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Mu-Induced Polarity in the *unc* Operon of *Escherichia coli*

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Mutant strains of *Escherichia coli* were isolated in which mutator (Mu) phage was inserted into various *unc* genes. Partial diploid strains were prepared from each of the Mu-induced *unc* mutants by using F-plasmids carrying mutations in one of the known *unc* genes (*uncA, uncB, uncC,* or *uncD*). The partial diploid strains and the corresponding segregant strains were examined for their ability to grow on succinate. The aerobic growth yields on limiting concentrations of glucose were also determined. Magnesium-stimulated adenosine triphosphatase activities, ATP-dependent transhydrogenase activities, and Atebrin fluorescence quenching activities were determined by using membrane preparations from each strain. Genetic complementation was assessed from the results obtained, and it was concluded that the four *unc* genes examined are part of a single transcriptional unit and that they are transcribed in the order *uncBADC*.

Oxidative phosphorylation is mediated by a membrane-bound magnesium-stimulated adenosine triphosphatase (Mg-ATPase) which in procaryotes, as in the mitochondria and chloroplasts of higher organisms, consists of two separable portions. One of these portions (F_1) can be dissociated from the membrane and possesses Mg-ATPase activity, whereas the other (F_0) appears to be an integral part of the membrane. F_1 is a complex containing at least five polypeptides (α, β, γ, δ, and ϵ); the number of polypeptide components in F_0 has not been definitely determined (17). Mutants of *Escherichia coli* in which oxidative phosphorylation is uncoupled from electron transport were first reported by Butlin et al. (3), and similar mutants have since been isolated in a number of laboratories and used in the study of energy-requiring processes (6, 22). Such mutations have been designated as occurring in *unc* genes and have been mapped near the *ilv* genes at about min 83 on the *E. coli* chromosome (6). The *unc* mutants described have been broadly classified by the presence or absence of membrane-bound Mg-ATPase activity and by in vitro reconstitution experiments (6).

A method has been developed recently for incorporating mutant *unc* alleles into plasmids (15). Using such plasmids, Cox et al. (5) and Gibson et al. (15) have identified four *unc* genes (*A, B, C,* and *D*) by genetic complementation tests. The availability of plasmids carrying mutant *unc* alleles for each of the *uncA, uncB, uncC,* and *uncD* genes, together with Mu-induced *unc* mutations, has made it possible to investigate whether or not these four genes form part of an operon. Mutator (Mu) phage can insert randomly into the *E. coli* chromosome (23) and confers a strong transcriptional polarity effect in distal genes of any operon in which it inserts (2, 10, 11). In the present paper, the arrangement of *unc* genes has been determined by an examination of the properties of partial diploid strains with known mutant *unc* alleles on the plasmid and with Mu-induced *unc* mutant alleles on the chromosome.

MATERIALS AND METHODS

Organisms. All of the bacterial strains used are derived from *E. coli* K-12 and are described in Tables 1 and 2.

Media and growth of organisms. The mineral salts minimal medium used and additions, unless otherwise indicated, were as described previously (15).

Cells for the preparation of membranes were grown in 14-liter New Brunswick fermentors essentially as described by Cox et al. (9). The inoculum was grown in 1 liter of glucose-mineral salts medium at 37°C overnight with shaking. The inoculum was then added to 10 liters of medium in the fermentor and incubated at 37°C with aeration until the cells were in mid-logarithmic phase. The medium in the fermentor vessels was supplemented with 5% (vol/vol) Luria broth (18).

Solid media were prepared by the addition of 2% (wt/vol) agar to the mineral salts minimal medium. For agar plates containing succinate as the sole source of carbon, glucose was replaced by sodium succinate (30 mM) and, in addition to the appropriate growth requirements, the medium was supplemented with 0.06% acid-hydrolyzed casein (Difco).

