JOURNAL OF BACTERIOLOGY, May 1992, p. 3370–3376
0021-9193/92/103370-07$02.00/0
Copyright © 1992, American Society for Microbiology
Vol. 174, No. 10

Effects of Inducing Expression of Cloned Genes for the F_0 Proton Channel of the *Escherichia coli* F_1F_0 ATPase

ROBERT A. MONTICELLO, EVELINA ANGOV,† AND WILLIAM S. A. BRUSILOW*

Department of Biochemistry, Scott Hall, Wayne State University School of Medicine, 540 E. Canfield Avenue, Detroit, Michigan 48201

Received 23 January 1992/Accepted 12 March 1992

To evaluate whether expression of cloned genes for the F_0 proton channel of the *Escherichia coli* F_1F_0 ATPase is sufficient to cause membrane proton permeability, plasmids carrying different combinations of the *uncB*, *E*, and *F* genes, encoding the a, c, and b subunits of the F_0 sector, cloned behind the inducible *lac* promoter in pUC9 or pUC18, were constructed. The effects of inducing F_0 synthesis in an *unc* deletion strain were monitored by measuring cell growth rate, quantitating F_0 subunits by immunoblotting, and measuring the ability of membranes to maintain a respiration-induced proton gradient and to bind F_1 and carry out energy-coupling reactions. The levels of functional reconstitutable F_0 in membranes could be increased four- to sixfold with no change in cellular growth rate or membrane proton permeability (assayed by fluorescence quenching). These results were obtained in uninduced cultures, so the F_0 genes were presumably being transcribed from some promoter besides *lac*. Induction of transcription of all three F_0 genes produced increased amounts of F_0 subunits in membranes as determined by immunoblot and F_1-binding assays, but, when reconstituted with F_1, the F_0 in membranes isolated from induced cultures was significantly less functional than the F_0 in membranes isolated from uninduced cultures. Such induction did result in growth inhibition, but there was no correlation between growth inhibition and either increased membrane proton permeability or the presence of functional, reconstitutable F_0.

The F_1F_0 proton translocating ATPase of *Escherichia coli* consists of an intrinsic membrane-bound F_0 sector to which an extrinsic F_1 sector is bound. The F_0 sector constitutes the transmembrane proton channel and is made up of three subunits: a, b, and c. The F_1 sector consists of five subunits: α, β, γ, δ, and ε. The subunit stoichiometry of the assembled F_1F_0 ATPase has been determined to be $a_1b_2c_{10}\alpha_3\beta_3\gamma_1\delta_1\varepsilon_1$ (8). The genes encoding these subunits are located in the *unc* operon, positioned at 84 min on the *E. coli* chromosome. The gene arrangement is *uncI*, *B*, *E*, *F*, *H*, *A*, *G*, *D*, *C*, corresponding, respectively, to i, a protein of unknown function, and subunits a, c, b, δ, α, γ, β, and ε (28). The different numbers of subunits in the assembled complex are believed to be the result of differential translation of the *unc* genes, each of which is present in a single copy in a 7-kb polycistronic mRNA (5, 12, 16).

The pathway of protons through F_0 is not known. All three subunits are required for reconstitution of a functional F_0 (22, 23). Various residues in the a and c subunits are believed to constitute a proton pathway from one side of the membrane to the other (24). There is evidence, however, that the proton conductivity of F_0 is strongly influenced by the presence or absence of certain F_1 subunits. Genetic studies indicated that F_0-dependent growth inhibition requires the F_0 genes plus the genes for several of the F_1 subunits (4, 18). Purified F_0 sectors synthesized and assembled in the absence of F_1 subunits were significantly less proton permeable when reconstituted into liposomes than were F_0 sectors synthesized and assembled in the presence of F_1 subunits (19).

We proposed a model for F_0 assembly in which F_0 was synthesized and assembled in a relatively proton-impermeable form and then converted to its active, proton-conducting form by specific interactions with F_1 subunits (18). As one test of this hypothesis, we constructed several plasmids carrying the genes for different F_0 subunits cloned behind the inducible *lac* promoter and measured the effects of increasing amounts of F_0 subunits on cell growth, membrane proton permeability, and the ability of membranes to bind purified F_1 and carry out energy coupling reactions. The results support the model described above and also suggest that overexpression of F_0 genes produces unassembled subunits that inhibit cell growth without necessarily affecting membrane proton permeability.

MATERIALS AND METHODS

Strains and plasmids. These studies were done in *E. coli* JM103 Δ(*uncB-uncD*), which is strain JM103 (13) in which seven of the nine *unc* genes (11), including all of the F_0 genes, are deleted. Plasmid pEA4, which carries the three F_0 genes in pACYC184, was described previously (2). Plasmid pEA5, which carries the three F_0 genes cloned behind the inducible *lac* promoter, was constructed by cloning the *Hin*dIII-*Sal*I fragment from pEA4 into pUC9 (25). Plasmid pRM2 was constructed by deleting the *Hpa*I-*Sma*I fragment containing *uncF* from pEA5. Plasmid pRM2 was then digested with *Bam*HI and religated to delete 617 bases from *uncB*, producing plasmid pRM3, which codes for the c subunit only. Plasmid pRM4 was constructed by cloning the *Hin*dIII-*Sal*I fragment from pEA4 into pUC18. The resultant plasmid carries the same insert as pEA5, but the insert is in the opposite orientation to the *lac* promoter. When it is important for clarity, the subunits contained in each plasmid are given within parentheses after the plasmid designation.

