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Action at a Distance for Negative Control of Transcription of the *glpD* Gene Encoding *sn*-Glycerol 3-Phosphate Dehydrogenase of *Escherichia coli* K-12

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Aerobic *sn*-glycerol 3-phosphate dehydrogenase is a cytoplasmic membrane-associated respiratory enzyme encoded by the *glpD* gene of *Escherichia coli*. The *glpD* operon is tightly controlled by cooperative binding of the *glp* repressor to tandem operators (O_D1 and O_D2) that cover the −10 promoter element and 30 bp downstream of the transcription start site. In this work, two additional operators were identified within the *glpD* structural gene at positions 568 to 587 (O_D3) and 609 to 628 (O_D4). The two internal operators bound the *glp* repressor in the presence or absence of the tandem operators (O_D1 and O_D2) in vitro, as shown by DNase I footprinting. To assess a potential regulatory role for the two internal operators in vivo, a *glpD-lacZ* transcriptional fusion containing all four operators was constructed. The response of this fusion to the *glp* repressor was compared with those of fusion constructs in which O_D3 and O_D4 were inactivated by either deletion or site-directed mutagenesis. It was found that the repression conferred by binding of the *glp* repressor to O_D1 and O_D2 was increased five- to sevenfold upon introduction of the internal operators. A regulatory role for HU was suggested when it was found that repressor-mediated control of *glpD* transcription was increased fourfold in strains containing HU compared with that of strains deficient in HU. The effect of HU was apparent only in the presence of all four *glpD* operators. The results suggest that *glpD* is controlled by formation of a repression loop between the tandem and internal operators. HU may assist repression by bending the DNA to facilitate loop formation.

sn-Glycerol 3-phosphate (glycerol-P) is a ubiquitous compound that can be used by *Escherichia coli* and other organisms. Glycerol-P is a direct precursor for phospholipid biosynthesis (33). Glycerol-P and its precursors, when present in excess, are used as carbon and energy sources. The reactions in the catabolic pathways are catalyzed by the proteins encoded by the genes of the *glp* regulon (23, 24). To maintain a cellular concentration of glycerol-P optimal for phospholipid biosynthesis, organisms must coordinate the levels of the catabolic activities with the levels of the phospholipid biosynthetic activities. Specific negative regulation of the *glp* regulon is exerted by the *glp* repressor (GlpR) (22–24). The affinity of GlpR for each *glp* operator site is reduced upon the binding of glycerol-P, the inducer for the *glp* regulon (23, 24).

The *glp* genes have been mapped to three different regions on the *E. coli* chromosome and are arranged in five different operons (24). The *glpFKX* operon is located near 89 min on the linkage map of *E. coli* (4, 42, 45). The *glpF* gene encodes the glycerol diffusion facilitator, a cytoplasmic membrane protein, and the *glpK* gene encodes the cytoplasmic glycerol kinase. The function of the protein encoded by *glpX* is unknown. The *glpTQ* operon and divergently transcribed *glpACB* operon are located near 51 min (4, 9–11, 21, 24, 44). The *glpT* gene encodes the cytoplasmic membrane-associated glycerol-P permease required for active transport of glycerol-P into the cytoplasm, and the *glpQ* gene encodes the periplasmic glycerophosphodiesterase that catalyzes the hydrolysis of glycerophosphodiesters to glycerol-P and an alcohol. The *glpACB* operon encodes the three subunits of anaerobic glycerol-P dehydrogenase. Under anaerobic growth conditions, this enzyme is essential for dissimilation of glycerol-P.

The *glpD* gene and divergently transcribed *glpEGR* operon are located near 77 min (4, 24). The *glpEGR* genes encode the specific repressor for the *glp* regulon and two other proteins (GlpE and GlpG) with unknown functions (36, 38, 49). The *glpD* gene encodes the aerobic glycerol-P dehydrogenase associated with the cytoplasmic membrane (3, 37). Under aerobic conditions, this dehydrogenase catalyzes the oxidation of glycerol-P to dihydroxyacetone phosphate with the concurrent formation of reduced flavin adenine dinucleotide used in the respiratory chain. Anaerobic repression of *glpD* expression is mediated by the two-component regulatory system encoded by *arcA* and *arcB* (19).

Lin and coworkers found that the *glpD* operon was controlled more tightly by GlpR than was either the *glpTQ* or *glpFKX* operon (30-, 26.5-, and 3.2-fold, respectively [12]). This conclusion was reached after measuring the activities of enzymes encoded by these genes (glycerol-P dehydrogenase, glycerol-P permease, and glycerol kinase) in extracts of a strain harboring a thermolabile *glp* repressor after growth at various temperatures (12). In contrast, it was recently found that the *glpFKX* operon was more sensitive than the *glpD* operon to repressor by using transcriptional fusions of the promoter-operator regions to *lacZ* (46). The discrepancy may be due to the presence of other potential operators within the *glpD* coding region that contribute additionally to negative regulation or to differential posttranscriptional regulation. Recently, it was found that three operators within the coding region of *glpT* contributed to about threefold negative control of *glpT* transcription by GlpR (47). Computer analysis revealed four potential operator sites within the *glpD* structural gene by using the published consensus *glp* operator sequence (half-site consensus is WATKYTCGWW, where W is A or T, K is G or T,

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TABLE 1. Oligonucleotides used in this study

Oligonucleotide	Sequence[a]	Positions[b]
85488	CGTTAATACATTCGAACgGATCCATGCGTCTC	−10–22
168198	GGCGTTAACCAAGCCGCGCGCTTGCCAGC	820–791
104322	AACGAACATTTAacAGaTTTAAgcAAAGTGAATG	167–196
85436	GGTCTGTTTATGTAtGAcCATCTGGGTAAACGC	514–546
Bing2	GCGTTTACCCAGATGgTCaTACATAAACAGACC	546–514
85423	CGAATATTCTGACTGcTGGGTgGACGACGCCC	621–652
Bing3	GGGCGTCGTCcACCCAgCAGTCAGAATATTCG	652–621
Bing1[c]	GAACGCCGGAGGATCCATGCGTCTCTC	9–22
207153	CCGGAAATTAAaCGgGGATTtGAATATTCTGAC	601–633
181347	GTCAGAATATTCaAATCCcCGtTTAATTTCCGG	633–601
241326	CTTTGCTAATAgGTagcATAAgcAACATTTAACAG	144–178
241183	TAAATGTTgcTTATgctACcTATTAGCAAAGAATCG	174–139
207055	CCGAGTAAGCACTTCGCCGCCTTTACGCAC	711–682
254069	CATATCACTCTAAGATCTTTTTTCAATGTTA	98–129