The nutrient broth used for genetic experiments

TABLE 1. *Strains and plasmids of E. coli K-12 used*[a]

Strain/ plasmid	Relevant genetic loci[b]	Other information
Strain		
AN180	F⁻ *argE thi-1*	Butlin et al. (3)
Mu-35	F⁻ *argE thi-1 unc-413*::Mu	Mu-induced mutant of AN180
AN346	F⁻ *ilvC argH pyrE entA*	Gibson et al. (14)
AN875	F⁻ *argH pyrE entA unc-413*::Mu	Isolated after transduction with strain Mu-35 as donor and strain AN346 as recipient
KL163	Hfr *nalA recA*	Obtained from J. Pittard
AN887	F⁻ *argH pyrE entA unc-413*::Mu *nalA recA*	Isolated after mating between strains KL163 and AN875
Mu-123	F⁻ *argE thi-1 unc-418*::Mu	Mu-induced mutant of AN180
AN872	F⁻ *argH pyrE entA unc-418*::Mu	Isolated after transduction with strain Mu-123 as donor and strain AN346 as recipient
AN884	F⁻ *argH pyrE entA unc-418*::Mu *nalA recA*	Isolated after mating between strains KL163 and AN872
Mu-107	F⁻ *argE thi-1 unc-415*::Mu	Mu-induced mutant of AN180
AN808	F⁻ *argH pyrE entA unc-415*::Mu	Isolated after transduction with strain Mu-107 as donor and strain AN346 as recipient
AN869	F⁻ *argH pyrE entA unc-415*::Mu *nalA recA*	Isolated after mating between strains KL163 and AN808
AN248	F⁻ *ilvC argH entA*	Butlin et al. (4)
Mu-82	F⁻ *argE thi-1 unc-421*::Mu	Mu-induced mutant of AN180
AN506	F⁻ *argH entA unc-421*::Mu	Isolated after transduction with strain Mu-82 as donor and strain AN248 as recipient
AN557	F⁻ *argH entA unc-421*::Mu *nalA recA*	Isolated after mating between strains KL163 and AN506
Diploid		
AN862	pAN11 *ilvC argH pyrE purE recA nalA*	Cox et al. (5)
AN780	pAN2 *ilvC argH pyrE purE recA nalA*	Cox et al. (5)
AN797	pAN5 *ilvC argH pyrE purE recA nalA*	Cox et al. (5)
AN805	pAN6 *ilvC argH pyrE purE recA nalA*	Gibson et al. (15)
AN819	pAN7 *ilvC argH pyrE purE recA nalA*	Cox et al. (5)
Plasmid		
pAN11	F′ *unc*⁺ *ilvC*⁺ *argH*⁺ *pyrE*⁺	Cox et al. (5)
pAN2	F′ *uncA401 ilvC*⁺ *argH*⁺ *pyrE*⁺	Cox et al. (5)
pAN5	F′ *uncB402 ilvC*⁺ *argH*⁺ *pyrE*⁺	Gibson et al. (15)
pAN6	F′ *uncC424 ilvC*⁺ *argH*⁺ *pyrE*⁺	Gibson et al. (15)
pAN7	F′ *uncD409 ilvC*⁺ *argH*⁺ *pyrE*⁺	Cox et al. (5)

[a] To simplify the list of strains, the strain numbers and *unc* alleles for the partial diploid and segregant strains characterized in this paper are given in Table 2.

[b] The gene designations follow those of Bachmann et al. (1), and the plasmid nomenclature is that of Novick et al. (21).

was Luria broth, with 10 mM glucose included as indicated.

Growth yields were measured, as described previously (9), in glucose-mineral salts medium supplemented with 5% (vol/vol) Luria broth. Turbidities of cultures were measured with a Klett-Summerson colorimeter.

Genetic techniques. The techniques used for genetic experiments were as outlined previously (14, 15). Tests for transfer of plasmids and for complementation were carried out as follows. Donor and recipient strains were grown to logarithmic phase (about 100 Klett units, using a blue filter) in 10 ml of Luria broth containing 10 mM glucose. After the culture of the donor strain was centrifuged, cells were suspended in 1 ml of mineral salts medium. Recipient cells were then cross-streaked against donor cells on glucose and succinate media and examined for the appearance of transconjugant colonies after 2 days of incubation at 37°C.

