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Gene order and gene–polypeptide relationships of the proton-translocating ATPase operon (*unc*) of *Escherichia coli*

(H$^+$-ATPase/cloned *unc* genes/protein synthesis *in vitro*/membrane assembly/membrane energetics)

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ABSTRACT **We have constructed an extensive set of plasmids that carry the genes specifying the eight polypeptides of the proton-translocating ATPase of *Escherichia coli*. Using detailed restriction analysis and *in vitro* protein synthesis directed by these plasmids, we have established the order of the eight *unc* genes to be *BEFHAGDC* and the corresponding polypeptides to be a, c, b, δ, α, γ, β, and ε. These analyses include determining the location of the gene coding for the δ subunit of the F_1 portion of the complex. We call this gene *uncH*. We have now established the gene order and gene–polypeptide relationships of the *unc* operon. This approach should be of use for study of other multigene bacterial operons, especially those with genes coding for polypeptides with unknown or unmeasurable catalytic activity.**

The proton-translocating ATPase (H$^+$-ATPase) of *Escherichia coli* is composed of eight polypeptide subunits (1, 2). Three of the polypeptides (a, b, and c) are tightly bound to the membrane in a structure called the F_0. The other five (α, β, γ, δ, and ε), composing the F_1 portion, are bound to the F_0, but can be removed from the F_0, and thus from the membrane, by relatively mild procedures (3). The genes that specify these eight polypeptides exist in an operon, called *unc*, located at 82.5 minutes on the *E. coli* chromosome. Six of these genes, designated *uncB*, *uncE*, *uncA*, *uncG*, *uncD*, and *uncC*, have been located on the chromosome by conventional genetic techniques (4). The location of the *uncF* gene (γ subunit) was determined by a combination of cloning, gene fusion, and *in vitro* transcription–translation procedures (5). Recently, several laboratories have begun to use recombinant DNA techniques to examine the molecular and structural details of the *unc* operon and eventually to understand the function of the H$^+$-ATPase (4–9). We have employed these techniques to extend our earlier work (7) and describe the gene–polypeptide relationships and the order of all eight genes of the operon. We have cloned the eight genes onto various amplifiable plasmid vectors and examined the gene–polypeptide relationships of the operon in detail by performing *in vitro* protein synthesis. This approach enabled us to identify and locate the gene for the δ subunit, which we designate *uncH*. In addition, we have shown the gene order of the operon to be *BEFHAGDC*, corresponding to a polypeptide order of a, c, b, δ, α, γ, β, and ε. This determination of the gene order differs from the previously reported order (5).

MATERIALS AND METHODS

Materials. [^{35}S]Methionine (1000 Ci/mmol; 1 Ci = 3.7 × 10^{10} becquerels) was purchased from New England Nuclear. All other chemicals were of highest purity commercially available.

***In Vitro* Protein Synthesis.** DNA-dependent protein synthesis was conducted by using the *in vitro* transcription–translation system described previously (10). Sodium dodecyl sulfate/polyacrylamide gels were run as described (7). Antibody precipitations were performed as described (7), using rabbit anti-F_1 antiserum.

Bacterial Strains, Phage, and Plasmids. The *E. coli* strain LE 392 was used for transformation and plasmid preparation. The λ transducing phage carrying the wild-type *unc* operon, λ_{unc} (7), was used for initial plasmid construction. Plasmid vectors pACYC184 (11), pBR322 (12), and pJJS100 (gift of John Sninsky) were used as indicated.

Restriction Endonuclease Digestion and Ligation of DNA Fragments. Endonuclease digestions, phosphatase treatment, ligation, analysis of restriction fragments, cell transformations, and DNA preparation were carried out as described (10).

