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Mutational Analysis of the *Escherichia coli glpFK* Region with Tn*5* Mutagenesis and the Polymerase Chain Reaction

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Transposon Tn*5* mutagenesis of the *Escherichia coli* chromosome was used to isolate 21 independent insertion mutations conferring an altered colony color phenotype on MacConkey-glycerol plates. The polymerase chain reaction was used to map 16 of these Tn*5* insertions within the *glpFK* region at 88 min. The most polar Tn*5* insertion was shown by nucleotide sequencing to be in the proposed *glpF* open reading frame. The data suggest that the *glpF* and *glpK* genes are in an operon with a bent DNA segment (BENT-6) involved in transcriptional regulation of this operon.

The glycerol facilitator (GlpF) is the only known pore-type protein in the *Escherichia coli* cytoplasmic membrane. It has recently been demonstrated to be in a family of proteins which passively allow transport of small molecules across membranes (3). This family includes nodulin-126, a soybean peribacteroid membrane protein that is induced by symbiosis with the bacterium *Bradyrhizobium japonicum* (10); big brain, the *Drosophila* neurogenic gene (25); and the major intrinsic protein (MIP) from bovine lens fiber membrane (11), one of the first putative gap junction proteins to be sequenced. Identical amino acids at the same position in procaryotic, plant, invertebrate, and mammalian proteins suggest a common structure and function of these proteins (3). The *glpF* gene appears to be in an operon with *glpK* (29).

The present studies were undertaken to evaluate the effects of insertions in the *glpFK* region. While we have investigated the effects of these transposon Tn*5* insertion mutations on glycerol kinase catalytic function and glycerol transport, the results also provide the opportunity to identify protein regions responsible for specific ligand binding. The polymerase chain reaction (PCR) was used to map the individual Tn*5* insertions within the *glpFK* region. This method used the insertion sequence (Tn*5*) for one primer and a known chromosomal DNA sequence (*glpK*) as an anchor sequence for the other primer. It is similar to a method recently used for *Drosophila* spp. with P elements (4) and a general method for amplifying human-specific sequences from a rodent background with primers directed to human *Alu* sequences (*Alu*-PCR) (23). The PCR products were sized on an agarose gel to allow a precise physical map to be constructed.

E. coli HB101 (6) was mutagenized with Tn*5* by using λ467 (λ::Tn*5*) to deliver the transposon (14a). To identify Tn*5* insertions into genes involved in glycerol metabolism, the Tn*5*-carrying *E. coli* (*E. coli*::Tn*5*) cells were plated on modified MacConkey medium with glycerol as the carbon source (24). Colonies with a white to yellow or light pink rather than bright pink color were identified, purified, and subjected to further analysis. Glycerol kinase activity was assayed by a previously described radiochemical method (16) which was optimized for these bacterial extracts. The method used for glycerol uptake was adapted from that of Sweet et al. (29).

Genomic Southern (28) blotting was performed with approximately 3 μg of purified *E. coli*::Tn*5* chromosomal DNA digested with the appropriate restriction endonuclease and electrophoresed on a 1% agarose gel. The DNA was transferred to GeneScreen Plus, and the blots were hybridized with plasmid probes labeled by the random hexanucleotide priming method as described previously (17). Plasmid pCJ102 (24) was used to detect *glpK*-specific sequences, while pL088 (15) was used to detect Tn*5* sequences.

For the PCR (26), primers were synthesized with an Applied Biosystems oligonucleotide synthesizer and DNA sequence information from the published sequence for *glpK* (24) and the ends of Tn*5* (1). The primers constructed from the *glpK* sequence include primer A, 5′-TAGTCATATTAC AGCGAAGCTT-3′, which is the 5′ reverse primer directed toward the region upstream of *glpK* and corresponding to nucleotides −15 to −36 on the nonsense strand; primer B, 5′-AAGCTTCGCTGTAATATGACTA-3′, is at the 5′ end directed toward the *glpK* gene and corresponds to nucleotides −36 to −15 on the sense strand; primer C, 5′-CACGCT CGCGAGAGCCTTCCAC-3′, is the reverse primer near the *Bam*HI site within the *glpK* gene and directed towards the 5′ end of *glpK*; primer D, 5′-TTATTCGTCGTGTTCTTCCC AC-3′, is located at the 3′ end of the *glpK* gene and is directed toward the 5′ end of the *glpK* coding sequence; and primer E, 5′-CTGGAAAACGGGAAAGGTTCCG-3′, corresponds to a sequence 30 base pairs (bp) from the end of Tn*5* (Fig. 1).

