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Constitutive Activation of the *fucAO* Operon and Silencing of the Divergently Transcribed *fucPIK* Operon by an IS*5* Element in *Escherichia coli* Mutants Selected for Growth on L-1,2-Propanediol

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L-1,2-Propanediol is an irretrievable end product of L-fucose fermentation by *Escherichia coli*. Selection for increased aerobic growth rate on propanediol results in the escalation of basal synthesis of the NAD^+-linked oxidoreductase encoded by *fucO*, a member of the *fuc* regulon for the utilization of L-fucose. In general, when *fucO* becomes constitutively expressed, two other simultaneous changes occur: the *fucA* gene encoding fuculose-1-phosphate aldolase becomes constitutively expressed and the *fucPIK* operon encoding fucose permease, fucose isomerase, and fuculose kinase becomes noninducible. In the present study, we show that *fucO* and *fucA* form an operon which is divergently transcribed from the adjacent *fucPIK* operon. In propanediol-positive and fucose-negative mutants the *cis*-controlling region shared by the operons *fucAO* and *fucPIK* is lengthened by 1.2 kilobases. DNA hybridization identified the insertion element to be IS5. This element, always oriented in the same direction with the left end (the *BglII* end) proximal to *fucA*, apparently causes constitutive expression of *fucAO* and noninducibility of *fucPIK*. The DNA of the *fucAO* operon and a part of the adjacent *fucP* was sequenced.

Following the discovery that a genetic derepression of ribitol dehydrogenase in *Klebsiella pneumoniae* conferred a growth ability on xylitol (27, 33), we tried to find other models of experimental evolution for assessing the importance of regulatory mutations in the acquisition of novel functions (for a review, see reference 30). During this search, we discovered that *Escherichia coli* can give rise to mutants that utilize L-1,2-propanediol aerobically as a sole source of carbon and energy. Serial selection on the compound resulted in the emergence of a mutant (ECL3) which grew on the novel carbon and energy source at a rate close to that on glycerol. This mutant produced constitutively an NAD^+-linked oxidoreductase active on L-1,2-propanediol (46). The proximity of the locus specifying this enzyme activity and the *fuc* locus (at min 60) specifying the growth ability on L-fucose led us to ask whether there was a link between the metabolism of the two compounds. When the propanediol-positive mutant was tested with fucose as a carbon and energy source, growth no longer occurred (13).

Previous work, together with our further studies (13), revealed the biochemical connection between the two metabolic pathways (Fig. 1). The dissimilation of fucose by wild-type *E. coli* requires the sequential action of fucose permease (encoded by *fucP*), fucose isomerase (encoded by *fucI*), fucose kinase (encoded by *fucK*), and fuculose-1-phosphate aldolase (encoded by *fucA*). The last enzyme catalyzes the formation of dihydroxyacetone phosphate and L-lactaldehyde. Under anaerobic conditions, the aldehyde is reduced to L-1,2-propanediol by an NAD^+-linked oxidoreductase (encoded by *fucO*) and excreted into the medium. Under aerobic conditions, the aldehyde is oxidized to L-lactate and then to pyruvate which enters the general metabolic pool. The inducer of the fucose system is not the substrate but the remote metabolite fuculose-1-phosphate (for reviews, see references 29 and 31).

Although the NAD^+-linked oxidoreductase catalyzes a reversible reaction, the wild-type strain fails to grow aerobically on propanediol because the compound is unable to induce the fucose regulon. By synthesizing the oxidoreductase constitutively at an elevated level, the mutant is able to grow rapidly on propanediol, despite the instability of the enzyme during aerobic metabolism (6; J. Aguilar, personal communication). Failure of the mutant to grow on fucose is due to the noninducibility of its permease, isomerase, and kinase. Associated with this change is the constitutive synthesis of the aldolase, whose activity is not necessary for growth on propanediol (20). The genetic basis for the complex mutant phenotype was obscure.

To test whether the acquisition of the novel growth ability is necessarily at the expense of the established metabolic pathway, nine additional independent selections for aerobic growth on propanediol were carried out. After about 40 mass doublings, a random clone from each of the nine lines was isolated. Seven out of the nine clones were fucose negative. One fucose-positive clone was further selected on propanediol for over 200 mass doublings. A progeny clone (ECL418) was then tested and found to remain fucose positive. In this mutant, the basal activity level of propanediol oxidoreductase was increased about 40-fold. Induction by fucose further increased the activity level by almost twofold. However, the basal and induced levels of the permease, isomerase, kinase, and aldolase remained normal. Enzyme analysis of one of the propanediol-positive but fucose-negative mutants (strain ECL421) revealed a pattern resembling that of the mutant first described (ECL3): the oxidoreductase and the aldolase were both constitutively synthesized, whereas the permease, isomerase, and kinase were noninducible (21).

When the propanediol-positive but fucose-negative mutants were selected for growth on fucose, two classes of suppressions were found: *fuc* linked and *fuc* unlinked. In both kinds of pseudorevertants, all the *fuc* structural genes

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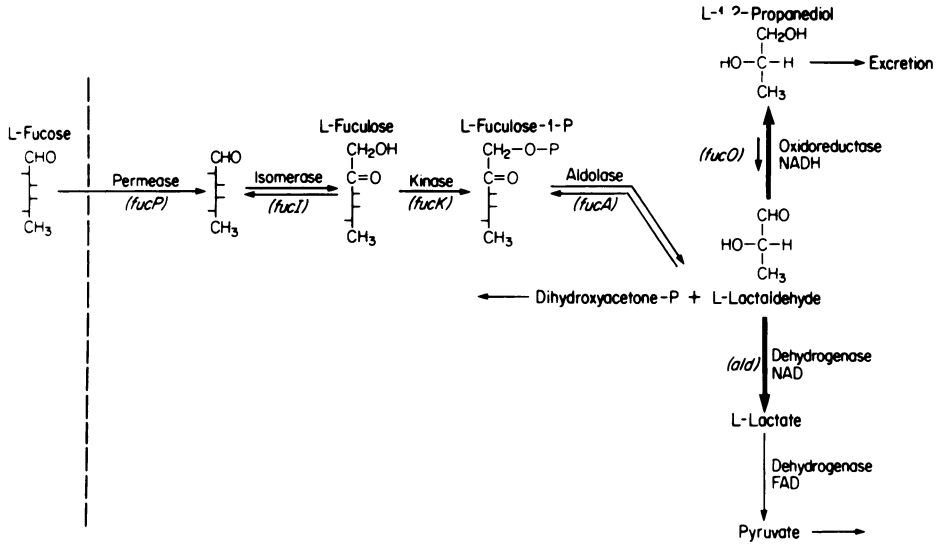