Growth and *lac* induction. Cells were grown in LB medium (14) containing 100 mg of ampicillin per liter, and growth was measured by monitoring cell turbidity (optical density at 550

* Corresponding author.
† Present address: Genetics and Biochemistry Branch, National Institutes of Diabetes and Digestive and Kidney Diseases, Bethesda, MD 20892.

nm [OD_{550}]). When cells reached an OD_{550} of 0.4, transcription from the *lac* promoter was induced by the addition of isopropyl thio-β-galactopyranoside (IPTG) to a final concentration of 1 mM. As reported by Fillingame et al. (7), induced cultures rapidly lost plasmids, as measured by the fraction of the culture that lost resistance to ampicillin. The addition of ampicillin to a final concentration of 400 mg/liter was reported to overcome this problem. We also found this to be the case, and so we added more ampicillin at the same time as IPTG.

Membrane preparation. Cells were chilled, centrifuged, and suspended in 50 mM morpholinepropanesulfonic acid (MOPS)–10 mM $MgCl_2$ (pH 7) (MOPS-Mg buffer) to a final concentration of 0.25 g of wet cells per ml. Cells were lysed in a French press at 16,000 lb/in^2. Unlysed cells were removed by centrifugation at 5,000 × *g* for 10 min. The membrane-containing supernatant fractions were centrifuged at 100,000 × *g* for 1 h. Membranes were suspended in MOPS-Mg at a final concentration of 20 to 40 mg of membrane protein per ml.

Fluorescence quenching. The use of 9-amino 6-chloro 2-methoxyacridine (ACMA) fluorescence quenching as an assay of membrane proton permeability was described previously (17). ACMA was obtained from Molecular Probes Inc. (Eugene, Oreg.). Samples of 0.5 mg of membrane protein were assayed in 2 ml of 20 mM Tris-HCl (pH 7.8)–200 mM KCl–5 mM $MgSO_4$ (fluorescence quenching buffer). Final concentrations of ACMA, NADH, and KCN were 5 μM, 250 μM, and 1 mM, respectively, for NADH-driven quenching. Final concentrations of ACMA, ATP, and NH_4Cl were 2.5 μM, 250 μM, and 20 mM, respectively, for ATP-driven quenching. Membrane suspensions were excited at 410 nm, and emission was measured at 490 nm in an SLM model 8000 fluorimeter.

F_1 binding, ATPase, and ATP synthase assays. A 1-mg sample of membrane protein was incubated with 1 or 2 U of purified F_1 ATPase (specific activity, 40 to 50 U/mg) and an equal volume of 2× fluorescence quenching buffer for 15 min at 30°C. The membranes were then centrifuged at 100,000 × *g* for 1 h, washed once with MOPS-Mg buffer, suspended in 200 μl of the same buffer, and assayed. For ATP-dependent fluorescence quenching assays, 0.25 mg of reconstituted membrane protein was assayed directly without washing. In vitro ATPase and ATP synthase assays were conducted as described previously (21).

Immunoblots. Sodium dodecyl sulfate-gel electrophoresis of crude membranes (15 μg of each), transfer to nitrocellulose, and immunoblotting were carried out as described previously (4). The primary polyclonal antibodies against the a, b, and c subunits were obtained from Karlheinz Altendorf, University of Osnabrück, Osnabrück, Germany.

RESULTS

The plasmids constructed for these studies are shown in Fig. 1. Plasmid pEA5 consists of *uncB*, *E*, and *F* cloned behind the *lac* promoter in pUC9. Plasmids pRM2, pRM3, and pRM4 carry subsets of the F_0 genes cloned into pUC plasmids. We studied the effects of these plasmids on the growth of their host *E. coli* and the synthesis and assembly of F_0 sectors.

Figure 2 shows the effects of each of these plasmids on the growth of *E. coli* JM103 in which the chromosomal *unc* genes are deleted. The addition of IPTG to induce transcription of the cloned genes from the *lac* promoter resulted in significant inhibition of growth in cells carrying pEA5, which

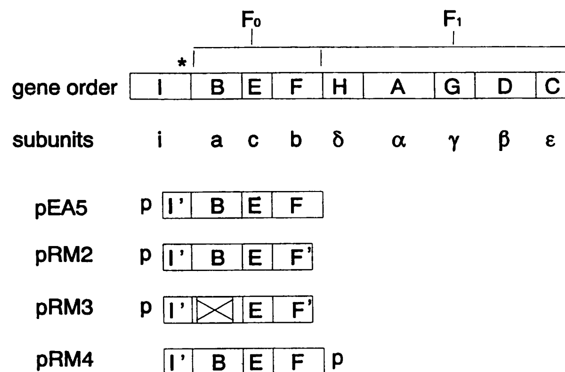

FIG. 1. Plasmids constructed for these studies. The boxes at the top indicate the order of the genes of the *unc* operon. The letter designations for each gene are within each box, and the F_0 and F_1 genes are labeled. The subunits coded for by each gene are indicated below the boxes. The four plasmids are listed on the left, and the F_0 genes cloned in each plasmid are indicated by the boxed letter gene designations. In each, the F_0 genes are cloned into a pUC vector so that transcription can be initiated at the *lac* promoter. The location of that promoter relative to the cloned genes is indicated. Note that the F_0 genes of pRM4 are cloned in the wrong orientation relative to the promoter so that transcription from the *lac* promoter produces antisense *unc* mRNA. The constructions of each plasmid and the restriction enzyme recognition sites at each end of the cloned DNA are described in Materials and Methods. The asterisk indicates the region containing a putative promoter (see the text). This region is bounded by a *Hin*dIII site in *uncI*, which is the left-end (as drawn) limit in pEA5, pRM2, and pRM3, and by the start of *uncB*.

