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Identification of the *glpT*-Encoded *sn*-Glycerol-3-Phosphate Permease of *Escherichia coli*, an Oligomeric Integral Membrane Protein

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A collection of hybrid plasmids carrying either the wild-type or mutated *glpT* gene was generated in vitro and used to characterize the *glpT*-dependent active transport system for *sn*-glycerol-3-phosphate in *Escherichia coli* K-12. Restriction endonuclease analysis and recloning of DNA fragments localized *glpT* to a 3-kilobase pair *Pst*I-*Hpa*I segment of DNA. Comparison of DNA carrying *glpT-lacZ* fusions with DNA carrying intact *glpT* allowed determination of the direction of transcription. Through characterization of the proteins synthesized by strains harboring hybrid plasmids carrying amber, missense, or deletion mutations in *glpT*, it was shown that *glpT* is a promoter-proximal gene in an operon consisting of at least two genes. The gene product of *glpT*, the *sn*-glycerol-3-phosphate permease, was found associated with the inner membrane. It could be solubilized by treatment with sodium dodecyl sulfate at 50°C. Its molecular weight, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, was dependent upon sample treatment before electrophoresis. The apparent molecular weight was 44,000 when membrane fractions were heated to 50°C; subsequent treatment at 95°C modified the protein such that it migrated faster (apparent molecular weight = 33,000). Several missense mutations in *glpT* were negatively dominant over wild-type *glpT*, indicating that the active form of the permease is multimeric. A gene (named *glpQ*) promoter distal to *glpT* codes for a periplasmic protein. This protein had previously been named GLPT protein to indicate its relationship to the *glpT* gene. The present report demonstrates that it is not the gene product of *glpT* and is not required for active transport of *sn*-glycerol-3-phosphate.

In *Escherichia coli*, *sn*-glycerol-3-phosphate (G3P) is actively accumulated by the cell via several transport systems. The highly specific *ugp* transport system is part of the *pho* regulon and is therefore functional in *pho* constitutive strains or when phosphate is limiting (2). G3P can also enter the cell via the relatively nonspecific hexose phosphate transport system (*uhp*) in *uhp* constitutive or induced strains (16). The *glpT* transport system, on the other hand, is geared specifically for the utilization of G3P; both *glpT* and the catabolic enzymes of the *glp* regulon are governed in parallel via inducer (G3P)-repressor interactions, as well as by catabolite repression and respiratory control (25).

Previous studies have demonstrated a close correlation between *glpT*-encoded transport activity and the presence of a periplasmic protein, which was named GLPT (3, 38, 39). The *glpT* system did not appear to be a binding protein-dependent transport system, however, since no affinity between GLPT and G3P could be demonstrated (8). In addition, transport activity was present in membrane vesicles lacking the GLPT protein (8). Transport in such vesicles was energized by the proton motive force. Hence, the *glpT* system seemed to resemble transport systems such as *lacY*, which have only an inner membrane permease. The possible role of the periplasmic GLPT protein was further clouded by the finding of only one genetic complementation group among *glpT* mutations (26a). Could this one gene be coding for the periplasmic GLPT protein?

To characterize the *glpT* system in greater detail with respect to the genes and proteins involved, a collection of hybrid plasmids carrying either the intact or the mutated *glpT* region was constructed in vitro. Characterization of strains harboring these plasmids has allowed

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identification of the G3P permease in the inner membrane and has also demonstrated the dispensability of the periplasmic GLPT protein with regard to active transport of G3P.

MATERIALS AND METHODS

Bacterial strains, phage, and plasmids. The bacterial strains, phage, and plasmids used are shown in Table 1.

Δ*glpT* was introduced via P1 transduction (29), using a Δ*glpT gyrA* donor with selection for Nal^r or a Δ*glpT zei-724*::Tn*10* donor with selection for Tet^r. Tet^s derivatives of the latter transductants were obtained as described by Bochner et al. (6).

DS410T, a Δ*glpT* derivative of the minicell-producing strain DS410, was constructed so that the G3P phenotype conferred on this strain by various hybrid plasmids could be determined before analysis of plasmid-encoded proteins with purified minicells (13, 24).

Plasmid DNA-transformed cells (12) were selected on antibiotic medium 3 (Difco Laboratories), using either 10 μg of tetracycline or 50 μg of ampicillin per ml.

TABLE 1. Bacterial strains, phage, and plasmids

Strain	Genotype[a]	Source
E. coli K-12		
TS100	F$^-$ *araD139 ΔlacU169 rpsL relA thiA glpR*	39
LA5000	TS100 *gyrA*	39
DL39	LA5000 Δ*(glpT-glpA)593 zei-724*::Tn*10*	26a
DL291	TS100 *gyrA* Δ*(glpT-glpA)593 recA1*	26a
TL45	TS100 *gyrA* Δ*(glpT-glpA)593*	26a
TL73	TS100 *recA1*	(i) P1 · MS367→TS100 (ii) Tet^s selection
TL74	LA5000 *recA1*	(i) P1 · MS367→LA5000 (ii) Tet^s selection
MS367	Hfr KL1699 *thi1 relA1 drm-3 recA1 srl*::Tn*10*	R. Isberg (17)
DS410	F$^-$ *minA minB ara lacY malA mtl xyl rpsL thi tonA azi*	13
DS410T	DS410 *gyrA* Δ*(glpT-glpA)593*	P1 · DL291→DS410
MX397	F$^-$ *metB1 leu^b trp^b lacZ^b galK^b galE sueA^c sueC^c tsx relA supD43,74 rpsL*	33
MX399	F$^-$ *metB1 leu^b trp^b lacZ^b galK^b galE sueB^c sueC^c tsx relA supD43,74 rpsL*	33
TL11	MX397 *gyrA* Δ*(glpT-glpA)593*	P1 · DL291→MX397
TL14	MX399 *gyrA* Δ*(glpT-glpA)593*	P1 · DL291→MX399
XA100	F$^-$ *ara* Δ*(lac-pro) gyrA metB argE^b rif thi*	31
XA101, 2, 3, 5, B, and C	XA100 with *sup*D,*E*,*F*,*G*,*B*,*C*, respectively	31
XA96	F$^-$ *ara* Δ*(lac-pro) gyrA argE^b rif thi supP*	31
TXA100	XA100 Δ*(glpT-glpA)593*	(i) P1 · DL39→XA100 (ii) Tet^s selection
TXA101, 2, 3, 5, B, and C	XA101, 2, 3, 5, B, and C Δ*(glpT-glpA)593*	(i) P1 · DL39→XA101 (ii) Tet^s selection
Phage		
λ p*glpT669*	ϕ*(glpT-lacZ)*[d]	26a
Plasmid[e]		
pBR322	*tet bla*	7
pB1[f]	*bla*	U. Rüther, U. Cologne
pMLB524[g]	*bla lac"ZY"*	5
pLC3-46	Colicin E1^{imm} *glpT glpQ glpA*	3, 11

[a] Genetic nomenclature according to Bachmann and Low (4).

[b] Amber mutation.

[c] Suppressor-enhancing mutation.

[d] *glpT-lacZ* operon fusion.

[e] Wild-type genes are indicated.

[f] Derivative of pBR322 containing the *lacZ*UV5 promoter on a 95-base pair fragment inserted after the *Eco*RI site; this promoter initiates transcription toward the nearby *Bam*HI site.