[a] Lowercase letters indicate bases substituted for the creation of mutations or restriction sites.
[b] See Fig. 1.
[c] The first 8 bases of this oligonucleotide are from the vector pACYC177.

and Y is C or T [50]). These sites were labeled box 1 (positions 522 to 541), box 2 (positions 632 to 651), O_D3 (positions 568 to 587), and O_D4 (positions 609 to 628) (see Fig. 1). Since each of these potential operator regions is located several hundred base pairs from the tandem operators O_D1 and O_D2, the binding of accessory proteins such as integration host factor (IHF) and HU protein may facilitate the bending of DNA to assist GlpR function at distal operators by DNA looping (28, 47). IHF is a sequence-specific, asymmetric, histone-like, heterodimeric protein (encoded by *himA* and *himD*) that plays important roles in replication, site-specific recombination, and transcription (13). The binding of IHF (consensus sequence is WATCAANNNTTR, where W is A or T and R is A or G [14]) is known to bend the DNA to facilitate the formation of a local nucleoprotein complex. IHF therefore plays an important auxiliary role in transcriptional regulation of certain genes (15). Another accessory protein, HU protein, is a sequence-nonspecific DNA-binding protein with two heterosubunits encoded by *hupA* and *hupB* genes. HU was found to play important roles in the transposition of bacteriophage Mu by formation of a higher-order structure and site-specific inversion of the flagellin promoter by assisting DNA loop formation (28). Recently, it was found that the binding of HU increased by two- to threefold the repression of the P2 promoter of the *gal* operon while IHF had no effect (2). This is in contrast to our recent finding that IHF contributed twofold to repression of *glpT* in the presence of internal *glpT* operators while HU had no effect (47). Although it is well-known that either IHF or HU can assist Int in the assembly of a functional *attL* site in lambda recombination, the roles of these two proteins are not necessarily interchangeable (28). In the present study, internal operators O_D3 and O_D4 were found to contribute sevenfold to repression of *glpD*. HU protein was found to contribute about fourfold to repression while IHF had no effect. These results suggest that repression of *glpD* is mediated by a repression loop, with HU facilitating repression by bending the DNA.

MATERIALS AND METHODS

Materials. The reagents for standard recombinant DNA techniques and DNA sequencing were obtained from New England Biolabs or Amersham Life Science, Inc. Oligonucleotides (Table 1) were synthesized on an Applied Biosystems 381A DNA synthesizer and purified as recommended by the manufacturer. New England Nuclear Corp. supplied [γ-^{32}P]ATP and α-^{35}S-dATP. The *glp* repressor was purified to homogeneity as previously described (22).

Construction of recombinant plasmids. Standard molecular cloning methods were used for the construction of recombinant plasmids. The plasmids used or constructed for this study are listed in Table 2. Plasmids pDW65, pDA1104,

TABLE 2. Plasmids used or constructed in this study

Plasmid	Insert	Source (reference)	Vector	Part of *glpD* contained (positions)
pDW1	*Alu*I-*Hin*P1I	pSY12 (48)	M13mp18	139–178
pDW65	*Hin*dIII-*Eco*RI	pDW1	pBS	139–178
pDA1104	*Eco*RI-*Eco*RV	pDA1101 (3)	pBS	387–768
pSY15	*Sau*3A-*Xba*I	pSY12 (48)	pBS	10–218
pBY100	*Hin*fI-*Eco*RI	pSH79 (48)	pDA1104	140–768
pBY102	*Bam*HI-*Bss*HII	PCR	pSH56	Entire *glpD*
pBY101	*Bam*HI-*Bss*HII	PCR	pSH56	Entire *glpD*
pBY103	*Bam*HI-*Bss*HII	PCR	pSH56	Entire *glpD*
pBY104	*Bam*HI-*Bss*HII	PCR	pSH56	Entire *glpD*
pBY105	*Bam*HI-*Bss*HII	PCR	pSH56	Entire *glpD*
pBY109	*Bam*HI-*Bss*HII	PCR	pSH56	Entire *glpD*
pBY150	*Bam*HI-*Bss*HII	PCR	pSH56	Entire *glpD*
pBY112	*Bam*HI-*Eco*RV	pBY102	pSP417	11–768
pBY159	*Bam*HI-*Eco*RV	pBY109	pSP417	11–768
pBY160	*Bam*HI-*Eco*RV	pBY150	pSP417	11–768
pBY162	*Bam*HI-*Eco*RI	pBY150	pBY159	11–768
pBY128	*Bam*HI-*Bgl*II	pBY102	pSP417	11–218

TABLE 3. Strains of *E. coli* K-12 used in this study

Strain	Genotype	Source or derivation
IH1-6	Δ*himA81 zdi-3123*::Tn*10kan lysA rpsL polA1 ilv*P_G1P_G2::*galK*	30
A5427	N99 *galK hupA16*::*kan hupB11*::*cat* Δ*himA82*::Tn*10*	25
DH5αF′	F′ ϕ80d*lacZ*Δ*M15 recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1* Δ(*argF-lacZ*)*U169*	50
MC4100	F$^-$ *araD139* Δ(*argF-lacZ*)*U169 rpsL150 deoC1 relA1 rbsR ptsF25 flbB5301*	5
TS100	MC4100 *glpR2*	21
SH305	MC4100 Δ*glpD102 recA1 srl*::Tn*10*	36
TST3	MC4100 *malT*::Tn*10*	39
WO331	MC4100 *glpR12* (*glpR^n*) *recA1 srl*::Tn*10*	50
BY4000[a]	MC4100 Φ(*glpD′-lacZ*)	λBY112 lysogen of MC4100[b]
BY1000[a]	TS100 Φ(*glpD′-lacZ*)	λBY112 lysogen of TS100[b]
BY4159[a]	MC4100 Φ($glpD'_{M4}$-*lacZ*)	λBY159 lysogen of MC4100[b]
BY1159[a]	TS100 Φ($glpD'_{M4}$-*lacZ*)	λBY159 lysogen of TS100[b]
BY4128[a]	MC4100 Φ(*glpD″-lacZ*)	λBY128 lysogen of MC4100[b]
BY1128[a]	TS100 Φ(*glpD″-lacZ*)	λBY128 lysogen of TS100[b]
BY4160[a]	MC4100 Φ($glpD'_{M1,2}$-*lacZ*)	λBY160 lysogen of MC4100[b]
BY1160[a]	TS100 Φ($glpD'_{M1,2}$-*lacZ*)	λBY160 lysogen of TS100[b]
BY4162[a]	MC4100 Φ($glpD'_{M1,2,4}$-*lacZ*)	λBY162 lysogen of MC4100[b]
BY1162[a]	TS100 Φ($glpD'_{M1,2,4}$-*lacZ*)	λBY162 lysogen of TS100[b]
BY4006	BY4000 *hupA16*::*kan hupB11*::*cat*	P1(A5427)[c] → BY4000
BY1006	BY1000 *hupA16*::*kan hupB11*::*cat*	P1(A5427)[c] → BY1000
BY4459	BY4159 *hupA16*::*kan hupB11*::*cat*	P1(A5427)[c] → BY4159
BY1459	BY1159 *hupA16*::*kan hupB11*::*cat*	P1(A5427)[c] → BY1159
BY4004	BY4000 Δ*himA82*::Tn*10*	P1(A5427) → BY4000(pUHS2)
BY1004	BY1000 Δ*himA82*::Tn*10*	P1(A5427) → BY1000(pUHS2)
BY4259	BY4159 Δ*himA81 zdi-3123*::Tn*10kan*	P1(IH1-6) → BY4159(pSC101)
BY1259	BY1159 Δ*himA81 zdi-3123*::Tn*10kan*	P1(IH1-6) → BY1159(pSC101)

