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Proton Leakiness Caused by Cloned Genes for the F_0 Sector of the Proton-Translocating ATPase of *Escherichia coli*: Requirement for F_1 Genes

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To study expression of *uncG*, the gene coding for the γ subunit of the *Escherichia coli* proton-translocating ATPase, deletions were made in the intergenic region between *uncA*, the gene coding for the α subunit, and *uncG*. Two deletions which fused *uncA* and *uncG* coded for α-γ fusion polypeptides which were synthesized well both in vitro and in vivo, demonstrating that *uncG* expression is normally controlled by nucleotides in the intergenic region. Multicopy plasmids carrying these fusion genes and the genes for the other subunits of the ATPase had a harmful effect on the growth of *E. coli*. The effect was overcome by *N,N'*-dicyclohexylcarbodiimide, indicating that the cells probably leaked protons. The deleterious effect was eliminated by making a nonpolar deletion in the upstream F_0 gene *uncB*, or by cloning each of the *uncA-uncG* fusion genes onto a separate plasmid, removed from the F_0 genes, thus demonstrating that the fusion genes were not primarily responsible for the proton permeability. A plasmid which carried F_0 genes and the gene for the δ subunit caused deleterious proton leakiness in *unc*$^+$ cells but not in cells from which the *unc* operon was deleted. The proton leakiness caused by these different plasmids was therefore due to the production of a leaky F_0 proton channel and required the presence of F_1 genes. The results support a model for ATPase assembly in which F_1 genes or polypeptides are involved in the formation or opening of the F_0 proton channel.

The *unc* operon of *Escherichia coli* is located at 84 min on the *E. coli* chromosome and consists of nine genes, eight of which code for the subunits of the proton-translocating ATPase. The gene order and gene-polypeptide relationships have been established as *uncIBEFHAGDC*, coding for, respectively, protein i, a polypeptide of unknown function, and ATPase subunits a, c, b, δ, α, γ, β, and ε. Subunits a, b, and c form the F_0 sector of the ATPase, an integral membrane sector which conducts protons across the lipid bilayer. Subunits α, β, γ, δ, and ε form the F_1 sector which contains the catalytic sites for ATP synthesis and hydrolysis (for a review, see reference 10). Although the genes are present in equal numbers in the operon and the operon is transcribed as a single polycistronic mRNA (13), the polypeptides are present in different numbers in the ATPase complex. The stoichiometry is believed to be a1, c10, b2, δ1, α3, γ1, β3, ε1 (9). The genes have been shown to be differentially expressed both in vitro and in minicells, probably as the result of differential translation of the genes in the mRNA transcript (4, 18).

One particularly interesting question involving expression of *unc* genes concerns control over *uncG* expression. This gene codes for the γ subunit, which is present in one copy per ATPase, but *uncG* is situated between *uncA* and *uncD*, which code for the α and β subunits, respectively, both of which are present in three copies per ATPase. The explanation for this curious gene arrangement is not clear. It is also not clear how *E. coli* regulates the synthesis and assembly of the different ATPase subunits to produce a functional membrane-bound enzyme without forming either an unblocked proton channel or a functional soluble ATPase. Experiments by different groups of investigators have differed in their conclusions about requirements for the assembly of a functional F_0. Cox et al. (6) showed that the F_1 subunits α and β were both required for the F_0 sector to assemble. In those studies the integration of F_0 subunits into membranes was examined in cells which contained chromosomal mutations in specific *unc* genes. Results of initial studies by Klionsky et al. (15) on F_0 assembly in cells carrying plasmids containing different F_1 and F_0 genes appeared to have confirmed this result; but results of subsequent investigations by Aris et al. (1) and Fillingame et al. (8) demonstrated that F_0 genes on multicopy plasmids are sufficient to make a functional F_0 sector in the absence of F_1 genes.

The study described here was initiated to investigate the role of the nucleotides in the 50-base-pair intergenic region between *uncA* and *uncG*. Deletions of the DNA in this gap included two which fused *uncA* to *uncG*. Each fusion, when present in a plasmid carrying the genes for the rest of the subunits, had a deleterious effect on the cells that carried the plasmid. The effects of these fusions on *uncG* expression, the deleterious effect of these plasmids on cellular proton permeability, and the involvement of F_0 and F_1 genes in this effect are also described.

MATERIALS AND METHODS

Materials. Restriction and DNA-modifying enzymes were purchased from Bethesda Research Laboratories (Gaithersburg, Md.), New England BioLabs (Beverly, Mass.), and Boehringer Mannheim Biochemicals (Indianapolis, Ind.). [^{35}S]methionine (1,000 Ci/mmol) and [^{35}S]ATP (500 Ci/mmol) were purchased from New England Nuclear Corp. (Boston, Mass.). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.), Aldrich Chemical Co., Inc. (Milwaukee, Wis.), or J. T. Baker Chemical Co. (Phillipsburg, N.J.).

