JOURNAL OF BACTERIOLOGY, Jan. 1991, p. 549–558
0021-9193/91/020549-10$02.00/0

Vol. 173, No. 2

Dual Regulation of the *ugp* Operon by Phosphate and Carbon Starvation at Two Interspaced Promoters

MEGUMI KASAHARA, KOZO MAKINO, MITSUKO AMEMURA, ATSUO NAKATA, AND HIDEO SHINAGAWA*

Department of Experimental Chemotherapy, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565, Japan

Received 20 August 1990/Accepted 14 November 1990

The *ugp* operon of *Escherichia coli* includes genes involved in the uptake of *sn*-glycerol-3-phosphate and glycerophosphoryl diesters and belongs to the *pho* regulon which is induced by phosphate limitation. This operon has two transcriptional initiation sites, as determined by S1 nuclease mapping of the in vivo transcripts. The downstream promoter has multiple copies of the *pho* box, the consensus sequence shared by the *pho* promoters; the upstream promoter has a consensus sequence for the promoters regulated by cyclic AMP and its receptor protein, CRP. PhoB protein, which is the transcriptional activator for the *pho* regulon, protected the regulatory region with the *pho* boxes in DNase I footprinting experiments and activated transcription from the downstream promoter in vitro. Studies with transcriptional fusions between *ugp* and a promoterless gene for chloramphenicol acetyltransferase show that the upstream promoter is induced by carbon starvation in a manner that required the *cya* and *crp* genes. PhoB protein may act as a repressor for this upstream promoter, which also overlaps the upstream third *pho* box. The downstream promoter was induced by phosphate starvation and requires the PhoB protein for its activation as do the other *pho* regulon promoters. These results suggest that the two promoters function alternately in responding to phosphate or carbon starvation, thus providing the cell with a means to adapt to these physiological stresses.

The *ugp* operon encodes proteins involved in the uptake of *sn*-glycerol-3-phosphate and glycerophosphoryl diesters and an enzyme hydrolyzing glycerophosphoryl diesters (6, 22). The *ugp* operon is induced by phosphate starvation and positively regulated by PhoB protein, as are other genes in the *pho* regulon. The nucleotide sequences of the first four genes from the promoter, which code for the binding-protein-dependent transport system, were analyzed by Overduin et al. (22), and the nucleotide sequence of the last gene, coding for the phosphodiesterase, was analyzed by Kasahara et al. (10). Promoters in the *pho* regulon share unique features, namely, the sequence characteristic of the −10 region and the 18-bp consensus *pho* box sequence 10 bp upstream of the −10 region. The activator protein PhoB binds the *pho* box in a way which activates transcription of the *pho* genes (14). The activity of PhoB as a transcriptional activater is modified by PhoR protein in response to the phosphate concentration in the medium. When the external phosphate is limiting for growth, PhoR activates PhoB by phosphorylation (13). The phosphate in the medium is monitored by the phosphate-binding protein, the *pstS* gene product, and the signal is transferred to PhoR via the proteins encoded by the *pst* operon (21).

In this work, we analyzed regulation of the *ugp* operon in vivo and in vitro, and identified two promoters, one inducible by carbon starvation and the other inducible by phosphate starvation. We show that the former is regulated by cyclic AMP (cAMP) and its receptor protein, CRP, and that the latter is regulated by phosphorylated PhoB.

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophages. *Escherichia coli* K-12 strains and plasmids used in this study are listed in Table 1.

Media. LB broth, LB agar, and T broth were as described by Miller (19). Tris-glucose (TG) medium with either high or low phosphate was described previously (2). Glucose-limited medium contained 0.02% glucose (30). Ampicillin was added to LB broth or T broth at 100 μg/ml and to TG medium at 2 mg/ml. Kanamycin was added to LB agar at 20 μg/ml.

Recombinant DNA methods. Standard methods for recombinant DNA were generally used as described by Maniatis et al. (16).

DNA sequencing. The manipulation of M13 phage was as described by Messing et al. (18). A series of phage clones with DNA fragments with one fixed end and the other end formed by successive deletions at the 5′ end was prepared as described by Hong (8). The DNA sequences were analyzed by the method of Sanger et al. (23).