Mutagenesis with Mu. Mu phage was added to a culture of strain AN180 (*unc*⁺) in logarithmic phase (ca. 6×10^8 cells per ml) in Z broth (Luria broth plus 2.5 mM $CaCl_2$) to give a multiplicity of infection of about 0.1. The culture was shaken at 37°C for 1 h and then diluted 1:10 with fresh Z broth, and the incubation was continued for 5 h, during which time the turbidity of the culture increased and then dropped to a minimum level. The culture was then centrifuged, and the cells were washed once in mineral salts medium and suspended in 10 ml of Luria broth plus glucose. The culture was grown for two generations to

TABLE 2. *unc alleles present in partial diploid and segregant strains*

Recipient strains	Donor strain[a]				
	AN862 (*unc*$^+$/*unc*$^+$)	AN797 (*uncB402*/*unc*$^+$)	AN780 (*uncA401*/*unc*$^+$)	AN819 (*uncD409*/*unc*$^+$)	AN805 (*uncC424*/*unc*$^+$)
AN887 (*unc-413*::Mu)	AN910 (*unc*$^+$/*unc-413*) Seg.[b] AN911 (*unc-413*)	AN914 (*uncB402*/*unc-413*) Seg. AN915 (*unc-413*)	AN912 (*uncA401*/*unc-413*) Seg. AN913 (*unc-413*)	AN918 (*uncD409*/*unc-413*) Seg. AN919 (*unc-413*)	AN916 (*uncC424*/*unc-413*) Seg. AN917 (*unc-413*)
AN884 (*unc-418*::Mu)	AN900 (*unc*$^+$/*unc-418*) Seg. AN901 (*unc-418*)	AN904 (*uncB402*/*unc-418*) Seg. AN905 (*unc-418*)	AN902 (*uncA401*/*unc-418*) Seg. AN903 (*unc-418*)	AN908 (*uncD409*/*unc-418*) Seg. AN909 (*unc-418*)	AN906 (*uncC424*/*unc-418*) Seg. AN907 (*unc-418*)
AN557 (*unc-421*::Mu)	AN962 (*unc*$^+$/*unc-421*) Seg. AN963 (*unc-421*)	AN966 (*uncB402*/*unc-421*) Seg. AN967 (*unc-421*)	AN964 (*uncA401*/*unc-421*) Seg. AN965 (*unc-421*)	AN970 (*uncD409*/*unc-421*) Seg. AN971 (*unc-421*)	AN968 (*uncC424*/*unc-421*) Seg. AN969 (*unc-421*)
AN869 (*unc-415*::Mu)	AN972 (*unc*$^+$/*unc-415*) Seg. AN973 (*unc-415*)	AN976 (*uncB402*/*unc-415*) Seg. AN977 (*unc-415*)	AN974 (*uncA401*/*unc-415*) Seg. AN975 (*unc-415*)	AN980 (*uncD409*/*unc-415*) Seg. AN981 (*unc-415*)	AN978 (*uncC424*/*unc-415*) Seg. AN979 (*unc-415*)

[a] For the diploid strains, the *unc* alleles shown first in parentheses are those on the plasmid and those shown second are on the chromosome.
[b] Seg., Segregant.

allow segregation and phenotypic expression; the cells were then washed twice with 10 ml of mineral salts medium and finally resuspended in 3 ml of the same medium. A penicillin selection procedure (19) was then used to enrich for strains which would grow with glucose, but not with succinate, as the sole source of carbon.

Preparation of cell membranes. Membranes were prepared as described previously (7). Briefly, washed cells were disintegrated by using a Sorvall Ribi cell fractionator, cell debris was removed by centrifugation, and the membranes were separated by ultracentrifugation. The membranes were then suspended in a 0.1 M *N*-tris(hydroxymethyl)methyl-2-aminoethane-sulfonic acid buffer system (pH 7.0) containing magnesium acetate, sucrose, and ethanedioxybis(ethylamine)tetraacetate. The protease inhibitors *p*-aminobenzamidine and ϵ-amino-*N*-caproic acid were included in all buffers at final concentrations of 6 and 40 mM, respectively.

Assay of Mg-ATPase. Assays for Mg-ATPase activity were carried out as described previously (14).

Measurement of the ATP-dependent transhydrogenase activity. The formation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) was assayed by coupling the transhydrogenase reaction to the NADPH-dependent enzyme glutathione reductase and measuring the decrease in absorbance at 340 nm (8, 13).

Measurement of Atebrin fluorescence. Atebrin fluorescence was measured at 30°C, as described by Haddock and Downie (16), by using an Aminco Bowman fluorimeter with the excitation wavelength set at 450 nm and the emission wavelength set at 510 nm.