Amplification of *E. coli*::Tn*5* chromosomal regions was performed in a 100-μl reaction volume with the above primers in an automated Thermal Cycler (Perkin Elmer Cetus). The buffers and concentrations of primers, dimethyl sulfoxide, and deoxyribonucleotide triphosphates were as recommended in the AmpliTaq (Cetus) kit. The amplification conditions were as follows: initial denaturing step at 95°C for 7 min followed by 30 cycles of denaturing at 90°C for 30 s, hybridizing at 60°C for 45 s, and polymerase extension at 68°C for 5 min. *Thermus aquaticus* polymerase (2.5 U; Cetus) was used in each reaction mix. The PCR products were analyzed by electrophoresis in a 1% agarose gel and staining with ethidium bromide.

The DNA sequence was determined by the dideoxy chain

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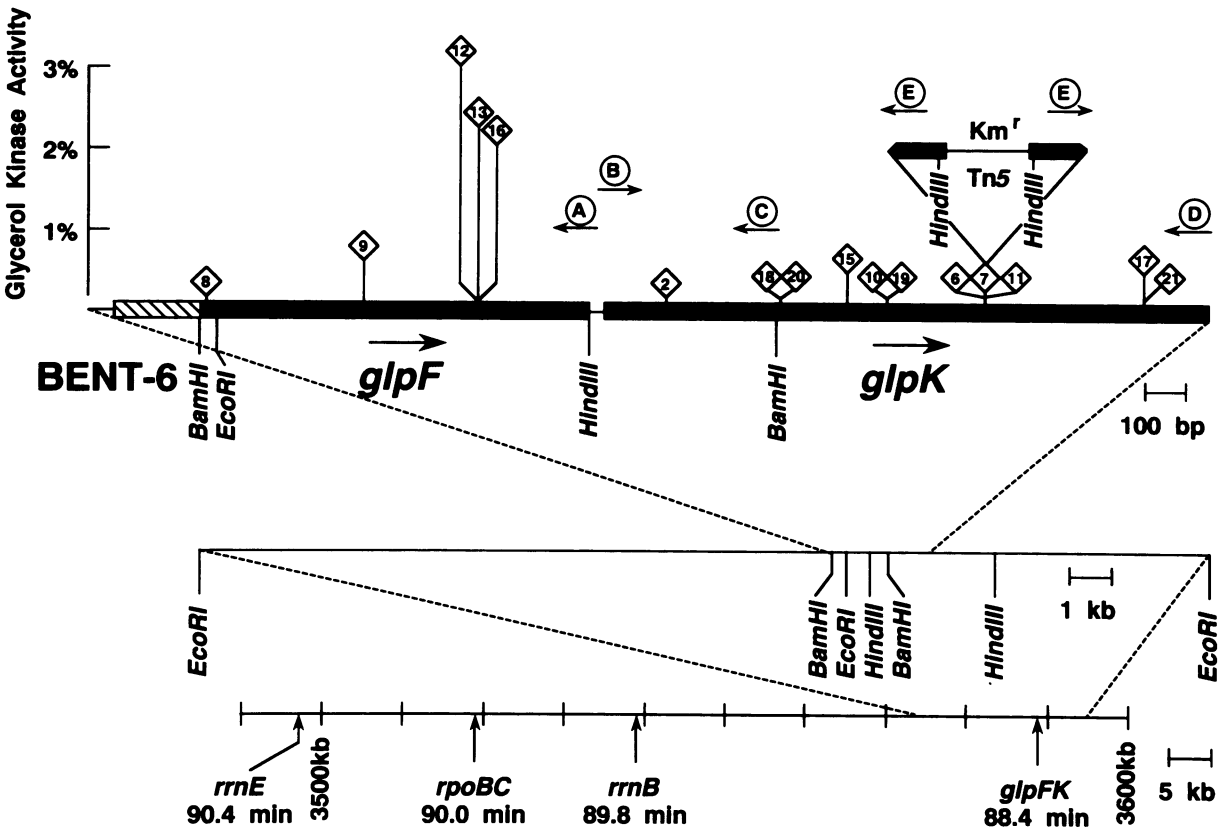

