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Effect of the δ Subunit on Assembly and Proton Permeability of the F_0 Proton Channel of *Escherichia coli* F_1F_0 ATPase

EVELINA ANGOV,[1]† THOMAS C. N. NG,[1] AND WILLIAM S. A. BRUSILOW[2]*

Department of Biochemistry, Wayne State University School of Medicine, 540 East Canfield Avenue, Detroit, Michigan 48201,[2] and Department of Chemistry and Biochemistry, University of Maryland, College Park, Maryland 20742[1]

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During the assembly of the *Escherichia coli* proton-translocating ATPase, the subunits of F_1 interact with F_0 to increase the proton permeability of the transmembrane proton channel. We tested the involvement of the δ subunit in this process by partially and completely deleting *uncH* (δ subunit) from a plasmid carrying the genes for the F_0 subunits and δ and testing the effects of those F_0 plasmids on the growth of *unc*$^+$ and *unc* mutant *E. coli* strains. We found that the δ subunit was required for inhibition of growth of *unc*$^+$ cells. We also tested membranes isolated from *unc*-deleted cells containing F_0 plasmids for F_1-binding ability. In *unc*-deleted cells, these plasmids produced F_0 in amounts comparable to those found in normal *unc*$^+$ *E. coli* cells, while having only small effects on cell growth. These studies demonstrate that the δ subunit plays an important role in opening the F_0 proton channel but that it does not serve as a temporary plug of F_0 during assembly, as had been previously speculated (S. Pati and W. S. A. Brusilow, J. Biol. Chem. 264:2640–2644, 1989).

The proton-translocating ATPase of *Escherichia coli* consists of two sectors, F_0 and F_1. F_0 forms a proton channel across the cytoplasmic membrane, and F_1 is an ATPase or ATP synthase, able to interconvert the synthesis or hydrolysis of ATP by using the energy of a transmembrane proton gradient, according to the chemiosmotic hypothesis. In *E. coli*, F_0 consists of three subunits—a, b, and c. F_1 consists of five subunits—α, β, γ, δ, and ε. The genes coding for these subunits exist in an operon designated *unc* (for uncoupled) which is located between 83 and 84 min on the chromosome (8, 9, 16, 17).

A number of studies have addressed the question of how the F_0 proton channel is assembled without the formation of any deleterious intermediates. Cox et al. (3, 4) described a model in which the assembly of F_0 depends upon the presence of certain F_1 subunits. Studies by Aris et al. (1) and Fillingame et al. (7), however, showed that a reconstitutable F_0 could be synthesized and assembled in the absence of F_1 subunits.

Our studies have shown that cloned F_0 genes on multicopy plasmids can produce lethal proton permeability but only in the presence of certain F_1 genes. In the absence of those F_1 genes, most importantly *uncA*, the gene for the α subunit, cloned F_0 genes code for the synthesis and assembly of a reconstitutable but closed F_0 sector (2, 15, 20). In those studies, the *uncH* gene, coding for the δ subunit, was present on all F_0 plasmids, leaving open the possibility that the δ subunit was acting as a temporary plug of the proton channel during assembly. The *E. coli* δ subunit has been shown to be involved in the attachment of F_1 to F_0 in reconstitution studies (19). Additionally, in chloroplasts, the δ subunit has been shown to block the proton channel and to restore photophosphorylation to partially stripped thylakoid membranes (5, 6, 13). In this paper, we describe genetic and biochemical experiments which test the role of the δ subunit in the F_1-F_0 channel-opening interaction during the assembly of the ATPase from plasmid-borne genes.

Strains and media. *E. coli* LE392 (18) or LE392Δ(*uncB-uncD*) (12) was used for all genetic and biochemical studies. Cells were grown in LB medium (14).