RESULTS

Classification of Mu-induced mutants by growth of partial diploid strains on succinate. Mu-induced mutants unable to grow on succinate but able to grow on glucose were isolated as described above. Such mutants were then screened to detect strains with low aerobic growth yields on limiting concentrations of glucose (6). Lysates were prepared, using the transducing phage P1, from each of the Mu-induced mutants giving low growth yields and used to transduce *ilv*$^+$ into strain AN346. The *unc* genes are normally cotransducible with the *ilv* genes at a frequency of between 40 and 60%, but, because of the presence of the mutator phage, Mu-induced *unc* mutant alleles cotransduced with the *ilv* genes at a frequency of about 1%. Fifteen transductant strains carrying Mu-induced *unc* mutant alleles were isolated, and a *recA* mutant allele was introduced as described previously (14). These strains were then used in genetic complementation tests with strains that each carry a known mutant *unc* allele incorporated into an F-plasmid. The Mu-induced *unc* mutants were divided into four groups on the basis of the formation of transconjugant colonies

on succinate medium after cross-streaking of each of the Mu-induced mutants with partial diploids carrying one of the mutant *unc* alleles (*uncA401, uncB402, uncC424,* or *uncD409*) on the plasmid. The results for one strain from each group are shown in Table 3. The four classes obtained indicated that the known *unc* genes formed a transcriptional unit and that the gene order was *uncB,A,D,C,* with the *uncB* gene being transcribed first. Thus, one strain failed to form transconjugant colonies with partial diploids carrying any of the four mutant *unc* alleles tested and was therefore $uncB^-A^-D^-C^-$; a second class of eight strains formed transconjugant colonies only with the partial diploid carrying the *uncB402* allele on the plasmid and was therefore $uncB^+A^-D^-C^-$; likewise, a third class of four strains was identified as $uncB^+A^+D^-C^-$ and a fourth class of four strains was identified as $uncB^+A^+D^+C^-$. Similar experiments were carried out by using the Mu-induced *unc* mutant alleles in a $recA^+$ background. Colonies were formed on the succinate medium in all cases, confirming that the lack of complementation in the *recA* background was due to Mu-induced polarity and not due to deletions.

Partial diploids representing each of the four classes of Mu-induced *unc* alleles in combination with each of the *uncA401, uncB402, uncC424,* and *uncD409* alleles, as well as the unc^+ allele, were isolated (Table 2). Segregant strains were also isolated from each of the partial diploids, giving a total of 40 strains retained for further work (Table 2).

Growth yields. The growth yields on limiting glucose of the 20 partial diploid strains and of the 20 segregant strains are shown in Table 4. In general, the values obtained were clearly those expected either for a normal strain or for an uncoupled strain (6) and were consistent with the ability of the particular strains to grow, or not to grow, on succinate. Strain AN980 (*uncD409/unc-415*::Mu), which grew poorly on succinate, gave an intermediate growth yield. The growth yields of strains AN968 (*uncC424/unc-421*::Mu) and AN978 (*uncC424/unc-415*::Mu) and of the segregant strains carrying the *unc-415*::Mu (e.g., AN973) allele were all lower than is normally found in an uncoupled mutant strain. These lower values may be correlated with the Mg-ATPase activities in the cytoplasmic fractions obtained from these strains (Mg-ATPase specific activities [micromoles of P_i released per minute per milligram of protein]: AN968, 0.69; AN978, 1.1; AN973, 0.35).

Complementation of Mg-ATPase and energy-linked activities in membranes prepared from partial diploid strains. The results shown in Table 5a are the values for the ATP-dependent transhydrogenase, NADH- and ATP-induced Atebrin fluorescence quenching, and Mg-ATPase activities, obtained by using membranes prepared from partial diploids which carry the *unc-413*::Mu mutation on the chromosome and various *unc* alleles on the plasmid. The *unc-413*::Mu mutant is an example of the $uncB^-A^-D^-C^-$ class. As judged from the values for ATP-induced Atebrin fluorescence quenching or ATP-dependent transhydrogenase activity, no significant complementation had occurred between the *uncA401* or the *uncD409* allele and the *unc-413*::Mu allele of the partial diploid strain as compared with control strain AN910 which has normal *unc* genes on the plasmid. However, membranes prepared from strain AN914 (*uncB402/unc-413*::Mu) had a normal amount of ATP-dependent transhydrogenase activity, and the ATP-dependent Atebrin fluorescence quenching, although low, was above the value normally found for a haploid strain carrying the *uncB402* allele. Complementation probably did not occur in strain AN916 (*uncC424/unc-413*::Mu), since the level of ATP-dependent Atebrin fluorescence quenching in membranes from haploid strains carrying the *uncC424* allele varied with the genetic back-