[a] Lysogens BY4000, BY1000, BY4159, BY1159, BY4128, BY1128, BY4160, BY1160, BY4162, and BY1162 have the same *glpD-lacZ* fusions as the parental plasmids pBY112, pBY112, pBY159, pBY159, pBY128, pBY128, pBY160, pBY160, pBY162, and pBY162, respectively.
[b] Single lysogens of each lysogenic strain were identified by assays of the β-galactosidase activities in at least eight independently isolated lysogens.
[c] The *hupA* and *hupB* alleles were introduced by two sequential transductions using A5427 as the P1 donor.

pSY15, and pBY100 contained various regions of the *glpD* gene cloned into pBluescript KS$^+$ (pBS; Stratagene) as indicated. Plasmids pBY101 (O_D2 mutated), pBY102 (wild type), pBY103 (box 1 mutated), pBY104 (box 2 mutated), pBY105 (O_D2, box 1, and box 2 mutated), pBY109 (O_D4 mutated), and pBY150 (O_D1 and O_D2 mutated) contained an intact *glpD* gene cloned into the vector pACYC177 (34), except for the indicated point mutations introduced by PCR primers (Table 1). During the construction of these plasmids, replacing the *Bam*HI-*Bss*HII region of pSH56 (37) with each PCR product deleted about 1,500 bp of the *glpEGR′* region of pSH56 and left *glpD* intact. The product of PCR using primers 85488 and 168198 with pSH56 as the template was used to construct pBY102, which carries the wild-type *glpD* gene. The construction of pBY101, which harbors an altered O_D2, was accomplished by a previously published method (43). Primers 104322 and 168198 were used for first-round PCR with pSH56 as the template. The product of the first-round PCR was used as a megaprimer together with primer 85488 and with pSH56 as the template in a second-round PCR. Plasmids pBY103, pBY104, pBY105, pBY109, and pBY150 were constructed by a modification of a previously published method (18). The construction of pBY103, pBY104, and pBY150 used pBY101 as the template, the construction of pBY105 used pBY103 as the template, and the construction of pBY109 used pSH56 as the template for PCR. Two first-round PCRs were used to produce two overlapping fragments of DNA by using the primer pairs as follows: primers Bing1 and Bing2 and primers 85436 and 168198 for pBY103; primers Bing1 and Bing3 and primers 85423 and 168198 for pBY104; primers Bing1 and Bing3 and primers 85423 and 168198 for pBY105; and primers 85488 and 181347 and primers 207153 and 168198 for pBY109. After the first-round PCR, the two products were mixed, denatured, and reannealed to be used as the template for the second-round PCR, using primers 168198 and 85488 for pBY109 and primers 168198 and Bing1 for pBY103, pBY104, pBY105, and pBY150. The oligonucleotides used for creating operator mutations contained base substitutions only in the wobble positions of affected codons. Therefore, each plasmid was predicted to encode wild-type GlpD, which was verified with strain SH305 (Δ*glpD102*) (Table 3). All of the plasmids complemented Δ*glpD102*. Plasmids pBY112, pBY159, pBY160, and pBY162 contained the same *Bam*HI-*Eco*RV region of *glpD* (except the point mutations from the parental plasmid [Table 2]) cloned into vector pSP417 (32), which created *glpD-lacZ* transcriptional fusions. Plasmid pBY128 contained a shorter region of *glpD* (*Bam*HI-*Bgl*II) cloned into vector pSP417. All plasmids were verified by DNA sequence analysis (35).

Growth media and bacterial strains. Luria-Bertani (LB) medium (26) with appropriate antibiotics (ampicillin, 100 μg/ml; tetracycline, 10 μg/ml; kanamycin, 50 μg/ml; and chloramphenicol, 50 μg/ml) was used for the growth of *E. coli* strains. Transformation of competent cells was carried out as previously described (39), with selection on LB plates containing ampicillin and 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) per ml where appropriate. LB medium containing 20 mM sodium citrate and appropriate antibiotics was used for the selection and scoring of transductants. For minimal medium, the A and B salts of Clark and Maaløe (7) were supplemented with 2 μg of thiamine per ml, 0.2% Casamino Acids, 0.2% maltose, and appropriate antibiotics at one-half strength.

The bacterial strains used or constructed are listed in Table 3. Single λ lysogens containing *glpD-lacZ* transcriptional fusions were constructed as follows. The various *glpD* control regions were cloned upstream of *lacZ* in pSP417 as described above. Promoter-probe vector pSP417 (32), a derivative of pRS415 (40), contains a promoterless *lacZ* gene downstream of the multiple cloning site, four transcriptional terminators upstream of the multiple cloning site, and a divergently transcribed *bla* gene. Each plasmid-borne *glpD-lacZ* fusion was transferred to phage λRS45 (containing a segment of the *bla* gene and divergent promoterless *lacZ* gene, as in pSP417) by homologous recombination (39, 40). Strain SH305 (Δ*glpD102 recA*) was used as the host (avoiding recombination between the mutated plasmid and the chromosome) for selection and purification of the resulting phages (λBY128, λBY112, λBY159, λBY160, and λBY162), which contain the same fusions as pBY128, pBY112, pBY159, pBY160, and pBY162, respectively. Single lysogens of MC4100 (*glpR$^+$*) and TS100 (*glpR2*) with each phage are listed in Table 3.