Plasmid constructions. The plasmids used in these studies are diagrammed in Fig. 1. Plasmids pWSB35, pRPG28, pRPG23, and pRPG51 have been described previously (2, 10). Plasmid pTN1 was constructed by digesting pWSB35 with *Nru*I and religating. The resultant plasmid, pTN1, coded for the F_0 genes *uncB*, *uncE*, and *uncF* and more than half of *uncH*. Plasmid pTN2 was constructed by digesting pWSB35 with *Bam*HI and religating, resulting in a 617-bp deletion within *uncB*. Plasmid pEA4 was constructed by using site-directed mutagenesis to insert a *Sal*I site within the second codon of *uncH*. A 1,499-bp *Bam*HI-*Eco*RI fragment which carried *uncE*, *uncF*, and *uncH* (c, b, and δ subunits) was cloned from pRPG23 into M13mp18. A 36-base synthetic oligonucleotide (5′-GGAGGGAGGGGCT GATGTCGACTTTATTACGGTAGC-3′) was annealed to single-stranded DNA isolated from phage produced by cells containing the resultant recombinant phage DNA. This primer contains a sequence complementary to the *unc* sequence around the ribosome-binding site and initiation codon for *uncH*, except that it contains a single-base deletion at base 6 (T) of *uncH* and a single-base substitution at base 9 (A to C) of *uncH*. We used the Amersham mutagenesis kit (Amersham Corp.) to replace the wild-type *uncH* sequence with this mutagenized sequence, which resulted in the creation of a *Sal*I site within the second codon of *uncH*. The *Bam*HI-*Sal*I fragment (containing all of *uncE*) from the mutagenized plasmid was sequenced in its entirety to ensure the absence of other mutations. This fragment was then cloned into plasmid pTN2, which had been digested with *Bam*HI and *Sal*I, to produce a plasmid, designated pEA3, coding for *uncE* and *uncF* and carrying a 617-bp *Bam*HI deletion within *uncB*. This missing *Bam*HI fragment was restored by cloning it from pRPG28 into *Bam*HI-digested pEA3. If an F_0 plasmid missing *uncH* were lethal, all viable clones carrying the correct *Bam*HI fragment would have carried it in the wrong orientation. We found, however, that