FIG. 1. Scheme for L-fucose utilization by *E. coli*. Heavy arrows indicate the divergence of the aerobic and anaerobic branches of the pathway.

were constitutively expressed (9). The nature of the linked suppression has not yet been defined. The unlinked suppression occurred in the *crp* gene encoding the cyclic AMP binding protein (55). Subsequently, it was shown that the *fuc* genes belong to a regulon under positive control of the *fucR* product, that the *P*, *I*, and *K* genes constitute an operon, and that the *fuc* genes are arranged in the clockwise order *O-A-P-I-K-R* (8, 11).

The objective of this study was to discover the genetic connection between the propanediol-positive phenotype and the fucose-negative phenotype. During the course of the work, we found that *fucA* and *fucO* genes also constitute an operon which is transcribed divergently from *fucPIK*.

MATERIALS AND METHODS

Bacterial strains and media. Sources and relevant properties of the *Escherichia coli* K-12 strains used in this study are summarized in Table 1. Strain JM109 (53) was used for the preparation of M13 bacteriophages. For selecting propanediol-positive mutants, MacConkey-propanediol agar medium containing 1% DL-1,2-propanediol was used. Minimal agar (51) was supplemented with 0.2% carbon source. When required, ampicillin was added at 200 μg/ml and tetracycline was added at 20 μg/ml.

Plasmids. The plasmids used for complementation, preparation of probes, and subcloning segments of the *fuc* region into M13 sequencing vectors were previously described (11). Plasmid pJE100, a pBR322 derivative containing the entire IS*5* element, was provided by Hamilton Smith (12).

Enzymes and chemicals. Restriction enzymes, T4 DNA ligase, and DNA polymerase I (Klenow fragment) were purchased from New England BioLabs, Beverly, Mass. RNase-free DNase and RNA markers were from Bethesda Research Laboratories, Inc., Gaithersburg, Md. The random primer DNA labeling kit was from Boehringer Mannheim Biochemicals, Indianapolis, Ind. Nylon filter (Nytran) and Elutip-d column were from Schleicher & Schuell, Inc., Keene, N.H. Sequenase DNA sequencing kit was from United States Biochemical Corp., Cleveland, Ohio.

Southern blot hybridization. Chromosomal DNA was prepared from 3 ml of an overnight LB (Luria-Bertani) culture by a lysozyme-sodium dodecyl sulfate method (32) and digested with restriction enzymes overnight at 37°C. The DNA (0.5 μg) was fractionated by electrophoresis on a 1% agarose gel which was then stained with ethidium bromide

TABLE 1. Genealogy of *E. coli* K-12 strains

Strain	Derived from strain:	Selection or isolation	Genotype[a]	Reference
ECL1	HfrC	Deletion of *phoA*	HfrC *phoA8 relA1 tonA22* $T2^r$ (λ)	28
ECL3	ECL1	Growth on propanediol (EMS^b-induced and spontaneous mutations)	*fucO*(Con) *fucA*(Con) *fucPIK*(Non)	46
ECL56	ECL3	Fucose-positive revertant (spontaneous)	*fucO*(Con) *fucA*(Con) *fucPIK*(Con) *crp-201*	20, 55
ECL418	ECL1	Growth on propanediol (spontaneous mutations)	*fucO*(Con) $fucA^+$ $fucPIK^+$	21
ECL421	ECL1	Growth on propanediol (spontaneous mutations)	*fucO*(Con) *fucA*(Con) *fucPIK*(Non)	21
ECL459	ECL421	Fucose-positive revertant (spontaneous)	*fucO*(Con) *fucA*(Con) *fucPIK*(Con)	9
ECL477	ECL1	Fucose-negative mutant (EMS-induced)	HfrC *fucR501 phoA8 relA1 tonA22* $T2^r$ (λ)	11
ECL706	ECL701	Transductional cross	*crp-201 zhd4*::Tn*10 fucO*(Con) *fucA*(Con) *fucPIK*(Con)	55

[a] Con, Constitutivity; Non, noninducibility.
[b] EMS, Ethyl methanesulfonate.

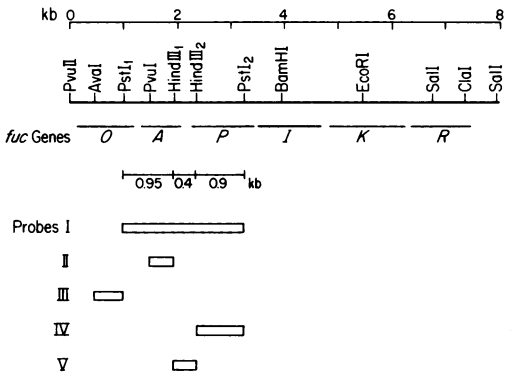

FIG. 2. Restriction map of the *fuc* regulon and probes used for Southern and Northern blot hybridizations. The order of the genes and the positions of the pertinent restriction sites were determined in a previous study (11). The open boxes under the map represent DNA fragments used as probes.

for photography. The DNA was blotted to a nylon membrane overnight by a capillary transfer method and hybridized with the radioactive DNA probe under high-stringency conditions (32). DNA fragments to be used as probes were electroeluted from the agarose gel. The preparation, after purification and concentration by the use of an Elutip-d column, was labeled with [α-^{32}P]dATP by the random primer method (18).