The *himA* deletion strains were constructed as follows. Plasmid pSC101 was introduced into strains BY4159 and BY1159 by selection for Tet^r, and the resulting transformants were used as recipients in P1 crosses with strain IH1-6 (Δ*himA81* [30]) as the donor and with Kan^r selection. Transductants containing the *himA* deletion were identified by their Tet^s phenotype (replication of plasmids with the pSC101 origin requires IHF protein [8, 30]). The resulting strains were named BY4259 and BY1259, respectively (Table 3). The Δ*himA* derivatives of strains BY4000 and BY1000 were constructed by the same method, except that plasmid pUHS2 (a Kan^r pSC101 derivative; R. Lutz and H. Bujard, University of Heidelberg) was used for transformation, and the resulting transformants selected by Kan^r were used as recipients in P1 crosses with strain A5427 (Δ*himA82*::Tn*10* [25]) as the donor and with Tet^r selection followed by screening to identify Kan^s transductants. The resulting strains were named BY4004 and BY1004, respectively (Table 3). To construct strains lacking HU, strains BY4000, BY1000, BY4159, and BY1159 were used as recipients in two consecutive P1 crosses with strain A5427 (*hupA16*::*kan hupB11*::*cat* [25]) as the donor and with Kan^r selection followed by Cam^r selection. The resulting strains were named BY4006, BY1006, BY4459, and BY1459, respectively (Table 3).

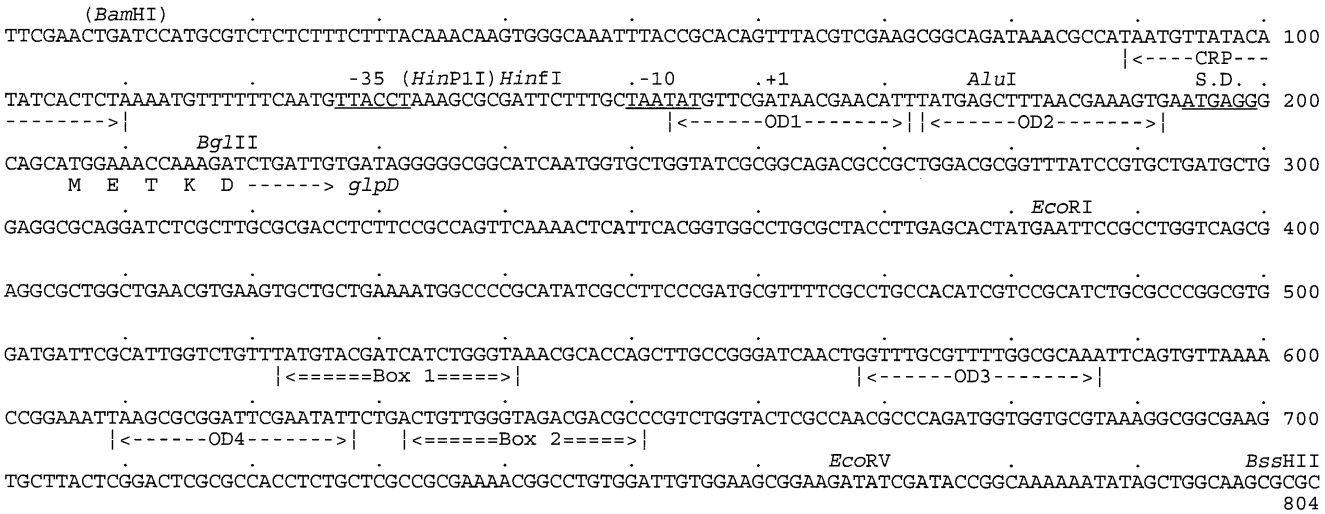

FIG. 1. Nucleotide sequence of the *glpD* control region. The transcriptional start site (+1) with the −10 and −35 promoter elements, the CRP site, and the translational initiation region with the ribosome binding site are indicated (48). The internal operators (O_D3 and O_D4) protected from DNase I digestion are indicated by dashed arrows. Box 1 and box 2 are indicated by double dashed arrows. The *Bam*HI site used for cloning purposes was introduced by using a T-to-G substitution at position 8. The restriction sites used for the construction of plasmids (Table 2) are also indicated.

DNase I footprinting. The conditions used for footprinting were modifications of previously described methods (46–48). The end-labeled DNA substrates were prepared by PCR using appropriate templates, one unlabeled primer, and one ^{32}P-end-labeled primer as indicated. Purified repressor was added to the radiolabeled DNA in a reaction mixture containing 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 50 mM KCl, 2 mM $CaCl_2$, 4 mM $MgCl_2$, 5% glycerol, 0.025% Triton X-100, 100 μg of bovine serum albumin per ml, and 5 μg of sonicated salmon sperm DNA per ml in a total volume of 0.1 ml. The reaction mixtures were incubated for 30 min at 37°C, with the subsequent addition of 0.5 ng of DNase I (Boehringer Mannheim). Incubation was continued for 3.5 min. The DNA was precipitated by the addition of 5 μg of sonicated salmon sperm DNA, 75 μl of 6.5 M ammonium acetate plus 34 mM EDTA, and 0.4 ml of 95% ethanol. The mixture was placed at −70°C for 20 min, centrifuged at 4°C, and rinsed with 80% ethanol. The samples were dried, dissolved in 4 μl of 10 mM EDTA in 95% formamide, and analyzed on a sequencing gel.

Assay of β-galactosidase. Cells were grown in minimal medium containing 0.2% maltose and 0.2% Casamino Acids as carbon sources by vigorous aeration. Enzyme activities were determined by using at least four different logarithmically growing cultures on at least two different occasions and were expressed in Miller units (26). Because it is possible that cells with differences in the *hup*, *himA*, and *glpR* alleles differ in size, thereby introducing error into the calculation of β-galactosidase specific activity when culture optical density is used as the measure of protein content, the specific activities were also determined relative to protein concentrations measured in extracts of sonicated cells. Repression ratios essentially the same as those reported in Table 5 were found.

Nucleotide sequence accession number. The nucleotide sequence used in this study is the first portion of GenBank accession number M55989, starting at nucleotide +1.