[g] Vector in which the *Eco*RI-*Ava*I fragment of pBR322 has been replaced by a 309-base pair *Eco*RI-*Ava*I fragment carrying the carboxyl-terminal end of *lacZ* (beginning at the *Eco*RI site) and the first part of *lacY* (ending at *Ava*I). Insertion in the correct orientation of an *Eco*RI fragment carrying an exogenous promoter fused to *lacZ* results in a hybrid plasmid carrying the exogenous promoter fused to *lacZ* (5).

LB medium (29) containing 0.4% glycerol and the appropriate antibiotic was used for growth of cells before preparation of minicells, measurement of G3P transport activity, or preparation of cold osmotic shock and membrane fractions. Cells in the logarithmic phase of growth were used for these purposes.

For minimal media, the A and B salts of Clark and Maaløe (10) were supplemented with 2 μg of thiamine per ml and 0.4% of the various carbon sources, unless otherwise indicated.

Isolation of mutations in cloned *glpT*. Point mutations in pGS31 harboring the wild-type *glpT* gene were generated in vitro, using hydroxylamine (0.4 M, 35 min, 68°C) as described by Humphreys et al. (18). Strain DL291 (Δ*glpT recA*) was transformed with the mutagenized DNA, and the resulting Tet^r transformants were replica plated onto minimal medium containing 5 μg of tetracycline per ml and either G3P or glycerol as carbon source. Lack of G3P transport activity confirmed the *glpT* nature of mutations in 12 independent G3P$^-$ transformants [designated DL291(pGS3100 to pGS3114); Table 2]. Reversion of the plasmid mutations was observed when 10^8 to 10^9 cells were plated on G3P.

To isolate amber mutations in cloned *glpT*, advantage was taken of the high-efficiency temperature-sensitive amber suppressor strains described by Oeschger et al. (33). A *supD*(Ts) Δ*glpT* recipient (TL11 or TL14) was transformed with pGS31, which had been treated with hydroxylamine as described above. Tet^r transformants were pooled in 10 independent groups, and two cycles of enrichment for *glpT* mutations were carried out at 42°C in minimal medium containing 0.4% succinate, 1% Casamino Acids, 0.5 mM tryptophan, 5 μg of tetracycline per ml, 5 mM glycerol, and 2.5 mM 3,4-dihydroxybutyl-1-phosphonate, a G3P analog bacteriostatic toward cells which possess a transport system for G3P (16). After the enrichment procedure, single colonies were obtained on antibiotic medium 3 containing 10 μg of tetracycline per ml (37°C). The colonies were replica plated onto G3P minimal medium at 32°C. Most of the resulting G3P$^+$ colonies contained amber mutations in plasmid-encoded *glpT*, which was verified by measuring transport of G3P after growth at 30 or 42°C. Ten independent mutants were saved for characterization [TL11 (pGS3151 to pGS3166); Table 2]. Transfer of the mutant plasmids into a nonsuppressing Δ*glpT* strain, DL291, resulted in a G3P$^-$ phenotype at high and low temperatures.

Deletions affecting cloned *glpT* were generated in pGS31H5 (Fig. 1), using restriction endonuclease *Sau*3A. The DNA was digested until fragments of approximately 1 to 6 kilobase pairs (kb) were obtained. The partially digested DNA was religated, and Tet^r transformants of DL291 were obtained. Analysis of plasmid DNA isolated from several G3P$^-$ transformants verified that various amounts of *glpT* DNA had been deleted (pGS32 and pGS34; Fig. 1). These plasmids no longer produced periplasmic GLPT protein (not shown) or the membrane-associated G3P permease (see Results). The partially digested pGS31H5 DNA was also used for construction of plasmids pB13, pB15, and pB112. In these plasmids *Sau*3A treatment shortened the recloned piece of DNA but left the *glpT* gene intact.

Isolation and analysis of plasmid DNA. When large quantities of highly purified DNA were required (in vitro protein synthesis), plasmids were isolated from amplified cultures by centrifugation to equilibrium in CsCl (12). For screening purposes, small amounts of DNA were isolated from 10-ml cultures (12, 22). Plasmids and restriction fragments were analyzed by electrophoresis on 0.7% agarose gels, using a Tris-acetate-EDTA buffer system (12). λ *c*I857 DNA cleaved with *Hin*dIII or *Eco*RI served as size markers. Conditions for restriction endonuclease cleavage and ligation of DNA were those recommended by the supplier (Boehringer) or described elsewhere (5, 12).

Analytical techniques. The *glpT*-encoded transport activity was assessed by measuring accumulation of [^{14}C]G3P (New England Nuclear) at a 0.3 μM concentration, as previously described (39).

Plasmid-encoded proteins were radioactively labeled with L-[^{35}S]methionine (Amersham Corp.) in minicells isolated by sucrose gradient centrifugation (24). Alternatively, a maxicell system (40) that uses the *recA* strain DL291 or the plasmid DNA-directed cell-free protein-synthesizing system (38, 45) was used.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was carried out overnight at 110 to 150 V on 12% polyacrylamide slab gels (16 by 20 cm), using the buffer system of Laemmli (23). The ratio of acrylamide to *N*,*N*'-methylene bisacrylamide was 60:1. Details concerning sample preparation are given

TABLE 2. Properties conferred by point mutations in cloned *glpT*

Plasmid	Allele no.	Characteristics[a]
pGS3100	600	Missense mutations which are negatively dominant over wild-type *glpT*$^+$ function (*glpT*$^{-d}$)
pGS3102	602	
pGS3107	607	
pGS3108	608	
pGS3109	609	
pGS3113	613	
pGS3114	614	
pGS3105	605	Missense mutation, not negatively dominant
pGS3101	601	Polar mutations not suppressible by ochre or amber suppressors
pGS3111	611	
pGS3110	610	Amber fragment not assessed[b]
pGS3162	662	No amber fragment observed[b]
pGS3151	651	12,000[b]
pGS3155	655	12,000[b]
pGS3163	663	13,800[b]
pGS3165	665	15,100[b]
pGS3166	666	15,100[b]
pGS3103	603	15,800[b]
pGS3152	652	17,400[b]
pGS3156	656	17,400[b]
pGS3153	653	27,000[b]
pGS3164	664	27,000[b]

[a] All mutations were isolated on pGS31 and therefore carry the genes encoding tetracycline resistance.

[b] Amber mutations. Amber fragments having the indicated apparent molecular weights are synthesized in vitro (Fig. 5).