Bacterial strains, plasmids, and media. *E. coli* LE392 (F$^-$ *supF supE hsdR galK trpR metB lacY tonA*) or LE392 Δ(*uncB-uncD*) (15) were used for all cloning experiments. Strains 1100, RH304, RH343, and RH344 were described

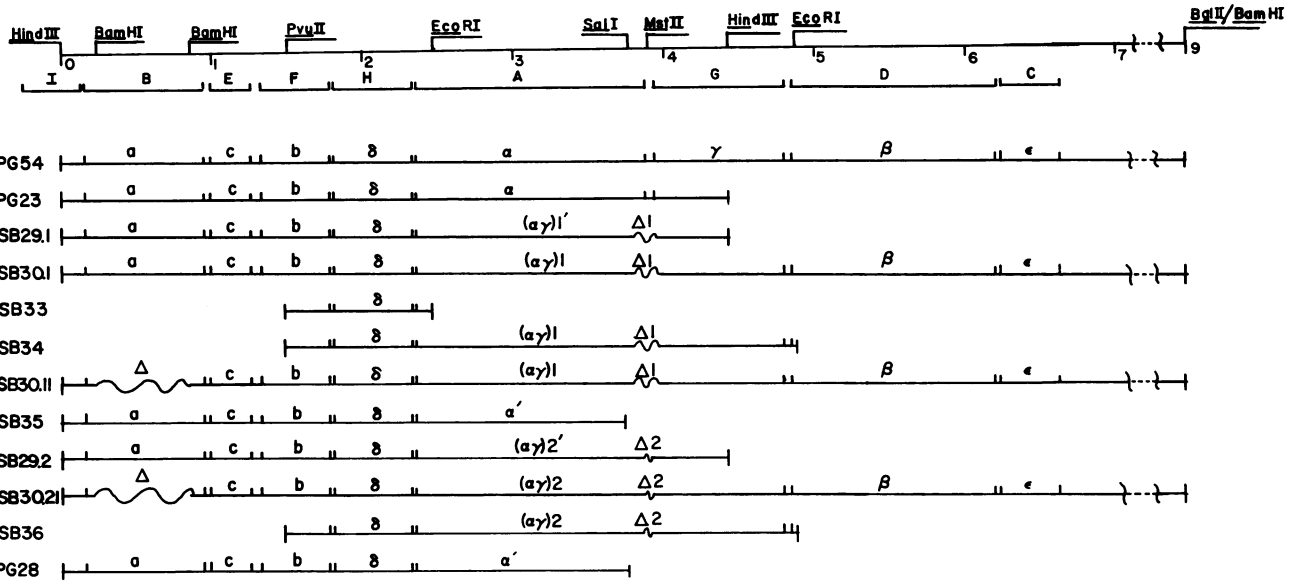

FIG. 1. Plasmids used or constructed for this study. The upper line indicates the restriction sites that are present in the *unc* operon (above the line) and length, in kilobases (below the line). Only those sites used to construct the various plasmids are indicated, so several *Pvu*II and *Sal*I sites and a second *Mst*II site, all of which were present in the operon, are not indicated. The second line shows the limits of the various genes of the operon. The *unc* promoter is located to the left of *uncI*. The designation for each plasmid is followed by a line indicating the limits of *unc* DNA present in that plasmid. The subunits coded for by each plasmid are shown above the location of the gene coding for each subunit. Partial subunits are indicated with a '. Deletions are indicated by a wavy line under a Δ. The two deletions described in the text are indicated by Δ1 and Δ2, which produced the fusion proteins (α-γ)1 and (α-γ)2, respectively. Plasmids pRPG23, pRPG28, and pRPG54 have been described previously (3, 11). Plasmids pWSB29.1, pWSB29.2, pWSB30.1, pWSB30.11, and pWSB30.21 are described in the text.

previously (12). Plasmids pWSB29.1 and pWSB29.2, which contained deletions between *uncA* and *uncG*, were constructed as follows. Plasmid pRPG23 was digested with the restriction endonuclease *Mst*II, which cut the plasmid once between *uncA* and *uncG*. The linearized DNA was digested with *Bal* 31 exonuclease. The digested DNA was ligated with T4 DNA ligase and used to transform *E. coli* LE392. Plasmid DNA from the transformants was prepared by the method described by Birnboim and Doly (2) and digested with *Mst*II, followed by agarose gel electrophoresis to identify those transformants that lost the *Mst*II site. To move those deletions into plasmids containing all the genes for the structural subunits of the ATPase (complete operon plasmids), the *Hin*dIII fragment containing each deletion was substituted for the equivalent, intact *Hin*dIII fragment in the complete operon plasmid pRPG54 as follows. Plasmid pRPG54 was digested with *Hin*dIII and treated with calf intestinal phosphatase. The resultant fragments were ligated to fragments from the *Hin*dIII-digested *uncA-uncG'* deletion plasmids described above. The ligated DNA was used to transform either *E. coli* LE392 or LE392 Δ(*uncB-uncD*). The DNA that was isolated from the transformants was digested with *Mst*II to identify those which contained a deletion. Plasmid pWSB30.0 carried the intact, unmodified *Hin*dIII fragment from pRPG23 and was thus identical to pRPG54. Plasmid pWSB30.1 carried the deletion originally constructed in pWSB29.1; and pWSB30.21, which is described in more detail below, carried the deletion that was originally constructed in pWSB29.2. All other plasmids were constructed as follows and are shown in Fig. 1. Plasmid pWSB33 was constructed by cloning the *Pvu*II-*Eco*RI fragment from pRPG23 into pUC9, which was digested with *Hin*cII and *Eco*RI. Plasmid pWSB34 was constructed by cloning the 2.4-kilobase-pair *Eco*RI fragment from pWSB30.1 into the *Eco*RI site of pWSB33. Plasmid pWSB35 was constructed by treating pWSB30.1 with *Sal*I and religating, thus deleting all the DNA between the *Sal*I site (Fig. 1) and a *Sal*I site in the pACYC184 vector DNA located to the right of the cloned *E. coli* DNA (Fig. 1). Plasmid pWSB36 was constructed by cloning the 2.4-kilobase-pair *Eco*RI fragment from pWSB30.21 into the *Eco*RI site of pWSB33. Of the plasmids that were not described, all were pACYC184 derivatives and thus carried resistance to chloramphenicol, except for pWSB29.1, pWSB29.2, pWSB33, pWSB34, and pWSB36, which were either pBR322 or pUC9 derivatives and thus carried resistance to ampicillin.