FIG. 1. Correlated physical and genetic map of the *E. coli glpFK* region. Below is shown the *E. coli* genetic linkage map, with some relevant genetic markers and positions given. The genetic map is expanded and a physical map of the *glpFK* region is shown (14). The physical map is expanded further, and DNA sequences corresponding to *glpK* (24), *glpF* (22; this work), and BENT-6 (20) are depicted by boxes. The direction of transcription is indicated by an arrow and is towards the *rha* locus at 88 min (2, 29). Note the scale for each map (100 bp, 1 kb, 5 kb) as given on the right. Above the *glpFK* sequences are shown the locations of the individual Tn*5* insertions (numbers within diamonds). The location was determined by combining the data from Southern blotting experiments (not shown) and Fig. 2 and 3. The height of the diamond corresponds to the percent GlpK activity with respect to controls (Table 1). Above *glpK*::Tn*5*-7 is a blow-up of the Km^r Tn*5* sequences (not to scale) showing the relevant *Hin*dIII restriction sites within the inverted repeat used for mapping purposes. The circled letters refer to the positions of the primers used in PCR, and the arrow depicts the direction of synthesis (5′→3′). Note the difference in distance between the *Bam*HI within BENT-6 and the *Eco*RI site within *glpF* as calculated for the physical map of Kohara et al. (14) (500 bp) and compare that determined from our map (50 nucleotides). This may reflect anomalous migration of bent DNA through an agarose matrix.

termination method (27) with a reverse sequencing primer for the vector pTZ19R (19), 5′-CAGGAAACAGCTATGAC-3′. Sequence analysis was performed with a Sequenase (U.S. Biochemicals Corp.) kit according to the manufacturer's instructions.

After Tn*5* mutagenesis, 21 strains carrying independent Tn*5* chromosomal insertions (HB101::Tn*5*-1 to HB101::Tn*5*-21) with altered colony color phenotype were isolated (Table 1). These mutants occurred at a frequency of approximately 1 per 12,000 colonies screened. Sixteen of the 21 insertion strains had a yellow or yellow-white colony color, and eventually the insertions were shown to map to the *glpFK* region. Interestingly, four of five insertion strains carrying insertions that were subsequently shown not to map to the *glpFK* region all had a light pink colony color (strains harboring insertions Tn*5*-3, Tn*5*-4, Tn*5*-5, and Tn*5*-14). Only one strain with an insertion mapping outside of the *glpFK* region, HB101::Tn*5*-1, had a yellow colony color.

E. coli strains harboring the individual Tn*5* insertion mutations were transformed with plasmid pCJ102, which contained an intact *glpK* gene, and the Ap^r transformants were assayed for colony color (Table 1). Two insertion strains, HB101::Tn*5*-5 and HB101::Tn*5*-14, were not transformed to Ap^r with pCJ102 after repeated (six times) experiments, while all other control and insertion strains were transformed to Ap^r. The absence of transformation was consistently noted regardless of the type of plates (MacConkey-glycerol, MacConkey-glucose, or LB, all containing ampicillin and kanamycin) used to select transformed cells. HB101::Tn*5*-5 and HB101::Tn*5*-14 were transformed to Ap^r with pBR322 (the vector for pCJ102) and other β-lactamase-producing plasmids, suggesting a lethal effect of increased copy number of the *glpK* gene or other *E. coli* chromosomal sequences contained on pCJ102.

In addition to functional complementation by a *glpK*-containing plasmid, extracts from strains harboring the individual Tn*5* insertion mutations without pCJ102 were assayed for glycerol kinase activity (Table 1). In this experiment, the glycerol kinase activity was expressed as a percentage of the values in controls. The controls (HB101::Tn*5*-101 and HB101::Tn*5*-102) were Km^r strains harboring a Tn*5* insertion presumably into regions of the *E. coli* chromosome not involved in glycerol metabolism. Their color phenotype on MacConkey-glycerol plates was pink, the same as that for wild-type HB101.