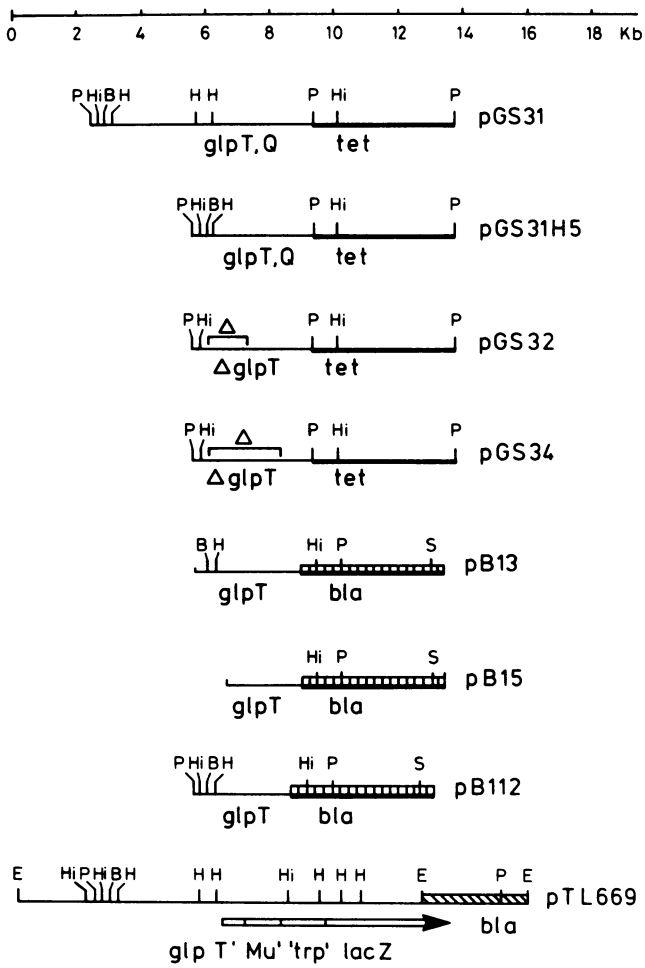

FIG. 1. Restriction endonuclease maps of recombinant plasmids containing *glpT* DNA. The plasmids are shown in linear form, opened at a restriction site between the vector and cloned DNA. The restriction endonucleases utilized were *Pst*I (P), *Hin*dIII (Hi), *Bgl*II (B), *Hpa*I (H), *Sal*I (S), and *Eco*RI (E). A common *Hpa*I site, when present, is used to align the plasmids vertically. The source of DNA is indicated as follows: pBR322, ▬ ; pB1, ▥ ; pMLB524, ▭ ; chromosomal DNA, ———. Approximately 1 kb of the left end of pTL669 is from λ.

below. The molecular weight standards used were bovine serum albumin (67,000), ovalbumin (43,000), aldolase (40,000), α-chymotrypsinogen (25,700), and lysozyme (14,300). Sample buffer contained 0.06 M Tris-hydrochloride (pH 6.8)–2% SDS–10% glycerol–3% 2-mercaptoethanol–0.001% bromophenol blue.

Analytical isoelectric focusing of proteins in cold osmotic shock fractions was done on LKB Ampholine PAG plates, using the pH range 4 to 6.5 as recommended by the manufacturer.

Protein concentrations were estimated by the method of Lowry et al. (26), with bovine serum albumin as the standard.

Subcellular fractionation. Periplasmic proteins were released by using the cold osmotic shock procedure of Neu and Heppel (32) as modified for binding proteins (35).

The cytoplasmic and outer membrane fractions were obtained essentially as described by Ito et al. (20). After disruption of the cells by sonication and removal of undisrupted cells, a total membrane fraction was obtained by sedimentation through 15% sucrose onto a 1.5-ml 70% cushion (SW41 rotor, 35,000 rpm, 90 min). This fraction was diluted and then layered on top of a second SW41 tube containing 1.5 ml of 70% sucrose and 8.5 ml of 53% sucrose. After centrifugation at 35,000 rpm for 4 h, the inner or outer membrane fractions were withdrawn from the upper or lower interface region, respectively. All steps were carried out at 0 to 4°C.

RESULTS

For cloning of the gene(s) encoding the *glpT* transport system, various derivatives of plasmid pLC3-46 (11) were introduced by transformation into strain DL291 [Δ(*glpT-glpA*)*593 recA*]. Because the *glpT* region is totally deleted in this

strain (26a), growth on G3P as carbon source must be due to the presence of the entire *glpT* transport system on the hybrid plasmid. Thus, for the subcloning experiments described below, appropriately digested plasmids were religated and transformed into DL291, with selection for $G3P^+$ Tet^r colonies (or in some cases, $G3P^+$ Amp^r).

The Clarke and Carbon plasmid pLC3-46 (11) corrects defects in both *glpT* and *glpA* (3, 37, 43). Furthermore, pLC3-46 directs in vitro the synthesis of the periplasmic GLPT protein (38). To obtain *glpT* DNA on a vector carrying a convenient antibiotic resistance marker, pLC3-46 was subjected to cleavage with various restriction endonucleases. It was found that cleavage with *Eco*RI, *Hin*dIII, or *Bam*HI resulted in only one, three, or three fragments, respectively. *Pst*I, however, cleaved this plasmid into five fragments of 7.3, 5.9, 5.3, 4.6, and 1.1 kb. To determine whether any of them carried *glpT*, the *Pst*I fragments were ligated into the *Pst*I site of pBR322. Transformation of DL291 with the ligation mixture resulted in $G3P^+$ Tet^r colonies. Analysis of plasmid DNA isolated from these clones showed that the largest *Pst*I fragment (7.3 kb) contained *glpT*. It did not contain *glpA*. The hybrid plasmid containing this fragment was named pGS31 (Fig. 1). The periplasmic fraction of DL291(pGS31) contained the GLPT protein (Fig. 2A). In addition, minicells containing pGS31 directed the synthesis of this and several other proteins (see Fig. 2B).

To eliminate DNA not required for *glpT* function, restriction enzymes were sought which cleave the recloned *Pst*I fragment of pGS31. It was found that *Hpa*I cleaved pGS31 into three fragments of 8.2, 2.9, and 0.5 kb. Religation of an *Hpa*I digest of pGS31 resulted in formation of pGS31H5, in which the two smaller *Hpa*I fragments were eliminated (Fig. 1). DL291(pGS31H5) was $G3P^+$ and contained the GLPT protein in its periplasm (Fig. 2A). This plasmid directed the synthesis of the GLPT protein in minicells and in vitro but had lost the gene coding for a protein of M_r 65,000 (Fig. 2B).

The M_r of the GLPT protein synthesized in vitro with pGS31H5 as the template was slightly larger than that of GLPT synthesized in minicells (Fig. 2B). These results are consistent with the conclusion (38) that this protein is synthesized with an amino-terminal signal sequence which is removed during the secretion process. Such amino-terminal extensions have been found for all exported bacterial proteins thus far studied.

To find out whether the 3-kb *Hpa*I-*Pst*I fragment of pGS31H5 could be shortened without losing G3P transport function, the plasmid was partially digested with endonuclease *Sau*3A; pGS31H5 has more than 30 restriction sites for *Sau*3A (data not shown). The resulting fragments were ligated into the *Bam*HI site of pB1 (Table 1). Transformation of DL291 yielded $G3P^+$ Amp^r Tet^s colonies. The structures of three plasmids isolated from such transformants are shown in Fig. 1 (pB13, pB15, and pB112). In these plasmids, *glpT* has been localized to approximately 2.4 kb. The periplasmic fractions obtained from DL291(pB13, pB15, or pB112) did not contain GLPT (Fig. 3). Furthermore, these plasmids did not direct the synthesis of GLPT in the minicell system (not shown). Strains harboring these plasmids were capable of accumulating [^{14}C]G3P at rates indistinguishable from those observed in DL291(pGS31H5). Therefore, neither growth on high concentrations of G3P (20 mM) nor transport of G3P (measured at 0.3 μM) required the presence of the periplasmic GLPT protein.