RESULTS

Apparent binding affinity of the *glp* repressor to various segments of *glpD*. Computer analysis revealed four potential *glp* operators within the *glpD* coding region, box 1, box 2, O_D3, and O_D4 (Fig. 1). To find out if any of them bind GlpR in vivo, plasmids containing different combinations of *glpD* operator sequences were constructed (Table 2). These plasmids were introduced into strain WO331 (Table 3) (50), a strain that contains a *glpD-lacZ* fusion and a noninducible repressor gene, *glpR*^{n}. Binding of GlpR^n to operators carried by multicopy plasmids releases repression of the *glpD-lacZ* fusion (50). Therefore, the level of expression of *lacZ* is proportional to the binding strength of the operators carried by plasmids introduced into this strain (50). From the results shown in Table 4, pSY15, which harbors the tandem operators O_D1 and O_D2, bound the repressor 10 times better than did pDW65, which harbors only O_D1 (2,210 versus 190), consistent with the previous discovery that GlpR binds tandem operators cooperatively (50). Plasmid pDA1104 contains only the potential internal *glpD* operator sequences and had almost no apparent binding affinity for GlpR^n, because the level of β-galactosidase expression was almost the same as that of WO331, which contains the vector pBS (80 versus 50). Plasmid pBY100 contains the potential internal *glpD* operator sequences along with O_D1 and O_D2. Apparently, pBY100 bound GlpR twice as strongly as did pSY15, which contains only O_D1 and O_D2 (3,800 versus 2,210). The effect seen with pBY100 was much higher than the sum of pDA1104 and pBY15. This synergistic effect may be due to the formation of a DNA loop, similar to that for repression of the *lac* system in which the effect of O_1 and O_2 together was much higher than the additive effect of the individual operators (29).

In order to find out which potential operators function in

TABLE 4. Titration of chromosomally encoded GlpR^n by different combinations of multicopy *glpD* operators

Plasmid	*glpD* operator(s) present	β-Galactosidase sp act[a]
pBS	None	50 ± 3
pDW65[b]	O_D1	190 ± 9
pSY15[b]	O_D1, O_D2	2,210 ± 20
pDA1104[b]	O_D3, O_D4	80 ± 7
pBY100[b]	O_D1, O_D2, O_D3, O_D4	3,800 ± 140
pACYC177	None	50 ± 1
pBY102[c]	O_D1, O_D2, O_D3, O_D4	380 ± 6
pBY101[c]	O_D2 mutated	200 ± 3
pBY103[c]	O_D2 and box 1 mutated	200 ± 8
pBY104[c]	O_D2 and box 2 mutated	250 ± 5
pBY105[c]	O_D2, box 1, and box 2 mutated	200 ± 12
pBY106[c]	O_D2, box 1, box 2, and O_D4 mutated	90 ± 4
pBY109[c]	O_D4 mutated	100 ± 1

[a] Data are the means ± standard deviations derived from at least four independently grown cultures of WO331 containing the indicated plasmids.
[b] Plasmid was constructed by using vector pBS.
[c] Plasmid was constructed by using vector pACYC177 and contained the same *glpD* sequence except for the indicated mutation(s).

TABLE 5. Effects of internal *glpD* operators on the regulation of *glpD-lacZ* transcriptional fusions

Strain	Relevant genotype[a]	β-Galactosidase sp act	Repression[b]
BY4000	*glpR*$^+$ Φ(*glpD′-lacZ*)	110 ± 5	
BY1000	*glpR2* Φ(*glpD′-lacZ*)	6,200 ± 150	56
BY4159	*glpR*$^+$ Φ($glpD'_{M4}$-*lacZ*)	130 ± 5	
BY1159	*glpR2* Φ($glpD'_{M4}$-*lacZ*)	1,000 ± 50	8
BY4128	*glpR*$^+$ Φ(*glpD″-lacZ*)	190 ± 14	
BY1128	*glpR2* Φ(*glpD″-lacZ*)	2,070 ± 6	11
BY4160	*glpR*$^+$ Φ($glpD'_{M1,2}$-*lacZ*)	150 ± 21	
BY1160	*glpR2* Φ($glpD'_{M1,2}$-*lacZ*)	150 ± 15	1
BY4162	*glpR*$^+$ Φ($glpD'_{M1,2,4}$-*lacZ*)	132 ± 2	
BY1162	*glpR2* Φ($glpD'_{M1,2,4}$-*lacZ*)	100 ± 11	1
BY4006	*glpR*$^+$ Φ(*glpD′-lacZ*) *hupA16 hupB11*	370 ± 10	
BY1006	*glpR2* Φ(*glpD′-lacZ*) *hupA16 hupB11*	5,720 ± 30	15
BY4459	*glpR*$^+$ Φ($glpD'_{M4}$-*lacZ*) *hupA16 hupB11*	320 ± 7	
BY1459	*glpR2* Φ($glpD'_{M4}$-*lacZ*) *hupA16 hupB11*	1,690 ± 34	5
BY4004	*glpR*$^+$ Φ(*glpD′-lacZ*) Δ*himA82*	124 ± 3	
BY1004	*glpR2* Φ(*glpD′-lacZ*) Δ*himA82*	6,300 ± 104	50
BY4259	*glpR*$^+$ Φ($glpD'_{M4}$-*lacZ*) Δ*himA81*	132 ± 2	
BY1259	*glpR2* Φ($glpD'_{M4}$-*lacZ*) Δ*himA81*	960 ± 50	7

[a] The sequences present in *glpD′-lacZ*, $glpD'_{M4}$-*lacZ*, and $glpD'_{M1,2}$-*lacZ* are the same except that $glpD'_{M4}$ contains 3 substitutions in O_D4 and $glpD'_{M1,2}$ contains 11 substitutions in O_D1 and O_D2. The *glpD″-lacZ* fusion contains a shorter *glpD* sequence with only O_D1 and O_D2.

[b] Repression was defined as the specific activity in the *glpR2* strain divided by the specific activity in the *glpR*$^+$ strain.

vivo, several plasmids in which each potential operator was mutated on the basis of the previous finding that substitutions at positions 3, 4, 5, and 8 from the center of symmetry of the palindromic operator caused severe decreases in GlpR binding (50) were constructed. These plasmids had the same *glpD* sequences except for multiple substitutions predicted to severely impair operator function (Tables 1 and 2). The assay system used was the same as that described above with strain WO331, except that the vector used in this case was a lower-copy-number plasmid (pACYC177). Therefore, the titration effect on GlpR^n is much less than that obtained with the high-copy-number vector pBS. From the results shown in Table 4, mutations in O_D2 caused a twofold decrease in apparent GlpR^n binding affinity (380 for pBY102 versus 200 for pBY101). Further mutation of box 1 and/or box 2 did not have significant effects on GlpR^n binding (compare the expression in WO331 harboring pBY103, pBY104, or pBY105 with that in WO331 harboring pBY101), indicating that these two sites probably are not functional repressor binding sites. However, mutation of O_D4 (pBY109) caused a fourfold decrease in the binding of GlpR^n compared with that of the wild-type plasmid pBY102 (100 versus 380). The effect of mutating O_D4 was stronger than was the effect of mutating the tandem operator O_D2 (pBY101), suggesting that cooperative interaction between O_D1 and O_D4 is equivalent or better than interaction between O_D1 and O_D2, as determined by using this plasmid system.