codes for the a, c, and b subunits of F_0. However, we also observed growth inhibition when IPTG was added to cells carrying pRM2, which codes only for the a and c subunits of F_0. Induction of transcription in a control plasmid, the vector pUC9, had no effect on growth; nor did induction of transcription of plasmid pRM3, carrying the gene for the c subunit, or plasmid pRM4, carrying the F_0 genes cloned into pUC18 in the opposite orientation to pEA5. Induction of transcription of the *uncE* and *F* genes, coding for the c and b subunits, respectively, or *uncF* and *H* genes, coding for the b and δ subunits, respectively, also had no effect on growth (data not shown).

We isolated membranes from these various cells and tested them for the presence of F_0 subunits in immunoblots (Fig. 3) and F_1-binding ability (Fig. 4), and for the ability of reconstituted membranes to carry out energy coupling reactions (Fig. 5, Table 1). Figure 3 shows immunoblots of membranes isolated from induced and uninduced cultures of the *unc* deletion strain carrying pEA5 (acb), pRM2 (ac), or pRM4 (acb, opposite orientation to pEA5). The levels of F_0 subunits in the 60-min sample isolated from uninduced cells carrying pEA5 were comparable to those in normal Unc^+ cells (data not shown). Both the induced and uninduced cultures of pEA5 produced significantly more of all of the F_0 subunits than did the initial uninduced culture. In reconstituted membranes from both induced and uninduced cultures treated with a given amount of purified F_1, the specific ATPase activity was 4 to 6 times higher than the specific ATPase activity of reconstituted membranes isolated from the culture before induction (t_0 membranes) Membranes isolated from the induced culture (induced membranes) consistently bound F_1 to a higher specific activity than did membranes isolated from the uninduced culture (uninduced membranes). Immunoblot and F_1 binding studies, therefore,

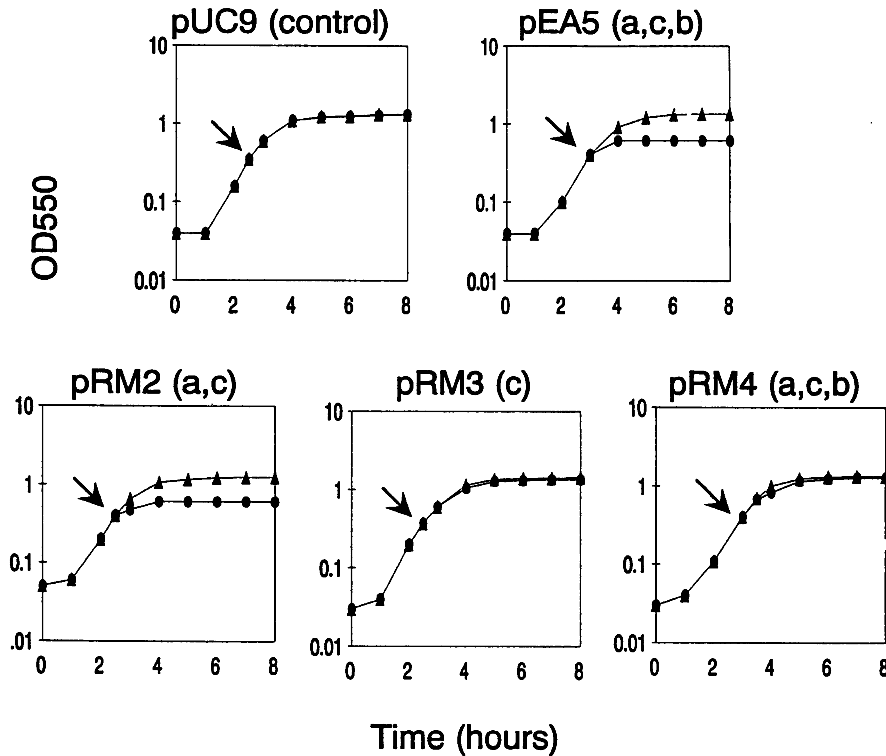

FIG. 2. Growth curves of induced (●) and uninduced (▲) cultures. *E. coli* JM103Δ(*uncB-uncD*) carrying each of the indicated plasmids was grown with shaking at 37°C in two 250-ml cultures of LB medium containing 100 μg of ampicillin per ml to an OD_{550} of approximately 0.4. Half of each culture was treated with 1 mM IPTG (arrow) to induce transcription from the *lac* promoter, and 300 μg of ampicillin per ml was added to both halves of the culture to prevent plasmid loss. The OD_{550} was monitored for an additional 6 h.