RESULTS

Determination of the Gene–Polypeptide Relationship and Gene Order by Cloning, Restriction Enzyme Mapping, and *in Vitro* Protein Synthesis. Our approach to the question of gene–polypeptide relationships and gene order of the *unc* operon has been to clone and reclone various portions of the operon by recombinant DNA techniques, to identify their physical locations by detailed restriction mapping, and then to determine which polypeptides are specified by the respective fragments by conducting *in vitro* protein synthesis. The constructions of the initial plasmids derived from λ_{unc} and their various derivatives are described in Fig. 1 and Table 1. The portions of the operon present on each plasmid are indicated in Fig. 2 along with a detailed restriction map. As described below, these results clearly show that the polypeptides are synthesized in the following order: a, c, b, δ, α, γ, β, and ε.

Location of the Genes for the a and c Polypeptides (*uncB* and *uncE*). The genes *uncB* and *uncE* code for the F_0 polypeptides a and c, respectively (5). A previous report (5) indicated that these genes were separated by *uncF*, which specifies the F_0 subunit b. *In vitro* transcription–translation of plasmid pRPG44 produced the same polypeptides as pRPG23 except for polypeptide a, demonstrating that *uncB* is indeed the first gene of the operon (Fig. 2 and Fig. 3, lane b). The products synthesized from pRPG38 and pRPG56 (for description, see Fig. 2) clearly demonstrate that the a and c polypeptides were synthesized even when the other three subunits (b, δ, and α) were not (Fig. 3, lane d), thus supporting a gene order of *uncBE*, rather

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Abbreviation: kb, kilobase(s).

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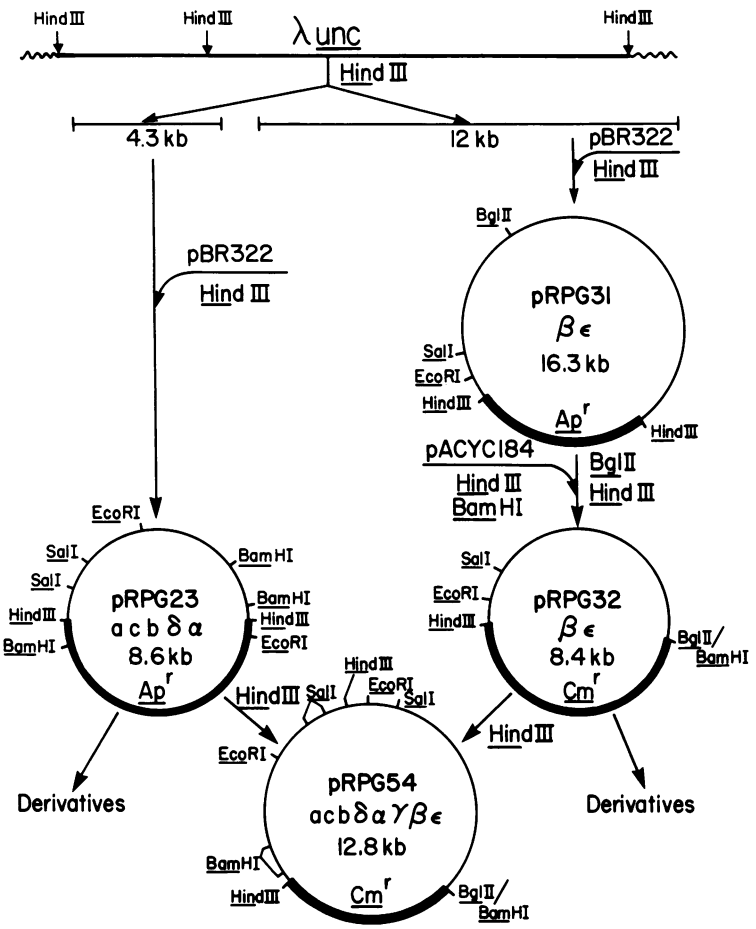

FIG. 1. Schematic illustration of the initial plasmid constructions from λ_{unc}. The plasmids pRPG23 and pRPG31 were constructed by recloning the 4.3- and 12-kb *Hin*dIII fragments from λ_{unc} into the single *Hin*dIII site of pBR322 to give plasmids of 8.6 and 16.3 kb, respectively. The locations of various restriction sites are shown for orientation in relation to Fig. 2. The heavy line of each plasmid represents the vector portion. The 4.7-kb *Bgl* II/*Hin*dIII fragment from pRPG31 was subsequently ligated with the 3.8-kb *Bam*HI/*Hin*dIII vector fragment of pACYC184 to give the 8.4-kb plasmid pRPG32. Subsequent opening of this plasmid by *Hin*dIII and insertion of the 4.3-kb *Hin*dIII fragment from pRPG23 yielded the 12.8-kb plasmid of pRPG54 containing the *unc* operon genes in the correct orientation such that all eight polypeptides are synthesized.