Plasmids pACYC184 (5) and pUC9 (21) were used as cloning vectors. LB medium contained the following (per liter): 10 g of tryptone (Difco Laboratories, Detroit, Mich.), 5 g of yeast extract (Difco), and 10 g of NaCl (17). Antibiotics were added to the medium at concentrations of 20 mg/liter for ampicillin and 25 mg/liter for chloramphenicol.

Fusion nomenclature. The fusion gene in plasmid pWSB30.1 contained the 5' 1,437 bases of *uncA* and the 3' 837 bases of *uncG*, is designated Φ(*uncA'-'uncG*)*1*(Hyb), and is referred to in this report as the *uncAG1* fusion gene. The hybrid polypeptide synthesized from this fusion gene contained the N-terminal 479 amino acids of the α subunit and the C-terminal 278 amino acids of the γ subunit and is called the (α-γ)1 polypeptide, with the parentheses designating the existence of a fusion between the subunits. The fusion gene in plasmid pWSB30.21 contained all of *uncA* except for the TAA stop codon, the last 30 bases of the *uncA-uncG* intergenic region, and all of *uncG* and is called the *uncAG2* fusion gene. The hybrid polypeptide that this gene codes for contained the entire α subunit, 10 amino acids coded for the DNA in the intergenic region, and the entire γ subunit and is called the (α-γ)2 polypeptide.

Other methods. DNA sequencing was carried out by the method described by Sanger et al. (19), as modified by

Bethesda Research Laboratories (Bethesda Research Laboratories Sequencing Manual), and [α-^{35}S]ATP was used to label the DNA. In vitro transcription-translation experiments were performed as described previously (3).

Immunoblotting procedure. Immunoblots of lysates from cells carrying various plasmids were developed with anti-F_1 antibody, which was kindly donated by Robert D. Simoni, Department of Biological Sciences, Stanford University, Stanford, Calif., followed by treatment with biotinylated goat anti-rabbit antibody and streptavidin-conjugated horseradish peroxidase, both of which were purchased from Bethesda Research Laboratories. The immunoblotting procedure was done as follows. Plasmid-containing *E. coli* was grown in LB medium to an optical density at 650 nm of 1.0, concentrated from 25 to 3 ml by centrifugation and resuspension, and lysed in a French press at 15,000 lb/in^2. The lysate was diluted 1:1 with 2× gel sample buffer, and 20 μl of each extract was electrophoresed on a 7.5% polyacrylamide gel (16). On this percentage of gel, only the α, β, and (α-γ) ATPase proteins were seen. The gel was soaked for 30 min in 25 mM Tris–192 mM glycine (pH 8.3), and the separated proteins were transferred to nitrocellulose by electrophoresis in 25 mM Tris–192 mM glycine (pH 8.3)–20% (vol/vol) methanol for 4 h at 60 V (0.25 to 0.35 A). The nitrocellulose paper was treated with Tween-saline (0.2% Tween 20, 0.9% NaCl, 10 mM Tris [pH 7.4]) for 12 h, and the ATPase proteins were visualized by incubating the nitrocellulose paper for 1 h with anti-F_1 antibodies (1/1,000 dilution of anti-F_1 antiserum in Tween-saline), washing with Tris-saline (0.9% NaCl, 10 mM Tris [pH 7.4]), incubating for 1 h with biotinylated goat anti-rabbit antibodies (diluted 1/1,000 in Tween-saline), washing with Tris-saline, incubating for 1 h with streptavidin-conjugated horseradish peroxidase (diluted 1/1,000 in Tween-saline), washing with Tris-saline, and finally treating with diaminobenzidine (0.5 mg/ml in 0.01% hydrogen peroxide, 10 mM Tris [pH 7.4]).