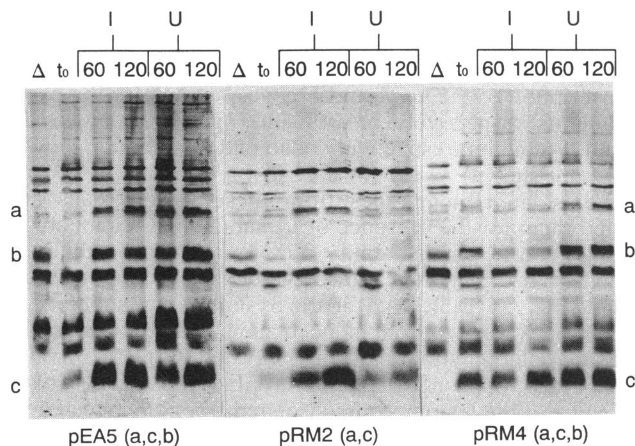

FIG. 3. Immunoblots of sodium dodecyl sulfate gels of membranes isolated from induced and uninduced plasmid-containing cultures. The three panels represent cultures carrying the plasmids indicated below each panel. Lanes: Δ, membranes from an *unc* deletion strain; t_0, membranes isolated from each culture at an OD_{550} of 0.4, prior to induction of transcription; I_{60} and I_{120}, membranes isolated 60 and 120 min, respectively, after the addition of IPTG to the induced culture; U_{60} and U_{120}, membranes isolated at the same times from the uninduced culture. Equal amounts of membrane protein (15 μg) were loaded in each lane. The immunoblots were visualized with antibodies to the F_0 subunits as described in Materials and Methods. The locations of the a, b, and c subunits are indicated.

demonstrated that both induced and uninduced membranes contained amounts of F_0 subunits that were comparable to what is found in membranes isolated from normal Unc^+ cells and 4 to 6 times more F_0 subunits than the amount in t_0 membranes.

Assays of energy coupling by the reconstituted F_1F_0 complexes, however, revealed significant differences between the F_0 sectors synthesized by the induced and uninduced cultures carrying pEA5. In ATP-driven fluorescence quenching studies, a decrease in ACMA fluorescence indicates the formation of a proton gradient due to proton pumping by the reconstituted F_1F_0. Although the induced membranes bound F_1 to a higher specific activity than did the uninduced membranes, reconstituted induced membranes displayed very poor ATP-driven fluorescence quenching when compared with that of the uninduced membranes, which displayed 50 to 75% of the ATP-driven fluoresence quenching of wild-type membranes (Fig. 5). In NADH-driven ATP synthesis assays (Table 1), the reconstituted membranes from the uninduced culture synthesized ATP significantly better than did membranes isolated from the induced culture. Therefore, even though the membranes isolated from the uninduced culture contained less F_0 and bound F_1 to a lower specific activity than did membranes isolated from the induced culture (60-min samples; Fig. 3 and 4), the F_1F_0 in those reconstituted uninduced membranes carried out energy coupling reactions significantly better than did the F_1F_0 in the reconstituted induced membranes. Induction of transcription produced more membrane-bound F_0 subunits but less functional F_0.

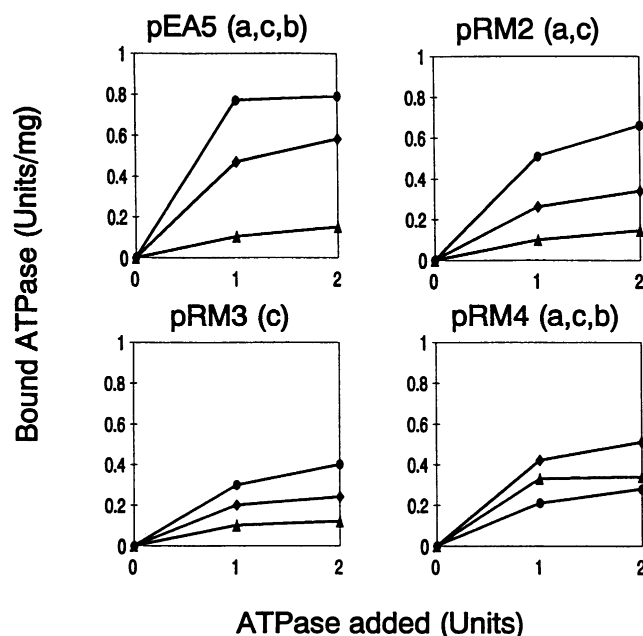

FIG. 4. Binding of purified F_1 ATPase to membranes isolated from induced and uninduced cultures. Membranes (1 mg) isolated from preinduced t_0 (▲), induced (●), or uninduced (◆) cultures were incubated with 0, 1, or 2 U of purified F_1 ATPase, washed, and assayed for bound ATPase activity. The final specific activities (units per milligram of membrane protein) are plotted on the ordinate as a function of added F_1 (abscissa). Each panel represents assays of membranes from a culture containing the indicated plasmid. Each point represents the average of duplicate samples. The error was typically less than 10%.

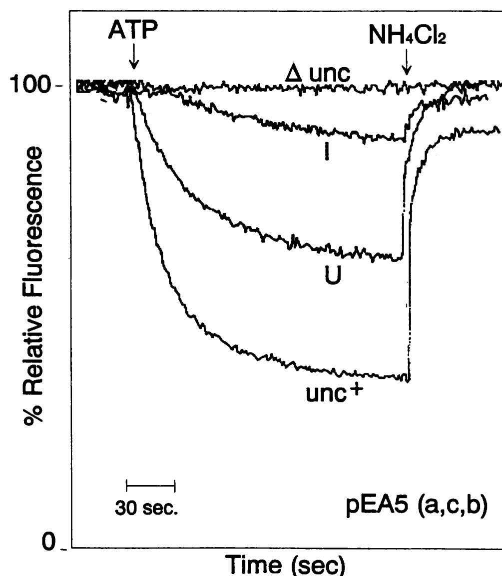

FIG. 5. ATP-dependent ACMA fluorescence quenching of reconstituted F_0-containing membranes. Control membranes and membranes isolated from induced (I) or uninduced (U) cultures of cells carrying pEA5 (acb) were treated with F_1-stripping buffer (1 mM Tris-Cl [pH 7.5], 0.5 mM EDTA, 2.5 mM 2-mercaptoethanol, 10% glycerol), reconstituted with F_1 as described in the legend to Fig. 4, and assayed for ATP-driven ACMA fluorescence quenching as described in Materials and Methods. Relative fluorescence is plotted versus time. Controls are membranes isolated from an *unc* deletion strain, JM103 Δ(*uncB-uncD*), and from an Unc^+ strain, JM103.