than *uncBF* as previously reported (5). Further support for this gene order was obtained by examining the products synthesized using pRPG51 as template. It directed the synthesis of the b and δ polypeptides, but not the a, c, or α polypeptides (Fig. 3, lane g), indicating that *uncF* (b polypeptide) does not lie between *uncB* and *uncE* (subunits a and c), as previously reported (5).

Location of the Gene for the c Polypeptide (*uncE*). The plasmid pRPG44 codes for the c polypeptide and therefore contains the *uncE* gene in its entirety. Plasmid pRPG38 also codes for the c subunit. The only region of the *unc* operon that these plasmids have in common, besides a 0.7-kb region near the *Hin*dIII site within the *unc* operon, is the 0.37-kb region between a *Bam*HI and a *Hpa* I site (see Fig. 2), so this small region must contain all of the *uncE* gene. This deduction has been confirmed by Futai and coworkers (13), who have recently determined the nucleotide sequence of the *uncE* gene.

Table 1. Plasmids used for protein synthesis *in vitro*

Plasmid	Subunits synthesized from plasmid *in vitro*	Plasmid vector	Antibiotic resistance	Size, kb
pRPG23	a c b δ α	pBR322	Ap	8.6
pRPG28	a c b δ α′	pBR322	Ap	7.4
pRPG31	β ε	pBR322	Ap	16.3
pRPG32	β ε	pACYC184	Cm	8.4
pRPG33	α′	pBR322	Ap	6.2
pRPG38	a c	pBR322	Ap	6.4
pRPG44	c b δ α	pACYC184	Cm	7.3
pRPG45	a c b δ	pACYC184	Cm	6.3
pRPG51	b δ	pJJS100	Ap	3.8
pRPG53	ε	pBR322	Tc	5.5
pRPG54	a c b δ α γ β ε	pACYC184	Cm	12.8
pRPG55	β′	pBR322	Tc	4.9
pRPG56	a c	pBR322	Ap	3.5
pRPG57	δ	pBR322	Ap	3.0
pRPG58	c	pJJS100	Ap	3.6
pWSB1	ε	pACYC184	Cm	8.5
pWSB2	β′	pACYC184	Cm	5.0

Ap, ampicillin; Cm, chloramphenicol; Tc, tetracycline; kb, kilobases. α′ and β′ indicate partial translation products.

Identification and Location of the Gene for the δ Polypeptide (*uncH*). Plasmid pRPG45 directed the *in vitro* synthesis of the a, c, b, and δ polypeptides (Fig. 3, lane f). From our previous conclusions concerning the location of the *uncB* and *uncE* genes for the a and c polypeptides, it is certain that the genes for the c, b, and δ polypeptides lie between the second of the two *Bam*HI sites and the *Eco*RI site in the middle of the 4.3-kb *unc* region contained in plasmid pRPG23 (Fig. 2). Plasmid pRPG51 also coded for both the b and δ subunits, further defining the location of the genes for the b and δ polypeptides (Fig. 2 and Fig. 3, lane g). The plasmid pRPG57 coded only for the δ polypeptide when used as a template in the *in vitro* protein synthesis system (Fig. 3, lane h), indicating that it contained the gene for this subunit. We propose that this gene be designated *uncH*. The order of polypeptide synthesis is, therefore, b δ, and the gene order is *uncFH*.