Role of the internal *glpD* operators in repression. As mentioned above, the potential *glpD* internal operators bound GlpR^n only in the presence of the tandem *glpD* operators O_D1 and O_D2. To determine the effects of the potential operators on *glpD* transcription in vivo, lysogens harboring *glpD-lacZ* fusions with various combinations of operators were constructed as described in Materials and Methods (Table 3). BY4000, BY1000, BY4159, and BY1159 have the same *glpD-lacZ* sequences, including the internal *glpD* region, except that both BY4159 and BY1159 carry the O_D4 mutation. BY4128 and BY1128 have a shorter *glpD* sequence, with only the tandem operators (O_D1 and O_D2) as the control. From the results shown in Table 5, mutations in O_D4 lowered the repression from 56 (expression of BY1000 divided by that of BY4000) to 8 (expression of BY1159 divided by that of BY4159), a repression ratio similar to that of strains with only the tandem operators (BY4128 and BY1128; repression ratio of 11). This suggests that the internal operator(s) contributes five- to sevenfold to repression (56 compared with 8 or 11) in vivo. The absolute level of *lacZ* expression in *glpR2* strains was not the same with or without the mutations for unknown reasons.

Tandem operators O_D1 and O_D2 dominate transcriptional repression. Plasmid-borne internal *glpD* operators O_D3 and O_D4 have no apparent affinity for GlpR^n in the absence of O_D1 and O_D2, as described above. In order to find out whether the in vivo effect of internal operators on negative regulation depends on the tandem *glpD* operators, lysogens BY4160 and BY1160 were constructed. These strains contain the same *glpD-lacZ* sequences except that the O_D1 and O_D2 operators were inactivated by specific mutations. The substitutions in O_D1 affected the −10 sequence of the promoter and resulted in decreased promoter activity. We found that it was necessary to decrease the level of *glpD* expression because constitutive expression of *glpD* from multicopy plasmids was toxic, resulting in random mutations in the *glpD* gene (data not shown). In addition to O_D1 and O_D2 mutations, strains BY4162 and BY1162 have O_D4 mutations. From the results shown in Table 5, when O_D1 and O_D2 were inactivated, there was no repression with or without the internal operators. Therefore, it is apparent that O_D1 and O_D2 are needed for repression and that the *glpD* internal operators are ineffective without the tandem *glpD* operators.

DNase I footprinting of the *glpD* operators. By a series of DNase I footprinting experiments, the exact positions of the *glpD* internal operators were identified. End-labeled DNA containing tandem operators O_D1 and O_D2 was used as a positive control for GlpR function (48). The results are shown in Fig. 2A. Repressor completely protected the tandem operators O_D1 and O_D2 at 1 nM GlpR (Fig. 2A, lanes 1 through 5), showing that GlpR is active and binds with high affinity to these tandem operators. In the presence of 2 mM inducer (glycerol-P), the binding affinity of GlpR was reduced (Fig. 2A, lane 6). Footprints of the internal operators, using the same fragment of DNA labeled at the other end, are shown in Fig. 2B. Starting at 4 nM GlpR, the internal operators O_D3 and O_D4 were protected. End-labeled DNA with O_D4 mutations was used for