The absence of GLPT in the periplasmic fractions of DL291(pB13, pB15, and pB112) was not artifactual due to the synthesis of vector-encoded β-lactamase. Both GLPT and β-lactamase were found in the periplasm of DL291(pB111) (Fig. 3, slot 4).

These results demonstrate that periplasmic GLPT is not the product of the *glpT* gene, but strongly suggest that it is instead the product of a gene distal to *glpT* in a bi- or polycistronic operon. This idea is consistent with the finding of only one genetic complementation group for *glpT* (26a), the lack of GLPT in some (but not all) *glpT* mutants (26a, 39), and the coinducibility of the periplasmic GLPT protein with the transport system (39).

Determination of the direction of transcription of *glpT* on the cloned DNA. To determine the direction of transcription of *glpT* on the cloned DNA, the *glpT* promoter was fused to the *lac* structural genes. DNA isolated from λ p*glpT669* (carrying a *glpT-lacZ* operon fusion [26a]) was cleaved with *Eco*RI, and the pattern obtained upon agarose gel electrophoresis was compared with that for λ *c*I857 DNA. A unique fragment of approximately 12.5 kb was present in λ p*glpT669* DNA.

To facilitate restriction endonuclease mapping of the *glpT-lacZ* fusion, the 12.5-kb fragment was recloned into the fusion-cloning vector pMLB524 (5). This was done by ligating *Eco*RI-digested pMLB524 and λ p*glpT669* DNA together. DL291 was transformed with the ligation mixture by selecting for Amp^r on LB medium containing 50 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside per ml. The 12.5-kb *Eco*RI fragment was present in all of the resulting $lacZ^+$ clones. Further restriction endonuclease analysis (pTL669, Fig. 1) revealed that the sequence *Pst*I-*Hin*dIII-*Bgl*II-*Hpa*I-*Hpa*I-

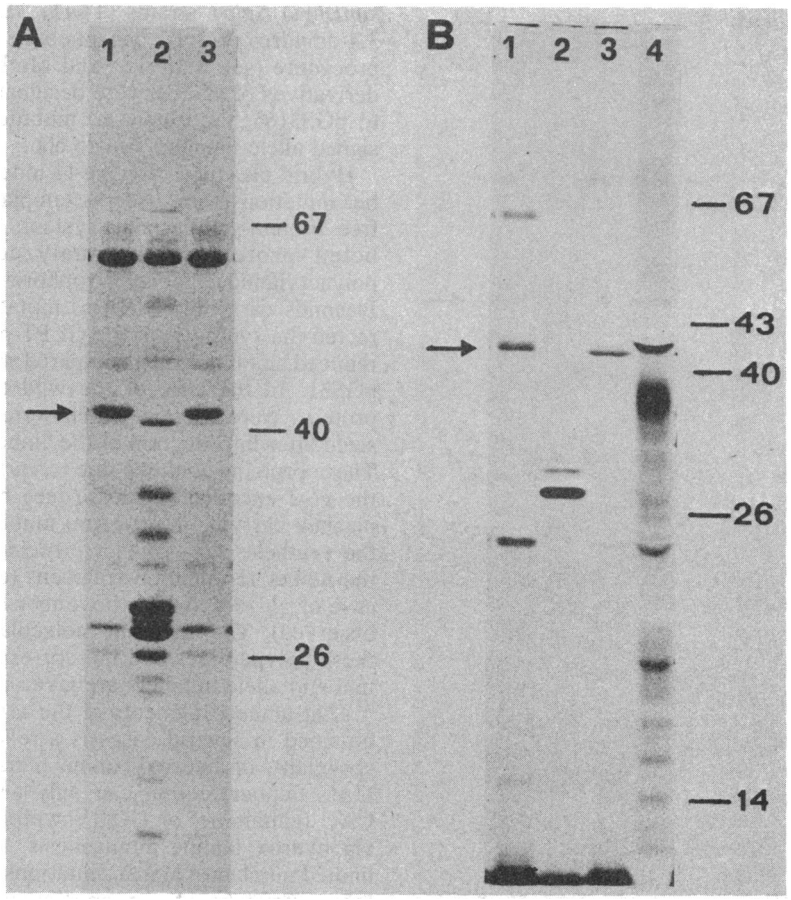

FIG. 2. SDS-polyacrylamide gel electrophoresis of cold osmotic shock fractions (A; Coomassie blue staining), and of proteins synthesized in minicells or in vitro (B; autoradiography). (A) A sample containing 40 μg of protein from the cold osmotic shock fractions of DL291(pGS31H5) (slot 1), pBR322 (slot 2), or pGS31 (slot 3) was diluted with sample buffer and heated (95°C, 5 min) before electrophoresis. (B) Minicells purified from 50-ml cultures were labeled (60 min, 37°C) with L-[^{35}S]methionine (20 μCi; 700 Ci/mmol) in 0.5 ml of minimal medium containing 0.2% glycerol and 0.01 ml of methionine assay medium (Difco). After a 15-min chase with cold methionine, the minicells were collected by centrifugation, washed, and then suspended in electrophoresis sample buffer. Samples were heated for 5 min at 95°C before electrophoresis. DS410T minicells contained the following plasmids: slot 1, pGS31; slot 2, pBR322; slot 3, pGS31H5. In vitro protein synthesis (slot 4) was carried out (38) in 0.2 ml, using 8 μg of pGS31H5 DNA and 10 μCi of L-[^{35}S]methionine (700 Ci/mmol). A sample from the reaction mixture was diluted with sample buffer and heated at 95°C before electrophoresis. In this and in the following gel photographs, the molecular weights of protein standards are indicated (× 10^3), and the position of the periplasmic GLPT protein is indicated by the arrow.

*Hpa*I found on pGS31 (containing *glpT*) was also present on the cloned *glpT-lacZ* fusion (pTL669). This demonstrates that transcription of *glpT* begins to the right of the third *Hpa*I site of pGS31, with transcription from left to right on the plasmids shown in Fig. 1.

Isolation of point mutations in cloned *glpT*. To provide further evidence for the bi- or polycistronic nature of the operon containing *glpT*, to solidify the argument that the periplasmic GLPT protein is not required for transport function, and to facilitate identification of the *glpT* gene product, point mutations affecting *glpT* function encoded by pGS31 were sought. Such mutations were readily isolated after hydroxylamine mutagenesis of pGS31 (see Materials and Methods). Plasmids having these *glpT* mutations were designated pGS3100 to pGS3114; the mutations were assigned allele numbers 600 to 614. SDS-polyacrylamide gel electrophoretic analysis of cold osmotic shock fractions prepared from DL291 harboring these plasmids allowed determination of the mutations' influence on the amount of GLPT produced (Fig. 4). It was found that many mutations had no effect on the amount of GLPT present in the periplasm (when com-

