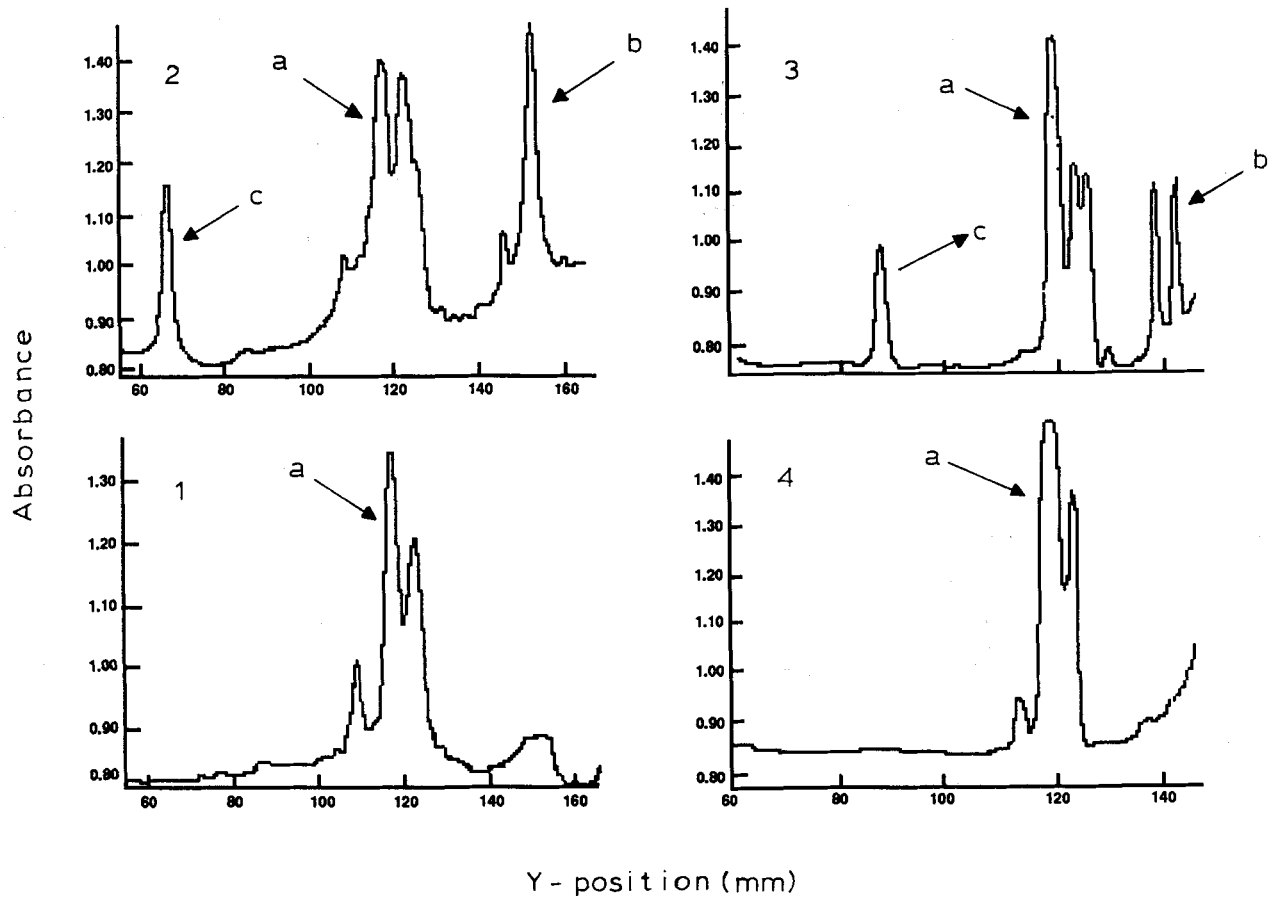

Fig. 1. Formation of chloroplast F_1-ATPase β (peak b) and ε-subunits (peak c) in *E. coli* minicells. The minicells (100 μl of suspension at A_{500} = 2) were incubated in the presence of 10 μCi of L-[^{35}S]methionine for 1 h at 37 °C, the labeled proteins resolved by SDS-PAGE on a 15% polyacrylamide gel and then visualised by fluorography using pre-flashed Kodak XRP film. Amounts of incorporation of radioactivity was determined using an LKB XL Ultrascan laser densitometer. 1, strain AN2967 (pUC9); 2, strain AN2965 (pAN480); 3, strain AN2947 (pAN369); 4, strain AN2967 (pUC9). Traces 1 and 2 were from experiments carried out at a different time to those shown in 3 and 4. The β- and ε-subunits were identified by comparison with molecular weight markers. Peak a is β-lactamase.