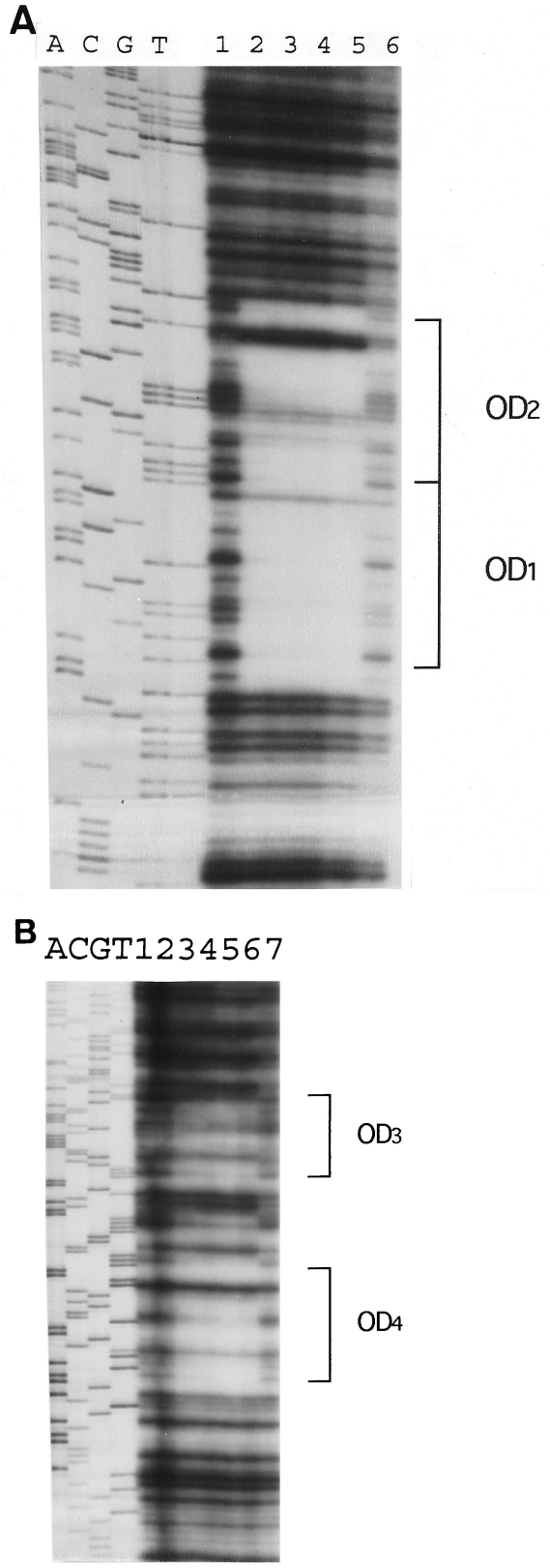

FIG. 2. Identification of *glpD* operators by DNase I footprinting. (A) O_D1 and O_D2. DNA for footprinting was labeled by PCR amplification of pBY100 with primer 207055 and ^{32}P-end-labeled SK primer. Reactions were carried out with the following concentrations of GlpR tetramers and analyzed: lane 1, no repressor; lane 2, 1 nM GlpR; lane 3, 2 nM GlpR; lane 4, 3 nM GlpR; lane 5, 4 nM GlpR; and lane 6, 4 nM GlpR and 2 mM glycerol-P. (B) O_D3 and O_D4 (in the presence of O_D1 and O_D2). DNA for footprinting was generated by PCR amplification of pBY100 with primer SK and ^{32}P-end-labeled primer 207055. Reactions were carried out with the following concentrations of *glp* repressor tetramers and analyzed: lane 1, no repressor; lane 2, 2 nM GlpR; lane 3, 4 nM GlpR; lane 4, 8 nM GlpR; lane 5, 20 nM GlpR; lane 6, 40 nM GlpR; and lane 7, 8 nM GlpR and 3 mM glycerol-P. The size markers (lanes A, C, G, and T) were generated by using pBY100 as the template in a standard sequencing reaction with primer SK (A) or 207055 (B).

footprinting, and the results are shown in Fig. 3. The protection previously shown for O_D3 and O_D4 disappeared, even when GlpR was present at 40 nM. The disappearance of O_D3 protection when O_D4 was mutated provides strong evidence that O_D3 and O_D4 bind GlpR cooperatively. When a truncated labeled DNA fragment without the tandem operators (O_D1 and O_D2) was used for footprinting (Fig. 4), the internal *glpD* operators still bound GlpR starting at 6 nM, a concentration slightly higher than that required for protection in the presence of O_D1 and O_D2 (lanes 1 through 6). This means that although the internal operators required O_D1 and O_D2 for repression in vivo, O_D1 and O_D2 were not required in order to demonstrate the binding of GlpR to O_D3 and O_D4 in vitro. Analogous footprinting experiments using labeled DNA containing box 1 and box 2 were carried out. Consistent with the in vivo evidence, box 1 and box 2 were not protected by GlpR, even at 50 nM GlpR (data not shown).

HU protein facilitates negative regulation of *glpD*. Since the distance between O_D2 and O_D3 is 395 bp, accessory proteins such as IHF and HU may assist in wrapping the intervening DNA to facilitate cooperative function of the distal *glpD* operators. To assess the potential regulatory roles of these proteins, the repression of *glpD* in strains deficient in IHF or HU was measured. Strains BY4006, BY1006, BY4459, and BY1459 (Table 3) were constructed by deletion of the *hupA* and *hupB* genes of strains BY4000, BY1000, BY4159, and BY1159, respectively (Table 3). As the results in Table 5 indicate, the loss of HU lowered repression fourfold (15 versus 56) when all four operators were present but had almost no effect on repression when O_D4 was nonfunctional (5 versus 8).

To study the influence of IHF on repression, strains BY4004, BY1004, BY4259 and BY1259 (Table 3) were contructed by deletion of *himA* in strains BY4000, BY1000, BY4159 and BY1159, respectively. Repression was measured as described above. From the results shown in Table 5, it was apparent that the loss of IHF had almost no effect on repression of either wild-type (50 versus 56) or O_D4-mutated (7 versus 8) *glpD-lacZ* fusions. These results are consistent with the lack of protection by IHF of potential IHF binding sites between the *glpD* operators by DNase I footprinting (data not shown).

DISCUSSION

Cooperative binding between two distant sites on DNA by a regulatory protein is a common mechanism for both negative and positive regulation in prokaryotic and eukaryotic organisms (1, 6, 16). In *E. coli*, it has been demonstrated that at least two spatially separated operators are necessary to give full repression of transcription of the *araBAD*, *gal*, *deo*, and *lac* operons (1) and the divergently transcribed *glpTQ-glpACB* operons (20, 47). The finding of a similar situation for repression of the *glpD* operon agrees with the suggestion that remote operator duplications are usually associated with multiple promoters subject to more than one system of regulation (16). In the case of *glpD*, besides regulation by GlpR, the promoter is subject to regulation by cyclic AMP-CRP and by the ArcA/ArcB systems (19). The adjacent, divergent *glpEGR* promoter

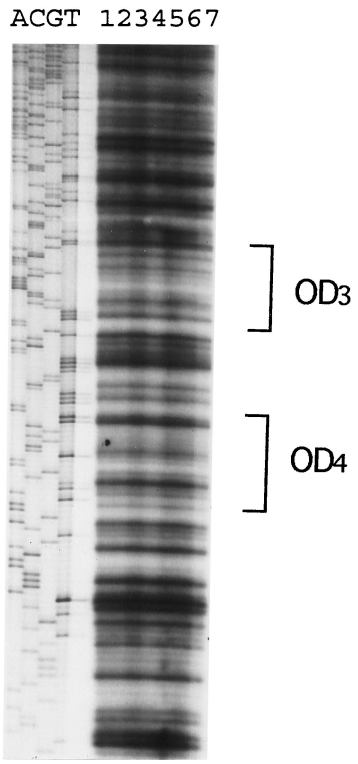

FIG. 3. Lack of binding of the *glp* repressor to mutated O_D4 in the presence of O_D1 and O_D2. DNA for footprinting was generated by PCR amplification of pBY109 with primer 254069 and ^{32}P-end-labeled primer 207055. Reactions were carried out with the following concentrations of the *glp* repressor and analyzed: lane 1, no repressor; lane 2, 2 nM GlpR; lane 3, 4 nM GlpR; lane 4, 8 nM GlpR; lane 5, 20 nM GlpR; lane 6, 40 nM GlpR; and lane 7, 40 nM GlpR and 3 mM glycerol-P. The size markers (lanes A, C, G, and T) were generated by using pBY109 as the template with primer 207055 in a standard sequencing reaction.