RESULTS

Construction of *uncA-uncG* fusion genes. Deletions of the intergenic region between *uncA* and *uncG* were constructed in two steps, as described above. First, pRPG23, a plasmid carrying *uncBEFHA* and part of *uncG*, was digested with the restriction endonuclease *Mst*II, which cut within the 50-base-pair *uncA-uncG* intergenic region. The linear DNA was treated with *Bal* 31 exonuclease and religated. Second, the *unc* DNA containing each deletion was transferred into a complete operon plasmid in a single *Hin*dIII cloning step. This procedure generated many different complete operon plasmids (although *uncI* was missing) carrying deletions of different sizes in the gap between *uncA* and *uncG*. There were two deletions, however, for which the second step of the procedure was initially unsuccessful, when the clonings were carried out in an *unc*$^+$ strain of *E. coli*. When I tried to reconstruct complete operon plasmids containing the *Hin*dIII fragment carrying these deletions, every resultant plasmid which contained the desired *Hin*dIII fragment carried it in the wrong orientation. DNA sequence analysis of the deletions revealed that both had fused *uncA* and *uncG* in frame, so in a complete operon plasmid the resultant *uncAG* fusion gene would code for a hybrid (α-γ) polypeptide with a molecular weight of 81,000 for one of these fusions and 88,000 for the other. The exact sequences of both deletions and the nomenclature used to describe them and the polypeptides they code for were given above.

I was able to clone the *uncAG1* fusion in a complete operon plasmid by carrying out the cloning using a strain

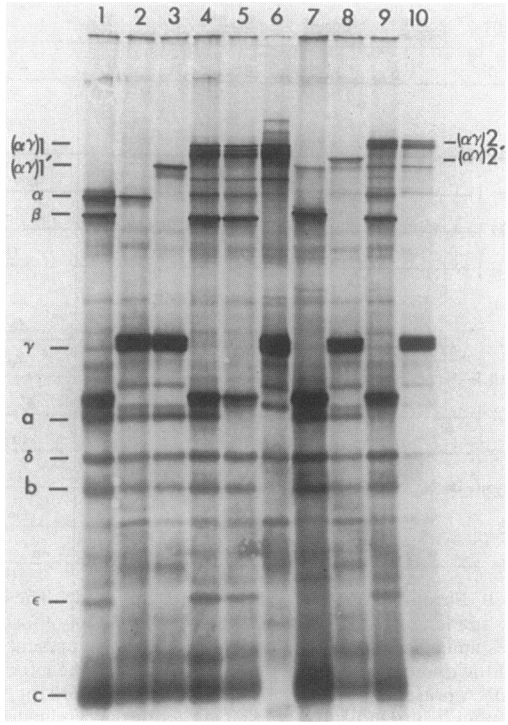

FIG. 2. In vitro transcription-translation products of plasmids. The plasmids described in the text were used to direct the in vitro synthesis of ^{35}S-labeled proteins. The proteins were separated on a sodium dodecyl sulfate-12.5% polyacrylamide gel and subjected to autoradiography. The locations of the ATPase polypeptide subunits are indicated to the left of the autoradiogram, as are the locations of the (α-γ)1 polypeptide and the (α-γ)1′ partial polypeptide. The locations of the (α-γ)2 polypeptide and the (α-γ)2′ partial polypeptide are indicated to the right of the autoradiogram. The plasmids used to direct synthesis of the proteins in each lane were pWSB30.0 (lane 1), pRPG23 (lane 2), pWSB29.1 (lane 3), pWSB30.1 (lane 4), pWSB30.11 (lane 5), pWSB34 (lane 6), pWSB35 (lane 7), pWSB29.2 (lane 8), pWSB30.21 (lane 9), and pWSB36 (lane 10). The proteins responsible for vector-encoded antibiotic resistance are the very dark bands running just above the a subunit in lanes 1, 4, 5, 7, and 9 (chloramphenicol resistance) or comigrating with the γ subunit in lanes 2, 3, 6, 8, and 10 (ampicillin resistance).

deleted for most of the ATPase genes, LE392 Δ(*uncB-uncD*). The resultant plasmid, pWSB30.1, coded for several proteins with molecular weights of approximately 80,000 (Fig. 2). These represent the hybrid (α-γ)1 fusion polypeptide (predicted molecular weight, 81,000) and smaller proteins which are probably either proteolytic products or prematurely terminated proteins. The fusion protein was synthesized very well in vitro compared with the γ subunit, which has been previously shown to be synthesized poorly or not at all in vitro (3, 7).

Deleterious effect of a complete operon plasmid carrying an *uncA-uncG* fusion gene. The presence of pWSB30.1 caused the deletion strain to grow more poorly on plates than the same strain carrying the vector plasmid pACYC184. When transformed into LE392 *unc*$^+$, however, pWSB30.1 was lethal or, at best, produced only pinpoint colonies on LB-antibiotic plates after several days of incubation at 37°C. These transformants did grow, however, if *N,N'*-dicyclohexylcarbodiimide (DCCD) was added to the plates. These observations indicate that the deleterious effect of pWSB30.1 is related to ATPase synthesis and assembly and

TABLE 1. Deleterious effect of pWSB30.1 on growth of *unc*$^+$ *E. coli*[a]

Host *unc* genotype	Plasmid	DCCD	Growth
unc$^+$	pWSB30.1	−	−
unc$^+$	pWSB30.1	+	+
Δ(*uncB-uncD*)	pWSB30.1	−	+
Δ(*uncB-uncD*)(pWSB30.1)	pRPG28	−	+ +
Δ(*uncB-uncD*)(pWSB30.1)	pRPG23	−	−
Δ(*uncB-uncD*)(pWSB30.1)	pRPG23	+	+