A combination of the physical data obtained from restric-

TABLE 1. Phenotype and glycerol kinase activity of Tn*5* insertion mutants of *E. coli* HB101

Insertion mutation	Colony color phenotype	Phenotype after transformation with pCJ102	Glycerol kinase activity (% of control)[a]	Site of Tn*5* insertion
Tn*5*-1	Yellow	Ap^r, bright pink	2.58	Not in *glpFK*
Tn*5*-2	Yellow	Ap^r, bright pink	0.02	*glpK*
Tn*5*-3	Light pink	Ap^r, bright pink	85.55	Not in *glpFK*
Tn*5*-4	Light pink-yellow	Ap^r, bright pink	9.49	Not in *glpFK*
Tn*5*-5	Light pink-white	No transformation	79.64	Not in *glpFK*
Tn*5*-6	Yellow	Ap^r, bright pink	0.03	*glpK*
Tn*5*-7	Yellow	Ap^r, bright pink	0.14	*glpK*
Tn*5*-8	Yellow	Ap^r, bright pink	0.30	*glpF*
Tn*5*-9	Yellow	Ap^r, bright pink	0.62	*glpF*
Tn*5*-10	Yellow	Ap^r, bright pink	0.30	*glpK*
Tn*5*-11	Yellow-white	Ap^r, bright pink	0.14	*glpK*
Tn*5*-12	Yellow	Ap^r, bright pink	3.48	*glpF*
Tn*5*-13	Yellow-white	Ap^r, bright pink	2.35	*glpF*
Tn*5*-14	Light pink	No transformation	104.50	Not in *glpFK*
Tn*5*-15	Yellow-white	Ap^r, bright pink	0.46	*glpK*
Tn*5*-16	Yellow-white	Ap^r, bright pink	1.82	*glpF*
Tn*5*-17	Yellow-white	Ap^r, bright pink	0.32	*glpK*
Tn*5*-18	Yellow-white	Ap^r, bright pink	0.23	*glpK*
Tn*5*-19	Yellow-white	Ap^r, bright pink	0.22	*glpK*
Tn*5*-20	Yellow	Ap^r, bright pink	0.14	*glpK*
Tn*5*-21	Yellow-white	Ap^r, bright pink	0.16	*glpK*
None (HB101 wild type)	Pink	Ap^r, bright pink	122.46	

[a] Glycerol kinase activity in Tn*5*-1 through Tn*5*-11 was compared with that in Tn*5*-101 as a control (100%), and activity in Tn*5*-12 through Tn*5*-21 as well as in HB101 was compared with that in Tn*5*-102 as a control (100%). Control strains HB101::Tn*5*-101 and HB101 Tn*5*-102 were Km^r strains harboring a Tn*5* insertion, presumably into regions of the *E. coli* chromosome not involved in glycerol metabolism. These strains give a pink colony color phenotype like that of wild-type HB101.

tion enzyme analysis and Southern blotting experiments coupled with the glycerol kinase specific activity data in Table 1 enabled us to construct a correlated physical and genetic map of the *glpFK* region (Fig. 1). Note that several insertions, Tn*5*-8, Tn*5*-9, Tn*5*-12, Tn*5*-13, and Tn*5*-16, mapped upstream of the *glpK* gene and that within this group there was a gradient of glycerol kinase activity, with the insertion located most 5′ giving the least activity relative to control levels. Tn*5*, like most transposons, exerts a polar effect on distally located genes in an operon (5). The data demonstrated that at least one other gene was located upstream of *glpK* and in an operon with *glpK*. The distance from Tn*5*-8 to the beginning of the *glpK* gene (≃840 bp) gave a minimum size for this gene. Since the λ transducing phage λd*glpK*100, which contains chromosomal DNA (≃7 kb) downstream from *glpK* (9), does not contain *glpF*, it is likely that the Tn*5* insertions into the region upstream were into the *glpF* gene (*glpF*::Tn*5*). The polarity data suggested that *glpF* and *glpK* were in an operon.

To further map the *glpK*::Tn*5* insertions and substantiate the Southern blotting data, we used the PCR with either 5′-specific (primer B) or 3′-specific (primer D) *glpK* sequences and a primer made to the Tn*5* end sequences, primer E (Fig. 1). Gel electrophoresis after PCR revealed fragments whose sizes corresponded to the distance of the Tn*5* insertion from either the 5′ or 3′ end of *glpK*. Since the Tn*5* ends contain a twofold axis of symmetry, a single primer could be used to initiate this PCR from both ends (IS*50*R and IS*50*L). We therefore also performed the PCR as a duplex amplification reaction, simultaneously adding primer B, primer D, and primer E, and obtained the results shown in Fig. 2A. Note that there were two bands which corresponded in size to the distance from the 5′ and 3′ ends.