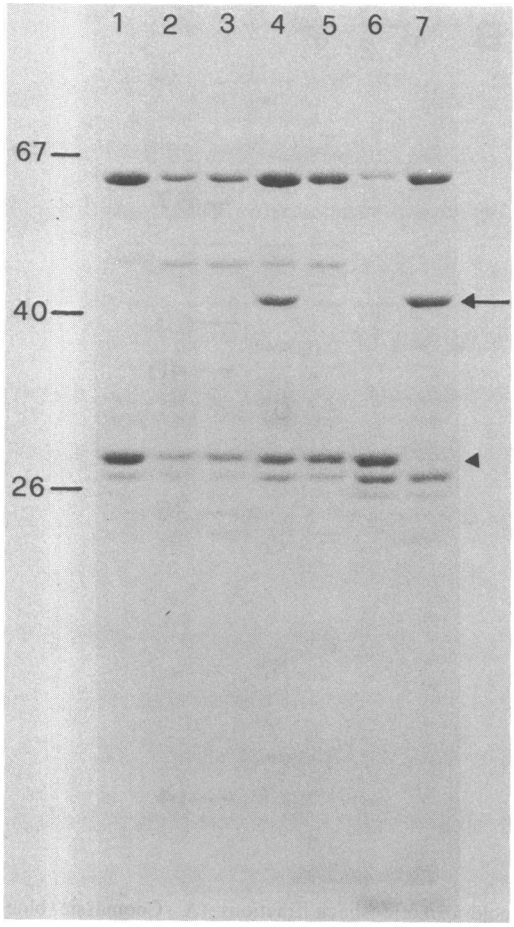

FIG. 3. SDS-polyacrylamide gel electrophoretic analysis of cold osmotic shock fractions from DL291 harboring *glpT*$^+$-Δ*glpQ* hybrid plasmids. Sample preparation and electrophoresis were carried out as in the legend to Fig. 2A. The arrowhead points to β-lactamase. DL291 contained the following plasmids: 1, pB13; 2, pB15; 3, pB110; 4, pB111; 5, pB112; 6, pBR322; 7, pGS31. Plasmids pB110 and pB111 were isolated as described for the other pB1 derivatives but are not shown in Fig. 1.

pared with wild type), whereas others markedly reduced (but did not eliminate) GLPT. G3P$^+$ revertants synthesized wild-type levels of GLPT. These results (including data obtained for other *glpT* mutations not shown in Fig. 4) are summarized in Table 2.

Mutations which allow production of wild-type levels of GLPT are most likely nonpolar missense mutations; some of these were negatively dominant (*glpT*$^{-d}$) over *glpT*$^+$ function (see below).

In addition to the above mutations, a series of *glpT* amber mutations on pGS31 were sought. Such mutations were readily isolated in *supD*(Ts) Δ*glpT* strains (TL11, TL14), using a 3,4-dihydroxybutyl-1-phosphonate enrichment procedure (see Materials and Methods). These derivatives of pGS31 were designated pGS3151 to pGS3166; the *glpT*(Am) mutations were assigned allele numbers 651 to 666.

Hybrid plasmids carrying 11 independent amber mutations were used as templates in a cell-free protein-synthesizing system. The radiolabeled proteins were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 5). Plasmids carrying *glpT*(Am) mutations still directed the synthesis of the GLPT protein but in reduced amounts when compared with wild-type pGS31. In the case of the wild type, several proteins were present which were not synthesized after introduction of the amber mutations. These proteins could be due to various forms of the *glpT*-encoded permease (see below). Each plasmid carrying a *glpT*(Am) mutation directed the synthesis of a smaller protein, presumably the amber termination fragment (except in the case of *glpT662*, where no amber fragment was observed). The apparent molecular weights of these polypeptides and the corresponding plasmid and allele numbers are given in Table 2.

That amber fragments of the same size were obtained in several cases is a reflection of the specificity of hydroxylamine mutagenesis; the UAG (amber) codon can only arise from the CAG (glutamine) or UGG (tryptophan) codons via hydroxylamine mutagenesis. Thus, only a limited number of amber mutations are expected (approximately 11 for an "average" *E. coli* protein of M_r 33,000 [19]). The seven different *glpT*(Am) mutations observed are therefore close to the maximum number obtainable with this mutagen.

To determine the influence of *glpT*(Am) mutations on in vivo function and on the synthesis of the GLPT protein, plasmids bearing them were transformed into various strains. Nonsuppressing strains DL291, TL45, and TXA100 remained G3P$^-$ in the presence of the *glpT*(Am) plasmids. Analysis of the periplasmic fractions obtained from these strains showed that production of the GLPT protein was strikingly reduced when compared with wild type but not totally eliminated (see Fig. 4; *glpT603*). When the *glpT*(Am) plasmids were placed into *supD*, *supE*, *supF*, *supG*, and *supP* backgrounds, the G3P$^-$ phenotype caused by the amber mutations was suppressed except in the cases of *glpT665* and *glpT666*; these mutations (which are presumably identical) were suppressed by all but the lysine inserter, *supG*. Cold osmotic shocks were obtained from the *supE* (TXA102) and *supG* (TXA105) strains harboring the *glpT*(Am) plasmids. These strains synthesized the periplasmic GLPT protein again. Since all ambers are derived from

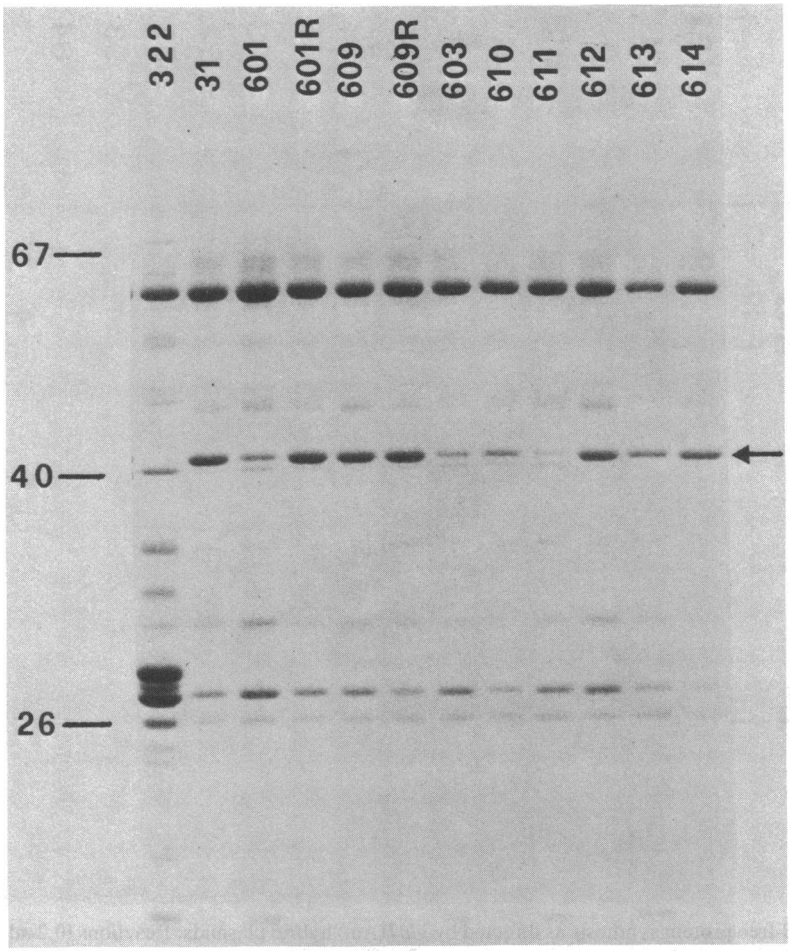

FIG. 4. Effect of *glpT* point mutations on the synthesis of periplasmic GLPT protein. The preparation and electrophoretic analysis of the cold osmotic shock fractions were carried out as described for Fig. 2A. DL291 contained the following plasmids: 322, pBR322; 31, pGS31; 601 through 614, mutant or revertant (R) derivatives of pGS31 with the indicated *glpT* allele (601 = pGS3101, 603 = pGS3103, etc.).