Northern (RNA) blot hybridization. Cells for isolation of total RNA (3) were harvested during exponential growth from LB broth with or without fucose as the inducer. RNA samples, along with a mixture of RNA markers for molecular weight, were loaded with 1 μl of ethidium bromide (1.0 μg/ml) into wells of a 1.5% agarose–6% formaldehyde gel. After electrophoresis for 17 h at 50 V, the gel was photographed. It was then directly blotted to a nylon membrane and hybridized with radioactive DNA probe under high-stringency conditions. The intensity of the hybridized band on the autoradiogram was measured by densitometric scanning (LKB Densitometer).

DNA sequencing. DNA sequence was determined by the dideoxynucleotide chain termination method (42). Restriction fragments of the *fuc* regulon (11) were subcloned into vectors M13mp18 and M13mp19. Overlapping fragments were sequenced in both directions with the Sequenase sequencing kit by using [α-^{35}S]dATP. The DNA sequences were compiled and analyzed with the University of Wisconsin Genetics Computer Group (UWGCG) programs on a VAX computer.

RESULTS

The *fucA* and the *fucO* genes constitute a counterclockwise-transcribed operon. In a previous study, the direction of transcription of *fucA* was tested by cloning the *Pst*I$_1$-*Pst*I$_2$ segment of 2.3 kilobases (kb) (encompassing the *fucA* gene, as shown by the restriction map in Fig. 2) into plasmid vector pUC19 in one or the other orientation. The gene was expressed only when the *Pst*I$_1$ end of the insert was proximal to the *lacZ* promoter on the plasmid (11). It was therefore concluded that the gene was transcribed clockwise. For reasons that are still unclear, the previous conclusion is in error. According to the findings of our present study, the transcription of *fucA* should be counterclockwise. First, DNA sequencing of the *Pvu*II-*Pst*I$_2$ segment, containing *fucO*, *fucA*, and part of *fucP* (11), revealed two tandem counterclockwise open reading frames (ORFs) and a part of a clockwise ORF (see section below). Second, Northern blot analysis with either probe II (an internal segment of *fucA*, as shown in Fig. 2) or probe III (an internal segment of *fucO*) revealed a fucose-inducible transcript of about 2 kb in the wild-type cells (see section below). The length of this transcript is close to the span of *fucA* plus *fucO*. Membership of the *fucA* and *fucO* genes in the same operon would explain the constitutive co-expression of these two genes in propanediol-positive but fucose-negative mutants.

The *fucR* gene is not required for the selection of propanediol-positive mutants. A simultaneous change in the expression of the *fucAO* and *fucPIK* operons suggested that the mutation might have occurred in the regulatory gene *fucR* (11, 20). To find out whether an altered *fucR* product caused the propanediol-positive and fucose-negative phenotype, we spread cells of strain ECL477 (*fucR*) on MacConkey-propanediol agar and incubated the plates at 37°C for 10 days. One papilla was picked from each of two random colonies and streaked on propanediol minimal agar for single colonies. When tested on fucose, both remained negative. Transformation of these two propanediol-positive but fucose-negative mutants with the multicopy plasmid pfuc39 bearing a *fucR*$^+$ gene did not restore the growth ability on fucose or abolish the growth ability on propanediol. A control transformation of the parental strain ECL477 (*fucR*) with the same plasmid did restore the growth ability on fucose. These results appear to exclude the differential effect of a mutant *fucR* product on the expression of *fucAO* and *fucPIK*.

The presence of an insertion sequence between *fucA* and *fucP* in the propanediol-positive and fucose-negative mutants. The failure to explain the complex mutant phenotype by a *fucR* mutation shifted our attention to a *cis*-acting controlling element. The promoters of both *fucAO* and *fucPIK* were shown to be within the *Hin*dIII$_1$-*Hin*dIII$_2$ region (Fig. 2), because its deletion resulted in the noninducibility of both operons (11). We compared the lengths of this region of the propanediol-positive but fucose-negative mutants with that of the wild-type strain by Southern blot analysis. Three restriction-enzyme digestions of chromosomal DNA of each strain were carried out (*Pst*I alone, *Hin*dIII alone, and *Pst*I plus *Hin*dIII), and the digests were hybridized with probe I (the 2.3-kb *Pst*I$_1$-*Pst*I$_2$ fragment covering the region which includes a part of *fucO*, the entire *fucA*, and most of *fucP*; Fig. 2). As shown in Fig. 3, the wild-type strain ECL1 gave a hybridization pattern expected from the restriction map: (i) the *Pst*I digest gave a 2.3-kb band, (ii) the *Hin*dIII digest gave a 0.4-kb band and two bands with slower migration rates (one generated from *Hin*dIII$_1$ to an external site and the other from *Hin*dIII$_2$ to an external site), and (iii) the *Pst*I-*Hin*dIII double digest gave three bands with sizes of 0.95, 0.9, and 0.4 kb. The patterns of strains ECL3 (propanediol positive and fucose negative), ECL421 (propanediol positive and fucose negative), ECL56 (a fucose-positive pseudorevertant of ECL3), and ECL459 (a fucose-positive pseudorevertant of ECL421) were the same but differed from that of the wild-type strain ECL1. In the *Pst*I digest of these mutants, a 3.5-kb band was seen instead of the 2.3-kb band. In the *Hin*dIII digest, a 1.6-kb band was seen instead of the 0.4-kb band. In the *Pst*I-*Hin*dIII double digest, an 1.6-kb band was seen instead of the 0.4-kb band but the 0.95-kb and the 0.9-kb bands exhibited by the wild-type strain remained. Thus, an insertion of about 1.2 kb occurred in the 400-base-pair (bp) *Hin*dIII$_1$-*Hin*dIII$_2$ region.