Enzyme assay. Chloramphenicol acetyltransferase (CAT) in the extracts of sonicated cells was assayed by the method of Shaw (26). The specific activity of CAT was expressed as nanomoles of 5-thio-2-nitrobenzoate liberated per min per unit of optical density at 450 nm (OD_{450}) of the cell culture.

S1 nuclease mapping. S1 nuclease mapping was done as described by Aiba et al. (1).

In vitro transcription. PhoB protein and PhoR1084 protein, which lacks the amino-terminal hydrophobic region and is constitutively active as a kinase (32), were purified, and in vitro transcription was done essentially as described by Makino et al. (13, 14). A reaction mixture (20 μl) contained the *Eco*RI-*Hin*dIII DNA fragment (1 pmol) carrying the *ugp* promoter region (Fig. 1C, line d), RNA polymerase holoenzyme (5 pmol), 1 mM ATP, and 10 pmol of PhoR protein in

TABLE 1. *Escherichia coli* strains and plasmids

Strain or plasmid	Relevant characteristics	Reference
E. coli		
ANCK10	F⁻ *leu lacY trp his argG rpsL ilv metA* (or *-B*) *thi*	15
ANCC75	As ANCK10 except *phoS64 purE ilv*⁺	2
ANCH1	As ANCK10 except Δ(*phoB-phoR*) Km^r	31
JM103	Δ(*pro-lac*) *supE thi* (F′ *traD36 proAB lacI^{q}Z M15*)	18
TP7811	F⁻ *xyl argH1 his*	4
TP7839	F⁻ *xyl argH1 his Δcrp-39*	4
TP7860	F⁻ *xyl argH1 his Δcya*	4
Plasmid		
pKK232-8	Vector for promoter cloning, Ap^r	5
pUC-4K	Plasmid with kanamycin resistance gene cartridge	28
pUC9	Vector for cloning, Ap^r	28

the presence or absence of 10 pmol of PhoB protein. The mixture was incubated at 37°C for 10 min for open complex formation. Then 10 μl of prewarmed solution containing 480 μM (each) ATP, GTP, and CTP, 40 μM [α-^{32}P]UTP (>400 Ci/mmol), and 15 μg of rifampin per ml was added to the mixture and incubated at 37°C for 5 min. Cold UTP (50 μM) was added and incubated at 37°C for 5 min. The *Bam*HI-*Hin*dIII fragment of plasmid pOS1 (11), which carries the *pstS* promoter region from −68 to +35 relative to the transcription start site, was used as a positive control for PhoB-dependent in vitro transcription.

Footprinting experiments. The DNase I and methylation protection experiments were done by the methods of Johnson et al. (9) and Craig and Nash (7), respectively.

Enzymes and radioisotopes. The restriction endonucleases, T4 DNA ligase, T4 DNA polymerase, T4 polynucleotide kinase, deoxyribonuclease I (DNase I), bacterial alkaline phosphatase, and an M13 sequencing kit including DNA polymerase (Klenow fragment) were obtained from Takara Shuzo (Kyoto, Japan). S1 nuclease and RNA polymerase holoenzyme were purchased from Pharmacia Biotechnology (Tokyo, Japan). [γ-^{32}P]ATP (>5,000 Ci/mmol), [α-^{32}P]dCTP (>400 Ci/mmol), and [α-^{32}P]UTP (>400 Ci/mmol) were purchased from Amersham (Tokyo, Japan).

RESULTS

Cloning of the promoter region of the *ugp* operon. We cloned the *ugp* operon from the *E. coli* genome library constructed by Kohara et al. (12) with phage vectors. We compared the physical maps of Kohara clones 1B6, 5B10, and 10F5, which contain chromosomal fragments around 75 min, where the *ugp* operon is located on the linkage map (25), with the physical map of the *ugp* region (24). The physical map of the chromosomal DNA carried on clone 5B10 indicated that it might contain the *ugp* region, so we isolated DNA from clone 5B10 and confirmed by restriction enzyme digestion that it does (Fig. 1). The physical map of the *Eco*RI fragment of clone 5B10 was identical with that of the *ugp* region cloned on plasmid pSH12, as determined by Schweizer and Boos (24). We sequenced the nucleotides of the entire *Eco*RI fragment (10; also unpublished data), and the sequence covering *ugpB*, *ugpA*, *ugpE*, and *ugpC* was identical to that reported by Overduin et al. (22).

