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Three Genes Coding for Subunits of the Membrane Sector (F_0) of the *Escherichia coli* Adenosine Triphosphatase Complex

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Two mutant *unc* alleles, *unc-469* and *unc-476*, have been characterized as affecting a previously undescribed gene, designated *uncF*. The *uncF* gene is part of the *unc* operon (with the gene order being *uncBFEAGDC*), although some uncertainty remains as to the relative order of the *uncF* and *uncE* genes. Mutant strains carrying the *uncF469* or *uncF476* allele lack the 18,000-molecular-weight component of the F_0 sector of the adenosine triphosphatase in the cell membrane but retain the dicyclohexylcarbodiimide-binding protein (molecular weight, 8,400). Conversely, strains carrying mutations in the *uncE* gene lack the dicyclohexylcarbodiimide-binding protein but retain the 18,000-molecular-weight protein in the cell membrane. Strains carrying mutations in the *uncB* gene have both the 18,000-molecular-weight protein and the dicyclohexylcarbodiimide-binding protein present in the cell membranes. The three proteins of the F_0 portion of the adenosine triphosphatase, viz., 24,000, 18,000, and 8,400 molecular weights, became membrane associated after in vitro transcription-translation with plasmid pAN51 as template. Plasmids carrying deletions which affected the *uncBFE* region were isolated from plasmid pAN51 and characterized genetically. A comparison of the genes that were absent from the various deletion plasmids with the membrane-associated products formed after in vitro transcription-translation indicated that the *uncB* gene coded for the 24,000-molecular-weight protein and that the gene order was probably *uncBFE*. A correlation between length of deoxyribonucleic acid, genes present, and their products is presented in relation to plasmid pAN51.

The genes forming the *unc* operon in *Escherichia coli* code for the components of the ATPase complex and map at about 82.5 min on the *E. coli* chromosome (1). Previous studies have led to the identification of six of the genes in the *unc* operon and established that the order of these genes is *uncBEAGDC*, the *uncB* gene being closest to the promoter (8, 9, 18).

The structure of the ATPase in *E. coli* appears to be similar to the structure of the ATPases in chloroplasts and mitochondria (31). The F_1 portion of the ATPase complex from *E. coli* consists of five subunits, designated α, β, γ, δ, and ϵ, in order of decreasing molecular weights, and these subunits form a complex of about 350,000 molecular weight (see reference 20). With mutant strains of *E. coli*, it was shown that the *uncA*, *uncD*, and *uncG* genes code for the α, β, and γ subunits of the F_1 ATPase, respectively (8, 10, 11, 22, 32). There is also some evidence that the *uncC* gene codes for the ϵ subunit (8).

Gel electrophoresis of preparations of the purified ATPase complex from *E. coli* have indicated that the complex contains three polypeptides attributable to the F_0 portion and that these polypeptides have molecular weights of 24,000, 19,000, and 8,400 (14). However, other workers have reported different molecular weights or number of subunits or both (15, 21, 29). The 19,000-molecular-weight polypeptide probably corresponds to the polypeptide, estimated to be of 18,000 molecular weight, formed from a plasmid (pAN51) carrying the known genes (*uncB* and *uncE*) coding for components of the F_0 ATPase (8). It was concluded, on the basis of membrane reconstitution experiments, that mutations in the *uncB* or *uncE* genes affect the F_0 portion of the ATPase complex (3, 9) although specific gene-polypeptide designations have not been made. The work described in the present paper characterizes a third gene, *uncF*, coding for a component of the membrane part of the ATPase, and evidence is presented for the

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identification of the polypeptides coded for by the *uncB*, *uncE*, and *uncF* genes.

MATERIALS AND METHODS

Enzymes and chemicals. Restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs (Beverly, Mass.). L-[^{35}S]methionine (1,200 Ci/mmol) was obtained from The Radiochemical Centre (Amersham, England). Agarose and chloramphenicol were obtained from Sigma Chemical Co. (St. Louis, Mo.), and cesium chloride was from British Drug Houses Chemicals Ltd., Poole, England.

Bacterial strains and plasmids. All of the bacterial strains used were derived from *E. coli* K-12 and are described in Table 1 or in the text. Strains used for the selection of recombinant plasmids were derivatives of *E. coli* K-12 C600. Plasmids used are described in Table 1, Fig. 4, and the text. Plasmid DNA was prepared as described by Selker et al. (30).

Genetic techniques. The techniques used for genetic experiments were as outlined previously (17, 18). Transformations were carried out as described by Lederberg and Cohen (23).

Analysis of plasmid DNA. Plasmid DNA was digested with restriction endonuclease *Bam*HI, *Bgl*II, *Eco*RI, or *Hin*dIII for 1 h at 37°C and with *Pst*I for 1 h at 30°C according to the manufacturer's (New England Biolabs) instructions. The digested DNA samples were electrophoresed in 1% agarose gels as described previously (8). We calculated the sizes of the DNA fragments, using as standards the fragments obtained after digestion of phage λ DNA with *Hin*dIII.

Polyacrylamide gel electrophoresis. Proteins were separated by two-dimensional gel electrophoresis as described previously (27, 28) or by sodium dodecyl sulfate slab gel electrophoresis (6). Proteins were stained with Coomassie blue R250, and peptides labeled with [^{35}S]methionine were identified by autoradiography of dried gels.

Identification of the DCCD-binding protein from membranes. The membranes were extracted and chromatographed on DEAE-cellulose as described by Fillingame (13). Membranes (about 40 mg of protein per ml) were reacted, where indicated, with dicyclohexylcarbodiimide (DCCD) for 24 h at 5°C.