Identification of the insertion element as IS5. The experi-

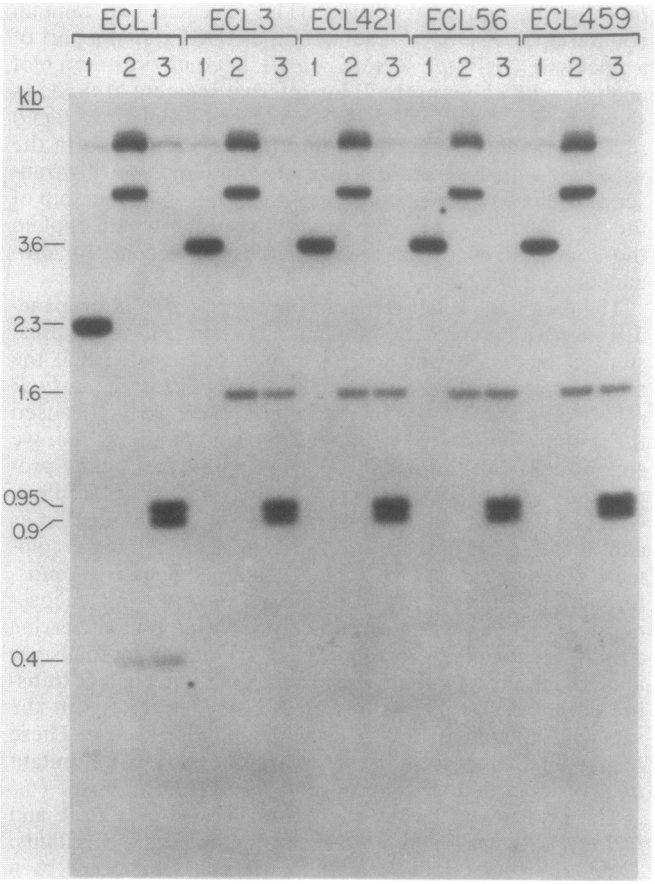

FIG. 3. Southern blot analysis of wild-type and mutant *fuc* regions. Chromosomal DNA from the wild-type strain ECL1, propanediol-positive but fucose-negative mutants ECL3 and ECL421, and the fucose-positive pseudorevertants ECL56 (from ECL3) and ECL459 (from ECL421) were prepared. The samples were digested with *Pst*I (lanes 1), *Hin*dIII (lanes 2), and *Pst*I-*Hin*dIII (lanes 3). Probe I was used for hybridization (see text).

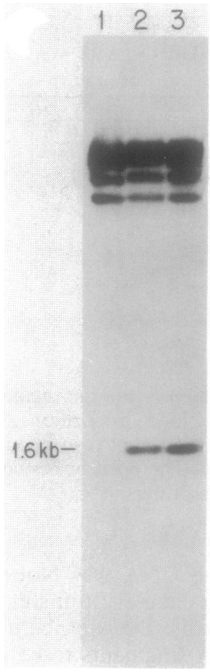

FIG. 4. Identification of the insertion sequence in the mutants ECL3 and ECL421 by Southern blot hybridization. Chromosomal DNA of wild-type strain ECL1 and mutants ECL3 and ECL421 were digested with *Hin*dIII. The probe was a 950-bp internal fragment of IS*5* (*Eco*RI-*Bgl*II) prepared by digesting plasmid pJE100 containing the entire insertion element (12) with *Eco*RI and *Bgl*II. Lane 1, ECL1; lane 2, ECL3; lane 3, ECL421.

ment described above showed that the 1.2-kb insertion sequence was not cut by *Hin*dIII or *Pst*I. Further restriction analysis by Southern blot hybridization revealed that the sequence contained sites for *Bgl*II, *Pvu*II, and *Eco*RI. This restriction pattern and the 1.2-kb size suggested that the insertion sequence was IS*5* (5, 17, 26, 37, 44, 50), at least 22 copies of which are present in the *E. coli* chromosome (34). An internal fragment of IS*5*, therefore, was used as a probe to hybridize with *Hin*dIII-digested chromosomal DNAs from the wild-type strain ECL1 and the propanediol-positive but fucose-negative mutants ECL3 and ECL421. The DNA from both the wild-type and mutant strains gave several bands as might be predicted. The mutants, however, showed an additional band of 1.6 kb. This band should be the *Hin*dIII$_1$-*Hin*dIII$_2$ segment containing the IS*5* (Fig. 4). Ten other independent propanediol-positive but fucose-negative mutants also showed the 1.6-kb band (data not shown). These mutants included a propanediol-positive and fucose-negative derivative of strain ECL477 (*fucR*).

Orientation and position of IS*5*. Since there is only one *Eco*RI site in IS*5* which is 103 bp from its right end, we asked whether this *Eco*RI site is proximal or distal to the chromosomal *Pst*I$_2$ site (0.9 kb from the *Hin*dIII$_2$ site) in the *fuc* region. DNA of the mutant strain ECL421 (propanediol positive and fucose negative) was doubly digested with the enzymes *Pst*I and *Eco*RI and analyzed by Southern blot hybridization with probe IV (Fig. 2). If the right end of the inserted IS*5* were proximal to the *Pst*I$_2$ site, the hybridized band should be 1 to 1.4 kb. On the other hand, if the right end of the inserted IS*5* were distal to the *Pst*I$_2$ site, the hybridized band should be 2 to 2.4 kb. The hybridized band was found to be about 1.1 kb, indicating that the right end of the IS*5* was on the side of *fucPIK*. Ten other independent propanediol-positive but fucose-negative mutants all gave a hybridized band of about 1.1 kb (data not shown). Thus, it appears that the IS*5* element constitutively activates *fucAO* in only one orientation and possibly at the same position. To locate the insertion in strain ECL421 more precisely, another Southern blot analysis was designed to measure the distances from each of the three restriction sites (*Bgl*II, *Pvu*II, and *Eco*RI) in IS*5* to each of the two *Hin*dIII sites in the *fuc* region. The chromosomal DNA was subjected to the following double digestions: *Hin*dIII-*Bgl*II, *Hin*dIII-*Pvu*II, and *Hin*dIII-*Eco*RI. The digests were hybridized with probe V (Fig. 2). The right end (*Eco*RI end) of the IS*5* was found to be about 100 bp from the *Hin*dIII$_2$ site (data not shown).