of the β-subunit. A similar pattern has been described resulting from the expression of the gene encoding the CF_1 β-subunit in a rabbit reticulocyte system [21]. The nucleotide sequence 5′ to the CF_1 β-subunit gene does not contain a recognisable Shine-Dalgarno sequence and the multiple bands apparently arising from the β-subunit gene might be attributed to initiation of translation at sites within the reading frame. A consensus Shine-Dalgarno sequence was therefore introduced by site-directed mutagenesis. A fragment carrying the CF_1 β- and ε-genes was prepared from the plasmid pAN379 and ligated with a *Kpn*I-*Bam*HI digest of the vector M13mp19 and the ligation mixture used to transform strain K37. A plasmid (pAN500) carrying the *atpB* and *atpE* genes was retained. An oligonucleotide with the sequence 5′-GACATACTTGAGGATATATTATG was used for mutagenesis and a corresponding mutant derivative (pAN501) of plasmid pAN500 was generated. The oligonucleotide sequence was identical to the region upstream of *atpB* except that the nucleotides −11 to −7, TACTA, were replaced by GAGGA. A *Kpn*I-*Bam*HI digest of plasmid pAN501 was ligated with a similar digest of the vector pUC18 and used to transform strain JM101. A plasmid (pAN480) carrying the correct insert and with the site-directed mutant sequence 5′ to the *atpB* gene was retained. Formation of the CF_1 β- and ε-subunits from plasmid pAN480 was tested in an *E. coli* minicell-producing strain. Incorporation of [^{35}S]methionine into the CF_1 β-subunit was increased about 4-fold for plasmid pAN480 compared with plasmid pAN369. (Fig. 1)

Complementation of E. coli uncD mutants

Two mutations affecting the *uncD* gene, encoding the *E. coli* β-subunit, were selected for complementation testing with plasmid pAN480. The first of these, the *uncD409* allele, encodes a β-subunit in which glycine-214 of the normal subunit is replaced by arginine [22]. This mutation interrupts assembly of the F_1 portion of the F_0F_1-ATPase. The second mutant allele (*uncD436*) results in lack of formation of either the β- or ε-subunits. This mutation also prevents the assembly of the F_0 portion of the F_0F_1-ATPase [23]. Plasmids pUC18 and pAN480 were used to transform strains AN1008 (*uncD436*) and AN2694 (*uncD*409) selecting for ampicillin resistance. Colonies from each of the four transformations were purified to give strains AN2809 (pUC18/*uncD436*), AN2811 (pAN480/*uncD436*), AN2822 (pUC18/*uncD409*) and AN2823 (pAN480/*uncD409*). All four strains were unable to grow on minimal medium containing succinate as carbon source and gave a growth yield on limiting concentrations of glucose equivalent to that obtained with an uncoupled mutant strain. These results indicated that the chloroplast β-subunit or β- plus ε-subunits were unable to replace the equivalent *E. coli* subunits to form a functional ATP synthase. Membrane and cytoplasmic fractions prepared from each of the strains also lacked ATPase activity (data not shown). Atebrin fluorescence quenching assays were also performed on the membrane preparations from the four strains and on membranes from the four strains treated by the method used to strip F_1-ATPase from normal membranes (Table III). All four membrane preparations lacked ATP-dependent atebrin fluorescence quenching activity but had normal NADH-dependent atebrin fluorescence quenching activity. These results indicated that the chloroplast subunits were also unable to replace the equivalent *E. coli* subunits in the generation of ATP-dependent proton pumping activity. Stripped membrane preparations from strains AN2809, AN2822 and AN2823 had similar NADH-dependent fluores-

TABLE III

Effects of the chloroplast β-subunit on atebrin fluorescence quenching activities of membranes from E. coli β-subunit mutant strains

Atebrin fluorescence quenching was measured as described previously [15]. A sample of membranes (about 1 mg protein) was diluted in 2.5 ml of 10 mM Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer containing 300 mM KCl and 5 mM $MgCl_2$. Atebrin was added to give a final concentration of 4 μM, NADH to 2 mM ATP to 1 mM, carbonyl cyanide-*m*-chlorophenylhydrazone to 20 μM and NaCN to 2.5 mM. The results of a typical experiment are shown.

	Atebrin fluorescence quenching (%)	
	NADH-dependent	ATP-dependent
AN2823 pAN480/*uncD409*	82	< 3
AN1846 [b] pAN36/*uncD436*	84	83
AN2823 Stripped [a]	76	< 3
AN2809 pUC18/*uncD436*	87	< 3
AN2809 Stripped [a]	88	< 3
AN2811 pAN480/*uncD436*	78	< 3
AN2811 Stripped	59	< 3
AN2811 Stripped and reconstituted with normal F_1	80	23
AN2811 Stripped and treated with DCCD	84	

[a] Native membranes washed with low ionic strength buffer to remove bound F_1-ATPase.
[b] See Ref. 26.