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

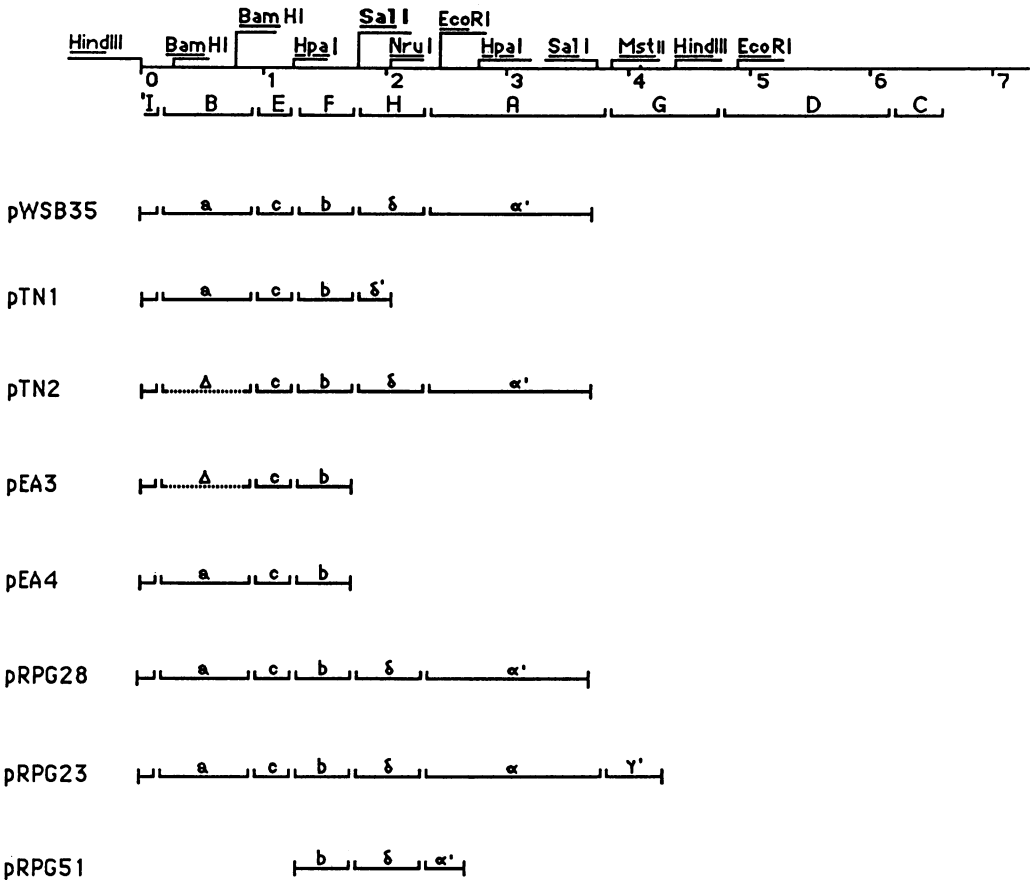

FIG. 1. Plasmids used in these studies. At the top, pertinent restriction enzyme recognition sites within the *unc* operon and the limits of the F_0 and F_1 genes are shown. The indicated scale is in kilobase pairs. B, E, F, H, A, G, D, and C are *unc* genes coding for F_0 subunits a, c, and b and F_1 subunits δ, α, γ, β, and ε, respectively. The *SalI* site created by site-directed mutagenesis is indicated in boldface. Plasmids and plasmid constructions are described in the text. Each plasmid and the ATPase subunits coded for by the *unc* genes present in each plasmid are indicated. Incomplete polypeptides are indicated (').

three of nine plasmids containing inserts carried the fragment in the correct orientation, indicating that the resultant F_0 plasmid, designated pEA4, was not harmful to an *unc* deletion strain.

Membrane preparation, F_1-binding assays, and fluorescence-quenching assays. *E. coli* growth and membrane preparation were carried out as described previously (12). Membranes were not treated with F_1 stripping buffer prior to reconstitution with F_1 or prior to fluorescence-quenching assays. 9-Amino-6-chloro-2-methoxyacridine (ACMA) was obtained from Molecular Probes, Inc. (Eugene, Oreg.). Fluorescence was measured with an SLM model 8000 fluorimeter.

For measurement of binding of F_1 to membranes, 2 mg of membrane protein was incubated with 0.5, 1, or 2 U of purified F_1 for 15 min at 30°C. The membranes were washed twice with 2 ml of 50 mM morpholinepropanesulfonic acid (MOPS)–10 mM $MgCl_2$ (pH 7), resuspended in 200 μl of the same MOPS-$MgCl_2$ buffer, and assayed for ATPase activity as described previously (20).

For the measurement of ATP-driven ACMA fluorescence quenching, 1 mg of membrane protein was reconstituted with 1 U of purified F_1 as described above and assayed for ATP-driven fluorescence quenching directly. The buffer used for these assays was 50 mM Tris hydrochloride–2.5 mM $MgCl_2$ (pH 7.8). The final concentrations of ACMA, ATP, and NH_4Cl were 4 μM, 1 mM, and 20 mM, respectively. The excitation and emission wavelengths were 410 nm and 490 nm, respectively.

Deletion of the δ subunit from F_0 plasmids. It has been shown previously that plasmid pWSB35 (acbδα') is harmful to *unc*$^+$ *E. coli* strains (2). Strain LE392, when transformed with pWSB35, produces pinpoint colonies on rich medium plates containing antibiotic after 24 h at 37°C. This gross deleterious effect on growth can be overcome by the presence of *N,N'*-dicyclohexylcarbodiimide (DCCD), which blocks the F_0 proton channel. F_1 genes or subunits, most importantly *uncA* (α subunit), are required for this deleterious effect, since the growth of chromosomal *unc* deletion strains or an *uncA* mutant is not affected by the presence of pWSB35 (2). In order to test the effect of the δ subunit on F_1-induced proton permeability, we deleted approximately one-third of the *uncH* gene from pWSB35 by digesting the plasmid with *NruI* and religating. The resultant plasmid, pTN1 (acbδ'), did not have any deleterious effect on the growth of *unc*$^+$ cells on plates. When we transformed pTN1 into *unc*$^+$ cells carrying pRPG51, a plasmid containing *uncF* and *uncH* cloned behind the *lac* promoter (10), the deleterious result was restored (Fig. 2). Additional genetic experiments demonstrated that restoration of the deleterious effect