To address the possibility that the induced cells were incorporating F_0 into inclusion bodies that might bind F_1 but might be incapable of energy coupling, we isolated membranes on a sucrose gradient; in immunoblots and in ATP-driven fluorescence quenching assays, the purified membranes behaved like crude membranes prepared as described in Materials and Methods (data not shown). The F_0 produced by the induced culture of pEA5 was therefore not sequestered in inclusion bodies.

Respiration-dependent fluorescence quenching studies (Fig. 6) to measure the proton permeability of these membranes did not reveal the cause of the growth inhibition or the differences between the F_0 sectors synthesized in induced or uninduced cultures of the *unc* deletion strain carrying pEA5 (acb). Uninduced membranes showed little change in proton permeability compared with that of t_0 membranes, although the F_1 binding and reconstituted ATP synthase activities both increased four- to sixfold. Induced membranes were leakier than the membranes isolated from uninduced cultures, but the differences varied from experiment to experiment, and significant differences in reconstituted energy coupling were seen even in samples of induced and uninduced membranes, which displayed little difference in NADH-driven fluorescence quenching.

The membranes isolated from either induced or uninduced cultures of pRM2 (ac) displayed no change in proton permeability despite the fact that induction resulted in both growth inhibition and an increased membrane content of the a and c subunits. (Fig. 2, 3, and 6). Combined with the results obtained from induced and uninduced cultures containing pEA5 (acb), these studies indicate that there was little or no correlation between growth inhibition and either an increase in F_0 content or changes in membrane proton permeability as measured by NADH-driven fluoresence quenching. Interestingly, membranes isolated from cells carrying either pRM2 (ac) or pRM3 (c) were capable of binding purified F_1 (Fig. 4). Neither of these reconstituted complexes was capable of ATP synthesis or ATP-driven proton pumping.

The immunoblots and assays of F_0 function revealed that, even in the uninduced cultures, the F_0 genes were being transcribed and translated. A certain amount of this transcription may have been due to leakiness of the *lac* promoter, even in a *lac*I^q background. However, from a genetic

TABLE 1. NADH-driven in vitro ATP synthesis activity of reconstitution membranes[a]

F_1-reconstituted membranes	ATP synthase activity (nmol/min/mg)
JM103 Δ(*uncB-uncD*)	0
JM103 Δ(*uncB-uncD*) + pEA5	
t_0	3 ± 1
I	5 ± 1
U	15 ± 1
JM103 Unc^+	90 ± 10

[a] Membranes isolated from the *unc* deletion strain carrying pEA5 were reconstituted with purified F_1 as described in the legend to Fig. 4 and assayed for in vitro ATP synthesis as described in Materials and Methods. t_0, I (induced), and U (uninduced) membranes were as described in the legend to Fig. 6. Membranes isolated from JM103 Unc^+ and JM103Δ(*uncB-uncD*) were the positive and negative controls, respectively.

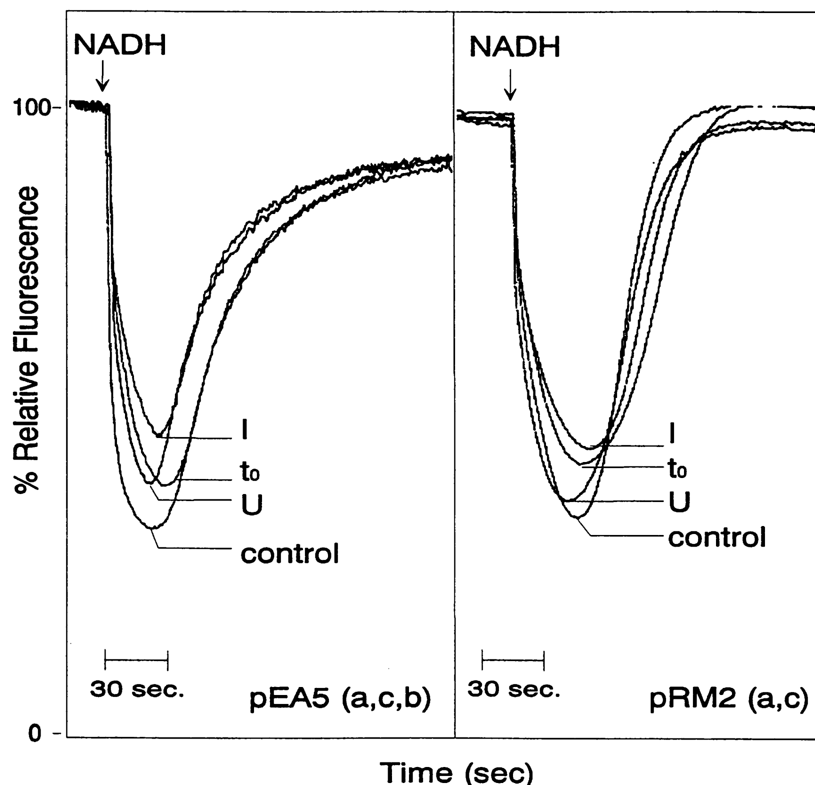

FIG. 6. NADH-dependent ACMA fluorescence quenching of F_0-containing membranes. Membranes isolated from the *unc* deletion strain carrying either pEA5 (acb) or pRM2 (ac) were treated with NADH in the presence of ACMA, and the resultant fluorescence quenching was measured versus time. Control membranes were isolated from the deletion strain containing pUC9 (uninduced; OD_{550} of ≈1). t_0 membranes were isolated from cells at an OD_{550} of 0.4 before induction of transcription. Induced (I) and uninduced (U) membranes were isolated from induced and uninduced cells 60 min after the addition of IPTG to the induced culture. For both the pEA5 and pRM2 cultures, induction resulted in significant growth inhibition (Fig. 2).