TABLE 3. *Formation of Suc^+ transconjugants in matings between $recA^-$ Mu-induced unc mutants and strains carrying plasmids with known mutant unc alleles*

Recipient carrying Mu-induced mutant *unc* allele on chromosome	Colonies formed on succinate[a] after mating with donor carrying indicated *unc* allele on plasmid				
	AN862 (*unc^+*)	AN797 (*uncB402*)	AN780 (*uncA401*)	AN819 (*uncD409*)	AN805 (*uncC424*)
AN887 (*unc-413*::Mu)	+	−	−	−	−
AN884 (*unc-418*::Mu)	+	+	−	−	−
AN557 (*unc-421*::Mu)	+	+	+	−	−
AN869 (*unc-415*::Mu)	+	+	+	(+)[b]	−

[a] Growth after 2 days at 37°C after cross-streaking of donor and recipient strains on succinate medium; +, transconjugant colonies formed; −, no transconjugant colonies formed. Results were confirmed by using the Suc phenotype as an unselected marker after cross-streaking on glucose medium.

[b] Very small colonies formed after cross-streaking.

TABLE 4. *Growth yields[a] of partial diploid strains and segregants*

Mu-induced mutant *unc* allele on the chromosome		*unc*[+][b]		*uncB402*		*uncA401*		*uncD409*		*uncC424*	
		Strain	Growth yield	Strain	Growth yield	Strain	Growth yield	Strain	Growth yield	Strain	Growth yield
unc-413::Mu	Diploid	AN910	200	AN914	147	AN912	125	AN918	122	AN916	137
	Segregant	AN911	141	AN915	144	AN913	149	AN919	147	AN917	149
unc-418::Mu	Diploid	AN900	212	AN904	212	AN902	125	AN908	131	AN906	115
	Segregant	AN901	149	AN905	140	AN903	137	AN909	144	AN907	137
unc-421::Mu	Diploid	AN962	196	AN966	200	AN964	195	AN970	120	AN968	98
	Segregant	AN963	139	AN967	131	AN965	135	AN971	131	AN969	132
unc-415::Mu	Diploid	AN972	210	AN976	200	AN974	204	AN980	152	AN978	83
	Segregant	AN973	102	AN977	109	AN975	102	AN981	116	AN979	102

[a] Expressed as turbidity (in Klett units) after growth on 5 mM glucose.
[b] *unc* allele on the plasmid.

ground (unpublished data). As would be expected for a diploid strain carrying a Mu-induced mutant allele of the $uncB^{-}A^{-}D^{-}C^{-}$ class, the Mg-ATPase activities (Table 5a) reflected the characteristics of the mutant *unc* allele present on the plasmid. Thus, the partial diploid strains carrying the *uncA401* and *uncD409* alleles on the plasmid lacked Mg-ATPase activity, whereas those carrying the *uncB402* and *uncC424* alleles retained Mg-ATPase activity (Table 5a).

Table 5b summarizes the results obtained with membranes prepared from partial diploids carrying the *unc-418*::Mu allele on the chromosome. (The *unc-418*::Mu allele is an example of the $uncB^{+}A^{-}D^{-}C^{-}$ class.) The values for ATP-dependent Atebrin fluorescence quenching and the ATP-dependent transhydrogenase activity in membranes from strain AN904 (*uncB402/unc-418*::Mu) were similar to those obtained for the control strain AN900 (*unc*[+]*/unc-418*::Mu) (Table 5b). The *uncB402* mutant allele carried on the plasmid was therefore complemented in a strain carrying the *unc-418*::Mu allele on the chromosome. However, the *uncA401, uncD409,* and *uncC424* alleles were not complemented in the presence of the *unc-418*::Mu allele (Table 5b), thus confirming the $uncB^{+}A^{-}D^{-}C^{-}$ classification made on the basis of growth studies. The lack of Mg-ATPase activity in membranes prepared from strain AN902 (*uncA401/unc-418*::Mu) or AN908 (*uncD409/unc-418*::Mu) is also consistent with this classification.