Northern blot analysis of *fucA* and *fucO* mRNA from wild-type and mutant strains. The lengths and abundance of *fucAO* transcripts of wild-type strain ECL1 and IS*5*-insertion mutants ECL3 and ECL421 were compared by using probes II and III (Fig. 2). When probe II (*fucA*) was used (Fig. 5A), uninduced wild-type strain ECL1 gave no band, whereas the induced cells gave a band of 2 kb. Longer exposure revealed a faint band of 1.5 kb (not shown). The mutants ECL3 and ECL421 grown under noninducing conditions gave a heavy and broad band (possibly two bands) spanning from 2 to 2.3 kb and another band of 1.5 kb. When probe III (*fucO*) was used (Fig. 5B), uninduced wild-type

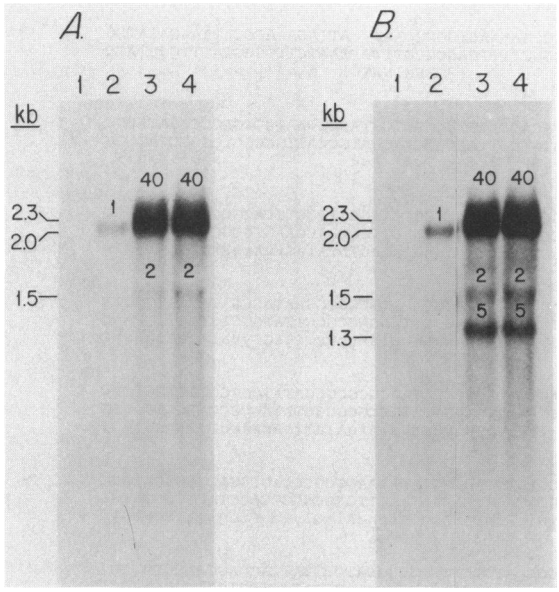

FIG. 5. Northern blot analysis of *fucA* and *fucO* transcripts from wild-type strain ECL1 and the propanediol-positive but fucose-negative mutants ECL3 and ECL421. The RNA was extracted from uninduced ECL1 cells, induced ECL1 cells, and uninduced cells of ECL3 and ECL421. Each RNA sample was loaded onto a 1.5% agarose–6% formaldehyde gel. For strain ECL1, 10 μg was used. For strains ECL3 and ECL421, 2 μg was used, but to this amount 8 μg of RNA from uninduced wild-type cells was added to make the total RNA 10 μg. After electrophoresis, the gel was blotted onto a nylon membrane and hybridized with probe II, an internal fragment of *fucA* (*Hin*dIII$_1$-*Pvu*I) (A), and probe III, an internal fragment of *fucO* (*Pst*I$_1$-*Ava*I) (B). The numbers above the bands represent the relative intensity measured by densitometric scanning. The band intensity of the induced wild-type strain ECL1 (10 μg of RNA) was taken as 1, and the intensities of the other bands were multiplied by 5 to correct for the smaller amount of mutant RNA used. Lanes: 1, ECL1 uninduced; 2, ECL1 induced; 3, ECL3 uninduced; 4, ECL421 uninduced.

strain ECL1 gave no band, whereas the induced cells gave a band of 2 kb. Longer exposure revealed one faint band of 1.5 kb and another of 1.3 kb (data not shown). The mutants ECL3 and ECL421 grown under noninducing conditions gave a heavy and broad band of 2 to 2.3 kb and another band of 1.5 kb (as with probe II). In addition, these mutants gave a band of 1.3 kb which was more intense than the 1.5-kb band.

Since a 2-kb band was revealed by both probes II and III, the *fucA* and *fucO* genes were apparently transcribed as a single messenger in wild-type cells. The increased intensity of the *fucAO* messenger in the mutants ECL3 and ECL421 grown under noninducing conditions indicated that the IS*5* insertion caused elevated constitutive transcription of the *fucAO* operon. The broadening of this band from 2 to 2.3 kb might be interpreted as the appearance of an additional transcription start site(s). The 1.5-kb band (hybridizable with both *fucA* and *fucO* probes) of the induced wild-type strain and the mutants grown under noninducing conditions might be the result of premature termination, processing, or degradation of the complete *fucAO* transcript.

The 1.3-kb band (hybridizable with the *fucO* but not the *fucA* probe) of the induced wild-type strain and the mutants ECL3 and ECL421 grown under noninducing conditions might represent a transcript of *fucO* initiated at a second promoter. During the course of multistep selection on propanediol, it was noted that the initial mutations increased basal activity levels of the oxidoreductase but not the aldolase. These mutants remained fucose positive (21). Since both strains ECL3 and ECL421 were isolated as multistep mutants (20, 21, 46), the increased amount of the 1.3-kb transcript might be attributable to a mutation(s) at the separate transcription start site for *fucO* prior to the IS*5* insertion event. The existence of a weak transcription start site just upstream of *fucO* in the wild-type strain is supported by the results of a previously reported experiment with plasmid pfuc25. The insert in this plasmid was a 6-kb *fuc* segment from *Pvu*II to *Eco*RI with a deletion in the internal *Hin*dIII$_1$-*Hin*dIII$_2$ region encompassing the promoters of *fucAO* and *fucPIK*. When present in large number of copies, this plasmid complemented a mutation in *fucO* but not in *fucA*. When present in only a few copies, this plasmid complemented neither mutation (11).

DNA sequences of the wild-type *fucAOP* region. The *Pvu*II to *Pst*I$_2$ segment of the *fuc* region was sequenced for both strands. Figure 6 shows that there are only two long ORFs (with more than 81 possible amino acids) in the counterclockwise direction. One starts at position 2061 and ends at position 1417, coding for a protein of 215 amino acid residues. This should be the ORF for the *fucA* protein, according to the map position previously published (11). Following closely in the same orientation is another ORF, from position 1389 to position 241, coding for a protein of 383 amino acid residues. This should be *fucO*. About 550 bp upstream to the coding region of *fucA* at position 2608, a third ORF begins in the clockwise direction. This should be part of *fucP*, the most proximal gene in the *fucPIK* operon. All three ORFs are preceded by good Shine-Dalgarno consensus ribosome-binding sites (45) before their ATG initiation codons. However, no consensus −10 and −35 hexamer pair is apparent, as expected for positively controlled promoters (35).