Location of the Gene Coding for the α Polypeptide (*uncA*). *In vitro* protein synthesis directed by pRPG28 (see legend of Fig. 2) showed that the α polypeptide (57,000 M_r) disappeared and was replaced by a band of slightly lower molecular weight (≈53,000) (Fig. 3, lane i). This new polypeptide was evidently a fusion product of the *uncA* gene and the vector (pBR322) after the *Sal* I site. An examination of the DNA sequence of pBR322 indicated the possible termination sites in all three reading frames after the *Sal* I site. From this information we estimate

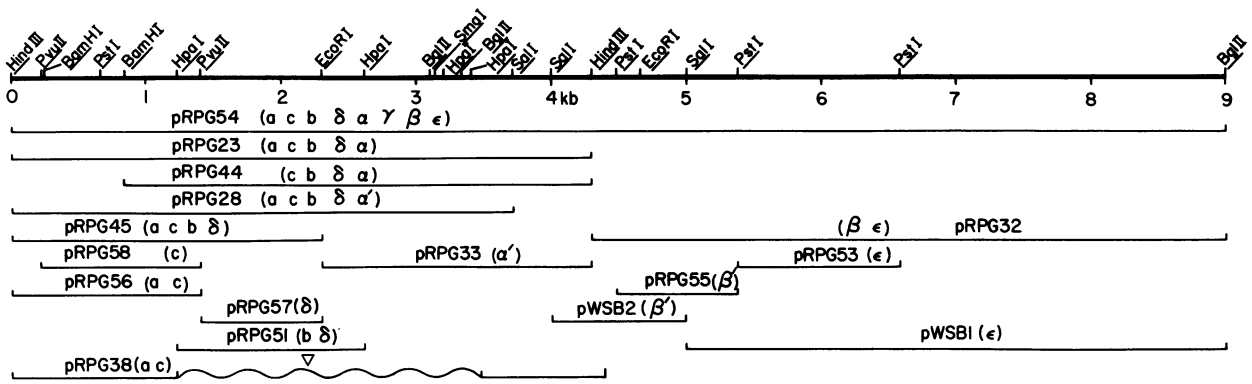

FIG. 2. Restriction map of plasmids containing various regions of the *unc* operon. The heavy line shows the location of the restriction endonuclease recognition sites with the distance given in kb. Regions of the *unc* operon present on the various plasmids are indicated. The ends of the lines indicate the restriction sites within the operon region used for recloning from the initial plasmids represented in Fig. 1. The vector used for each plasmid, the total size in kb, and the antibiotic resistance encoded are shown in Table 1. Individual H^+-ATPase polypeptides produced *in vitro* from each plasmid are also indicated, as are partial translation products (e.g., α'). Plasmid pRPG38 has undergone a 2.2-kb deletion of the 4.3-kb *Hin*dIII fragment shown by the wavy line at the bottom of the figure. The promoter region for the operon is at the left as drawn. Plasmids were constructed by standard methods (10) as described below. Additional properties of the plasmids are given in Table 1 and Fig. 1. *pRPG23.* The 4.3-kb *Hin*dIII fragment from λ_{unc} containing the genes for the a, b, c, δ, and α subunits was inserted at the *Hin*dIII site of pBR322, yielding an 8.6-kb plasmid designated pRPG23. We had previously designated this plasmid pUNC1 (7). *pRPG28.* The 3.7-kb *Hin*dIII/*Sal* I fragment from pRPG23 (Fig. 1) was ligated with the large *Hin*dIII/*Sal* I fragment of pBR322, yielding a 7.4-kb plasmid designated pRPG28. *pRPG31.* The 12-kb *Hin*dIII fragment of λ_{unc} was ligated into the *Hin*dIII site of pBR322, yielding the 16.3-kb plasmid pRPG31. *pRPG32.* The 4.7-kb *Hin*dIII/*Bgl* I fragment from the 12-kb insert of pRPG31 was ligated with the 3.8-kb *Hin*dIII/*Bam*HI fragment of pACYC184 to yield the 8.4-kb plasmid pRPG32. *pRPG33.* The 2.3-kb *Eco*RI fragment of pRPG23 (Fig. 1) was deleted to yield a 6.2-kb plasmid designated pRPG33. *pRPG38.* The 2.2-kb *Hpa* I fragment was deleted from the center of pRPG23 by cutting with *Hpa* I and religating to give the plasmid designated pRPG38. *pRPG44.* The 3.5-kb *Bam*HI/*Hin*dIII fragment of pRPG23 was ligated with the 3.8-kb *Hin*dIII/*Bam*HI fragment of pACYC184 to give the 7.3-kb plasmid pRPG44. *pRPG45.* The 2.3-kb *Eco*RI fragment of pRPG23 was ligated with *Eco*RI-digested pACYC184 to yield the 6.3-kb plasmid pRPG45. *pRPG51.