[a] *E. coli* LE392, with the indicated *unc* genotype, was transformed with the indicated plasmid and transferred to LB plates containing chloramphenicol. The hosts containing pWSB30.1 were transferred to plates that also contained ampicillin. DCCD was added to the indicated plates by the addition of a small chunk of DCCD (1 to 5 mg) to the center of each plate after the cells were transferred to the plates. A minus sign in the growth column indicates that no colonies were visible after 15 to 20 h of incubation at 37°C. A plus sign indicates that colonies were visible after 15 to 20 h of incubation but were noticeably smaller than colonies containing a control plasmid carrying the same antibiotic resistance gene as the indicated plasmid but no *unc* genes. The control plasmids were pACYC184 (chloramphenicol resistance) and pBR322 (ampicillin resistance). A double plus sign indicates that colonies were not noticeably different in size from those which contained control plasmids. Transformation efficiencies for all cells were within a factor of 5.

probably involves proton leakiness, since DCCD is known to block proton conduction through the F_0 (20). There was a strong selection against the harmful effects of pWSB30.1 in the *unc*$^+$ strain. Approximately 1 to 5% of transformants grew well; and analysis of the plasmids in those strains showed that pWSB30.1 was converted to pRPG54, the normal complete operon plasmid, probably by recombination with the chromosomal *unc* operon.

The next question addressed was which ATPase genes present in the *unc*$^+$ strain but absent in the *unc* deletion strain were required for the full deleterious effect. To answer this, plasmids carrying different ATPase genes were added to the deletion strain containing pWSB30.1. The results (Table 1) indicate that an added α subunit is required for the full deleterious effect. When plasmid pRPG23 was added to LE392 Δ(*uncB-uncD*)(pWSB30.1), the transformants did not grow on LB plates containing chloramphenicol and ampicillin. These cells did grow, however, if DCCD was added to the plates. When the same cells were transformed with pBR322 or pRPG28 instead of pRPG23, all the transformants grew well. The difference between pRPG23 and pRPG28, both of which have been described in detail previously (3, 11), is that pRPG28 is missing the 3′ 119 bases of *uncA* and all of the partial *uncG* that is present on pRPG23. Otherwise, pRPG28 and pRPG23 are identical, and neither by itself is harmful to cells.

To study the biochemistry of this deleterious effect, the *uncA-uncG* fusion gene was cloned behind the inducible *lac* promoter in the plasmid pUC9, as described above. Surprisingly, the resultant plasmid, pWSB34, was not harmful to either *unc*$^+$ or *unc*-deleted cells in the presence or absence of the inducer isopropyl-β-D-thiogalactopyranoside. The harmful effect of pWSB30.1, therefore, was not due solely to the production of the (α-γ)1 fusion protein. Other genes present on pWSB30.1 were required for the deleterious effect.

To investigate this requirement for other *unc* genes, I deleted the F_0 gene *uncB* from pWSB30.1 by digesting the plasmid with *Bam*HI and religating. The resultant plasmid, pWSB30.11 (Fig. 1), was not harmful to either *unc*$^+$ or *unc*-deleted cells. To ensure that no second-site mutations occurred, I restored the deleted *Bam*HI fragment to pWSB30.11 to recreate pWSB30.1, and the resultant plasmid was once again lethal to *unc*$^+$ *E. coli*. The deleterious effect was therefore caused at least in part by the F_0 genes present in pWSB30.1.

Effect of a second *uncA-uncG* fusion plasmid on the growth of *E. coli*. Subsequent studies on a second *uncA-uncG* fusion gene, *uncAG2*, produced similar results. This second fusion, which was constructed in the same way as the first, was only missing 20 bases from the gap between *uncA* and *uncG* and was missing the TAA stop codon for *uncA*. The resultant fusion gene consisted of the rest of *uncA*, 30 bases of the *uncA-uncG* intergenic region, and all of *uncG*. It therefore coded for an (α-γ)2 fusion polypeptide with a predicted molecular weight of 88,000. Attempts to carry out the second step of the fusion cloning, as described above, were unsuccessful even when the cloning was done in an *unc* deletion strain. The *Hin*dIII fragment carrying this second deletion could only be cloned into a complete operon plasmid in the wrong orientation with respect to the rest of the genes in the operon. The results from the experiments with the *uncAG1* fusion suggest that this second, larger *uncAG2* fusion gene might be harmful because of proton leakiness caused by the F_0 genes on the plasmid. A third step was therefore added to the complete operon construction to remove the F_0 gene *uncB*. After ligation of the *Hin*dIII fragments, the ligation mixture was treated with *Bam*HI restriction endonuclease and the resulting fragments were religated, thus deleting most of *uncB*. Cells containing the plasmid carrying the intact *uncAG2* fusion but missing *uncB* were then viable, and the resultant plasmid, pWSB30.21 (Fig. 1), coded for a set of (α-γ) 2 fusion polypeptides (Fig. 2, lane 9) of slightly higher molecular weight than those coded for by pWSB30.1. This plasmid did not affect the growth on LB antibiotic plates of either *unc*$^+$ or *unc*-deleted *E. coli*. When I attempted to reinsert the deleted *Bam*HI fragment into pWSB30.21 in an *unc* deletion strain, I found that of the 11 viable transformants which contained a plasmid carrying the small *Bam*HI fragment, all 11 carried it in the wrong orientation relative to the other *unc* genes, again demonstrating that the lethal effect involved F_0 function. I cloned the large *uncAG2* fusion gene onto a separate plasmid behind the inducible *lac* promoter; and the resultant plasmid pWSB36 (Fig. 2) had no extremely harmful effects on *unc*$^+$ or *unc*-deleted *E. coli* either in the presence or absence of the inducer isopropyl-β-D-thiogalactopyranoside, although the *unc*$^+$ strain carrying pWSB36 did grow slightly more poorly than the same strain carrying a control plasmid.