In vitro protein synthesis. The method used for in vitro protein synthesis was as described by Zalkin et al. (35) and Zubay et al. (36), except that methionine was omitted from the reaction mixture and carrier-free [^{35}S]methionine (100 to 150 μCi/ml) was included. Plasmid DNA was added to a final concentration of 100 to 200 μg of DNA per ml. The reaction mixture (200 μl) was incubated at 34°C for 30 min, and the reaction was terminated by the addition of 12.0 ml of buffer containing 5 mM *N*-tris(hydroxymethyl)-methyl-2-aminomethane sulfonic acid (pH 7.0), 15% glycerol, 0.5 mM dithiothreitol, 0.5 mM EDTA, and 40 mM ε-amino-*n*-caproic acid. A membrane fraction was then prepared by centrifugation at 160,000 × *g* for 2 h, and the membranes were suspended in 100 μl of the same buffer system.

Chloroform-methanol-soluble proteins were extracted from 100 μl of the reaction mixture by adding 1.4 ml of $CHCl_3$-CH_3OH (2:1) and incubating the mixture for 20 h at 5°C. Insoluble material was precipitated by centrifugation (5 min in a Beckman Microfuge), and the chloroform-methanol supernatant was evaporated to dryness. The residue was solubilized (12) and electrophoresed (6).

Other methods. Media and growth of organisms (16), preparation of cell membranes (9), assay of ATPase activity (17), measurements of quinacrine fluorescence quenching of membranes (19), and determination of proteins (24) were as described previously.

RESULTS

Isolation and genetic characterization of strains carrying mutations affecting the *uncF* gene. Two mutant strains of *E. coli* K-12 (G38 and G101), unable to grow on succinate and having growth yields characteristic of mutants in which electron transport was uncoupled from oxidative phosphorylation, were isolated after mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (5). The mutant alleles, *unc-469* and *unc-476*, present in strains G38 and G101, respectively, were then cotransduced with *ilv* genes into strain AN346 to give the strains AN1419 (*unc-469*) and AN1515 (*unc-476*). These alleles were also incorporated into F-plasmids, by the method described previously (16), to form the new F-plasmids pAN54 and pAN61, respectively. Genetic complementation tests on succinate medium (17), using F-plasmids carrying the appropriate *unc* alleles, indicated that the new mutations did not complement with each other but did complement with point mutations in any of the previously described *unc* genes (*BEAGDC*). It was concluded that the new mutations were in a previously undescribed gene, which was designated *uncF*.

Complementation tests were carried out on succinate medium with donor strains carrying the F-plasmid pAN54 (*uncF469*) or pAN61 (*uncF476*) and a series of female strains carrying Mu-induced polarity mutations in the *unc* operon. From the results of complementation tests (Table 2), it was concluded that the *uncF* gene was between the *uncB* and *uncA* genes, but it was not possible to order the *uncE* and the *uncF* genes in the operon with the Mu-induced *unc* mutants available.

Lack of the 18,000-molecular-weight F_0 protein in membranes from *uncF* mutant strains. Membrane preparations from both strains AN1419 (*uncF469*) and AN1515 (*uncF476*) lacked ATPase activity. The cytoplasmic fractions, however, had ATPase activities of between 0.3 and 0.5 μmol of P_i/min per mg of protein, compared with a normal cytoplasmic ATPase activity of <0.1 μmol of P_i/min per mg of protein. The ATP-dependent membrane energization, as judged by quinacrine flu-

TABLE 1. *Strains of E. coli and plasmids used*

Bacterial strain or plasmid	Relevant genotype[a]/phenotype	Other information (reference)
AN346	*ilvC argH pyrE entA*	(3)
AN732	*argH pyrE entA metE46*	Laboratory stock culture
G38	*uncF469 argH pyrE entA metE46*	Isolated from strain AN732 after treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine
AN1419	*uncF469 argH pyrE entA*	Isolated after transduction of strain AN346 with strain G38 as donor
AN1440	*uncF469 argH pyrE entA recA srl*::Tn*10*	Isolated after transduction of strain AN1419 with strain NK5304 as donor
G101	*uncF476 argH pyrE entA metE46*	Isolated from strain AN732 after treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine
AN1515	*uncF476 argH pyrE entA*	Isolated after transduction of strain AN346 with strain G101 as donor
AN1521	*uncF476 argH pyrE entA recA srl*::Tn*10*	Isolated after transduction of strain AN1515 with strain NK5304 as donor
NK5304	*srl-1300*::Tn*10 recA56*	Obtained from D. Botstein and N. Kleckner (M.I.T.)
AN1456	*uncE429 thr leu hsdR hsdM srl*::Tn*10 recA*	Laboratory stock
AN284	*uncE408 argH entA*	Laboratory stock
AN936	*uncE429 argH pyrE entA*	(9)
AN1115	*uncE463 argH pyrE entA*	Laboratory stock
AN283	*uncB402 argH entA*	(3)
AN1156	*uncB454 argH pyrE entA*	Laboratory stock
AN1136	*uncB455 argH pyrE entA*	Laboratory stock
AN1305	pAN32/*uncB454 argH pyrE entA recA gyrA*	Laboratory stock
AN1020	pAN13/*uncE429 argH pyrE entA recA gyrA*	Laboratory stock
AN943	*uncE429 argH pyrE entA recA gyrA*	(9)
AN727	*uncB402 argH pyrE entA recA gyrA*	(7)
AN730	*uncA401 argH pyrE entA recA gyrA*	(7)
pAN54	*pyrE*$^+$ *uncF469 ilvC*$^+$ *argH*$^+$	F-plasmid carrying the *uncF469* allele isolated as described previously (16)
pAN61	*pyrE*$^+$ *uncF476 ilvC*$^+$ *argH*$^+$	F-plasmid carrying the *uncF476* allele isolated as described previously (16)
pAN51	Cm Tc^s *uncB*$^+$*F*$^+$*E*$^+$*A*$^+$	(8); this paper
pAN94	Cm Tc^s *uncA*$^+$	Deleted pAN51; this paper
pAN95	Cm Tc^s *uncF*$^+$*E*$^+$*A*$^+$	Deleted pAN51; this paper
pAN97	Cm Tc^s *uncB*$^+$	Deleted pAN51; this paper
pAN32	*pyrE*$^+$ *uncB454 ilvC*$^+$ *argH*$^+$	F-plasmid carrying the *uncB454* allele isolated as described previously (16)
pAN13	*pyrE*$^+$ *uncE429 ilvC*$^+$ *argH*$^+$	F-plasmid carrying the *uncE429* allele isolated as described previously (16)
pACYC184	Cm Tc	(4)