For the Tn*5* insertions upstream of *glpK*, we used a primer to the *glpK* sequence immediately 3′ to the *Bam*HI site (primer C) directed upstream and the Tn*5*-specific primer (primer E) to obtain the result shown in Fig. 2B. To confirm these data, a primer (primer A) to the 5′ of the *glpK* gene was also synthesized and used with the Tn*5* primer (primer E) to obtain the results shown in Fig. 2C. The data in Fig. 2B and C are very similar but displaced 480 bp, which is the distance from the *Hin*dIII site upstream of *glpK* to the *Bam*HI site in the *glpK* gene, the distance between primers A and C.

To investigate the hypothesis that Tn*5* insertion mutations upstream of *glpK* and polar on *glpK* expression were in a gene responsible for facilitating the transport of glycerol (*glpF*), we measured glycerol uptake in *E. coli* HB101 strains harboring Tn*5*-8, Tn*5*-9, and Tn*5*-12. Uptake was determined at different times by using radiolabeled [^{14}C]glycerol. Controls included *E. coli* HB101 and HB101::Tn*5*-101, which contains an insertion in the *E. coli* chromosome that is not in any of the genes involved in glycerol metabolism. Additional controls included a strain with an insertion within the *glpK* gene (*glpK*::Tn*5*-2), and each insertion strain tested (HB101::Tn*5*-2, HB101::Tn*5*-8, HB101::Tn*5*-9, and HB101::Tn*5*-12) was transformed with the *glpK*-containing plasmid pCJ102 to relieve polarity effects.

[^{14}C]glycerol was taken up by 10^8 cells at a rate of approximately 20 to 50 pmol/min between 1 and 5 min, and uptake approached saturation after 5 min in the wild-type controls HB101 and HB101::Tn*5*-101 (Fig. 3). Glycerol uptake was essentially zero for strains harboring insertions Tn*5*-2, Tn*5*-8, Tn*5*-9, and Tn*5*-12. When each of these strains was transformed with the *glpK*-containing plasmid pCJ102, only the strain with insert Tn*5*-2 (*glpK*::Tn*5*-2) was corrected for glycerol uptake (Fig. 3A). This reflects the fact that the glycerol which is taken up into the cell must be phosphorylated by GlpK to be trapped within the cell (12). The fact that the uptake defect in strains harboring insertions Tn*5*-8, Tn*5*-9, and Tn*5*-12 was not corrected even after the *glpK* gene was added by transformation with pCJ102, as reflected by no difference in the time course of [^{14}C]glycerol uptake with or without pCJ102 (Fig. 3B, C, and D), demonstrated that these insertion mutations were in a gene (*glpF*) whose