complementation analysis of F_0 plasmids containing various deletions, Kanazawa et al. (9) concluded that a functional promoter exists immediately preceding *uncB*. This promoter is located in the region under the asterisk in Fig. 1. Plasmid pRM4 consists of the F_0 genes cloned backwards into pUC18, so that the transcription from the *lac* promoter produces antisense *unc* mRNA. If this region does carry a functional *unc* promoter, uninduced cells carrying pRM4 should synthesize amounts of F_0 comparable to those synthesized by the uninduced pEA5 culture. Figures 3 and 4 demonstrate that to be the case. Induction of transcription from the *lac* promoter resulted in a significant decrease in expression of F_0 genes from this plasmid. It is therefore likely that, as concluded by Kanazawa et al. (9), there is an active promoter other than the previously demonstrated *unc* promoter (10, 20, 29) immediately upstream of *uncB* but either within or immediately after *uncI*.

DISCUSSION

These results demonstrate that the amount of functional, reconstitutable F_0 in *E. coli* membranes can be increased four- to sixfold, from very low levels to levels approximately equal to those in normal Unc^+ membranes, with no change in cellular growth rate or membrane proton permeability, supporting the hypothesis that, in the absence of F_1 subunits, F_0 is synthesized and assembled in a form that is relatively impermeable to protons. Past results from our laboratory have shown that the presence of certain F_1 subunits, specifically δ and α, significantly increase the proton-conducting ability of F_0 (2, 4, 18). Studies involving purified F_0 sectors have shown that F_0 synthesized and assembled in the absence of F_1 is three to five times less permeable to protons when reconstituted into liposomes than F_0 made in the presence of F_1 (19).

The reconstituted F_1F_0 complexes of the induced and uninduced pEA5 (acb) membranes differed in their abilities to carry out the two energy coupling reactions. Uninduced membranes, when reconstituted with F_1, contained 50 to 75% of the specific ATPase activity of Unc^+ membranes and were capable of ATP-dependent proton pumping at levels 50 to 75% of those displayed by Unc^+ membranes, indicating that these F_0 sectors are structurally and functionally identical or very similar to normal F_0. The same reconstituted membranes, however, could synthesize ATP in vitro only 10 to 20% as well as Unc^+ membranes. It is not clear why reconstitution of ATP synthesis was not as efficient as reconstitution of ATP-driven proton pumping.

Induction of transcription from the *lac* promoter appeared to adversely affect the function of the resultant F_0. Membranes isolated from the induced culture of pEA5 contained increased amounts of F_0 subunits, as assayed by immunoblotting and F_1 binding, relative to those in membranes of the uninduced culture. When reconstituted with F_1, the membranes from the induced culture did not carry out either energy coupling reaction very well, either because the F_0

sectors were less active in energy coupling or because the membranes were more proton permeable. However, in experiments where the differences in apparent proton permeability of induced and uninduced membranes were very small, the reconstituted membranes still displayed the same differences in ATP synthase and ATP-driven proton pumping activities. For induced membranes which showed a significant difference in proton permeability, as assayed by NADH-driven fluorescence quenching, we adjusted the fluorescence quenching of the uninduced membranes to that of the induced membranes by adding KCN. Even then, the differences in energy coupling persisted (data not shown). The data suggest that the F_0 subunits in the membranes isolated from induced cultures were either degraded or less well assembled than the F_0 sectors present in the membranes of the uninduced culture. The immunoblots, however, did not reveal significant proteolysis of the F_0 subunits in the induced membranes.

These differences in F_0 sectors produced by induced and uninduced cultures might be explained if assembly of the F_0 required an additional factor or factors, such as has been shown to be necessary for assembly of the *Saccharomyces cerevisiae* mitochondrial F_0 (1). In the induced cultures, increasing expression from the *lac* promoter might titrate out that assembly factor, resulting in incorrect folding or assembly of additional F_0 subunits synthesized, whereas uninduced cultures might produce subunits slowly enough not to overwhelm the assembly machinery. Our experiments did not address this question, since we analyzed the differences in F_0 content of membranes 1 and 2 h after induction, rather than immediately after induction. If such an assembly factor exists, it might not be absolutely required, since measurable functional F_0 might be produced even in its absence. The presence of such an assembly factor might, however, either minimize incorrect subunit-subunit interactions or optimize the folding, membrane insertion, or assembly of one or more F_0 subunits. One model for F_0 assembly proposed that the α and β subunits of F_1 act as such assembly factors (6), and the recent demonstration that the entire operon can be overexpressed to produce an abundance of functional F_1F_0 may support that proposal (15). However, in these studies, we demonstrated wild-type levels of functional reconstitutable F_0 made in the absence of any F_1 subunits.