The *unc-421*::Mu allele is an example of the $uncB^{+}A^{+}D^{-}C^{-}$::Mu class, and the results obtained with membranes prepared from partial diploids carrying this mutant allele are presented in Table 5c. The ATP-dependent Atebrin fluorescence quenching, ATP-dependent transhydrogenase, and Mg-ATPase activities obtained with membranes from strains AN966 (*uncB402/unc-421*::Mu) and AN964 (*unc401/unc-421*::Mu) indicate that the *uncB402* and *uncA401* alleles were complemented in the presence of the *unc-421*::Mu allele. The *uncC424* and *uncD409* alleles were not complemented in the presence of the *unc-421*::Mu allele (Table 5c), thus confirming the $uncB^{+}A^{+}D^{-}C^{-}$ classification made on the basis of growth studies. The NADH-dependent Atebrin fluorescence quenching was less in strains AN968 (*uncC424/unc-421*::Mu) and AN970 (*uncD409/unc-421*::Mu), presumably due to increased proton permeability of membranes prepared from these strains (see below).

Table 5d summarizes the results obtained with membranes prepared from partial diploid

TABLE 5. *Mg-ATPase activities and energy-linked reactions in membranes prepared from partial diploid strains carrying various unc::Mu alleles on the chromosomes*

Strain	Atebrin fluorescence quenching (%)		ATP-dependent transhydrogenase (nmol of NADPH formed/min per mg of protein)	Mg-ATPase (μmol/min per mg of protein)
	ATP	NADH		
(a) Carrying *unc-413*::Mu ($B^-A^-D^-C^-$) on the chromosome				
AN910[a] (*unc*$^+$/*unc-413*::Mu)	76	73	24	0.60
AN911[b] (*unc-413*::Mu)	0	87	4	0.04
AN914 (*uncB402/unc-413*::Mu)	20	84	28	0.47
AN912 (*uncA401/unc-413*::Mu)	0	82	2	0.08
AN918 (*uncD409/unc-413*::Mu)	0	83	3	0.08
AN916 (*uncC424/unc-413*::Mu)	22	84	7	0.67
(b) Carrying *unc-418*::Mu ($B^+A^-D^-C^-$) on the chromosome				
AN900 (*unc*$^+$/*unc-418*::Mu)	61	81	19	0.41
AN901[b] (*unc-418*::Mu)	0	88	2	0.03
AN904 (*uncB402/unc-418*::Mu)	59	84	22	0.44
AN902 (*uncA401/unc-418*::Mu)	3	85	5	0.08
AN908 (*uncD409/unc-418*::Mu)	0	76	2	0.08
AN906 (*uncC424/unc-418*::Mu)	5	85	4	0.51
(c) Carrying *unc-421*::Mu ($B^+A^+D^-C^-$) on the chromosome				
AN962 (*unc*$^+$/*unc-421*::Mu)	80	84	20	0.98
AN963[b] (*unc-421*::Mu)	0	81	3	0.09
AN966 (*uncB402/unc-421*::Mu)	79	82	31	0.60
AN964 (*uncA401/unc-421*::Mu)	40	83	12	0.25
AN970 (*uncD409/unc-421*::Mu)	0	45	3	0.05
AN968 (*uncC424/unc-421*::Mu)	0	63	5	0.74
(d) Carrying *unc-415*::Mu ($B^+A^+D^+C^-$) on the chromosome				
AN972[a] (*unc*$^+$/*unc-415*::Mu)	78	83	24	1.1
AN973[b] (*unc-415*::Mu)	0	85	3	0.23
AN976 (*uncB402/unc-415*::Mu)	83	83	24	0.75
AN974 (*uncA401/unc-415*::Mu)	34	82	12	0.32
AN980 (*uncD409/unc-415*::Mu)	0	47	4	0.10
AN978 (*uncC424/unc-415*::Mu)	0	38	3	1.1

[a] The results shown for strains AN910 and AN972 are similar to those obtained with membranes from a normal haploid strain and a strain diploid for the *unc* region, respectively.