[a] Chromosomal gene nomenclature according to Bachmann and Low (1); plasmid gene nomenclature according to Novick et al. (26).

orescence-quenching activity, was absent from membrane preparations of both strains. NADH-dependent quinacrine fluorescence-quenching activity was normal (80%), indicating that the membranes were impermeable to protons (33). The membranes from the mutant strains, unlike those from normal strains (6), did not become permeable to protons after washing with low-ionic-strength buffer in the absence of *p*-aminobenzamidine. The binding of purified F_1 ATPase to washed membranes from both strains was less than 5% of that obtained with washed membranes from a normal strain. The above characteristics are similar to those obtained with membranes from strains carrying a mutation in the *uncE* gene (9), and it is similarly concluded that the *uncF* gene codes for a component of the F_0 portion of the ATPase complex.

Membrane preparations from strains carrying mutations in the *uncE*, *uncB*, or *uncF* gene were examined by two-dimensional gel electrophoresis. The electrophoretograms (Fig. 1) indicated

TABLE 2. *Complementation tests with plasmids carrying uncF alleles and Mu-induced polarity mutants affected in the unc operon*

Mu-induced *unc* mutant		Complementation[a] by plasmids carrying *uncF469* or *uncF476* alleles
Strain	Genotype	
AN887	*unc*::Mu($B^-E^-A^-G^-D^-C^-$)	−
AN888, AN1498	*unc*::Mu($B^+E^-A^-G^-D^-C^-$)	−
AN889, AN1495, AN1497, AN1499	*unc*::Mu($B^+E^+A^-G^-D^-C^-$)	+
AN1303, AN1314	*unc*::Mu($B^+E^+A^+G^-D^-C^-$)	+
AN1272, AN1313	*unc*::Mu($B^+E^+A^+G^+D^-C^-$)	+
AN869, AN883	*unc*::Mu($B^+E^+A^+G^+D^+C^-$)	+

[a] Complementation was indicated by the formation of transconjugant colonies on succinate medium after 2 days at 37°C.

that, whereas the membranes from three strains carrying different mutant *uncE* alleles and three strains carrying different mutant *uncB* alleles contained an apparently normal 18,000-molecular-weight component, membranes from the *uncF* mutants lacked this polypeptide, which is known to be a product of the *unc* genes (8). These results suggest that the *uncF* gene codes for the 18,000-molecular-weight polypeptide.

Lack of the DCCD-binding protein in membranes from *uncE* mutant strains. The effects of mutations in the *uncE* gene have been described previously, with the two mutant alleles studied being *uncE429* and *uncE408* (9). Membrane preparations from mutant strains carrying either the *uncE429* or *uncE408* allele contain an apparently normal 18,000-molecular-weight F_0 polypeptide (see above). Strains carrying mutations in the *uncB*, *uncE*, or *uncF* gene were examined for the presence of the DCCD-binding protein, using the chloroform-methanol extraction and chromatography procedures described previously (13). Membranes from strains carrying mutations in the *uncB* or *uncF* gene contained the DCCD-binding protein, but those membranes from strains carrying the *uncE429* allele or the *uncE408* allele did not (Fig. 2). These results are consistent with the *uncE* gene's being the structural gene for the DCCD-binding protein.

Construction of the plasmids, derived from pAN51 with deletions in the region of the *uncB*, *uncE*, or *uncF* gene. Plasmid pAN51, which carries the known genes coding for the F_0 portion of the ATPase complex (8), also carries the *uncF* gene since transformants which grew on succinate were obtained after transformation of strain AN1440 (*uncF469*) by this plasmid. Information concerning gene-polypeptide relationships for the *uncB*, *uncE*, and *uncF* genes could therefore be gained by using various deletion plasmids, derived from pAN51, in in vitro transcription-translation experiments.

A restriction map (Fig. 4) of the plasmid pAN51 was derived from the analysis of DNA fragments after digestion by the restriction endonucleases *Hin*dIII, *Bam*HI, *Eco*RI, *Pst*I, and/or *Bgl*II (Fig. 3; 8, 25; and data not shown). It has been shown previously that, if linear plasmid DNA is used in transformation experiments, deletions may occur at the ends of the DNA during ligation in vivo (34). This technique was used to form deletions around the *Pst*I site in pAN51. Thus, plasmid pAN51 was digested with *Pst*I restriction endonuclease, and this DNA was used to transform strain AN1456 (*uncE429*) to chloramphenicol resistance. The sizes of plasmids in the chloramphenicol-resistant transformants were determined by agarose gel electrophoresis of the DNA from lysed single colonies (2). Two of the transformants tested were found to contain plasmids smaller than plasmid pAN51; the smaller plasmids were designated pAN94 and pAN95, and the plasmid DNA was prepared. Neither of the plasmids could be digested by the restriction endonuclease *Pst*I (data not shown), indicating that these plasmids had deletions, including the *Pst*I site (see below).