Inducing expression of F_0 genes cloned behind the *lac* promoter did affect cell growth, but the effects of this induction on F_0 synthesis and assembly and the reasons for the resultant growth inhibition are not clear. In respiration-driven fluorescence quenching experiments, the membranes isolated from the induced culture of cells carrying pEA5 were occasionally leakier (quenched less well) than membranes from uninduced cultures, but the quenching was reduced by at most 50%. In most experiments the difference between respiration-driven fluorescence quenching in membranes from induced and that in membranes from uninduced samples was small, despite the difference in growth of the cultures. Also, induced cultures of cells carrying pRM2, which codes only for the a and c subunits of F_0, also stopped growing. Membranes isolated from those cells were no more permeable to protons than were membranes isolated from the uninduced culture. These membranes bound F_1 but were not capable of ATPase-dependent energy coupling, so they did not contain functional F_0. Combined with the results from the uninduced culture of pEA5, these results indicate that the growth inhibition seen in induced cells carrying F_0 plasmids is not necessarily the result of leaky F_0 sectors. Kanazawa et al. (9) demonstrated that the harmful effects of cloned F_0 genes were caused primarily by production of the a subunit; our results are consistent with that explanation, since it is clear that functional F_0 produced in the uninduced cultures does not affect cell growth or proton permeability. The growth inhibition observed upon induction of transcription of pRM2 (ac) was not seen when transcription was induced from pRM3, which codes only of for the c subunit. An excess of unassembled or misassembled a subunit might therefore be responsible for the observed growth inhibition. von Meyenberg et al. (26) demonstrated that overproduction of the a subunit inhibited growth by increasing the proton permeability of membranes. In these studies, however, we were unable to attribute growth inhibition to increased proton permeability.

The results and conclusions from this study are compatible with other studies on the synthesis and assembly of F_0 genes from multicopy plasmids. Aris et al. (3) concluded that the synthesis and assembly of F_0 from multicopy plasmids did not affect cell growth. Fillingame et al. (7) demonstrated that overproduction of the genes for F_0 plus δ resulted in harmful proton permeability in Unc^+ *E. coli*, but the effect was not as great in an *unc* deletion strain. When the genes were expressed in the deletion strain, as in this study, that study reported some growth inhibition and increased F_1 binding and proton permeability but little or no reconstituted ATP-dependent proton pumping in the reconstituted membranes. This inability to carry out energy coupling was attributed to possible proteolysis of the F_0 subunits synthesized in the absence of F_1. We observed similar results with membranes from our induced cultures, but we believe those cells carried misassembled or unassembled F_0 subunits, since we saw no significant proteolysis of F_0 subunits in the immunoblots, and the uninduced cultures, which contained fewer F_0 subunits, reconstituted energy coupling better. Our previous studies have shown that δ is opening the F_0 proton channel (2). When we constructed a plasmid carrying the F_0 genes and *uncH* (δ subunit) cloned behind the *lac* promoter, the presence of the δ subunit significantly affected the results of these various assays, making the membrane appear to be even more permeable to protons in both the induced and uninduced cultures (data not shown).

The uninduced cultures appeared to be expressing the F_0 genes from a promoter on the cloned region of the *unc* operon. Analyzing membranes isolated from cells carrying plasmid pRM4, in which the F_0 genes are cloned into pUC18 in the opposite orientation to the *lac* promoter, we still observed significant synthesis of F_0 genes in the uninduced culture, synthesis which was reduced by inducing transcription from the *lac* promoter. If the F_0 genes were being transcribed from a true *unc* promoter, such expression from that promoter may be growth dependent, since at optical density of 0.4, much less F_0 was being synthesized than at later times in the uninduced cultures. As referred to in Results, Kanazawa et al. (9) also concluded that an active promoter exists in a region identical to what we studied in these experiments.

ACKNOWLEDGMENTS

This research was supported by National Science Foundation grant DMB-9096159 and by American Heart Association Grant-in-aid 900799. W.S.A.B. is the recipient of Public Health Service research career development award K04-AI00882 from the National Institutes of Health.