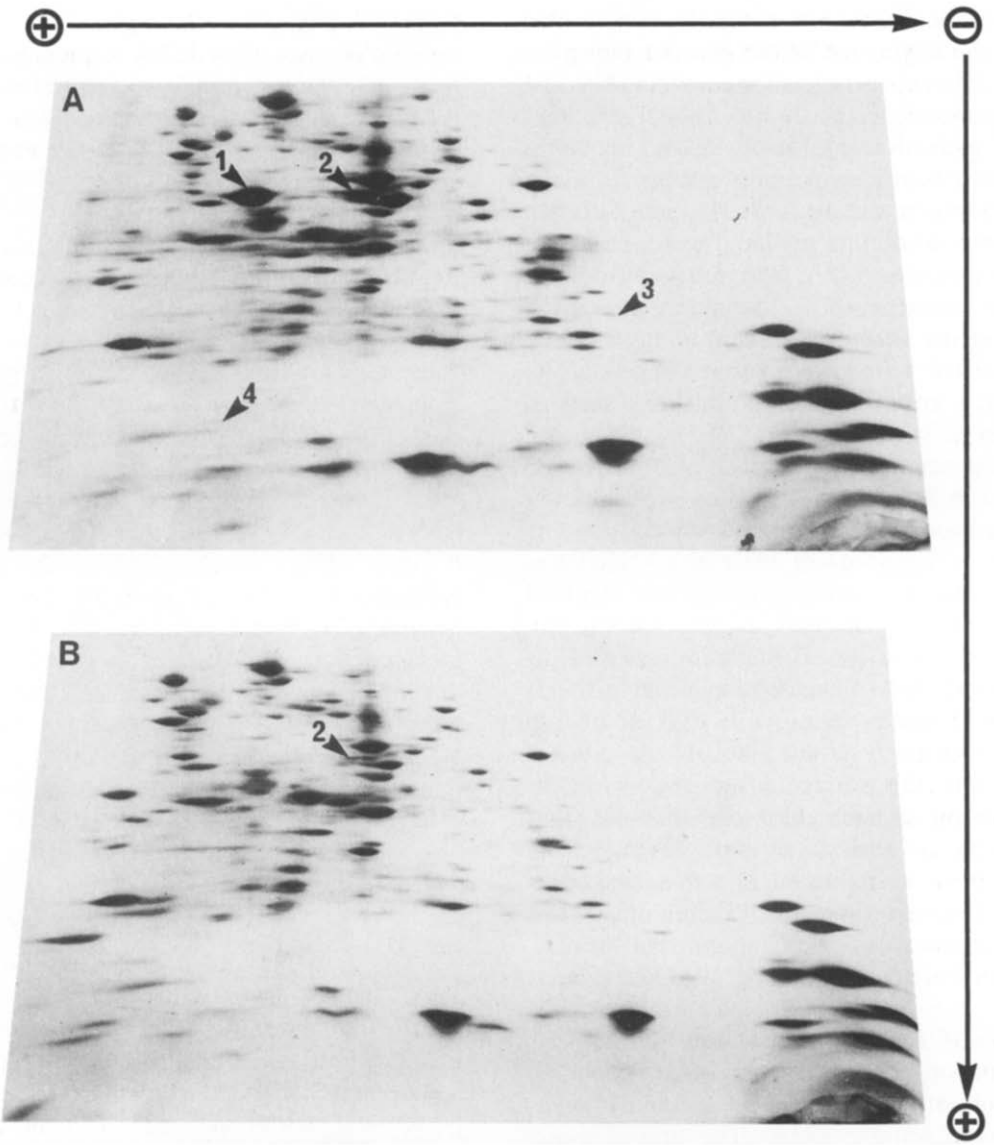

Fig. 2. Two-dimensional gel electrophoresis of membrane preparations from (A), strains AN2811 (pAN480/*uncD436*) and (B), AN2809 (pUC18/*uncD436*). In the first dimension, ampholines with a pH range from 5 to 7 and from 3.5 to 10 were present at 2.4% and 1.6% (w/v), respectively. In the second dimension, an acrylamide gradient of 10.5 to 24.5% (w/v) was used. Arrows indicate positions of the chloroplast β-subunit (1), the *E. coli* α-subunit (2), the *E. coli* γ-subunit (3) and the *E. coli b*-subunit (4).

cence quenching activities to the native membranes. However, stripped membranes from strain AN2811 (pAN480/*uncD436*) had comparatively low NADH-dependent atebrin fluorescence quenching activity indicating that these membranes were proton permeable. Addition of a purified *E. coli* F_1-ATPase preparation to the stripped membranes from strain AN2811 (pAN480/*uncD436*) reconstituted normal NADH-dependent atebrin fluorescence quenching activity and also reconstituted about 30% of normal ATP-dependent atebrin fluorescence quenching activity. The latter result, in particular, indicates the presence of a functional F_0. The addition of the purified *E. coli* F_1-ATPase had no effect on the membranes from the other three strains. The addition of the inhibitor DCCD to stripped membranes of strain AN2811 (pAN480/*uncD436*) also reconstituted normal NADH-dependent atebrin fluorescence quenching activity indicating that DCCD had sealed the proton pore.