Between the coding regions of *fucA* and *fucP*, there are five stretches that match to various degrees the consensus cyclic AMP receptor protein (CRP)-binding sequences (15)—all with a core TGTGA. It remains to be established which ones of these candidate sites actually bind CRP. Downstream of the *fucAO* coding region, there are three pairs of inverted repeats capable of forming stem-loop structures. The most distal one, followed by a stretch of six thymidine bases, is likely to be the transcription terminator (7).

A *crp* mutation can cause an IS*5*-silenced *fucPIK* operon to be expressed constitutively. Strain ECL56, the fucose-positive pseudorevertant of the IS*5* insertion mutant ECL3, synthesized fucose permease, fucose isomerase, and fuculose kinase constitutively (20) as a result of a *crp-201* mutation (9, 55). We transduced the *crp-201* allele (via strain ECL706) to the IS*5* insertion mutant ECL421 by selection for a closely linked Tn*10* marker. Inheritance of the *crp-201* allele converted the *fucPIK* from the noninducible to the constitutive state. In another experiment, it was found that such a suppression also occurred in the IS*5* insertion mutant derived from strain ECL477 (*fucR*). The action of the *crp-201* product therefore did not require the *fucR* product. Moreover, the suppressibility of three independent IS*5* insertion mutants by the *crp-201* allele supported the evidence obtained from Southern blot analysis that the IS*5* insertions in different mutants are at the same locus. When the fucose-positive pseudorevertants, ECL56 (from strain ECL3) and ECL459 (from strain ECL421), were probed for the presence

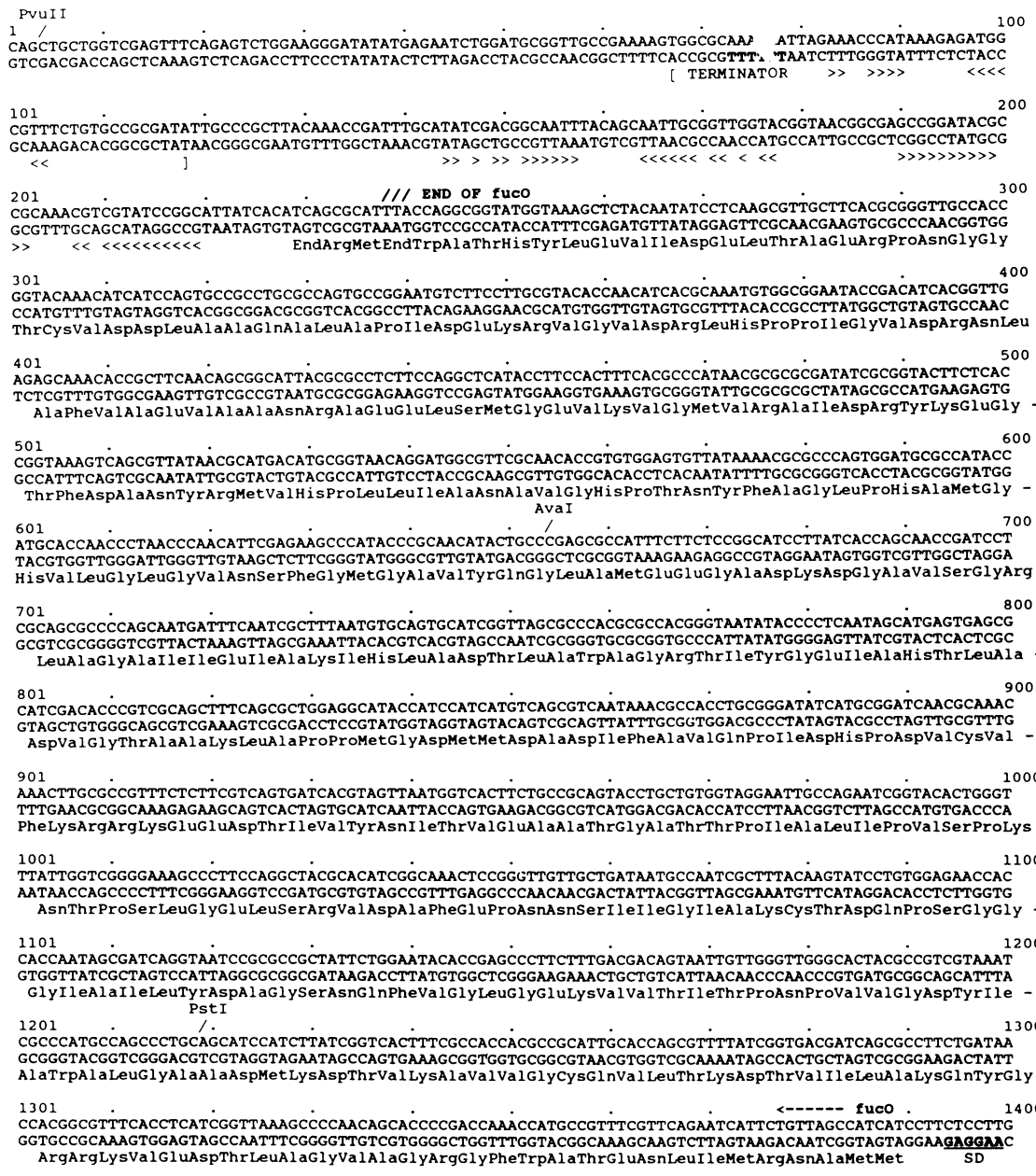

FIG. 6. DNA sequence of the *fucO*, *fucA*, and the start of *fucP*. Positions of pertinent restriction sites are indicated. The ORFs are delimited by the amino acid sequences given under DNA sequences. The potential ribosome binding sequences (indicated by SD) and *crp* sites (indicated by CRP) are underlined. Terminator sequences are marked by brackets. Conserved bases in consensus sequences are in boldface. >, <, Inverted repeats downstream of the *fucO* gene. The sequence of *fucP* from nucleotide 2700 to the $PstII_2$ site is not shown.

of IS*5* in the $HindIII_1$-$HindIII_2$ region, the insertion element was still there (Fig. 3).