Digestion of plasmid pAN95 with *Bam*HI indicated that all three *Bam*HI sites present in plasmid pAN51 were retained in plasmid pAN95. However, one of the *Bam*HI fragments of DNA (corresponding to the fragment carrying the *Pst*I site in plasmid pAN51) was reduced in size by about 100 base pairs (Fig. 3 and 4). Genetic complementation studies with plasmid pAN95 in transformation experiments (Table 3) indicated that the *uncB* gene was not expressed, whereas the *uncF*, *uncE*, and *uncA* genes were expressed.

The sizes of DNA fragments obtained after digestion of plasmid pAN94 with restriction endonuclease *Hin*dIII (Fig. 3) indicated that the cloned fragment of DNA carrying the *unc* genes was reduced in size from 4.3 to 3.5 kilobases. As judged from the DNA fragments present after digestion of plasmid pAN94 with *Bam*HI, the deletion does not extend beyond the first *Bam*HI site on the cloned fragment (Fig. 3 and 4). Genetic complementation studies with plasmid pAN94 (Table 3) indicated that the *uncA* gene was expressed but that the *uncB*, *uncE*, and *uncF* genes were not.

An additional plasmid, pAN97, resulting from a spontaneous deletion, was identified during the screening of a series of strains containing plasmid pAN51. Analysis of the plasmid pAN97

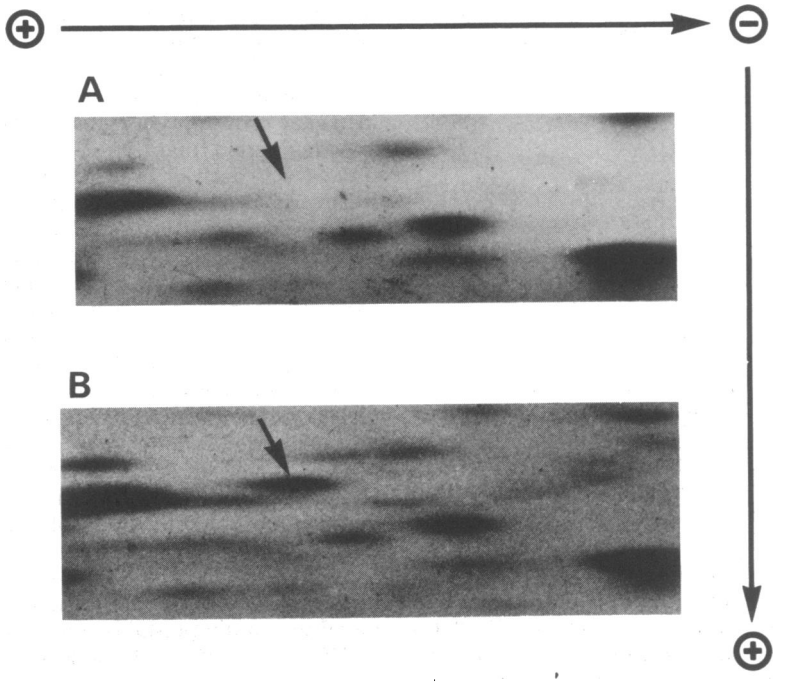

FIG. 1. *Two-dimensional gel electrophoresis of membrane proteins from (A) strain AN1419 (uncF469) and from (B) strain AN284 (uncE408). In the first-dimension isoelectric focusing gel, ampholines of pH ranges 5 to 7 and 3.5 to 10 were present at 1.2% (wt/vol) and 0.8%, respectively. In the second dimension, a sodium dodecyl sulfate-polyacrylamide slab gel was used with an acrylamide gradient of 10.5 to 24.5% (wt/vol). A similar protein pattern to (A) was obtained with membranes from strain AN1515 (uncF476), and protein patterns similar to (B) were obtained with membranes from strains AN936 (uncE429), AN1115 (uncE463), AN283 (uncB402), AN1156 (uncB454), and AN1136 (uncB455). The protein marked by an arrow and present in (B) but not in (A) has a molecular weight of 18,000 and an apparent isoelectric point of 5.8 and co-electrophoreses with a [^{35}S]methionine-labeled protein formed from the plasmid pAN51 (8). Only a portion of the gel is shown, and this portion covers a pH range of about 5.0 to 7.0 and a molecular weight range of about 23,000 to 11,000. About 500 μg of membrane protein was electrophoresed in each gel, and the proteins were stained with Coomassie blue R250.*

with the restriction endonuclease *Hin*dIII showed that the vector portion of the plasmid was unaffected by the deletion and that the deletion in the cloned fragment of DNA corresponded to about 1.4 kilobases (Fig. 3 and 4). Digestion of plasmid pAN97 with restriction endonucleases *Eco*RI, *Bam*HI, and *Bgl*II showed that the deletion eliminated the *Eco*RI site but did not extend into either the *Bam*HI site or *Bgl*II site (Fig. 3 and 4). Genetic complementation tests (Table 3) indicated that plasmid pAN97 expressed the *uncB* gene, but not the *uncF*, *uncE*, and *uncA* genes.