Assays for expression of the *uncA-uncG* fusion genes. Results of several experiments demonstrated that the *uncA-uncG* fusion genes were expressed even in the cells which were not affected by the presence of those genes, and that the *Bam*HI deletion in pWSB30.11 and pWSB30.21 had no polar effect on expression of downstream genes. In vitro transcription-translation of the plasmids carrying either of the *uncA-uncG* fusion genes (Fig. 2) revealed that both (α-γ) fusion proteins were synthesized very well in vitro, regardless of the presence or absence of the small *Bam*HI fragment in the plasmid. Each of the *uncA-uncG* fusion genes cloned behind the *lac* promoter and *uncH* in pWSB34 and pWSB36 was also expressed well in vitro. Finally, immunoblots of extracts of cells carrying the various fusion-containing plasmids revealed that the (α-γ) fusion proteins were synthesized well in all of the cells (Fig. 3). The absence of a harmful effect of these plasmids was not due to low or nonexistent expression of the fusion genes.

Effects on growth of *E. coli* of a plasmid carrying F_0 genes alone. The most straightforward explanation for the results of the effects of a plasmid carrying only F_0 genes on the

growth of *E. coli* is that without the presence of a normal *uncA*, *uncG*, or both, the high copy number of F_0 genes on the fusion plasmids results in the formation of an unplugged F_0, which leaks protons. Such an effect of overexpression of F_0 genes on multicopy plasmids has been reported previously (8). To test this possibility, I deleted the *uncAG1* fusion gene, *uncD*, and *uncC* from pWSB30.1 to produce a plasmid containing just the F_0 genes *uncB*, *uncE*, and *uncF* and the F_1 gene *uncH*. The resultant plasmid, pWSB35, which coded for the a, c, and b subunits of the F_0 and the δ subunit of the F_1 (Fig. 2, lane 7) did not affect the growth of *unc*-deleted cells but was harmful to *unc*$^+$ cells. This deleterious effect could be overcome by DCCD. Since pWSB35 appeared to have the same effect on *unc*$^+$ cells as pWSB30.1, the lethality of either of the plasmids carrying the *uncA-uncG* fusion genes along with F_0 genes was probably caused by the formation of a proton-leaky F_0 in those cells. Since an *unc* deletion strain was insensitive to pWSB30.1 and pWSB35, this deleterious effect required F_1 genes.

The effect of pWSB35 on *unc*$^+$ cells was strain dependent. In strain 1100, pWSB35 was lethal. In strain LE392, pWSB35 caused inhibition of growth, but cells carrying this plasmid did grow. This plasmid had no effect on the growth of an *unc*-deleted derivative of either strain; and for both *unc*$^+$ strains carrying pWSB35, the addition of DCCD significantly enhanced growth, so the effect of the plasmid was qualitatively the same but quantitatively different in these two strains of *E. coli*. Aris et al. (1) have noted that the amount of F_0 synthesized in cells carrying various F_0 plasmids was very strain dependent.

Requirement for F_1 genes of F_0-induced proton permeability. As an additional test of the requirement for F_1 genes, three different *unc* F_1 mutants were transformed with pWSB30.1 or pWSB35 and plated onto LB-chloramphenicol plates with or without DCCD. The results (Table 2) showed that pWSB30.1 is very harmful to the γ and β mutants and their *unc*$^+$ parent strain 1100, but the deletion strain and the α mutant containing this plasmid did grow. When the same strains were transformed with pWSB35, the deletion strain and the α mutant still grew, and strain 1100 and the β mutant did not grow, but the γ mutant did grow and thus appeared to be resistant to the lethal effects of this plasmid. Although growth of RH343(pWSB35) is scored ++ (Table 2), these cells did not grow quite as well as RH304(pWSB35). When the transformants were incubated for an additional day at 37°C, even the β mutant transformed with pWSB35 started to form small colonies. In every case, pWSB30.1 had a more severe effect on growth than did pWSB35, and in all cases the addition of DCCD to the plates resulted in significantly increased growth of colonies. The proton leakiness caused by the F_0 genes on pWSB30.1 and pWSB35 therefore required F_1 genes, with the strongest requirement being for *uncA*. Results of the studies with mutants indicated a weaker involvement of *uncG* in F_0 leakiness and an even weaker involvement of *uncD*. The requirement for a γ subunit in the deleterious effect could apparently be partially fulfilled by the (α-γ)1 fusion protein coded for by pWSB30.1.