DISCUSSION

Studies of insertion sequences showed that among the different effects exerted by these elements on local DNA are influences on gene expression (for reviews, see references 4, 16, 25, 47, and 49). The ability of insertion elements to turn on or turn off adjacent genes was discovered during the pioneering studies of IS*2* and IS*3*. The nature of the effect often depends on the orientation of the IS introduced (19, 40, 41). Constitutive gene activation might result from the creation of a new promoter that includes a section of resident DNA and a section of inserted DNA (19, 22, 23). In the case of the cryptic *bgl* operon, insertion of IS*1* or IS*5* in the *cis* regulatory region *bglR* can allow inducible expression (38). Activation by IS*5* is orientation independent (39). IS*5*, however, was reported to direct low levels of transcription of adjacent regions (*galK*) in only one orientation (36, 43).

Heterologous gene activation by IS*5* was also observed. Examples are the expression of the yeast *URA1* gene (24), the staphylococcal erythromycin resistance gene (2), and the *Bacillus subtilis* gene for xylose isomerase (52). The IS*5* that activated the yeast gene and the IS*5* that activated the *Staphylococcus* gene are oriented in the same direction as those that activated the eleven independent insertion muta-

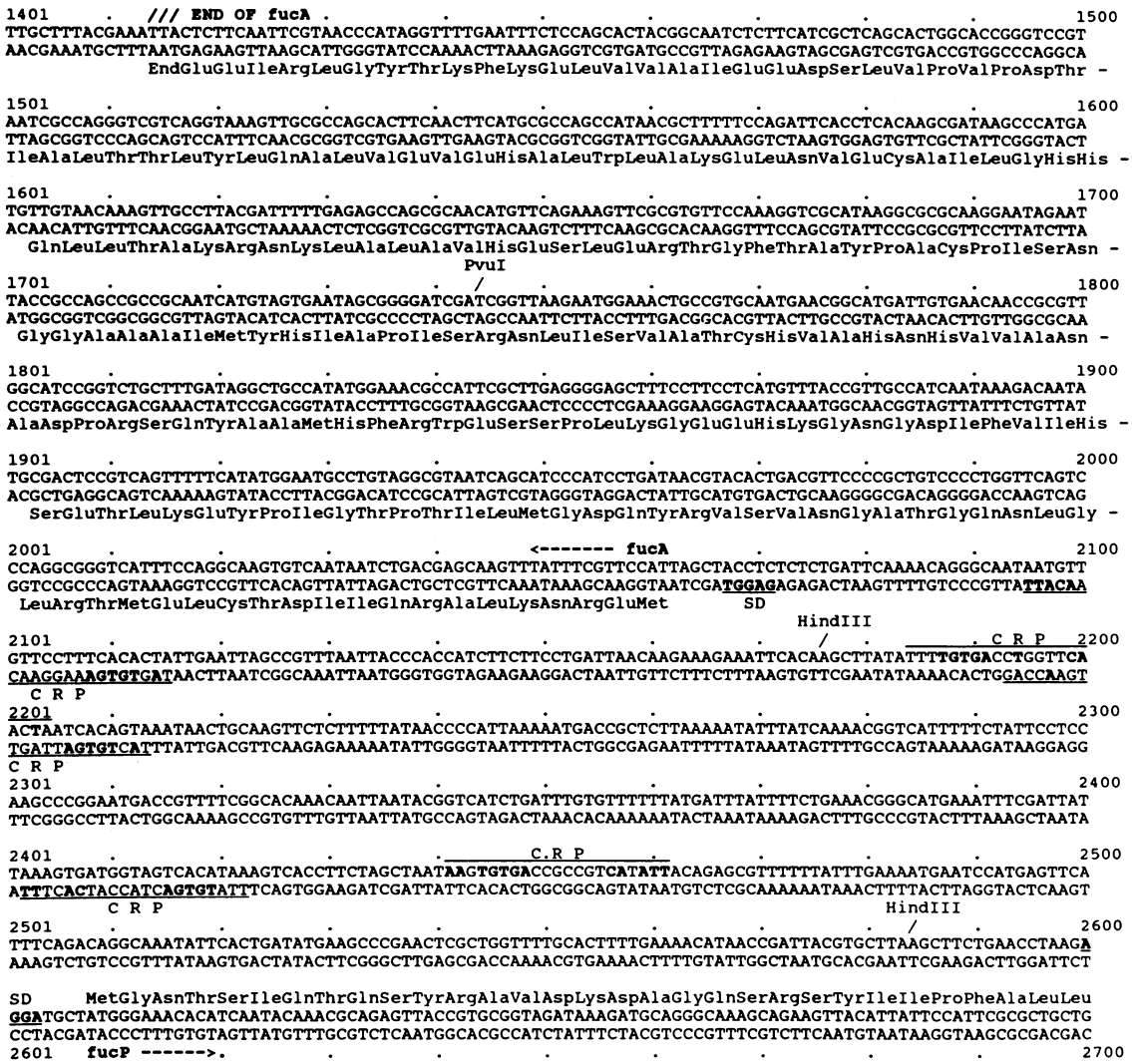

FIG. 6–*Continued*

tions of the *fuc* operon reported here. Thus available data mostly suggest that IS*5* effectively activates a downstream gene(s) only when the left (*Bgl*II) end of the element is proximal to the target.

In general, the mechanism of transcriptional activation or inactivation by IS elements is not well understood. It is noteworthy that to have an effect, the insertion does not always have to be close to or within the promoter region. For instance, insertions of *gamma-delta* in either orientation severely reduce *ebgA* expression from both sides of the gene several kilobases away by altering the helicity in the region of the promoter (48).