Analysis of membrane-bound proteins formed from plasmids pAN51, pAN94, pAN95, and pAN97. The 24,000- and 8,400-molecular-weight protein components of the F_0 portion of the ATPase complex do not enter the isoelectric focusing gel (8; R. H. Fillingame, personal communication) and, therefore, the two-dimensional gel system cannot be used to analyze for these proteins. The use of a one-dimensional sodium dodecyl sulfate gel electrophoresis system in the analysis of products from the in vitro transcription-translation experiments is complicated by the formation of a number of products derived from the vector portion of the plasmid DNA. To overcome these problems, we took advantage of the fact that the 24,000-, 18,000-, and 8,400-molecular-weight proteins are components of the membrane sector of the ATPase and become associated with the membranes in the in vitro transcription-translation system used. Thus, the plasmid pAN51 (*uncB*$^+$*F*$^+$*E*$^+$*A*$^+$), carrying the known genes coding for the membrane sector of the ATPase, and the plasmid vector pACYC184 were used as templates in an in vitro transcription-translation system containing [^{35}S]methionine as a radioactive label. After incubation, the reaction mixtures were diluted about 100-fold with buffer, and membrane fractions were prepared by cen-

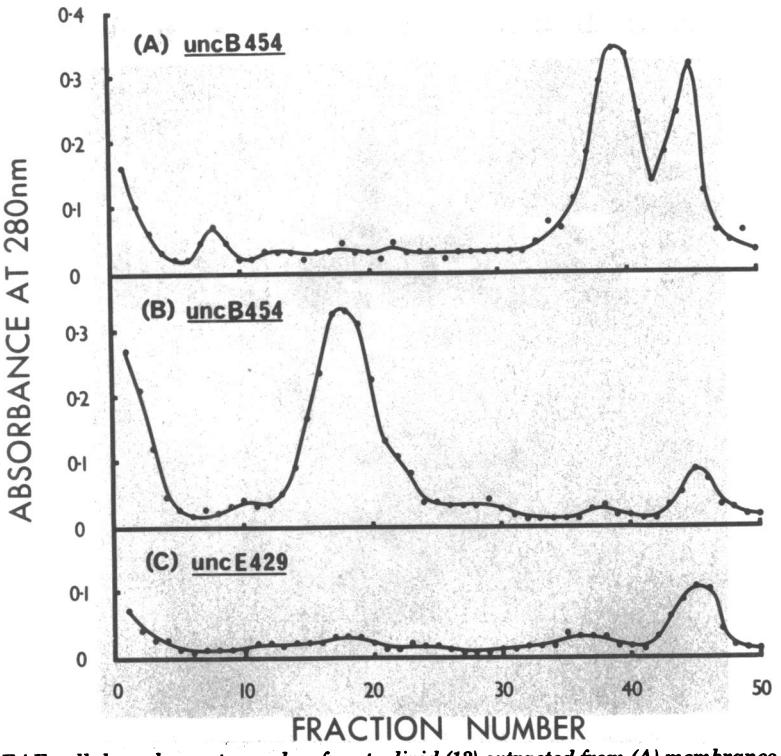

FIG. 2. *DEAE-cellulose chromatography of proteolipid (13) extracted from (A) membranes from the partial diploid strain AN1305 (uncB454/uncB454), (B) membranes from the partial diploid strain AN1305 (uncB454/uncB454) reacted for 24 h at 5°C with 1.0 mM DCCD, and (C) membranes from the partial diploid strain AN1020 (uncE429/uncE429) reacted for 24 h at 5°C with 1.0 mM DCCD. The membranes used for extraction were prepared from 300 g (wet weight) of cells. Crude proteolipid was applied to the DEAE-cellulose column, the column was washed with chloroform-methanol and chloroform-methanol-water mixtures, and the DCCD-binding proteins were eluted with a linear ammonium acetate gradient (0 to 45 mM) as described by Fillingame (13). Sixty 20-ml fractions were collected, and protein was detected by the absorbance at 280 nm. The shift in the protein peak (cf. A and B) was confirmed as being due to the reaction with the DCCD-binding protein with DCCD by the use of [^{14}C]DCCD; the peak of radioactivity cochromatographed with the major peak shown in B.*

trifugation. The membranes were solubilized, and the proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Radioactively labeled polypeptides were then identified by autoradiography of the dried gels. As shown in Fig. 5, a number of polypeptides fractionate with the membranes, and some are coded for by the vector pACYC184. Three components not coded for by the vector can be clearly identified (Fig. 5), and these components have molecular weights of 24,000, 18,000, and 8,400, corresponding to the proteins of the F_0 portion of the ATPase referred to above. Although the α and δ subunits of the F_1 portion of the ATPase complex are formed from plasmid pAN51 (8), they are not incorporated into the membranes during in vitro protein synthesis. The deletion plasmids pAN94, pAN95, and pAN97 were therefore used in an in vitro transcription-translation system, and the [^{35}S]methionine-labeled polypeptides were analyzed by one-dimensional sodium dodecyl sulfate gel electrophoresis of the membrane fractions prepared as described above.

The autoradiographs obtained (Fig. 5) when plasmid pAN95 (*uncF*$^+$*E*$^+$*A*$^+$) was used as a template in the in vitro transcription-translation system indicated that the 18,000-molecular-weight polypeptide was formed, but that the 24,000-molecular-weight component was not. A new polypeptide of 15,500 molecular weight was formed, and this polypeptide is presumably a fragment of the 24,000-molecular-weight polypeptide, shortened due to the deletion in the *uncB* gene (see above). The 8,400-molecular-weight polypeptide is also formed from plasmid pAN95 (data not shown), but to resolve the 24,000-molecular-weight polypeptide and the