* The 1.4-kb *Hpa* I fragment (Fig. 2) of pRPG23 was ligated at the *Pvu* II site of pJJS100, yielding the 3.8-kb plasmid designated pRPG51. *pRPG53.* The 1.2-kb *Pst* I fragment of pRPG32 was ligated at the *Pst* I site of pBR322 to yield the 5.5-kb plasmid pRPG53. *pRPG54.* The 4.3-kb *Hin*dIII insert of pRRPG23 was ligated with *Hin*dIII-cut pRPG32 to yield the 12.8-kb plasmid designated pRPG54. *pRPG55.* The 0.9-kb *Pst* I fragment from pRPG32 was ligated with *Pst* I-cut pBR322 to yield the 5.2-kb pRPG55. *pRPG56.* The 1.4-kb *Pvu* II/*Eco*RI fragment of pRPG23 was ligated with the 2.1-kb *Pvu*II/*Eco*RI fragment of pBR322 to yield the 3.5-kb plasmid pRPG56. *pRPG57.* The 0.9-kb *Pvu* II/*Eco*RI fragment of pRPG23 was ligated with the 2.1-kb *Pvu* II/*Eco*RI fragment of pBR322 to yield the 3.0-kb plasmid pRPG57. *pRPG58.* The 1.2-kb *Pvu* II insert of pRPG23 was ligated at the *Pvu* II site of pJJs100 to yield the 3.6-kb pRPG58. *pWSB1.* The 4.4-kb *Sal* I fragment from pRPG54 was ligated at the *Sal* I site of pACYC184 to give the 8.4-kb plasmid designated pWSB1. *pWSB2.* The 1.0-kb *Sal* I fragment from pRPG54 was ligated at the *Sal* I site of pACYC184 to give the 5.0-kb plasmid designated pWSB2.

the limits for the start of the *uncA* gene, and those limits are indicated in Fig. 5. This gene must start very close to the *Eco*RI site in the middle of the 4.3-kb *unc* insert in pRPG23. This region is also where gene *uncH* must end. This result has been confirmed by the nucleotide sequence studies of Gay and Walker (14) and Kanazawa *et al.* (13), who have placed the start of *uncA* three codons to the left of the *Eco*RI site (10). We have constructed a plasmid with a deletion of the *unc* DNA region on the left side of the *Eco*RI site. Interestingly enough, although apparently missing the codons for the first three amino acids of the α subunit, this plasmid, pRPG33, still coded for a polypeptide of the same size as the α subunit (Fig. 3, lane j), which was precipitable with anti-F_1 antibody, indicating that there was a fortuitous fusion of the plasmid DNA with the *uncA* DNA that permitted translation of the new hybrid α polypeptide.

Cloning the Genes for the β and ε Polypeptides (*uncD* and *C*). A 12-kb *Hin*dIII fragment from λ_{unc} DNA was inserted into the *Hin*dIII site of pBR322 (Fig. 1). *In vitro* protein synthesis directed by this plasmid, designated pRPG31, revealed the presence of the β and ε subunits (Fig. 4, lane b). A portion of this plasmid containing the distal portion of the *unc* operon was then transferred to pACYC184 as shown in Fig. 1. *In vitro* transcription–translation of the resultant plasmid, designated pRPG32, showed that it coded for the β and ε subunits (Fig. 4, lane c), but the high molecular weight protein (70,000 M_r) of unknown function made by pRPG31 had disappeared and presumably been replaced by a truncated polypeptide (60,000 M_r), slightly larger than the α polypeptide.