[b] These strains are representative of segregants from each of the groups of partial diploid strains shown.

strains carrying the *unc-415*::Mu allele on the chromosome. (The *unc-415*::Mu allele is an example of the *uncB*$^+$*A*$^+$*D*$^+$*C*$^-$ class.) Membranes prepared from the segregant strain AN973 (*unc-415*::Mu) retained a significant level of Mg-ATPase activity which was not coupled to membrane energization, as judged by the lack of ATP-dependent Atebrin fluorescence quenching or of ATP-dependent transhydrogenase activity (Table 5d). The Mg-ATPase activity was about one-third the level found in a normal strain grown under similar conditions. All of the partial diploid strains carrying the *unc-415*::Mu allele in combination with other *unc* alleles (Table 5d) had Mg-ATPase activity, although the activity in the diploid strain AN980 (*uncD409/unc-415*::Mu) was lower than that in the segregant strain AN973 (*unc-415*::Mu).

The values for the ATP-induced Atebrin fluorescence quenching and ATP-dependent transhydrogenase in membranes from strains AN978 (*uncC424/unc-415*::Mu) and AN980 (*uncD409/unc-415*::Mu) suggest that no complementation occurred in either strain. This is inconsistent with the data obtained from growth studies, which suggest that complementation did

occur in strain AN980 (*uncD409/unc-415*::Mu). However, the NADH-induced Atebrin fluorescence quenching was less in membranes from strain AN978 (*uncC424/unc-415*::Mu) and AN980 (*uncD409/unc-415*::Mu) due to increased proton permeability (see below), and this was probably sufficient to cause loss of the ATP-dependent Atebrin fluorescence quenching and transhydrogenase activities.

Proton permeability. It was observed that membranes prepared from some of the partial diploid strains gave low NADH-induced Atebrin fluorescence quenching (Table 5c and 5d). Quenching of Atebrin fluorescence has been shown to be related to the formation of a transmembrane pH gradient (12). To determine whether or not the low activity of the membranes from the partial diploid strains was due to increased proton permeability, the NADH-induced Atebrin fluorescence quenching was tested in the presence and absence of dicyclohexylcarbodiimide. Dicyclohexylcarbodiimide has been shown previously (20) to stimulate NADH-induced acridine dye fluorescence quenching in membranes which had become proton permeable either as a consequence of a mutation or by removal of the Mg-ATPase. The four strains giving low NADH-induced Atebrin fluorescence quenching carried either the *unc-421*::Mu or *unc-415*::Mu mutant allele on the chromosome and either the *uncC424* or *uncD409* mutant allele on the plasmid (Table 6). The quenching in membranes from each of these four strains could be restored to the normal level by the addition of dicyclohexylcarbodiimide (Table 6).

DISCUSSION

It is concluded, from the polarity effects shown by mutants in which Mu was inserted in various *unc* genes, that the known *unc* genes formed a transcriptional unit and that the genes were transcribed in the order *uncB,A,D,C*.

TABLE 6. *Effect of DCCD*[a] *on NADH-dependent Atebrin fluorescence quenching*

Strain	Atebrin fluorescence quenching (%)	
	−DCCD	+DCCD
AN968 (*uncC424/unc-421*::Mu)	63	82
AN970 (*uncD409/unc-421*::Mu)	45	79
AN978 (*uncC424/unc-415*::Mu)	38	84
AN980 (*uncD409/unc-415*::Mu)	47	79

[a] Dicyclohexylcarbodiimide (DCCD; 20 μM) was added to the assay mixture 5 min before the addition of Atebrin.

The *unc-413*::Mu ($B^-A^-D^-C^-$) allele, which caused the loss of complementation with all of the known *unc* mutant alleles, may have resulted from the insertion of the mutator phage in the *uncB* gene itself. Thus, while strain AN914 [*uncB402/unc-413*::Mu ($B^-A^-D^-C^-$)] did not grow on succinate and had a low growth yield on limiting concentrations of glucose, similar to the segregant strain, membrane preparations had a normal amount of ATP-dependent transhydrogenase activity and a higher level of ATP-dependent fluorescence quenching than would be expected for a mutant strain carrying the *uncB402* allele. This discrepancy is not understood; however, it is possible that an abnormal *uncB* gene product may be formed which complements the *uncB402* allele with respect to the ATP-dependent transhydrogenase activity but that the complementation is insufficient to give growth on succinate or a normal growth yield.