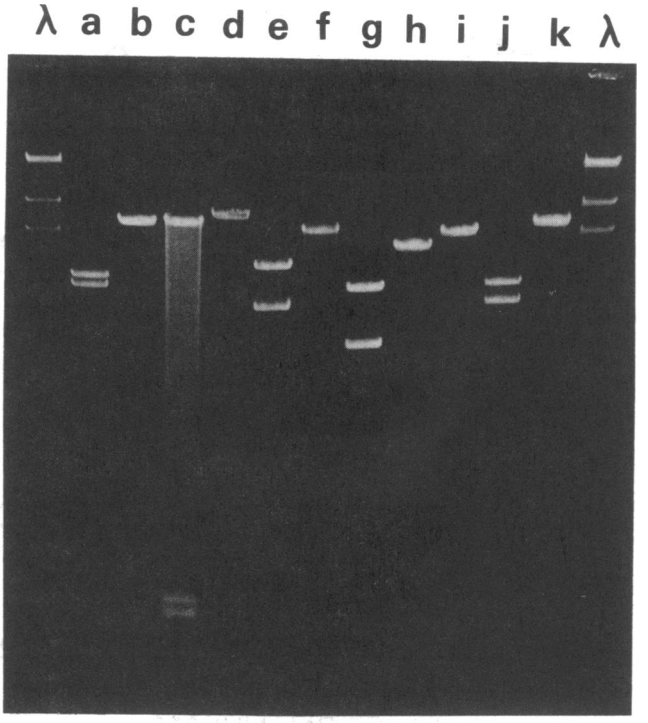

FIG. 3. *Electrophoresis of restriction endonuclease cleavage fragments of plasmid DNA. (a) pAN51 + HindIII; (b) pAN51 + BamHI; (c) pAN95 + BamHI; (d) pAN51 + BglII; (e) pAN51 + EcoRI; (f) pAN97 + EcoRI; (g) pAN97 + HindIII; (h) pAN97 + BamHI; (i) pAN97 + BglII; (j) pAN94 + HindIII; (k) pAN94 + BamHI; (λ) lambda DNA + HindIII. In lanes d and i, the BglII fragment of about 0.2 kilobases is not easily visualized.*

vector protein of similar molecular weight, it was necessary to electrophorese for a prolonged period; this resulted in the loss of the 8,400-molecular-weight polypeptide from the gel. The above results are consistent with the *uncB* gene's being the structural gene for the 24,000-molecular-weight component of the F_0 portion of the ATPase. The 24,000-molecular-weight polypeptide was formed when plasmid pAN97 (*uncB*$^+$) was used as a template in the in vitro transcription-translation system (Fig. 5). The subunits of molecular weights 18,000 or 8,400 were not formed. A polypeptide of a molecular weight of 14,000 was formed; this polypeptide had a net charge similar to that of the normal 18,000-molecular-weight subunit, as judged by its position after isoelectric focusing gel electrophoresis (data not shown). It is possible that this 14,000-molecular-weight polypeptide is a shortened form of the 18,000-molecular-weight subunit.

With plasmid pAN94 (*uncA*$^+$) as a template in the transcription-translation system, none of the subunits of the membrane sector of the ATPase was formed (Fig. 5), but a component of molecular weight 19,000 was formed. It appears possible that this component could be a hybrid polypeptide formed by a fusion of the *uncB* and *uncE* genes due to the deletion which spans the *uncB*, *uncF*, and *uncE* genes, since this polypeptide has one of the distinctive properties of the 8,400-molecular-weight subunit. Thus, the 8,400-molecular-weight subunit and the 19,000-molecular-weight hybrid polypeptide can be extracted from the transcription-translation reaction mixture with chloroform-methanol, whereas neither the 24,000- nor the 18,000-molecular-weight component is extracted under these conditions (Fig. 6).

DISCUSSION

The evidence described in this paper indicates that the three genes *uncB*, *uncF*, and *uncE* code for the three polypeptides, of molecular weights 24,000, 18,000, and 8,400, respectively, which form the F_0 sector of the ATPase complex in *E. coli* (14).

Strains carrying the *uncE429*, *uncE408*, or *uncE463* allele do not have the 8,400-molecular-weight DCCD-binding protein present in the cell membrane but do have the 18,000-molecular-