Order of the Genes for the β and ε Polypeptides (*uncD* and *C*). The genes for the β and ε polypeptides have been mapped by conventional genetic means, and their order has been determined to be *uncD* (β) *uncC* (ε) (15). Our mapping studies have confirmed this order. Plasmid pRPG53 contains a 1.3-kb *Pst* I fragment cloned from pRPG32 and codes for only the ε polypeptide (Fig. 2 and Fig. 4, lane d). We did not construct a plasmid containing just the gene for the β polypeptide, but we placed it to the left side of the gene for ε for the following reasons. Plasmid pWSB2 contains the small 1.0-kb *Sal* I fragment that spans the *Hin*dIII site in the middle of the *unc* operon. It codes for a 10,000 M_r soluble protein that is precipitable with anti-F_1 antiserum (Fig. 4, lanes f, g, and h). In addition, plasmid pRPG55 contains a 0.9-kb *Pst* I fragment from pRPG32 (Fig. 2) that codes for a 30,000 M_r soluble protein that is also precipitable with anti-F_1 antiserum (Fig. 4, lanes h, i, j, and k). The 10,000 M_r polypeptide and the 30,000 M_r polypeptide are probably both partial β polypeptides synthesized from the NH_2-terminal region of the gene for the β subunit. In addition, plasmid pWSB1, which contains all of the *unc* operon to the right of the *Sal* I site indicated in Fig. 2, contains the gene for the ε subunit and does not contain the gene for the β subunit. Thus, the gene for the β subunit starts before that *Sal* I site and precedes the gene for the ε subunit.

Reconstruction of the *unc* Operon. The strategy for the reconstruction of the operon, along with the resultant plasmids, is presented in Fig. 1. *In vitro* protein synthesis directed by pRPG54 indicated that seven of eight *unc* polypeptides were

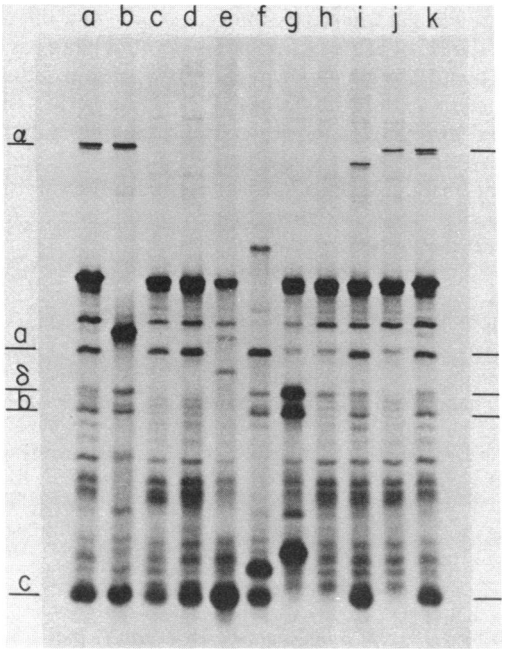

FIG. 3. *In vitro* protein synthesis product of plasmids constructed to determine the order of the first five genes of the *unc* operon. Plasmids were constructed as described in Fig. 2, and *in vitro* transcription–translation, followed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and autoradiography, was performed. The locations of the *unc* polypeptides α, a, δ, b, and c are indicated alongside the autoradiogram and were determined by running pure F_1F_0 alongside. All other proteins are either vector encoded or partial ATPase polypeptides. Lanes: a, pRPG23; b, pRPG44; c, pRPG38; d, pRPG56; e, pRPG58; f, pRPG45; g, pRPG51; h, pRPG57; i, pRPG28; j, pRPG33; and k, pRPG23.