REFERENCES

1. **Ackerman, S. H., and A. Zagoloff.** 1990. ATP10, a yeast nuclear gene required for the assembly of the mitochondrial F_1F_0 complex. J. Biol. Chem. **265:**9952–9959.
2. **Angov, E., T. C. N. Ng, and W. S. A. Brusilow.** 1991. Effect of the δ subunit on assembly and proton permeability of the F_0 proton channel of *Escherichia coli* F_1F_0 ATPase. J. Bacteriol. **173:**407–411.
3. **Aris, J. P., D. J. Klionsky, and R. D. Simoni.** 1985. The F_0 subunits of the *Escherichia coli* F_1F_0-ATP synthase are sufficient to form a functional proton pore. J. Biol. Chem. **260:** 11207–11215.
4. **Brusilow, W. S. A.** 1987. Proton leakiness caused by cloned genes for the F_0 sector of the proton-translocating ATPase of *Escherichia coli*: requirement for F_1 genes. J. Bacteriol. **169:** 4984–4990.
5. **Brusilow, W. S. A., D. J. Klionsky, and R. D. Simoni.** 1982. Differential polypeptide synthesis of the proton-translocating ATPase of *Escherichia coli*. J. Bacteriol. **151:**1363–1371.
6. **Cox, G. B., J. A. Downie, L. Langman, A. E. Senior, G. Ash, D. R. H. Fayle, and F. Gibson.** 1981. Assembly of the adenosine triphosphate complex in *Escherichia coli*: assembly of F_0 is dependent on the formation of specific F_1 subunits. J. Bacteriol. **148:**30–42.
7. **Fillingame, R. H., B. Porter, J. Hermolin, and L. K. White.** 1986. Synthesis of a functional F_0 sector of the *Escherichia coli* H^+-ATPase does not require synthesis of the alpha or beta subunits of F_1. J. Bacteriol. **165:**244–251.
8. **Foster, D. L., and R. H. Fillingame.** 1982. Stoichiometry of subunits in the H^+-ATPase complex of *Escherichia coli*. J. Biol. Chem. **257:**2009–2015.
9. **Kanazawa, H., T. Kiyasu, T. Noumi, and M. Futai.** 1984. Overproduction of subunit a of the F_0 component of proton translocating ATPase inhibits growth of *Escherichia coli* cells. J. Bacteriol. **158:**300–306.
10. **Kanazawa, H., K. Mabuchi, and M. Futai.** 1982. Nucleotide sequence of the promoter region of the gene cluster for proton-translocating ATPase from *Escherichia coli* and identification of the active promoter. Biochem. Biophys. Res. Commun. **107:** 568–575.
11. **Klionsky, D. J., W. S. A. Brusilow, and R. D. Simoni.** 1983. Assembly of a functional F_0 of the proton-translocating ATPase of *Escherichia coli*. J. Biol. Chem. **258:**10136–10143.
12. **McCarthy, J. E. G.** 1988. Expression of the *unc* genes in *Escherichia coli*. J. Bioenerg. Biomembr. **20:**19–39.
13. **Messing, J., R. Crea, and P. H. Seeburg.** 1981. A system for shotgun DNA sequencing. Nucleic Acids Res. **9:**309–321.
14. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
15. **Moriyama, Y., A. Iwamoto, H. Hanada, M. Maeda, and M. Futai.** 1991. One-step purification of *Escherichia coli* H^+-ATPase (F_0F_1) and its reconstitution into liposomes with neurotransmitter transporters. J. Biol. Chem. **266:**22141–22146.
16. **Nielsen, J., F. G. Hansen, J. Hoppe, P. Friedl, and K. von Meyenburg.** 1981. The nucleotide sequence of the *atp* genes coding for the F_0 subunits a, b, c, and the F_1 subunit δ of the membrane bound ATP synthase of *Escherichia coli*. Mol. Gen. Genet. **184:**33–39.
17. **Nieuwenhuis, F. J. R. M., B. I. Kanner, D. L. Gutnick, P. W. Postma, and K. Van Dam.** 1973. Energy conservation in membranes of mutants of *Escherichia coli* defective in oxidative phosphorylation. Biochim. Biophys. Acta **325:**62–71.
18. **Pati, S., and W. S. A. Brusilow.** 1989. The roles of the α and γ subunits in proton conduction through the F_0 sector of the proton-translocating ATPase of *Escherichia coli*. J. Biol. Chem. **264:**2640–2644.
19. **Pati, S., W. S. A. Brusilow, G. Deckers-Hebestreit, and K. Altendorf.** 1991. Assembly of the F_0 proton channel of the *Escherichia coli* F_1F_0 ATPase: low proton conductance of reconstituted F_0 sectors synthesized and assembled in the absence of F_1. Biochemistry **30:**4710–4714.
20. **Porter, A. C. G., W. S. A. Brusilow, and R. D. Simoni.** 1983. Promoter for the *unc* operon of *Escherichia coli*. J. Bacteriol. **155:**1271–1278.
21. **Scarpetta, M. A., C. A. Hawthorne, and W. S. A. Brusilow.** 1991. Characterization of semi-uncoupled hybrid *Escherichia coli-Bacillus megaterium* F_1F_0 proton translocating ATPases. J. Biol. Chem. **266:**18567–18572.
22. **Schneider, E., and K. Altendorf.** 1984. Subunit b of the membrane moiety (F_0) of ATP synthase (F_1F_0) from *Escherichia coli* is indispensible for H^+ translocation and binding of the water-soluble F_1 moiety. Proc. Natl. Acad. Sci. USA **81:**7279–7283.
23. **Schneider, E., and K. Altendorf.** 1985. All three subunits are required for the reconstitution of an active proton channel (F_0) of *Escherichia coli* ATP synthase (F_1F_0). EMBO J. **4:**515–518.
24. **Schneider, E., and K. Altendorf.** 1987. Bacterial adenosine 5′-triphosphate synthase (F_1F_0): purification and reconstitution of F_0 complexes and biochemical and functional characterization of their subunits. Microbiol. Rev. **51:**477–497.
25. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic primers. Gene **19:**259–268.
26. **von Meyenberg, K., B. B. Jørgensen, O. Michelsen, L. Sørensen, and J. E. G. McCarthy.** 1985. Proton conduction by subunit a of the membrane-bound ATP synthase of *Escherichia coli* revealed after induced overproduction. EMBO J. **4:**2357–2363.
27. **von Meyenburg, K., B. B. Jørgensen, J. Nielsen, and F. G. Hansen.** 1982. Promoters of the *atp* operon coding for the membrane-bound ATP synthase of *Escherichia coli* mapped by Tn10 insertion mutations. Mol. Gen. Genet. **188:**240–248.
28. **Walker, J. E., M. Saraste, and N. J. Gay.** 1984. The *unc* operon. Nucleotide sequence, regulation and structure of ATP-synthase. Biochim. Biophys. Acta. **768:**164–200.