The presence of the *uncD436* allele results in the lack of formation of both the β- and ε-subunits. The chloroplast β- and ε-subunits did not functionally replace the corresponding *E. coli* subunits. In order to test the effect of the chloroplast β-subunit alone, in the presence of the *uncD436* allele, a plasmid was constructed in which a fragment carrying the *uncC* gene was inserted into a unique *Hpa*I site in the plasmid pAN480. This *Hpa*I site was in the *atpE* gene downstream from the translation start site. No differ-

ences could be found between a strain carrying this plasmid and that carrying pAN480 (data not shown).

The membrane preparations of strains AN2809 and AN2811 were examined by two-dimensional gel electrophoresis (Fig. 2). A very low level of F_1-ATPase α-subunit was present in membranes from strain AN2809 (pUC18/*uncD436*) but all other F_1-ATPase subunits and the *b*-subunit of the F_0 were absent. However, strain AN2811 (pAN480/*uncD436*) had a relatively large amount of CF_1 β-subunit associated with the membranes plus an increased level of *E. coli* α- and a low level of the γ subunit (Fig. 2). The *b*-subunit was observable in the original gels but was present at only a very low level. The amount was consistent, however, with the low level of reconstituted ATP-dependent atebrin fluorescence quenching activity obtained for membranes from strain AN2811.

Discussion

Heterologous expression of the chloroplast *atpB* gene has previously been achieved either in vivo as a fusion protein in *E. coli* [24] or in vitro using an *E. coli* S30 extract for transcription and translation [7]. Westhoff et al. [21] reported expression, in a rabbit reticulocyte system, of the spinach chloroplast genes encoding both the CF_1 α- and β-subunits. Fragments of these subunits were also formed and these were attributed to premature termination of translation. The present work demonstrates that incorporation of a consensus Shine-Dalgarno sequence (GAGGA) seven bases from the start codon of the *atpB* gene increased expression levels of the chloroplast β-subunit in *E. coli* and reduced the synthesis of lower molecular weight polypeptides presumably by reducing translation initiation at internal sites. The *atpE* gene was also expressed. Although this gene overlaps the 3′ end of *atpB* by four bases, a Shine-Dalgarno sequence GGAGA is found embedded in the coding region of *atpB* 13 bases from the start codon [7].

In this study and a previous paper [9] we have attempted to substitute chloroplast homologues for subunits of *E. coli* ATP synthase in order to gain insights into the assembly, function and evolution of this membrane-bound complex. The similarities of subunit composition, amino acid sequences and gene operon structure (see Ref. 8) provided a basis for the hope that in the absence of a particular *E. coli* subunit, the expressed chloroplast gene could provide a replacement in vivo. Indeed, chloroplast subunit I was able to substitute for subunit *b* in the *E. coli* F_0 [9] despite only 19% identity of their amino acid sequences [6]. Predictions of secondary and tertiary structures of the *b*-subunit of *E. coli* and CF_0I were similar [6] and it would appear that structure rather than identity of amino acid sequence is important in these subunits. The chloroplast and *E. coli* β- and ϵ-subunits share 66 and 26% sequence identity, respectively [7]. Two *uncD* mutant strains were used as recipients for the plasmids expressing the chloroplast subunits. The *uncD409* allele produces a mutant β-subunit which affects assembly of the F_0F_1-ATPase [25]. The chloroplast β-subunit was presumably unable to displace this mutant polypeptide since neither proton pore nor ATPase function was observed. The second allele, *uncD436*, results in the lack of formation of both the β- and ϵ-subunits [26] and the equivalent chloroplast subunits were unable to replace them.

It has been shown previously that the β-subunit of the ATP synthase plays a role in coordinating the assembly of the membrane or F_0 sector such that at no stage during assembly is an open proton pore formed (see Ref. 23). This function of the β-subunit is quite distinct from its catalytic role in the fully assembled ATP synthase. The presence of the *uncD436* allele, because of its inability to form the β-subunit, does not form a functional F_0 sector. It is clear from the ATP-dependent atebrin fluorescence quenching obtained with the stripped and reconstituted membranes from strain AN2811 that the chloroplast β-subunit is able to replace the function of the *E. coli* β-subunit for the assembly of the F_0 portion of the ATP synthase.

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