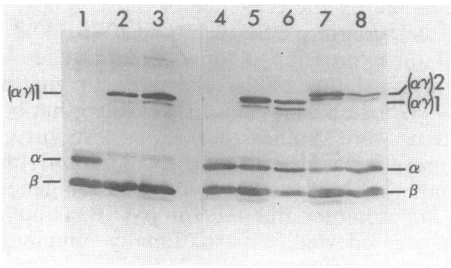

FIG. 3. Immunoblots of extracts of cells carrying *uncA-uncG* fusion plasmids. The locations of the α, β, (α-γ)1, and (α-γ)2 proteins are indicated next to the immunoblot. The proteins run in lanes 1 to 3 were from LE392 Δ(*uncB-uncD*) carrying pRPG54 (lane 1), pWSB30.1 (lane 2), and pWSB30.11 (lane 3). The proteins run in lanes 4 to 8 were from LE392 *unc*$^+$ carrying pRPG54 (lane 4), pWSB30.11 (lane 5), pWSB34 (lane 6), pWSB30.21 (lane 7), and pWSB36 (lane 8).

TABLE 2. Effects of plasmids on the growth of *unc* mutants[a]

E. coli strain	Effect of the following F_0 plasmids after the indicated times:			
	pWSB30.1		pWSB35	
	1 day	2 days	1 day	2 days
1100	−	−	−	−
1100 Δ(*uncB-uncD*)	+	+	++	++
RH304 (α$^-$)	+	+	++	++
RH343 (γ$^-$)	−	+	++	++
RH344 (β$^-$)	−	−	−	+

[a] The *unc* mutants RH304, RH343, and RH344, defective in *uncA* (α), *uncG* (γ), and *uncD* (β), respectively, were transformed with pWSB30.1 or pWSB35 and plated on LB-chloramphenicol plates. Growth was scored after 24 and 48 h, as described in footnote *a* of Table 1. As *unc*$^+$ and *unc* mutant controls, *E. coli* 1100, the parental strain for the mutants, and *E. coli* 1100 (*uncB-uncD*) were also transformed with these plasmids. The addition of DCCD to the plates converted all −'s to +'s. All strains were *recA56* to eliminate recombination with chromosomal *unc* genes.

DISCUSSION

This study was undertaken to determine the role of the nucleotides in the regions between *uncA* and *uncG* in the expression of *uncG*. The gene which codes for the γ subunit, present in one copy per ATPase complex, is located between two genes which code for the α and β subunits, each of which is present in three copies per complex. Understanding the regulation of synthesis of the γ subunit and the advantage of this particular arrangement of genes is thus important to the understanding of the biogenesis of ATPase. In vitro, *uncG* is expressed very poorly, and the low expression has been attributed to control of translation initiation (3), the frequency of rare codons present in *uncG* (14, 22), or to increased proteolytic sensitivity of the γ subunit (7). In-frame fusion of *uncG* to *uncA*, so that the *uncG* gene was expressed from whatever translational signals governed *uncA* expression, produced fusion polypeptides which were synthesized well both in vitro and in vivo. This result demonstrates that synthesis of the γ subunit is probably not limited by codon usage or gross proteolytic sensitivity of the γ subunit, since both fusion proteins contained all or almost all of the γ subunit. The DNA in the intergenic region, therefore, probably plays a role in the normal translational regulation of *uncG* expression. However, the fact that both (α-γ) fusion proteins were synthesized both in vitro and in vivo as multiple species demonstrates that perhaps there are specific signals in the DNA or mRNA near the 3′ end of *uncG* that result in the production of appreciable amounts of partial *uncG* gene product.

Both *uncA-uncG* fusion genes, however, when present in a plasmid containing the other *unc* genes, were lethal to *E. coli unc*$^+$. A plasmid containing the larger of the two fusion

genes was apparently lethal even to a deletion strain, since it could not be constructed. Results of several experiments demonstrated that the lethality is not a function of the fusion genes or the hybrid (α-γ) proteins for which they coded, but rather was probably due to a decontrol of F_0 assembly or function, resulting in harmful proton leakiness in cells carrying these plasmids.

The harmful effects of pWSB30.1 or pWSB35, which carries just the three F_0 genes and *uncH*, required F_1 genes, particularly *uncA*. Transformation of *uncA*, *uncG*, and *uncD* mutants with pWSB30.1 and pWSB35, however, showed that the γ and the β subunits were also involved in producing the proton permeability caused by these plasmids, although not to as great an extent as the α subunit. The observation that pWSB30.1, which carries the fusion genes and all the other ATPase genes, was in every case more harmful than pWSB35 also demonstrated that genes besides just the F_0 genes were involved in producing the harmful proton permeability.