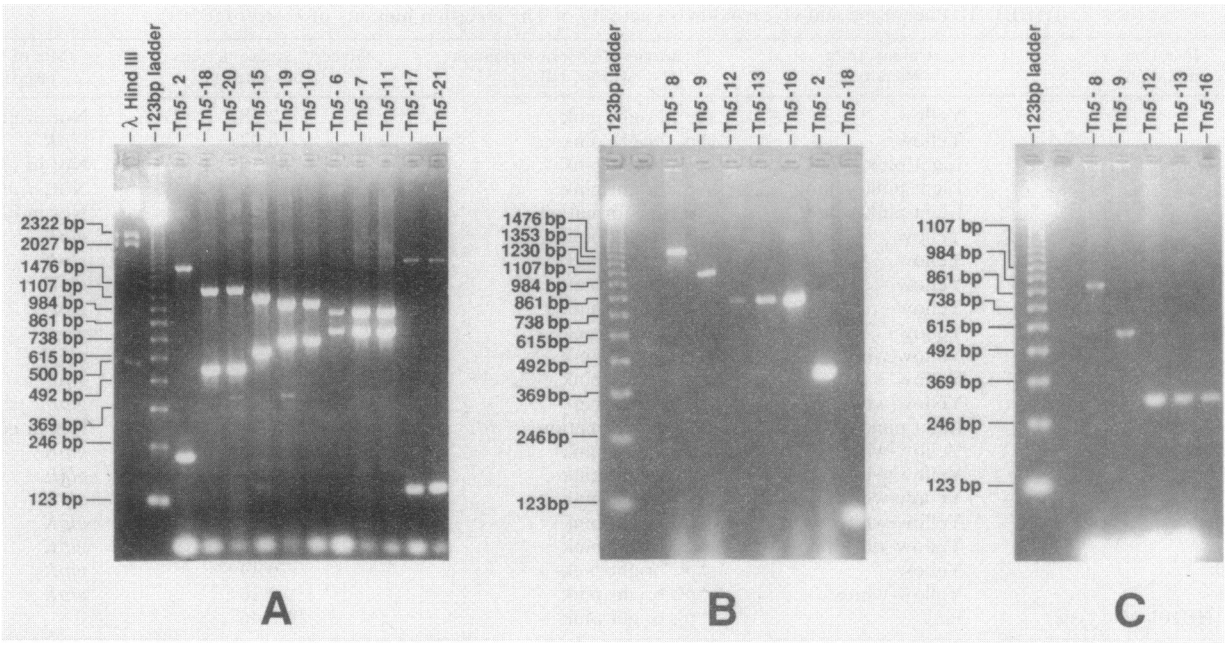

FIG. 2. Analysis of *glpFK*::Tn*5* insertions by PCR. For each gel, the size markers (phage λ digested with *Hin*dIII or 123-bp ladder) are shown at the left. (A) PCR products generated by using chromosomal DNA isolated from mutant strains harboring different Tn*5* insertions into *glpK* (*glpK*::Tn*5*) with primers B, D, and E. The individual insertions are shown at the top of the gel. Note that two PCR products were generated, one with primers B and E and the other with primers E and D. Primer E primed synthesis from both ends of Tn*5* because the ends are inverted repeats. (B) PCR products generated with insertion upstream of *glpK* and in the 5′ region of *glpK* with primers E and C. Primer C is located just 3′ of the *Bam*HI site in *glpK*. Note that the product from Tn*5*-18 migrated below the 123-bp marker. Southern analysis of *Bam*HI- and *Hin*dIII-digested chromosomal DNA from the strains carrying Tn*5*-18 and Tn*5*-20 demonstrated an intact 460-bp *Hin*dIII-*Bam*HI *glpK* fragment (data not shown). Thus, Tn*5*-18 and Tn*5*-20 must be located on the 3′ side of the *Bam*HI site within *glpK* and, in conjunction with the data in this figure, must be located between the *Bam*HI site and the 3′ end of primer C (Fig. 1). (C) PCR products generated with insertions upstream of *glpK* with primers E and A. Note that the relative positions of migration are the same as in panel B but displaced approximately 480 bp, which is the distance between primers A and C.

product (GlpF) is involved in glycerol uptake. Thus, it can be concluded that Tn*5*-8, Tn*5*-9, and Tn*5*-12 are insertions within the *glpF* structural gene.

The nucleotide sequence of the junction between Tn*5*-8 and the *E. coli* chromosome was determined to delineate the insertion site. Tn*5*-8 had inserted between the *Bam*HI site in BENT-6 (Fig. 1) and the *Eco*RI site within an open reading frame (ORF) proposed to be *glpF* (22). This insertion was 21 bp downstream of the ATG initiation codon and thus disrupted the ORF proposed to be *glpF*.

In conclusion, we have subjected the *E. coli* K-12 strain HB101 chromosome to mutagenesis with transposon Tn*5* and screened for insertions into genes involved in glycerol metabolism as evidenced by an altered colony color phenotype when plated on MacConkey-glycerol plates. Twenty-one independent Tn*5* insertions were identified, and 16 mapped to the *glpFK* region at 88.4 min on the *E. coli* genetic linkage map (2) and 3590 kb on the *E. coli* physical restriction map (14). The color phenotype of different strains harboring the 16 Tn*5* insertions into the *glpFK* region and Tn*5* insertions (Tn*5*-1, Tn*5*-3, and Tn*5*-4) outside the *glpFK* region was corrected by transformation with a *glpK*-containing plasmid. The fact that the colony color phenotype of strains harboring *glpF*::Tn*5* insertions (Tn*5*-8, Tn*5*-9, Tn*5*-12, Tn*5*-13, and Tn*5*-16) was corrected by a plasmid harboring *glpK* sequences suggests that the rate-limiting step for conversion of glycerol to glycerol 3-phosphate is not glycerol uptake. This also suggests the existence of a second transport system for glycerol and that increased activity of GlpK, secondary to increased gene dosage from a high-copy-