To identify the promoter region of the *ugp* operon, DNA prepared from clone 5B10 was digested with *Eco*RI and *Sal*I (Fig. 1), and the resultant staggered ends were converted to blunt ends by T4 DNA polymerase. The DNA fragments were first cloned into the *Hin*cII site of M13mp18, and then the 2.9-kb *Bam*HI-*Hin*dIII fragment from the recombinant phage was recloned into a promoter cloning vector, pKK232-8. The resultant plasmid contained the *ugp* promoter region in the same orientation as the *cat* gene. CAT activity of strain ANCK10 (wild type) harboring this plasmid grown in a limited phosphate medium was about 700 U per OD_{450} unit of the cell culture, and CAT activity for this strain when grown in a excess phosphate medium was about 40 U per OD_{450} unit. Similarly, we found that the 600-bp *Pvu*II-*Eco*RI fragment and the 240-bp *Sau*3AI-*Eco*RI (Fig. 1) fragment also had a phosphate-starvation-inducible promoter activity with an induction ratio similar to the above. Therefore, we concluded that the 240-bp *Sau*3AI-*Eco*RI fragment (Fig. 1) contains the *ugp* promoter that is induced by phosphate starvation.

Delineation of the *ugp* promoters by deletion analysis. To define the functional promoter region, we constructed a series of upstream deletion mutations of the cloned DNA containing the *ugp* promoter in vitro by a method previously described (11). The replicative-form DNA of M13mp18 phage carrying the blunt-ended 2.9-kb *Sal*I-*Eco*RI fragment containing the *ugp* promoter region in the *Hin*cII site was mildly digested with DNase I, and DNAs with a single cut were isolated by electroelution. Then they were digested with *Bam*HI, the cohesive ends were converted to blunt ends by T4 DNA polymerase, and they were self-ligated. Twenty phage clones that contained *Eco*RI-*Hin*dIII fragments from 50 to 260 bp long were selected, and the upstream endpoints of the deletions were analyzed by DNA sequencing after recloning the DNA fragments into M13mp18 (Fig. 2). The *Sma*I-*Hin*dIII fragments of the M13mp18 derivatives were recloned into pKK232-8 to construct fusions with the *cat* gene, and the resultant plasmids (pKD series in Table 2) were introduced into ANCK10. The promoter activity of the DNA fragments with various upstream deletions and the constant downstream end at +18 (Fig. 2) was measured by monitoring the CAT activity of the extracts of the cells carrying the fusion plasmids (Table 2). The endpoints of the upstream deletions and their promoter activity are graphically shown in Fig. 3. The CAT activity of the nondeleted fragment with the excess phosphate was 2% of that with limiting phosphate. The deletions up to nucleotide −88 from the transcription initiation site for the *ugp1* promoter (described below) had little effect on the relative promoter activities under the two conditions. However, when the deletion proceeded beyond −74, the promoter activity expressed under excess phosphate conditions was virtually lost, and the activity under limited phosphate conditions was substantially enhanced (Table 2; Fig. 3). These results suggest that the region just downstream of −88 is essential to the promoter activity functioning under excess phosphate conditions and that the upstream third *pho* box might have a negative regulatory function for the *pho* promoter. The putative promoter (P_{ugp2}) with the −35 and −10 sequences was identified in this region, and it overlaps the upstream *pho* box (Fig. 2). Further, the fusion genes with deletion endpoints between −56 and −41 still showed low but significant levels of the activity (3.6 U per OD_{450}), which was regulated by phosphate concentration in the medium