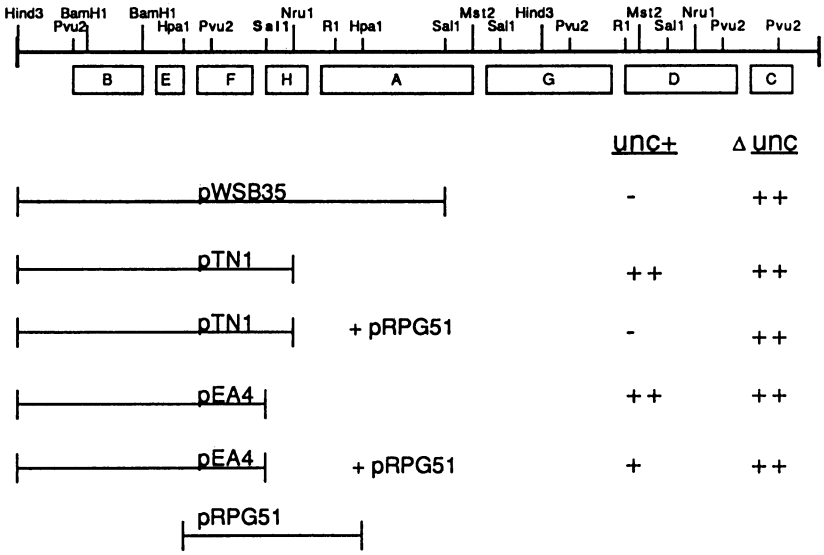

FIG. 2. Effects of F_0-containing plasmids on the growth of *unc*$^+$ and *unc*-deleted *E. coli* strains. The indicated plasmids were transformed into LE392 or LE392Δ(*uncI-uncC*) and plated onto LB-antibiotic plates. Growth was scored after 15 h at 37°C. Symbols: ++, transformation efficiency and colony size comparable to those of controls (pACYC184 for chloramphenicol-resistant plasmids and pBR322 for ampicillin-resistant plasmids); −, no growth or barely discernible growth of pinpoint colonies after 15 h; +, colony size larger than that indicated by − but clearly much poorer growth than indicated by ++. B through C are *unc* genes as described in the legend to Fig. 1.

could be caused by the δ subunit alone (not shown). As was the case with growth inhibition caused by pWSB35, the harmful effect of pRPG51 plus either pTN1 or pEA4 could be overcome by the presence of DCCD.

Plasmid pTN1 coded for almost two-thirds of the δ subunit, which may have been sufficient to block the channel but not sufficient for an effective interaction with other F_1 subunits to unblock the channel. We created a *Sal*I site in the second codon of *uncH* in pWSB35 so that all of *uncH* could be deleted by digesting the resultant plasmid with *Sal*I and religating. This construction, described above, produced plasmid pEA4, which contained only the F_0 genes *uncB*, *uncE*, and *uncF* and not *uncH* or *uncA*. We tested the effects of pEA4 on the growth of *unc*$^+$ and *unc*-deleted *E. coli* strains. Both strains grew equally well on plates, and neither exhibited any deleterious effects on growth compared with strains carrying control, non-*unc*-containing plasmids.

The genetic results obtained with pTN1 and pEA4 are summarized in Fig. 2 and demonstrate that the δ subunit does not act as a temporary plug of the proton channel during the assembly of the ATPase. These results do show, however, that δ is required for the harmful F_1-dependent effect of cloned F_0 genes in strain LE392. Moreover, the harmful effects of cloned F_0 genes apparently require the presence of more δ subunits than are produced by the single copy of *uncH* in the *E. coli* chromosome, since neither pTN1 nor pEA4 appeared to have harmful effects on *unc*$^+$ *E. coli* strains.