The *uncA* gene was transcribed after the *uncB* gene and before the *uncC* and *uncD* genes. Thus, no complementation of the *uncA401* allele occurred in the presence of the *unc-413*::Mu ($B^-A^-D^-C^-$) or *unc-418*::Mu ($B^+A^-D^-C^-$) allele as judged from the data obtained from both the growth studies and from the experiments with membrane preparations. Furthermore, complementation occurred between the *uncA401* allele and the *unc-421*::Mu ($B^+A^+D^-C^-$) or *unc-415*::Mu ($B^+A^+D^+C^-$) allele. The partial complementation found in the experiments with membrane preparations is similar to that obtained previously between the *uncA401* allele and the normal *uncA* gene (14).

The conclusion regarding the order of the *uncD* and *uncC* genes is based mainly on the complementation obtained between the *uncD409* mutant allele and the *unc-415*::Mu ($B^+A^+D^+C^-$) allele. Complementation was judged to occur both from the small colonies formed during complementation tests on succinate and from the intermediate growth yield obtained with strain AN980 [*uncD409/unc-415*::Mu ($B^+A^+D^+C^-$)]. In addition, membranes prepared from the segregant strains carrying the *unc-415*::Mu ($B^+A^+D^+C^-$) allele (e.g., strain AN973) had, unlike normal strains, Mg-ATPase activity both on the membrane and in the cytoplasmic fraction. Mutant strains carrying the *uncC424* allele have a similar distribution of Mg-ATPase activity but higher specific activities (15). Furthermore, the *uncD* gene has been shown to code for the β-subunit of the Mg-ATPase (D. R. H. Fayle, J. A. Downie, G. B. Cox, F. Gibson, and J. Radik, Biochem. J., in press), and a strain carrying a mutation in the *uncD* gene lacks Mg-ATPase activity (5). Thus,

it is unlikely that Mg-ATPase activity would be obtained in the absence of the β-subunit. The Mg-ATPase activity in membranes prepared from strain AN980 (*uncD409/unc-415*::Mu) was less than that found in the membranes prepared from the corresponding segregant strain. This is consistent with the previously observed effect of the *uncD409* allele when it is present in a partial diploid strain with the normal *uncD* gene (5).

An additional complication in using the results obtained from experiments with membrane preparations to determine the order of the *uncD* and *uncC* genes is the apparent increased permeability to protons, as inferred from the effect of dicyclohexylcarbodiimide on NADH-induced Atebrin fluorescence quenching, of some of the membrane preparations. The partial diploid strains giving such "leaky" membrane preparations carried either the *unc-421*::Mu ($B^+A^+D^-C^-$) or *unc-415*::Mu ($B^+A^+D^+C^-$) allele in combination with the *uncD409* or *uncC424* mutant allele. Partial genetic complementation in these strains was therefore probably not reflected in the values obtained for the ATP-induced Atebrin fluorescence quenching and ATP-dependent transhydrogenase activity. The reason for the apparent increased proton permeability in membranes from these particular strains is not known.

A correlation was observed between cytoplasmic Mg-ATPase activity and growth yields. Those strains with high levels of Mg-ATPase activity had lower growth yields than those normally observed for uncoupled strains (see, for example, the results obtained for the partial diploid strains AN968 [*uncC424/unc-421*::Mu] and AN978 [*uncC424/unc-415*::Mu] and the haploid strains carrying the *unc-415*::Mu allele). It seems likely that not only are these strains incapable of forming ATP by oxidative phosphorylation, but also the cytoplasmic Mg-ATPase activity hydrolyzes ATP produced by substrate-level phosphorylation.

It should be emphasized that it is not known in which genes the various Mu insertions occurred. The number of genes in the *unc* operon has yet to be determined, but this can presumably be accomplished by the genetic characterization of further point and polarity mutants. The possibility that all of the components of the Mg-ATPase complex are coded for by the *unc* operon may imply that the individual polypeptide components are present in equimolar proportions.

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