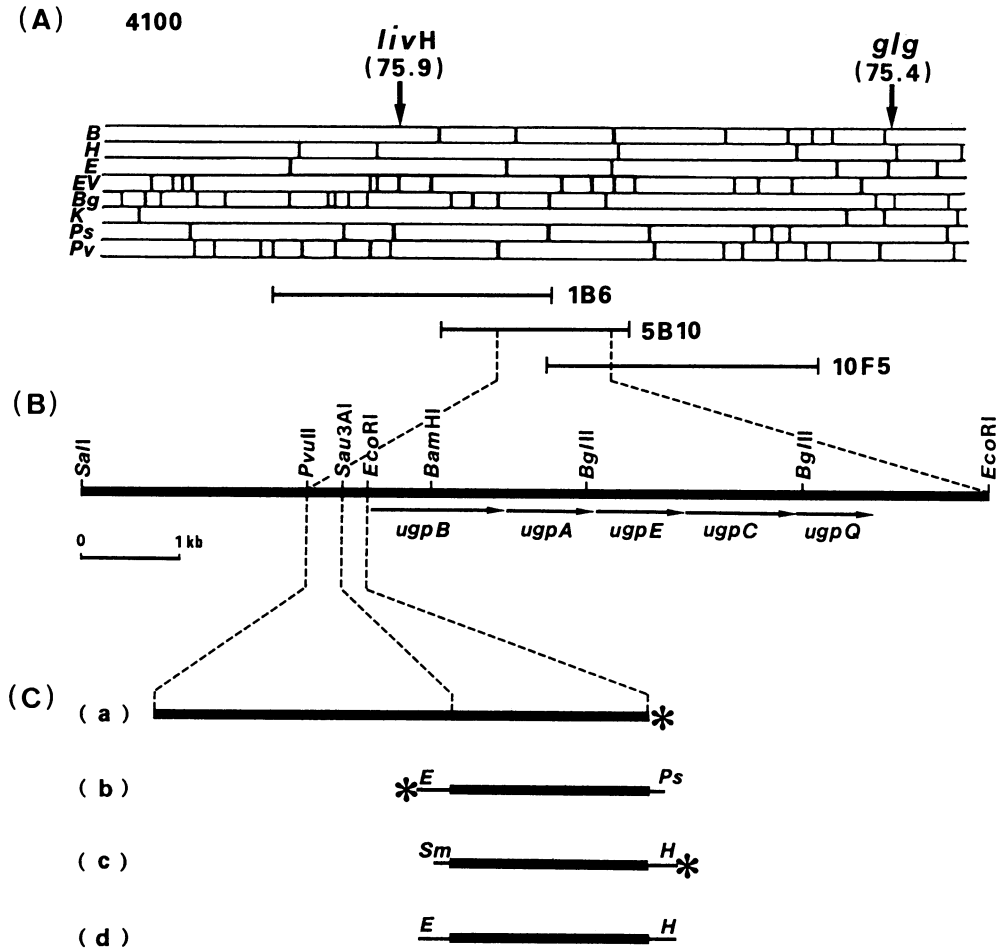

FIG. 1. Physical maps of the DNA fragment containing the *ugp* operon and the restriction fragments used in this work. (A) Restriction map of the *E. coli* chromosome segment that contains the *ugp* region carried by λ clones 1B6, 5B10, and 10F5, as described by Kohara et al. (12). (B) Enlarged restriction map of the *ugp* region. (C) Restriction fragments used in the footprinting and S1 nuclease mapping experiments. The portions of DNA derived from the chromosome and vector are indicated by thick and thin lines, respectively. Lines: a, *Pvu*II-*Eco*RI fragment (about 600 bp) labeled with [γ-^{32}P]ATP by T4 DNA kinase at the *Eco*RI end (*) used as the sense-strand probe in the DNase I and methylation protection experiments; b, *Eco*RI-*Pst*I fragment containing the *Sau*3AI-*Eco*RI chromosomal fragment (−225 to +18) labeled at the *Eco*RI end (*) used as the antisense-strand probe in the DNase I and methylation protection experiments; c, *Sma*I-*Hin*dIII fragment labeled at the *Hin*dIII end (*) used as the probe in the S1 nuclease mapping; d, *Eco*RI-*Hin*dIII fragment used as the template in in vitro transcription. B, *Bam*HI; Bg, *Bgl*I; E, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII; K, *Kpn*I; Ps, *Pst*I; Pv, *Pvu*II; Sm, *Sma*I.

(Table 2). These deletion mutants no longer contained the three intact *pho* boxes, but at least the downstream *pho* box was intact (Fig. 2). When the deletion extended into the last downstream *pho* box, no activity was observed (Table 2). These results suggest that the two *pho* boxes upstream of the −10 region are required for the efficient activity of the phosphate-starvation-inducible promoter.