It is not known if the observed proton permeability is due to the F_1 subunit-catalyzed formation of a functional proton channel or the opening of a nonleaky but functional F_0 by F_1 subunits. Relatively small differences in F_0 activity (proton conduction, reconstitution with F_1) have been attributed to F_1 subunits that protect F_0 subunits from proteolysis (1, 8). One or more of the F_0 subunits may be rapidly degraded in the absence of one or more of the F_1 subunits. Such a model could account for the results reported here. In this case, however, the presence of F_1 subunits produces dramatic physiological differences (lack of growth in the absence of DCCD) which imply a more significant role for F_1 subunits in F_0 proton permeability than the effects measured previously. A biochemical analysis of the leaky cell membranes awaits the development of an inducible lethal system. It has been shown previously that induction of the overexpression of F_0 genes in the absence of F_1 genes can produce a lethal leaky F_0 (8). To properly study the biochemistry of the F_1-dependent process described here, however, the synthesis of F_1 subunits, especially the α subunit, must be induced in the presence of a nonlethal pWSB30.1 (i.e., in an *unc* deletion strain).

In the initial studies on F_0 assembly by Cox et al. (6), two-dimensional gel electrophoresis of membrane proteins isolated from different *unc* mutants was used, combined with an analysis of how well stripped membranes from those mutants could be reconstituted with purified F_1, to determine the requirements for the assembly of an F_0. Aris et al. (1) and Fillingame et al. (8) both used fluorescence quenching assays to measure membrane energization in cells carrying various F_0 plasmids and to measure the reconstitution of F_1 with the F_0 in those membranes. The study reported here was genetic. *E. coli* that are *unc*$^+$ either fail to grow or grow poorly when carrying certain F_0 plasmids, plasmids which have little or no effect on the growth of cells from which the *unc* operon was deleted. This inhibition of growth is a function of ATPase genes, and its reversal by DCCD indicates that it involves proton conduction through the F_0 sector.

From the various studies on F_0 assembly from genes on plasmids, it is difficult to determine in which situation the F_0 subunits are synthesized and assembled in a fashion similar to their synthesis and assembly from chromosomal genes in vivo. Results of the studies by Aris et al. (1) and Fillingame et al. (8) demonstrated that F_1 genes or proteins are not essential for the synthesis and assembly of a functional F_0. Also, Fillingame et al. (8) were able to demonstrate that F_0 genes alone made cells leak protons when those genes were transcribed from a strong, inducible promoter (8). In neither study, however, was it concluded that in vivo assembly of the F_0 was not facilitated by F_1 genes or subunits. There was no discussion by Aris et al. (1) of the effects of their F_0 plasmids on the growth of *unc*$^+$ cells, although they reported several results consistent with the involvement of F_1 in F_0 assembly. It was noted that *unc*-deleted strains carrying an F_0 plasmid grew at the same rate as the same strain carrying a non-F_0 plasmid. It was also noted that a strain deleted for the chromosomal F_1 genes did not contain any membrane-bound F_0 subunits. Finally, the presence of F_1 genes resulted in a 50% increase in F_0 activity produced by an F_0 plasmid. In the study on F_0 assembly by Fillingame et al. (8), the amount of F_0 produced from a multicopy plasmid carrying the F_0 genes was less in an *unc* deletion strain than in an *unc*$^+$ strain (8). In both studies, these results were attributed to the possibility that F_1 subunits protected the F_0 subunits from proteolysis. It may be, however, that the absolute and relative amounts of F_0 and F_1 subunits are extremely important in the in vivo synthesis of an F_0. None of the plasmids used in those two studies or this study contained the true *unc* promoter. Gene expression has been driven by plasmid promoters, some of which are well defined and some of which are not. If the F_1 subunits serve a catalytic role in F_0 assembly, then the level of expression of F_0 genes would critically influence the effects of those F_1 subunits. Very high rates of synthesis of F_0 subunits, as were present in the study done by Fillingame et al. (8), might then be expected to result in F_1-independent F_0 assembly and proton leakiness. Results of my study indicate that F_1 genes or polypeptides are involved in proton permeability caused by F_0 genes cloned in the plasmids described here, a conclusion which supports the basic conclusions of Cox et al. (6). Perhaps these plasmids fortuitously express the ATPase genes at levels that more closely resemble those produced from the chromosomal genes than do the plasmids used in the other studies (6, 8). Such an explanation might also account for the nonlethality of pRPG23, pRGP28, pWSB29.1, and pWSB29.2 (Fig. 1), all of which contain F_0 genes, but none of which is lethal to *unc*$^+$ *E. coli*. These plasmids are pBR322 derivatives, and the lethal plasmids are pACYC184 derivatives. There might be some effect of plasmid copy number or transcription through the cloned region which makes one set harmful to an *unc*$^+$ strain and the other harmless.

Although both the γ and the β subunits are involved in F_0 gene-dependent proton leakiness, the α subunit appears to be the most important F_1 subunit involved in this phenomenon. Plasmid pWSB30.1, which synthesizes the (α-γ)1 polypeptide, requires an added α subunit for its harmful effect. Apparently, the same plasmid coding for the (α-γ)2 polypeptide, a plasmid that I was unable to construct, does not require an α subunit for its harmful effect. Since the (α-γ)1 plasmid requires an added α subunit to harm *unc*-deleted *E. coli*, but the (α-γ)2 plasmid apparently does not, it can be speculated that the important determinants in the α protein for assembly of a leaky F_0 are located in the final 35 amino acids of the polypeptide, which are present in the (α-γ)2 polypeptide but which are absent from the (α-γ)1 polypeptide.

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