A pivotal role of an IS element in the experimental evolution of the propanediol pathway was unsuspected because of the plethora of mutant phenotypes encountered. Initially, we thought that mutations in *fucR* might be responsible for most of the features observed. These include (i) the increase of *fucO* expression without noticeable change in the expression of the other *fuc* genes, (ii) the simultaneous constitutivity of *fucO* and *fucA* coupled to the noninducibility of *fucPIK*, (iii) the ability of linked or unlinked mutations to convert *fucPIK* from the noninducible to a constitutive state, and (iv) persisting normal inducibility of lactaladehyde dehydrogenase (9, 21, 55). Recent studies indicate a broad metabolic function for the dehydrogenase whose gene (*ald*) mapped at min 31 (1, 10, 54). The exclusion of this gene from the *fuc* regulon allowed us to focus on the organization of the *fuc* genes proper at min 60.

It now seems clear that at least two different mechanisms can lead to effective propanediol utilization, one dependent on IS*5* activation and the other not. Both evolutionary solutions, however, exploit intrinsic properties of domains of *fuc* structural genes.

The IS*5*-dependent evolutiontary pathway is apparently predestined by an insertion hot spot in the common *cis*-controlling region of the *fucAO* and *fucPIK* operons. As indicated in Fig. 5 and 7, insertions at this locus led to the constitutive synthesis of elevated levels of *fucAO* transcripts, a portion of which were longer than normal. A primer extension experiment indicated the IS*5* insertion created one or more transcription start points about 350 bp upstream from the normal one (unpublished results).

In wild-type cells, both the *fucR* product (activated by the inducer fuculose-1-phosphate) and the *crp* product are required for full expression of the *fuc* regulon (11, 55). In the IS*5* insertion mutants, the FucR-dependent transcription of

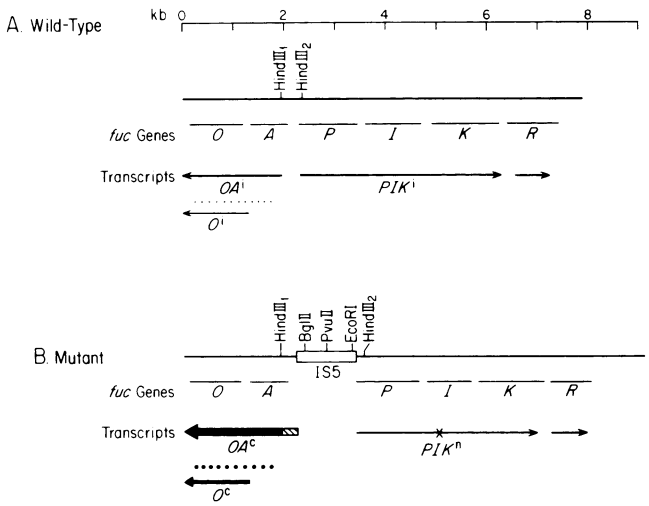

FIG. 7. Transcriptions of the *fuc* regulon in the wild-type strain and the IS*5* insertion mutant. Arrows represent the *fuc* transcripts. The direction of transcription of *fucR* is clockwise (unpublished data). The dotted lines represent the 1.5-kb transcript (see text). The thickness of the solid arrows and dotted lines indicates the relative abundance of the message. The hatched portion of the thick OA^c transcript indicates the additional part copied from a new upstream site in the IS*5* mutant. Superscripts: c, constitutive expression; i, inducible expression; n, noninducibility.

fucAO either is obscured by the copious constitutive transcription from the new start site or no longer occurs. The silencing of the *fucPIK* operon suggests that the function of its FucR or CRP site is disrupted. Constitutive expression of the *fucPIK*, however, could still occur as a result of a *crp-201* mutation which apparently strengthens the activating effect of the cyclic AMP-CRP complex. The exclusive dependence of the transcription from the CRP site is indicated by the observation that a deletion through the *fucR* gene and extending into the *fucK* gene in the IS*5* mutant ECL56 (*crp-201*) had no significant effect on the expression of *fucPI* (Y. Zhu, Ph.D. thesis, Harvard University, Cambridge, Mass., 1988). Surprisingly, when the *crp-201* allele was introduced into a wild-type strain, expression of the *fucPIK* operon in the recipient remained normal but *fucAO* could be only weakly induced (55). Better understanding of the regulation of the two divergent operons by the products of *fucR* and *crp* awaits identification of their sites and characterization of their operon specificity.

The IS*5*-independent evolutionary pathway for propanediol utilization appears to be built on a sequence upstream of *fucO* (possibly the weak transcriptional start site in the wild-type strain). This sequence provides a foundation for mutational enhancement of expression. Successive enhancements of *fucO* expression by this mechanism might foreclose the opportunity for IS*5* to play a role, because the oxidoreductase activity no longer limits the growth rate. A case in point is strain ECL418, which acquired effective growth ability on propanediol without losing the fucose growth ability after selection on the novel carbon and energy source for over 200 generations (21). We recently subjected strain ECL418 to further selection on propanediol (over 100 generations) without detecting fucose-negative derivatives. Southern blot analysis did not reveal any insertion sequence in the *fuc* region. In addition, it was found that a *fucO* transcript was produced constitutively, whereas the *fucAO* transcript was still produced inducibly (Y.-M. Chen, unpublished data).

In principle, mutations in *fucR* might also lead to constitutive activation of *fucAO*. No such mutant was seen during our studies. In retrospect, it appears that constitutivity resulting from a *fucR* mutation could not provide an adequate level of oxidoreductase activity under aerobic conditions (in part because of the instability of the enzyme). It might be noted that induction of the wild-type *fucAO* during aerobic growth merely increased the oxidoreductase activity 4-fold (instead of more than 10-fold during anaerobic growth). In contrast, the oxidoreductase activity in mutants that grew rapidly on propanediol under aerobic conditions was 20- to 70-fold of the normal basal level (20, 21). It is also of interest that gene amplification has not yet been discovered as a response to selection on propanediol.

Before submitting this work, we received a manuscript from T. Conway and L. O. Ingram giving the DNA sequence of *fucO* and suggesting that this gene was transcribed together with *fucA* (14). The coding sequences of our work are in accord with the work of the other authors.

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