Identification of the mRNA initiation sites of the *ugp* operon. To identify the *ugp* promoter regulated by the *pho* system, we tried to find the mRNA initiation site of the *ugp* operon by in vivo S1 nuclease mapping. The 240-bp *Sma*I-*Hin*dIII fragment containing the *ugp* promoter region was used as a probe for S1 nuclease mapping (Fig. 1C, line c). We used ANCK10 (wild type) carrying pKD507 and ANCC75 (*pstS64*) carrying pKD507 for RNA sources. When they are grown in excess phosphate medium such as T broth, strain ANCC75 constitutively expresses the *pho* genes, whereas wild-type strain ANCK10 does not express the *pho* genes. After S1 nuclease digestion, the size of the protected DNA was estimated by comparison with the products of the Maxam-Gilbert (17) sequencing reactions (Fig. 4, lanes 1 and 6). Two kinds of *ugp* transcripts, one in RNA prepared from ANCC75(pKD507) and the other in ANCK10(pKD507), were detected (Fig. 4). The shorter transcript detected in ANCC75(pKD507) cells should correspond to the transcript initiated from the *ugp* promoter induced by phosphate limitation (P_{ugpI}) and the start site guanine is numbered +1 throughout this study (Fig. 4, lanes 4 and 5). Six base pairs upstream from this transcription initiation site, we found a DNA sequence homologous to the consensus sequence for the −10 region of many promoters, suggesting that the sequence (cATgtT) is the −10 region of P_{ugpI}. No sequence homologous to the −35 sequence was detected at the appropriate position upstream from the −10 region. Instead we found three sets of the consensus sequence (*pho* box) shared by the regulatory regions of the *pho* genes (Fig. 2). On the other hand, the longer transcript was found only in mRNA prepared from ANCK10(pKD507) cells (Fig. 4, lanes 2 and

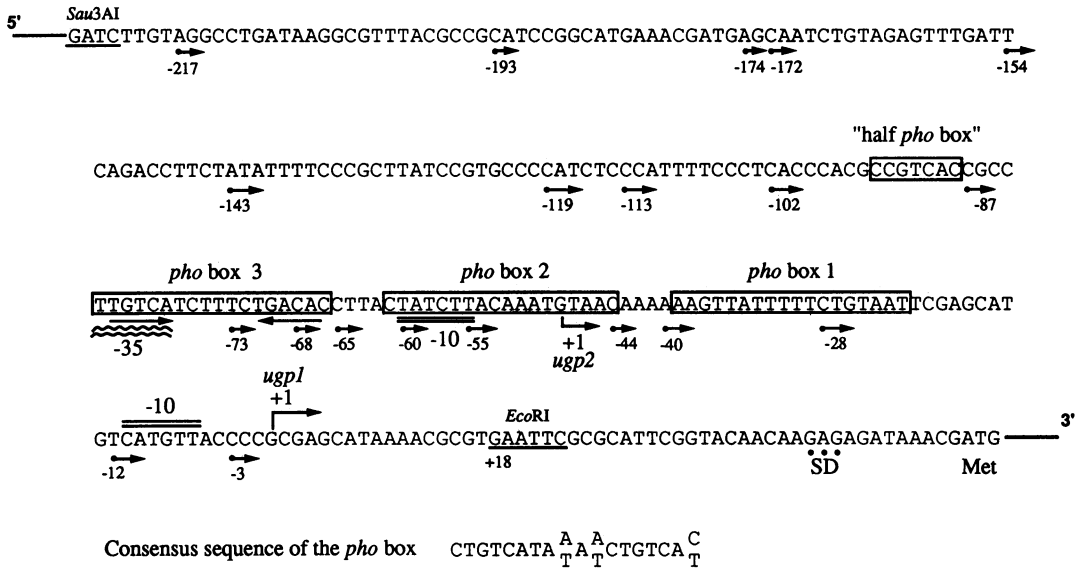

FIG. 2. Nucleotide sequence of the DNA fragment encompassing the *ugp* promoter region, and the endpoints of the upstream deletion mutations. Arrows indicate the endpoints of deletion mutations. The *pho* boxes and a "half *pho* box" are boxed, and the −10 regions is shown by double lines. The wavy line indicates the −35 region of the promoter that is active under the excess phosphate conditions (P_{ugp2}). +1 indicates the mRNA start point identified by S1 nuclease mapping of the *ugp* transcript produced under the limiting phosphate conditions (see Fig. 4). SD marks the ribosome-binding site. The consensus sequence of the *pho* box is adapted from Shinagawa et al. (27). The two convergent arrows under the *pho* box 3 indicate the putative consensus sequence for CRP-binding site.