triplets coding for either glutamine or tryptophan, it was interesting to see whether insertion of lysine by amber suppression (*supG*) would cause an alteration in the isoelectric point of GLPT. The above shock fluids were therefore analyzed by analytical isoelectric focusing. No matter which of the seven different amber positions was suppressed, the isoelectric point of GLPT was identical to that found for GLPT from the wild type (data not shown). Thus, none of the amber mutations is in the gene coding for GLPT, but instead must be in a gene encoding the G3P permease and must be promoter proximal to the gene encoding the periplasmic GLPT protein, as concluded above after isolation of pB13, pB15, and pB112. Hence, GLPT is a misnomer for the periplasmic protein, since mutations affecting G3P active transport are not in the gene encoding this protein, but rather are in the gene encoding the G3P permease. Polar mutations decrease the amount of GLPT produced. We therefore propose naming the gene encoding the periplasmic GLPT protein *glpQ* to distinguish it from *glpT*, which encodes the G3P permease.

Somewhat puzzling was the observation that the *glpT*(Am) mutations exerted no polar effect on production of GLPT in the minicell system. This result is explainable, however, if the polar effect of the *glpT*(Am) mutations observed in growing cells is mediated by the transcription termination factor Rho (1). It has been reported that minicells are Rho deficient (15). The polar effect of *glpT*(Am) mutations on *glpQ* was also not apparent with a maxicell system (40). The amber fragments were not observed in the minicell or the maxicell system.

Identification of the *glpT* gene product, the G3P permease. The results reported so far suggest that the periplasmic component is encoded for

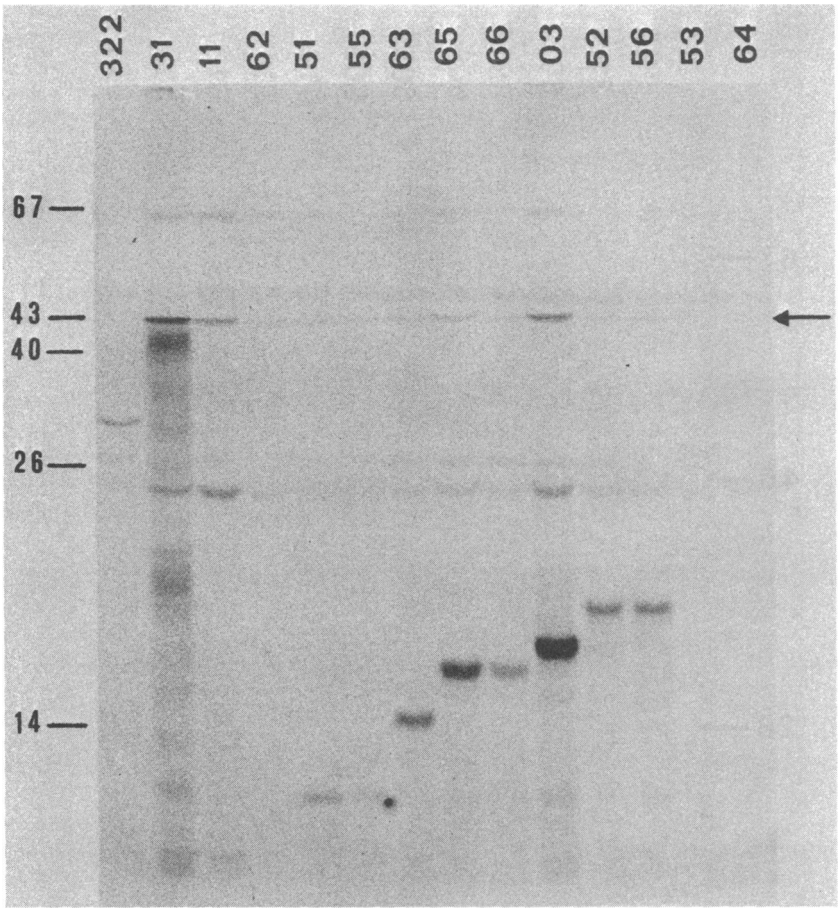

FIG. 5. Cell-free protein synthesis as directed by *glpT*(Am) hybrid plasmids. Reactions (0.2 ml) containing 20 μg of plasmid DNA and 10 μCi of L-[^{35}S]methionine (700 Ci/mmol) were incubated for 60 min at 36°C. After a 5-min chase with 0.25 mM methionine, DNase and RNase were added (50 μg/ml each), and incubation was continued for 10 min before addition of EDTA (25 mM). The samples were dialyzed, and then 25 μl of each was diluted with sample buffer and heated at 95°C before electrophoretic analysis on a 13% SDS-polyacrylamide slab gel. Protein molecular weight standards were located by Coomassie blue staining, and radiolabeled proteins were located by autoradiography of the dried gel. The plasmid DNAs used as templates were as follows: 322, pBR322; 31, pGS31; 11, pGS3111; 62, pGS3162; 51, pGS3151; 55, pGS3155; 63, pGS3163; 65, pGS3165; 66, pGS3166; 03, pGS3103; 52, pGS3152; 56, pGS3156; 53, pGS3153; 64, pGS3164.

by a gene (*glpQ*) located distal to *glpT* and is not required for growth on or transport of G3P. What, then, is required for *glpT* function? When the minicell or cell-free system for radioactively labeling plasmid-encoded proteins was used, no protein was found which correlated well with the presence or absence of the transport system. Because a permease function should be an inner membrane function, the inner and outer membrane fractions (20) were isolated from DL291 carrying either wild-type or mutated hybrid plasmids. Analysis of these fractions by SDS-polyacrylamide gel electrophoresis (Fig. 6) showed typical inner and outer membrane protein profiles (20). The outer membrane protein profiles were very similar in all strains tested. Initially, no difference in the protein pattern could be detected when inner membranes were solubilized in 95°C SDS (not shown). There was, however, a striking difference among the inner membrane protein profiles when the preparations were dissolved in SDS at 50°C before electrophoresis. Inner membranes from DL291(pGS31) (wild-type *glpT* plasmid) contained large amounts of a protein having an apparent M_r of 44,000 (Fig. 6A, slot 2). This protein was missing in inner membranes from DL291 harboring either pGS34 (Δ*glpT*) or pGS3103 [*glpT*(Am)] (Fig. 6A, slots 1 and 3, respectively). These results suggest that this protein is the G3P permease, the product of *glpT*. The apparent M_r of this protein is slightly larger than that of the periplasmic GLPT protein. To rule out the possibility that it is a