Effects of F_0 plasmids on F_1-binding ability. We prepared membranes from *unc*-deleted cells carrying pEA4, pWSB35, or the control plasmid vector pACYC184. At culture turbidities (optical density at 650 nm) of less than 0.5, all three cultures grew equally well in rich (LB) medium. At higher cell densities, the pACYC184 culture grew best and the pWSB35 culture grew worst, but the maximum difference we observed in the extent of growth was approximately 25 to 30%. In all of our experiments, we therefore used membranes from cells grown to an optical density at 650 nm of 0.7 to 0.9 to maximize any effects the cloned F_0 genes might have had on proton permeability and cell growth. We quantitated F_1 binding to membranes isolated from *unc*-deleted cells carrying each of the three plasmids (Fig. 3). The membranes from cells carrying either pEA4 or pWSB35 bound F_1 equally well, and the specific activities of the reconstituted membranes were comparable to those for membranes isolated from wild-type *E. coli* (0.4 to 0.7 U/mg with our assay). A similar reconstitution experiment done by Fillingame et al. (7) produced specific activities of 0.8 to 1.3 U/mg for reconstituted membranes from a strain carrying an F_0-overproducing plasmid and of 1 to 1.5 U/mg for reconstituted membranes which had been stripped of their F_1. The membranes used for our reconstitution experiments were isolated from cells carrying pEA4 or pWSB35, plasmids not engineered to overproduce F_0. These membranes were not stripped, so we would expect the specific activities of our reconstituted membranes to be lower than that for stripped and reconstituted membranes because of the latter's lower total protein concentration. This experiment demonstrated that membranes isolated from cells which grew well (compared with cells containing the control plasmid) did contain abundant reconstitutable F_0 sectors. We reconstituted these membranes and tested their abilities to carry out ATP-driven fluorescence quenching (Fig. 4). Membranes from cells carrying either pEA4 or pWSB35 carried out this reaction equally well, again demonstrating that these membranes contained functional reconstitutable F_0 sectors.

Aris et al. (1) and Fillingame et al. (7) have demonstrated that cloned F_0 genes on multicopy plasmids code for reconstitutable F_0 sectors in cells missing F_1 genes. Both studies correlated a decrease in respiration-induced quenching of membrane-associated ACMA fluorescence with the presence of an increased number of F_0 genes or with increased expression of those genes. We have seen gross inhibition of growth only in those cells which also contain certain F_1

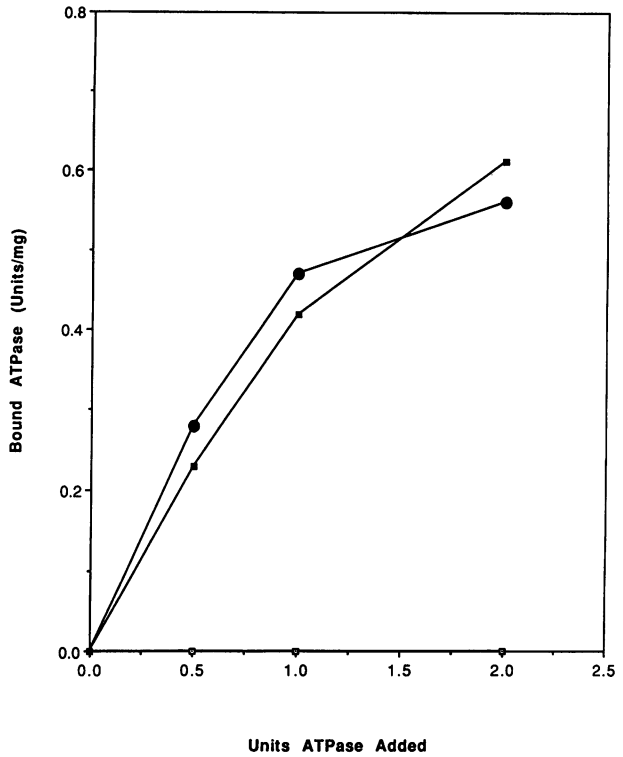

FIG. 3. F_1 binding to membranes of an *unc* deletion strain carrying pACYC184, pEA4, or pWSB35. Membranes were prepared from *E. coli* LE392Δ(*uncB-uncD*) carrying the vector pACYC184 (□), pEA4 (*uncBEF*) (●), or pWSB35 (*uncBEFHA'*) (■), grown in LB medium to an optical density at 650 nm of 0.7 to 0.9. Two milligrams of membrane protein was incubated with the indicated number of units of purified F_1 for 15 min at 30°C, and the mixture was washed twice with 50 mM MOPS–10 mM $MgCl_2$, resuspended in 200 μl of the same buffer, and assayed for membrane-bound ATPase activity.