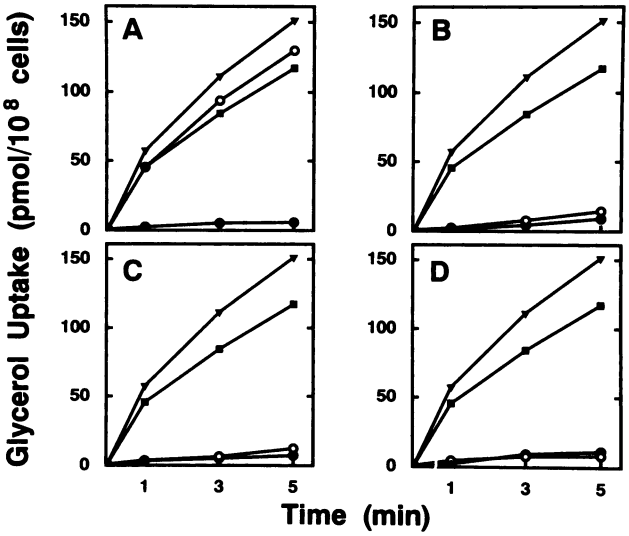

FIG. 3. Uptake of labeled glycerol by wild-type HB101 and strains harboring Tn*5* insertions. Uptake of [U-^{14}C]glycerol was measured at 1, 3, and 5 min by the method described in the text. Uptake by two strains with wild-type phenotypes, HB101 (▼) and HB101::Tn*5*-101 (■), is shown in each panel for comparison with strains containing mutations in the *glpK* and *glpF* loci before (●) and after (○) transformation with pCJ102. The mutant strains included the *glpK* mutant Tn*5*-2 (A) and the *glpF* mutants Tn*5*-8 (B), Tn*5*-9 (C), and Tn*5*-12 (D).

number plasmid containing *glpK*, can effectively utilize the alternative transport system and correct the color phenotype (13).

E. coli strains harboring two insertions, Tn*5*-5 and Tn*5*-14, were not transformed by the *glpK*-containing plasmid pCJ102 but were transformed by the pBR322 parent vector. This suggests that in strains harboring these insertion mutations, the excess glycerol kinase provided by the plasmid pCJ102 leads to the accumulation of a toxic metabolite(s). Strains harboring Tn*5*-5 or Tn*5*-14 have a single-copy functional *glpK*, as evidenced by normal GlpK activity and normal-size *glpK* gene by PCR, but indeed grow more slowly and have smaller colonies regardless of the medium used.

Five insertions (Tn*5*-8, Tn*5*-9, Tn*5*-12, Tn*5*-13, and Tn*5*-16) mapped upstream of *glpK* and were polar on *glpK*, which suggests that this region is in an operon with *glpK*. These mutations mapped within an ORF proposed to correspond to *glpF* (22), and indeed, mutations which mapped to these positions caused defective uptake of glycerol (Fig. 3). Since HB101::Tn*5*-8 is defective in glycerol uptake and strains with Tn*5* insertions into *glpK* are not, this clearly establishes the ORF upstream of *glpK* as the *glpF* gene. Our data, taken in conjunction with the DNA sequence data of Muramatsu and Mizuno (22), establish that *glpF* is in an operon with *glpK* and that a bent DNA segment (BENT-6) is immediately adjacent and is probably part of the *glpFK* operon (Fig. 1). Regulatory sequences for the *glpFK* operon are probably within BENT-6. The fact that no additional insertion mutations were found upstream or downstream of *glpFK* suggests that there are no additional structural genes in the *glpFK* operon with a function essential for fermentation of glycerol.

The position of BENT-6 with respect to the *glpFK* genes strongly suggests that this sequence may be involved in regulating the *glpFK* operon. Inspection of BENT-6 reveals a sequence (TACAAT) with homology to the procaryotic promoter consensus hexamer sequences for the −10 region (TATAAT), with only one mismatch (T instead of C) in the least-conserved (49%) position. There is also a sequence 18 bp upstream of the −10 region (ATGATC) with similarity to the consensus (TTGACA) for the −35 region of procaryotic promoters (18, 21). Recently, bent DNA sequences, even those with very limited similarity to consensus transcription initiation sites, have been demonstrated to function as promoters in vivo (8). In addition, the requirement for the in vivo activation of the *E. coli gal* promoter can be eliminated by replacing the catabolite gene activator protein-binding site by bent DNA (7). These published data, together with the *glpFK* operon structure deduced in this study (Fig. 1), suggest that BENT-6 may be the functional promoter for the *glpFK* operon.

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