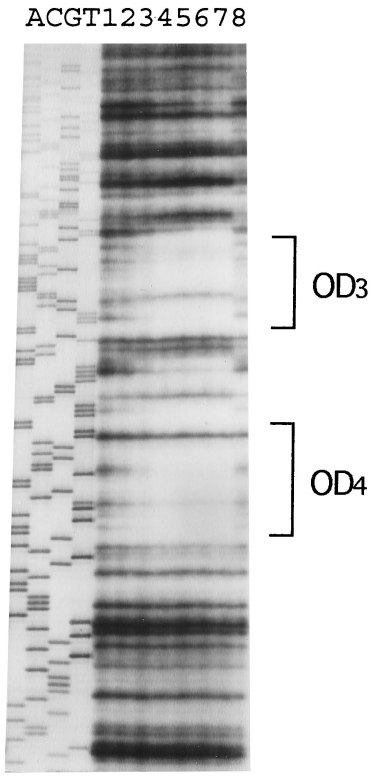

FIG. 4. DNase I footprinting of the *glpD* internal operators (in the absence of O_D1 and O_D2). DNA for footprinting was generated by PCR amplification of pDA1104 using SK primer and ^{32}P-end-labeled primer 207055. Reactions were carried out with the following concentrations of *glp* repressor tetramers and analyzed: lane 1, no repressor; lane 2, 1 nM GlpR; lane 3, 3 nM GlpR; lane 4, 6 nM GlpR; lane 5, 9 nM GlpR; lane 6, 20 nM GlpR; lane 7, 40 nM GlpR; and lane 8, 9 nM GlpR and 3 mM glycerol-P. The size markers (lanes A, C, G, and T) were generated by using pDA1104 as the template with primer 207055 in a standard sequencing reaction.

may share some of these *cis*-acting regulatory elements; therefore, it may be argued that the remote placement of operators O_D3 and O_D4 avoids crowding of regulatory elements near the promoters (16). An emerging general feature of negative control by GlpR is that GlpR binds cooperatively to tandem operators (O_D1 and O_D2 or O_A2 and O_A3) located at or near the promoter, with additional contributions to repression provided by remote operators (O_D3 and O_D4 or O_T1 to O_T3), to form a dense nucleoprotein complex with the intervening DNA forming a loop.

A typical operator that binds a dimeric repressor contains a palindromic sequence, with each half of the symmetric site binding one monomer of the repressor. In the case of a repressor that binds tandem operators, the repressor dimers or tetramers lie adjacent to each other and cooperative binding increases the local concentration of the second repressor dimer. It is apparent that a plasmid containing tandem operators O_D1 and O_D2 binds the repressor 10 times better than does a plasmid harboring only O_D1. In addition, although 21 bp intervene between O_D3 and O_D4, the observation that substitutions in O_D4 eliminated protection of O_D3 in vitro is strongly suggestive of cooperative binding of the repressor at O_D3 and O_D4. O_D1 matches the *glp* consensus operator perfectly, but O_D2, O_D3, and O_D4 have 5, 6, and 4 mismatches, respectively, compared with the 20-bp consensus *glp* operator sequence (50). It is reasonable that GlpR binding at a better operator like O_D1 or O_D4 facilitates cooperative binding of the weak operator, O_D2 or O_D3, respectively. It is known that GlpR binds cooperatively to tandem consensus operators (50). Cooperative binding of the repressor to the two internal operators may be possible because the distance between the centers of symmetry of O_D3 and O_D4 is 41 bp (about four helical turns), a distance that would allow GlpR binding to the same face of DNA. The fact that the internal operators were unable to function alone in vivo even though these operators did bind GlpR in vitro (the amount of GlpR needed for footprinting of the internal operators was essentially independent of the presence of O_D1 and O_D2) suggested a further cooperative mechanism, with GlpR simultaneously binding the four operators. It is reasonable that the binding of GlpR to the internal operators does not repress RNA polymerase because it is known that operators located in the downstream coding regions normally are not effective in repression since they are far away from the RNA polymerase binding site or other essential activation sites (1, 16). This is probably the reason that most functional operators overlap or are close to the transcription initiation sites (16). The results of the present study indicate that the internal operators O_D3 and O_D4 exert their function in vivo by cooperative binding of GlpR tetramer(s) with O_D1 and O_D2, since they had no effect in vivo in the absence of O_D1 and O_D2 but contributed five- to sevenfold to repression in the presence of O_D1 and O_D2. By using plasmid-encoded transcriptional fusions of the promoter-operator regions to *lacZ* and repressor provided by a multicopy plasmid, a repression ratio of 393 was found for transcriptional control of *glpFKX*, a ratio four times

higher than that of an analogous *glpD-lacZ* construct containing only the promoter and tandem operators O_D1 and O_D2 (46). By using a *glpD* fusion containing the internal operators, it is now clear that O_D3 and O_D4 contribute additionally five- to sevenfold to repression. Therefore, *glpD* is likely to be repressed more tightly than is *glpFKX*, consistent with the earlier discovery that the *glpD* operon is controlled more tightly than is either the *glpTQ* or *glpFKX* operon, as determined by measuring enzyme activities encoded by each operon (12, 23, 24).

Multiple operators are separated by up to several hundred base pairs in the *lac*, *deo*, *gal*, and *ara* systems (1, 6). Similar evidence of repressor binding to widely separated operators has been found for control of the *glpTQ-glpACB* operons (20, 47) and the *put* operon of *Salmonella typhimurium* (41). In all cases, DNA loop formation mediated by repressor binding to distal operators seems to be the mechanism for repression (1, 16). Like the eukaryotic enhancers, DNA seems to form a loop within a long range (6, 16), as further supported by the recent report that a twofold increase in repression was exerted by a second distal *lac* operator when two *lac* operators were separated by distances ranging from 600 to 1,500 bp (27). It was shown that even without the supporting proteins, intrinsic curving and bending play an essential role in protein-DNA recognition (17). The distance between O_D2 and O_D3 is 395 bp, a distance suitable for DNA loop formation as GlpR binds to the four *glpD* operators. There is a predicted intrinsic bend in the DNA within *glpD* (from positions 521 to 661, covering the internal *glpD* operators [Fig. 1]) with a predicted bend of 77.42° (31). The intrinsic bend might contribute additionally to wrapping of the DNA to form a loop. However, the accessory protein HU contributes additionally to repression, presumably by assisting in DNA bending. Despite the fact that HU and IHF have many structural and functional similarities, it was shown that IHF had little or no effect on *glpD* transcription while HU increased repression about fourfold. The influence of HU was similar to that found for repression of the *gal* operon promoter P2, in which a two- to threefold effect was seen (2). The mechanisms for the binding of IHF and HU are predicted to be very similar, and these proteins act interchangeably in some cases (28). Nevertheless, each protein functions specifically in one operon but has no effect in another operon, arguing that selective action of HU and IHF in different operons might enhance the ability for specific regulation.

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