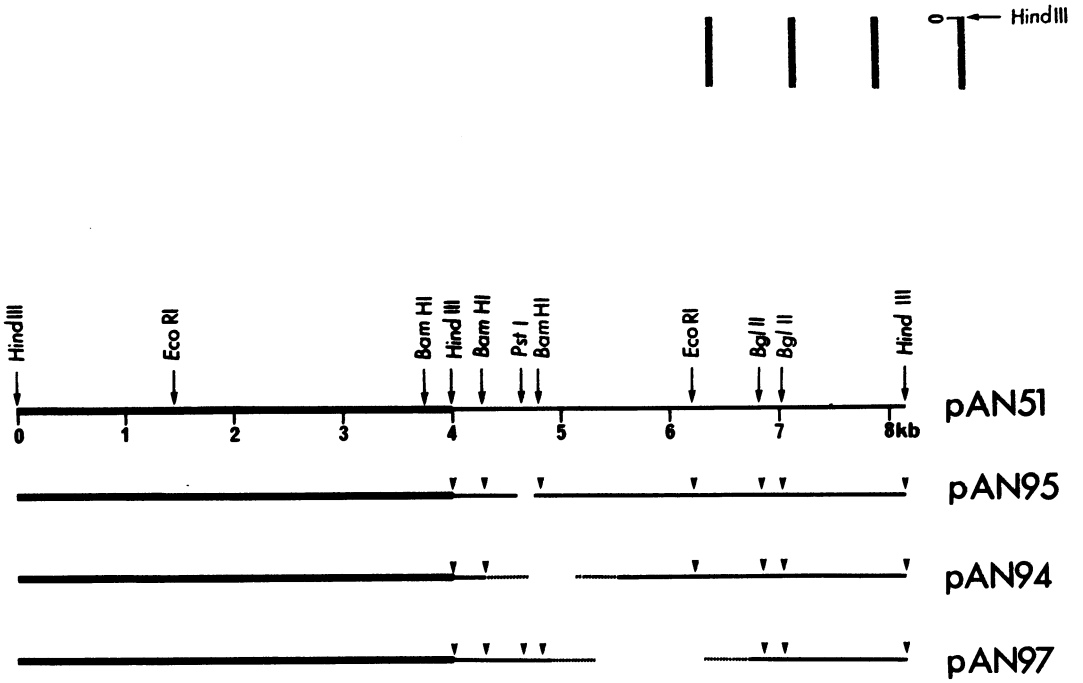

FIG. 4. *Restriction endonuclease cleavage maps of plasmids pAN51, pAN95, pAN94, and pAN97. The vector portion (pACYC184) of the plasmids is indicated by the heavy line, and the restriction endonuclease sites in the vector are taken from Chang and Cohen (4). The positions of the restriction endonuclease sites on plasmid pAN51 were estimated from the sizes of DNA fragments obtained after sequential digestions with the restriction endonucleases indicated. The sizes and location of the deleted regions of plasmids pAN94, pAN95, and pAN97 were calculated from the data in Fig. 3. The locations of the ends of the deletions are uncertain and are indicated by broken lines.*

weight protein. Conversely, strains carrying the *uncF469* or *uncF476* allele do not have the 18,000-molecular-weight protein but do have the DCCD-binding protein present in the cell membrane. These five mutant alleles do not exert polar effects on distal genes of the operon and are therefore not chain-terminating (nonsense) mutations. All five mutant alleles are revertible and are therefore not deletions. It appears, therefore, that these mutant alleles result in the particular F_0 proteins affected by the mutation not being incorporated into the membranes. No attempt has been made to identify the altered proteins in the cytoplasmic fraction. The presence or absence, in membranes from mutants, of the 24,000-molecular-weight protein coded for by the *uncB* gene could not be ascertained since this protein cannot be detected by the two-dimensional electrophoresis procedure used. However, mutations affecting any one of the *uncB*, *uncF*, and *uncE* genes cause the loss of proton permeability in washed membrane preparations, indicating that all three proteins are probably required for the formation of a functional proton pore.

TABLE 3. *Complementation between various plasmids and strains carrying known unc alleles*[a]

Plasmid	Recipient strain (chromosomal *unc* allele)			
	AN727 (*uncB402*)	AN1440 (*uncF469*)	AN943 (*uncE429*)	AN730 (*uncA401*)
pAN51	+	+	+	+
pAN94	−	−	−	+
pAN95	−	+	+	+
pAN97	+	−	−	−

[a] Mutant strains were transformed with plasmid DNA, chloramphenicol-resistant transformants were selected on nutrient medium (brain heart infusion, Oxoid Ltd., London, England) containing 100 μg of chloramphenicol per ml, and then complementation was determined after 1 to 3 days of growth on succinate-minimal medium plates containing 100 μg of chloramphenicol per ml.

Only the 24,000-molecular-weight protein of the three proteins of the F_0 portion of the ATPase was not formed from plasmid pAN95. This plasmid carries a deletion of about 100 base pairs, affecting the *uncB* gene but not the *uncE* and *uncF* genes. The *uncB* gene, therefore, probably codes for the 24,000-molecular-weight sub-

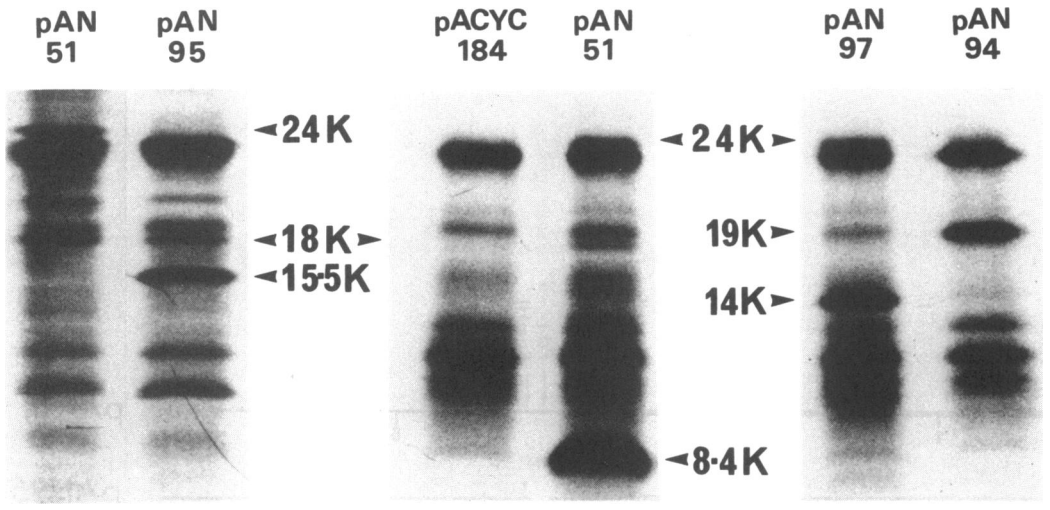

FIG. 5. *Autoradiographs of gels of [^{35}S]methionine-labeled peptides incorporated into membranes during in vitro transcription-translation. The plasmids indicated were used as templates in an in vitro transcription-translation system; a membrane fraction was prepared and then electrophoresed. The two gels on the left were electrophoresed for 16 h at 12 mA, and the component of 8,400 molecular weight (8.4K), coded for by plasmids pAN95 and pAN51, was electrophoresed off the gel. The other gels were run for 16 h at 8 mA.*

unit, and the 15,500-molecular-weight polypeptide formed from plasmid pAN95 is presumably a shortened *uncB* gene product. Since 100 base pairs would be equivalent to a polypeptide of about 4,000 molecular weight, it is possible that premature termination of protein synthesis occurred due to a frameshift at the site of the deletion. Similarly, plasmid pAN50, reported previously (8) to have a deletion corresponding to about the first 0.9 kilobases of the cloned fragment, complements *uncF* and *uncE* mutant strains, but not *uncB* mutant strains (8; J. A. Downie, unpublished data). The 18,000- and 8,400-molecular-weight proteins are formed in the transcription-translation system using this plasmid, but the 24,000-molecular-weight protein is not. Additional evidence that the *uncB* gene codes for the 24,000-molecular-weight protein is the observation that this protein was formed from the plasmid pAN97 (*uncB*$^+$), whereas the 18,000- and 8,400-molecular-weight proteins were not.