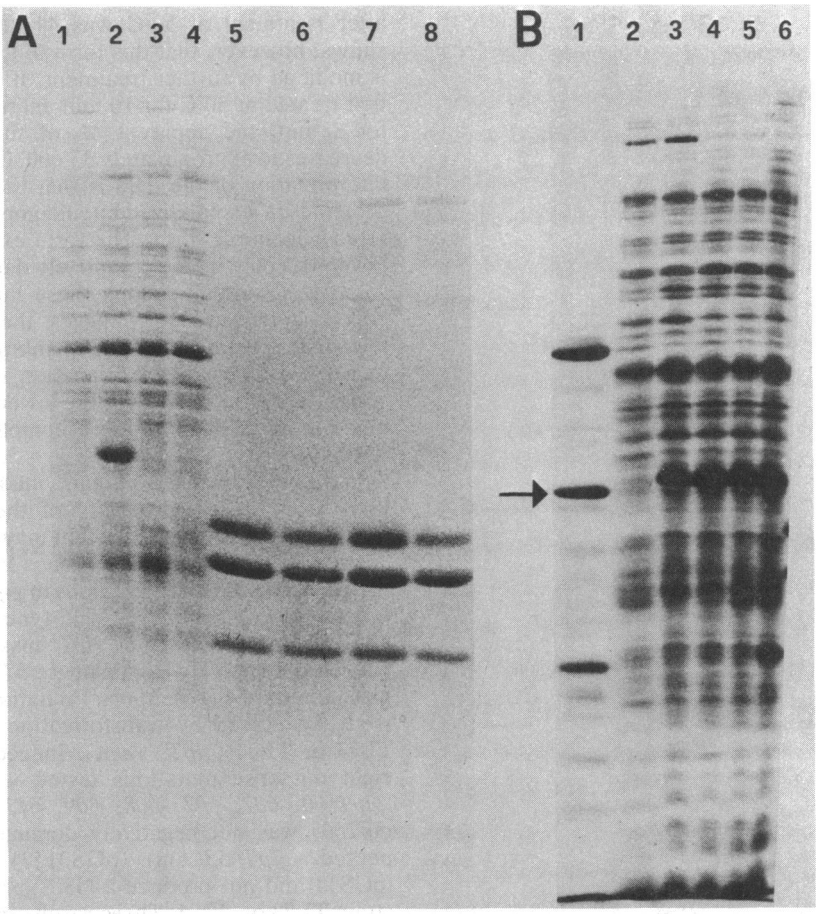

FIG. 6. SDS-polyacrylamide gel electrophoresis of cytoplasmic and outer membrane fractions from DL291 harboring *glpT* wild-type and mutant hybrid plasmids (Coomassie blue staining). Samples (40 μg of protein) of inner membrane fractions were treated at 50°C for 10 min in the presence of 2% SDS–10mM dithiothreitol. Outer membrane proteins (40 μg per slot) were precipitated with 10% trichloroacetic acid. After an acetone wash, sample buffer was added and the samples were heated (95°C, 5 min). (A) Inner membranes from DL291(pGS34) (Δ*glpT*, slot 1), pGS31 (*glpT*$^+$, slot 2), pGS3103 [*glpT*(Am), slot 3], and pGS3107 (*glpT*[missense], slot 4). Slots 5 through 8 contained the corresponding outer membrane fractions. (B) Slot 1 contained cold osmotic shock fraction from DL291(pGS31) prepared as described for Fig. 2A. Inner membranes (slots 2 through 6) from DL291(pGS34) (Δ*glpT*), pB15 and pB13 (both *glpT*$^+$ Δ*glpQ*), pGS3100 (*glpT*[missense]), and pGS31 (wild type), respectively, were prepared for electrophoresis as described for (A).

membrane-associated precursor to GLPT, inner membrane fractions were prepared from DL291(pB13) and DL291(pB15) (*glpT*$^+$ Δ*glpQ*). The 44,000 M_r protein was also present in these cases (Fig. 6B, slots 3 and 4), further supporting the conclusion that this protein is the G3P permease.

Inner and outer membranes from DL291 harboring two plasmids with *glpT* missense mutations were also prepared and analyzed. The missense mutation *glpT600*(pGS3100) allowed production and assembly of the G3P permease into the inner membrane (Fig. 6B, slot 5). The permease was not found, however, in inner membranes isolated from DL291(pGS3107) (*glpT607*; Fig. 6A, slot 4). It is possible that *glpT607* affects assembly of the permease into the membrane or that the defective permease is degraded, which does occur for certain *lacZ* missense mutations (44), or both.

Taken together, all of the results provide strong evidence for the *glpT-glpQ* operon, with promoter-proximal *glpT* coding for the cytoplasmic membrane-associated G3P permease, which is solely responsible for active accumulation of G3P.

Thermal modification of the G3P permease. Early attempts to identify the G3P permease were hampered by the protein's apparent aggregation upon treatment at high temperatures (95 to 100°C) in typical sample buffers used for SDS-polyacrylamide gel electrophoresis. Because the

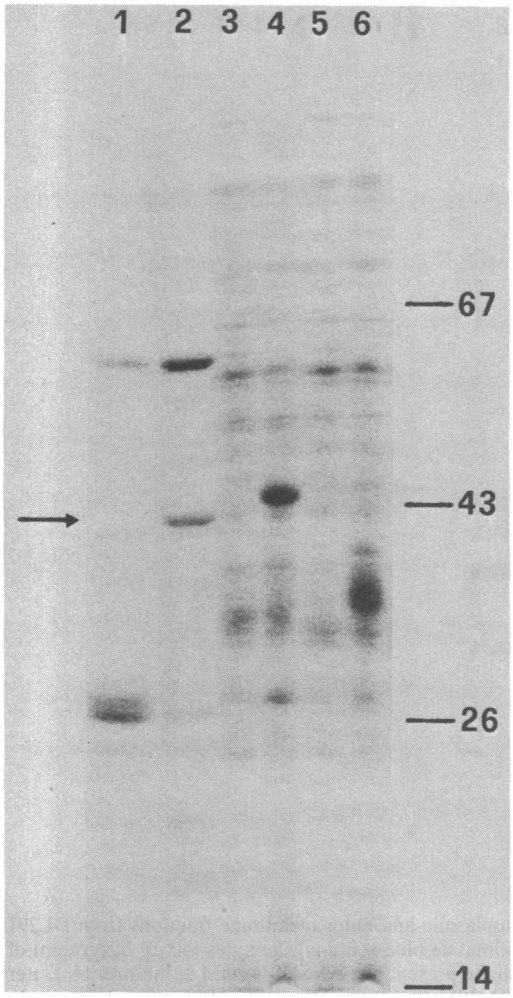

FIG. 7. Thermal modification of the G3P permease. For reference, cold osmotic shock fractions from DL291(pBR322) and pGS31 (slots 1 and 2) were prepared as for Fig. 2A. Just before electrophoresis, inner membranes (40 μg of protein) from DL291(pGS3103) [*glpT*(Am), slot 3] and DL291(pGS31) (*glpT*$^+$, slot 4) were heated at 50°C for 10 min. Portions of the same samples were subsequently heated at 95°C for 5 min (slots 5 and 6). The samples contained 0.8% SDS–10 mM dithiothreitol.