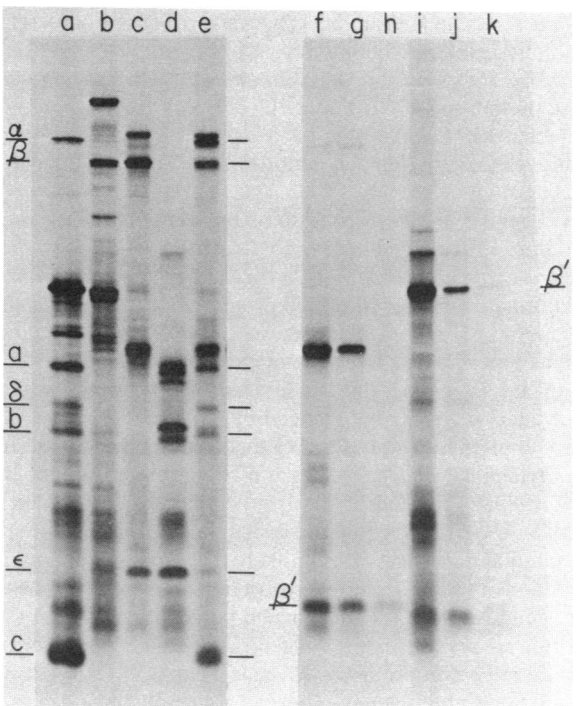

FIG. 4. *In vitro* protein synthesis products of plasmids containing the final two genes of *unc* operon and the complete operon. See Fig. 3 for details. Lanes: a, pRPG23; b, pRPG31; c, pRPG32; d, pRPG53; e, pRPG54; f, pWSB2; g, pWSB2 soluble products; h, anti-F_1-precipitable protein coded for by pWSB2; i, pRPG55; j, pRPG55 soluble products; k, anti-F_1-precipitable protein coded for by pRPG55.

synthesized. The γ subunit, however, was not demonstrable (Fig. 4, lane d).

Location of the Gene for the γ Subunit (*uncG*). Using the approach outlined here, Downie *et al.* have mapped the gene for the γ subunit (*uncG*) as spanning the *Hin*dIII site within the operon (5). We previously reported our inability to detect the *in vitro* synthesis of the γ polypeptide under a variety of conditions (7), but we have now been able to clearly detect its production *in vivo* by using minicells carrying pRPG54 (unpublished data). By examining the products synthesized by minicells containing pRPG23, pRPG32, and pRPG54 on sodium dodecyl sulfate gels, we have determined that the gene for the γ polypeptide (*uncG*) spans the *Hin*dIII site (data not shown).

DISCUSSION

We determined the order of the eight genes of the *unc* operon by examining the *in vitro* protein synthesis products of plasmids carrying various portions of the operon. Our restriction map and gene order is shown in Figs. 2 and 5. The novel features of this map, compared to previous results, are the gene order of *uncBEF* rather than *uncBFE* and the identification and mapping of the *uncH* gene, which codes for the δ subunit.

The approach described here for gene mapping by plasmid construction followed by *in vitro* protein synthesis is straightforward and avoids potential problems inherent in using genetic complementation because, other than antibiotic resistance, no