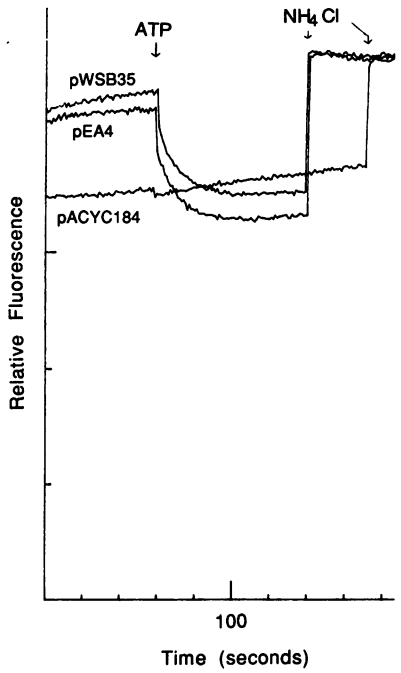

FIG. 4. ATP-driven fluorescence-quenching assays of membranes isolated from cells carrying pACYC184, pEA4, or pWSB35 reconstituted with purified F_1 ATPase. Each division on the ordinate represents 20% relative fluorescence. One milligram of membrane protein was incubated with 1 U of ATPase for 15 min at 30°C and assayed for ATP-driven quenching of ACMA fluorescence, as described in the text. ATP and NH_4Cl were added at the indicated times.

genes in addition to the cloned F_0 genes. Two of the plasmids studied by Aris et al. (1), pDJK19 and pDJK20, were similar to our pEA4 plasmid in that they carried the F_0 genes with very little of the *uncH* gene. Those two plasmids, however, also contained a mutation (at the time undiscovered) in the Shine-Dalgarno region for *uncE* which lowered the synthesis of the c subunit and which we have shown to affect the harmful proton leakiness caused by pWSB35 in *unc*$^+$ cells (20). Fluorescence-quenching experiments with membranes isolated from an *unc* deletion strain carrying either of those plasmids demonstrated that the cloned F_0 genes did not make the membranes grossly permeable to protons but decreased the NADH-driven quenching by approximately one-third (1). The studies of Fillingame et al. (7) demonstrated the lethality of induced F_0 plus δ genes cloned behind the *lac* promoter in pUC9. The results of both studies were interpreted as indicating that F_0 synthesized from cloned genes in the absence of F_1 genes was proton permeable, although Aris et al. (1) concluded that the presence of plasmid-encoded, membrane-bound F_0 did not affect the growth rates of *E. coli*. Our studies demonstrate that *unc*-deleted cells grow well even when carrying F_0 plasmids which code for membrane-bound, reconstitutable F_0 sectors in numbers comparable to those found in *unc*$^+$ *E. coli* cells.

Our interpretation of these various results is that in the absence of F_1 subunits, F_0 can be synthesized and assembled in a form which does not affect proton permeability enough to inhibit cell growth. We speculate that if F_0 plus δ were overproduced, as in the experiments of Fillingame et al. (7), the presence of the δ subunit might increase the proton permeability of cells enough to significantly inhibit growth. In our experiments with cloned F_0 genes in plasmid derivatives of pACYC184, gross inhibition of growth was seen only when F_0 was made in the presence of δ, α, and other F_1 subunits (2, 15). Since all of the experiments discussed above were done on F_0 genes lacking the true *unc* promoter or *uncI* (the first gene of the operon, which has no known function), cloned into different vectors with (undoubtedly) different copy numbers, the conclusions might not apply perfectly to the in vivo situation of F_0 sectors synthesized from single-copy genes. This criticism applies equally to all of these studies except the original study of Cox et al. (3).

Humbert and Altendorf have demonstrated that F_0-dependent resistance to aminoglycosides, caused by increased proton permeability of the cytoplasmic membrane, requires the presence of F_1 genes (11). One conclusion from that study was that the structure of F_0 synthesized in the absence of certain F_1 genes might be different from that synthesized in the presence of those F_1 genes, a conclusion which agrees with our assembly hypothesis. Since many studies have shown that F_0 channel remains open when F_1 is stripped from the membranes, the interaction of F_0 with F_1 subunits may result in an irreversible structural change in F_0 to produce the open proton channel.

Although these results indicate that the δ subunit is not a

temporary plug during assembly, they do not necessarily contradict the conclusion obtained from studies on the chloroplast F_1F_0 that the assembled, functional F_0 proton channel is blocked by the δ subunit (5, 6, 13). The δ subunit, once it has acted to open the F_0 channel, might then be positioned to act as a plug, or part of a plug, of that open channel.

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