The deletion of about 800 base pairs in plasmid pAN94 (*uncA*$^+$) spans the *uncB*, *uncF*, and *uncE* genes, and a product of molecular weight 19,000 is formed from this plasmid. This product could be the result of a fusion between the *uncB* and *uncE* genes. Thus, the 19,000-molecular-weight product is extractable into chloroform-methanol, whereas the polypeptides of molecular weights 24,000 and 18,000 are not. Therefore, it is likely that the 19,000-molecular-weight polypeptide is formed as the result of a fusion of a major part of the 8,400-molecular-weight protein (which is soluble in chloroform-methanol) with part of the 24,000-molecular-weight protein. The above would suggest that the gene order is *uncBFE*. This gene order is consistent

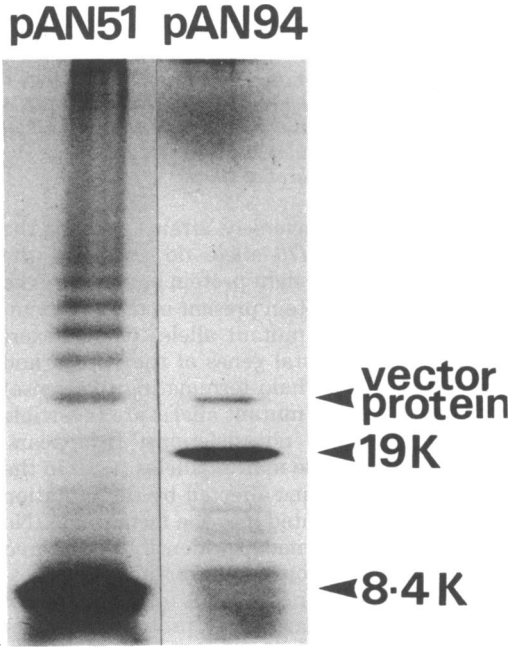

FIG. 6. *Autoradiograph of electrophoretogram of chloroform-methanol-soluble proteins formed by plasmids pAN51 and pAN94 during in vitro protein synthesis. The gel was run for 16 h at 8 mA.*

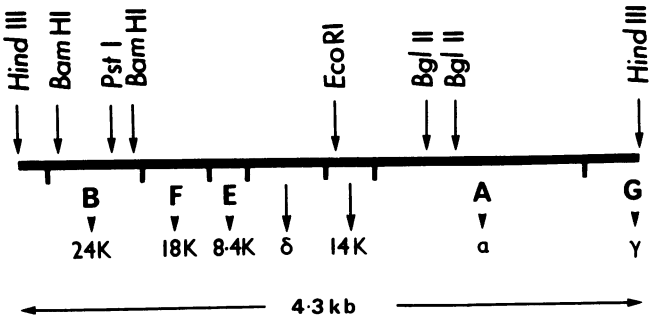

FIG. 7. *Gene-polypeptide relationships for the plasmid pAN51. The sizes of the genes were estimated from the molecular weights of their products, taking the value of 115 as the average molecular weight of an amino acid. The order of the genes and the subunits formed from them is as described in the text and as described previously (8, 10, 11, 22, 32). The order and identity of the genes forming the δ subunit and the 14,000-molecular-weight polypeptide are uncertain.*

with the observation that plasmid pAN97 (*uncB*$^+$) forms a protein which appears to have the characteristics of a shortened form of the 18,000-molecular-weight subunit. However, this 14,000-molecular-weight protein was present in membranes at a higher level than would be expected, judging from the amounts of the normal 18,000-molecular-weight subunit present. It is possible that the shortened form was more efficiently incorporated into the membrane fraction under the conditions of the experiments described here.

Two proteins, in addition to those referred to above, of molecular weights 17,000 and 14,000, have been reported previously as being products of genes located between the *uncB* and *uncG* genes. It is possible to detect the 17,000-molecular-weight protein on two-dimensional gels of solubilized membrane preparations if the membranes have previously been extracted with acetone (G. B. Cox, unpublished data). This protein is not detectable in acetone-extracted membranes prepared from mutant strains carrying the alleles *uncE408* and *uncE429*. It seems likely, therefore, that the 17,000-molecular-weight protein is the DCCD-binding protein electrophoresing as a dimer. We have been unable to detect the 14,000-molecular-weight polypeptide in membrane preparations, even from cells grown on succinate as carbon source (14), and this protein may be modified before assembly or may not be a structural component of the ATPase complex.

Strains carrying mutations in the genes coding for the δ subunit of the F_1 ATPase and for the 14,000-molecular-weight protein remain to be isolated, but these genes appear to be situated between the *uncE* and *uncA* genes. Gene-polypeptide relationships have now been suggested for all seven of the known *unc* genes, and these are summarized in Fig. 7.

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ADDENDUM IN PROOF

The 18,000-molecular-weight subunit reported here focuses at the same isoelectric point as the subunit of 19,000 molecular weight previously reported (14) to be present in the purified ATPase complex (R. H. Fillingame, personal communication), providing further evidence that these two polypeptides are the same.

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