GlpQ (GLPT) protein requires treatment at high temperature for its dissociation into subunits (8, 38), plasmid-encoded proteins labeled in minicells or in vitro during early stages of this work were routinely treated at 95°C before electrophoretic analysis. The inability to find a permease protein which correlated with the genetic analysis and knowledge of the insolubility of the lactose permease after heat treatment (14, 41) led us to explore different regimens of sample treatment before electrophoresis. As described above, the apparent M_r of the G3P permease after treatment at 50°C was 44,000. Figure 7 shows, however, that this form of the permease is modified by further treatment. If samples are first treated at 50°C for 10 min and then at 95°C for 3 min, the apparent M_r of the permease decreases to approximately 33,000. One possible interpretation of the data is that the 44,000 M_r protein is in a solubilized but incompletely denatured oligomeric form. The form exhibiting the lower M_r could be the completely denatured and solubilized subunit. Along these lines, genetic experiments (see below) suggest that the active form of the permease in the membrane is oligomeric. An alternative explanation is that both species are monomeric and the lower M_r species binds more SDS or is more completely denatured or both.

Negative dominance of *glpT* missense mutations. The heat modifiability of the G3P permease suggests that the protein may be a dimer or an oligomer in the membrane. If this is so, then certain missense mutations in *glpT* could be negatively dominant over wild-type *glpT* function. To find out whether *glpT* missense mutations are negatively dominant (*glpT*$^{-d}$), hybrid plasmids carrying nonpolar mutations in *glpT* were introduced by transformation into strain TL73 or TL74 (*glpT*$^+$ *recA*). Indeed, seven of eight missense mutations tested were *glpT*$^{-d}$ (*glpT600*, *-602*, *-607*, *-608*, *-609*, *-613*, and *-614*). *glpT605* was not negatively dominant. As expected, *glpT653*(Am) (pGS3153) or Δ*glpT* (pGS34) did not produce a G3P$^-$ phenotype in strain TL73 or TL74. These results indicate that the active form of the G3P permease is a dimer or an oligomer. The negative dominance effect could not be attributed to a general membrane disturbance due to a large amount of the *glpT* missense products encoded by the multicopy plasmids, since these strains were readily transduced to Lac$^+$ and thus exhibit normal lactose permease function. There is probably not a limited number of assembly sites in the cytoplasmic membrane, since large amounts of the G3P permease could be accommodated.

DISCUSSION

The experiments reported here show that *glpT* and *glpQ* are organized in a transcriptional unit. The promoter-proximal gene *glpT* codes for a cytoplasmic membrane-associated permease which is responsible for active accumulation of G3P. *glpQ* is distal to *glpT* and encodes a periplasmic protein. The finding that *glpQ* is not required for transport of G3P helps to clarify the seemingly paradoxical findings of earlier experiments which suggested that the periplasmic protein (which was named GLPT) was involved in the transport process (39), but only one genetic

complementation group (which should code for a membrane permease) was found for transport function (26a). It is now clear that all mutations in this complementation group are in *glpT*; none are in *glpQ*, but those in *glpT* can influence production of the periplasmic protein due to polar effects. Thus, the G3P permease alone is capable of active accumulation of G3P via the *glpT*-dependent transport system.

The anaerobic G3P dehydrogenase is defined by *glpA*, a genetic locus closely linked to *glpT* (21) but in a separate operon (28). The purified dehydrogenase is composed of subunits of 62,000 and 43,000 daltons (36). Genetic information required for correction of the *glpA* defect in strain DL291 was not present on the 7.3-kb *Pst*I DNA fragment which contained *glpT* (pGS31). This *Pst*I fragment did contain a gene coding for a 65,000 M_r protein which was lost during construction of pGS31H5 (Fig. 1 and 2B). It is likely that this protein is the large subunit of the dehydrogenase. Schryvers and Weiner (37) have recently proposed the same physical location for the gene encoding the 62,000 M_r subunit of the enzyme.

The G3P permease, the product of *glpT*, has been identified in cytoplasmic membrane preparations from strains carrying *glpT* on multicopy plasmids. The permease protein exhibits an apparent molecular weight of 33,000 upon SDS-polyacrylamide gel electrophoresis after sample treatment at 95°C. A protein of similar size was seen with pLC3-46 DNA-directed protein synthesis in a cell-free system (38). Smaller plasmids containing recloned *glpT* also directed the synthesis of the same protein in vitro (Fig. 2B).

The permease was first identified after solubilization of inner membrane preparations in SDS at intermediate temperatures (50°C). After such treatment, it exhibited a molecular weight of 44,000 on SDS-polyacrylamide gels. The heat modifiability of this higher-molecular-weight form of the protein suggested an oligomeric structure for the permease. Evidence supporting this idea was obtained by showing that some *glpT* missense mutations were negatively dominant over wild-type function. Similar results have been reported for the lactose permease (27). In most cases where negative dominance was observed, the G3P$^-$ phenotype was tight, since there was a large excess of *glpT* missense product (encoded by the multicopy plasmid) over the wild-type permease (encoded by the chromosome). A complete missense protein was required to confer negative dominance. Amber mutations which allowed synthesis of up to 80% of the polypeptide chain were not *glpT*$^{-d}$. The amber fragments were unstable, however; they were observed only in the cell-free protein-synthesizing system and not in the minicell or maxicell system even when labeling mixtures were boiled immediately in SDS. The missense products which caused negative dominance were stably incorporated into cytoplasmic membrane fractions of strain TL74 (not shown).

Suppression of nonsense mutations can be used to introduce defined amino acid substitutions in a protein, as has been elegantly described by Miller and co-workers in their studies on *lacI* (30). Among the seven different *glpT*(Am) mutations isolated in the present work, only the insertion of lysine at *glpT665* and *glpT666* drastically impaired transport function. The other positions were rather insensitive to various amino acid substitutions when tested at 37°C. Insertion of tyrosine at four positions (*glpT651* and *glpT655*; *-663*; *-603*; *-652*; and *-656*) resulted in a temperature-sensitive phenotype (tested at 43°C). Thus, substitutions causing an altered growth phenotype are in the 12,000 to 17,400 M_r region of the permease. In contrast, most of the missense mutations (Table 2) cluster near the carboxyl-terminal end of *glpT*. This conclusion is based on mapping these plasmid-carried *glpT* mutations against λ transducing phages carrying various portions of *glpT*. Most missense mutations map in the same or a more promoter-distal deletion interval than the amber mutation giving rise to the 27,000 M_r amber peptide and must therefore be near the carboxyl-terminal end of the permease protein (unpublished data).

Introduction into *E. coli* of certain genes on multicopy plasmids can be detrimental to cell growth due to hyperproduction of the plasmid-encoded proteins (9, 34, 42). This was apparently not a problem when *glpT-glpQ* was cloned into pBR322, even though constitutive strains were used. Strains harboring plasmids with polar mutations in *glpT* did grow better on minimal-glycerol medium than those harboring either the wild-type or missense plasmids, however. Therefore, strains were routinely maintained on rich media containing glucose to minimize, by catabolite repression, hyperproduction of the *glpT* and *glpQ* gene products. Although not studied in detail, a high frequency of spontaneous mutation eliminating *glpT* function was not observed.

The identification of the G3P permease as an oligomer of a single polypeptide chain is the second such example (27, 41) among *E. coli* active transport systems energized by the proton motive force. It will be challenging to incorporate the protein into black lipid films and study its G3P transport properties in response to pH gradients and electrical potentials.

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