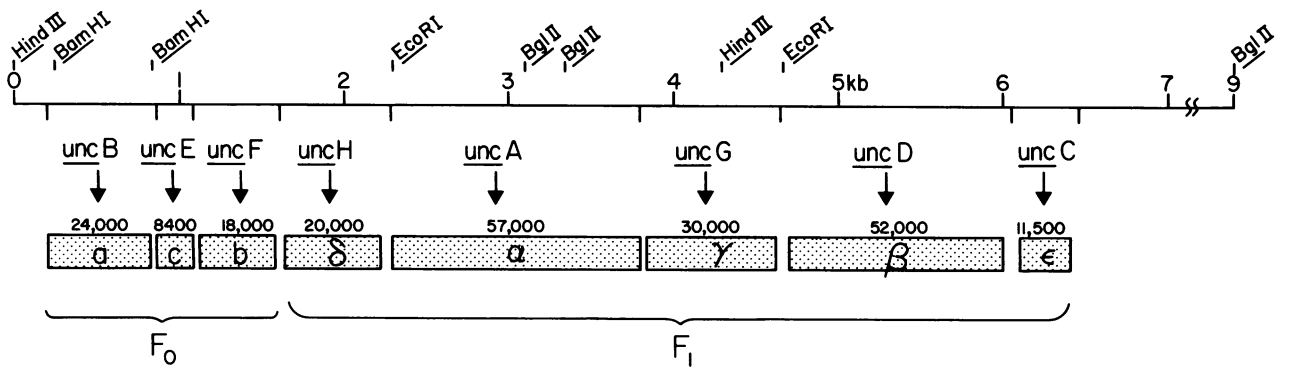

FIG. 5. Gene–polypeptide relationships and gene order for the *unc* operon. The partial restriction map for the 9-kb region containing the operon is shown at the top of the figure for reference, with distance indicated in kb. The physical sizes of the genes were estimated from the molecular weights of the individual polypeptides as shown, assuming an average amino acid molecular weight of 115. The precise position of the final gene, *uncC*, is uncertain and could possibly extend to the right by about 0.1 kb. Similarly, *uncB* at the promoter-proximal end (upper left of the figure) may extend left by no more than 0.15 kb. The polypeptides composing the F_0 and F_1 portions of the H^+-ATPase complex are indicated at the bottom of the figure.

genetic selection and scoring is necessary. We merely screened antibiotic-resistant colonies for plasmids with inserts of the desired size and then performed *in vitro* protein synthesis using those plasmids as DNA template. This approach is potentially useful for the study of other bacterial operons, especially those with genes coding for polypeptides with unknown or unmeasurable catalytic activity.

Although there is believed to be but a single message for all the genes in the *unc* operon (15), we had no difficulty seeing *in vitro* expression of genes that had been removed from the *unc* promoter. Either there are promoters present throughout the operon or, more likely, transcription of the recloned *unc* regions was initiated from vector promoters (16). In addition, the upstream regions did not interfere with expression of the following *unc* genes as evidenced by our results.

An additional complication of this analysis could arise from identifying fusion polypeptides as ATPase subunits on the basis of their gel mobility and antibody precipitation. We avoided this pitfall by verifying polypeptide assignments by using more than one plasmid derivative. It is unlikely that independent plasmid constructions using different restriction sites or different vectors would result in identical fusion polypeptides.

There are several features of gene arrangement in this operon that are interesting to note. There is a gap (<0.1 kb) between the *uncE* and *uncF* genes. There is also probably a gap (<0.2 kb) between the *uncB* and *uncE* genes. But between *uncB* and *uncC* there do not appear to be any spaces large enough to code for additional polypeptides of significant size. For example, the 14,000 M_r polypeptide postulated by Downie *et al.* (5) to exist between the *uncE* and *uncA* genes would require a region of approximately 300 bases. Such a region does not exist within the operon.

With regard to assembly of the complex, it is interesting to consider that because the genes for the F_0 polypeptides biosynthetically precede those of the F_1 polypeptides, insertion of the F_0 polypeptides into the membrane may be the initial step in the assembly sequence. In this regard, it is noteworthy that gene *uncH*, which codes for the δ polypeptide, is the first of the genes for the F_1 polypeptides, because Smith and Sternweiss have indicated that this polypeptide is involved in attachment of F_1 to F_0 (17). It is also interesting to note that the γ subunit does not appear to be synthesized in our *in vitro* system yet is synthesized well in minicells (data not shown). The reasons for this difference are not yet known.

In addition to the study of gene order, these plasmids containing various portions of the *unc* operon should be of use to study the regulation of polypeptide synthesis and the assembly of the eight polypeptides into a functional ATPase complex in the cytoplasmic membrane.

Nomenclature for the Genes Coding for the H^+-ATPase. The designation *unc* (uncoupled) has been established by Gibson and colleagues to signify the genes coding for the polypeptides of the H^+-ATPase of *E. coli*. Recently, as more has become known about the gene–polypeptide relationship, others have introduced the designations *atp* (9, 14) and *pap* (13), which are intended to replace *unc* with abbreviations that are more informative with regard to polypeptide function. It is our feeling, however, that the designation of *unc* is so firmly established that introduction of new designations is confusing, and we urge others to adhere to the usage of *unc*.

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