3), and the initiation site of this transcript expressed under the excess phosphate conditions was guanine at −48. Seven base pairs upstream from this initiation site, we found the −10 region (TATctT), and 17 bp upstream from the −10 region, we found the −35 region (TTGtCA) (Fig. 2). This promoter (P_{ugp2}) may be responsible for the promoter activity expressed under the excess phosphate conditions (Table 2; Fig. 3), and is indicated upstream of P_{ugp1} in Fig. 2. These results show that the *ugp* operon has two promoters.

TABLE 2. Promoter activity of the *ugp* regions with various upstream deletion mutations

Plasmid[a]	Upstream endpoint	CAT activity[b] (U/OD_{450})	
		Ex Pi	Li Pi
pKD501	−217	20.7	1,068
pKD502	−193	28.9	1,098
pKD503	−174	22.9	851
pKD504	−172	27.9	973
pKD505	−154	27.3	1,098
pKD506	−143	24.7	919
pKD507	−119	15.7	828
pKD508	−113	15.4	1,163
pKD509	−102	11.0	986
pKD510	−87	26.0	1,226
pKD511	−73	0.2	1,630
pKD512	−68	0.2	2,037
pKD513	−65	0.3	2,196
pKD514	−60	0.2	1,747
pKD515	−55	0.2	660
pKD516	−44	<0.1	3.6
pKD517	−40	<0.1	3.6
pKD518	−28	<0.1	<0.1
pKD519	−12	<0.1	<0.1
pKD520	−3	<0.1	<0.1
pKK232-8		<0.1	<0.1

[a] pKD plasmids carry the *ugp* regulatory regions with various upstream endpoints and the constant downstream endpoint at +18 (Fig. 2) on promoter assay vector pKK232-8.

[b] The CAT activity in ANCK10 (wild-type) carrying plasmids which contain the various *cat* fusion genes. The values are the average of duplicated assays. Ex Pi, Excess phosphate conditions; Li Pi, limiting phosphate conditions.

Activation of transcription of the *ugp* operon by phosphorylated PhoB protein in vitro. We examined whether the phosphorylated PhoB protein activates transcription initiation of the *ugp* operon by RNA polymerase. In these experiments, PhoR protein and ATP were added to the reaction mixture for phosphorylation of PhoB protein. The reaction mixtures containing a linear DNA template (Fig. 1C, line d), PhoR1084 protein, ATP, and RNA polymerase, with or without PhoB protein, were incubated at 37°C for 10 min to form a transcription-initiation complex. The nucleoside triphosphates and rifampin, which inhibits reinitiation of transcription, were added, and the mixtures were incubated at 37°C for 5 min. The in vitro transcripts were analyzed by 8% polyacrylamide gel containing 8 M urea (Fig. 5). Strong bands of the *ugp1* transcript were observed only in the reaction mixture with phosphorylated PhoB protein added (Fig. 5, lane 3), and weak bands of the *ugp2* transcript were found only in the mixture without PhoB protein (Fig. 5, lane 2). The *ugp1* transcript was calculated to be 49 bp by comparing it with the *pstS* transcript (Fig. 5, lane 1). Therefore, phosphorylated PhoB protein greatly stimulated the *ugp* transcription initiated from the *ugp1* site, which is the same as the start site of the transcript found in the *pho* constitutive strain (Fig. 4), and appeared to inhibit the *ugp2* transcription (Fig. 5, lane 3). The *ugp2* transcript corresponded to the transcript found in the wild-type strain grown in T broth (Fig. 4).

Binding sites of PhoB protein on the regulatory region of the *ugp* operon. The binding sites of PhoB protein on the regulatory region of the *ugp* operon were studied by footprinting experiments. DNase I and methylation protection

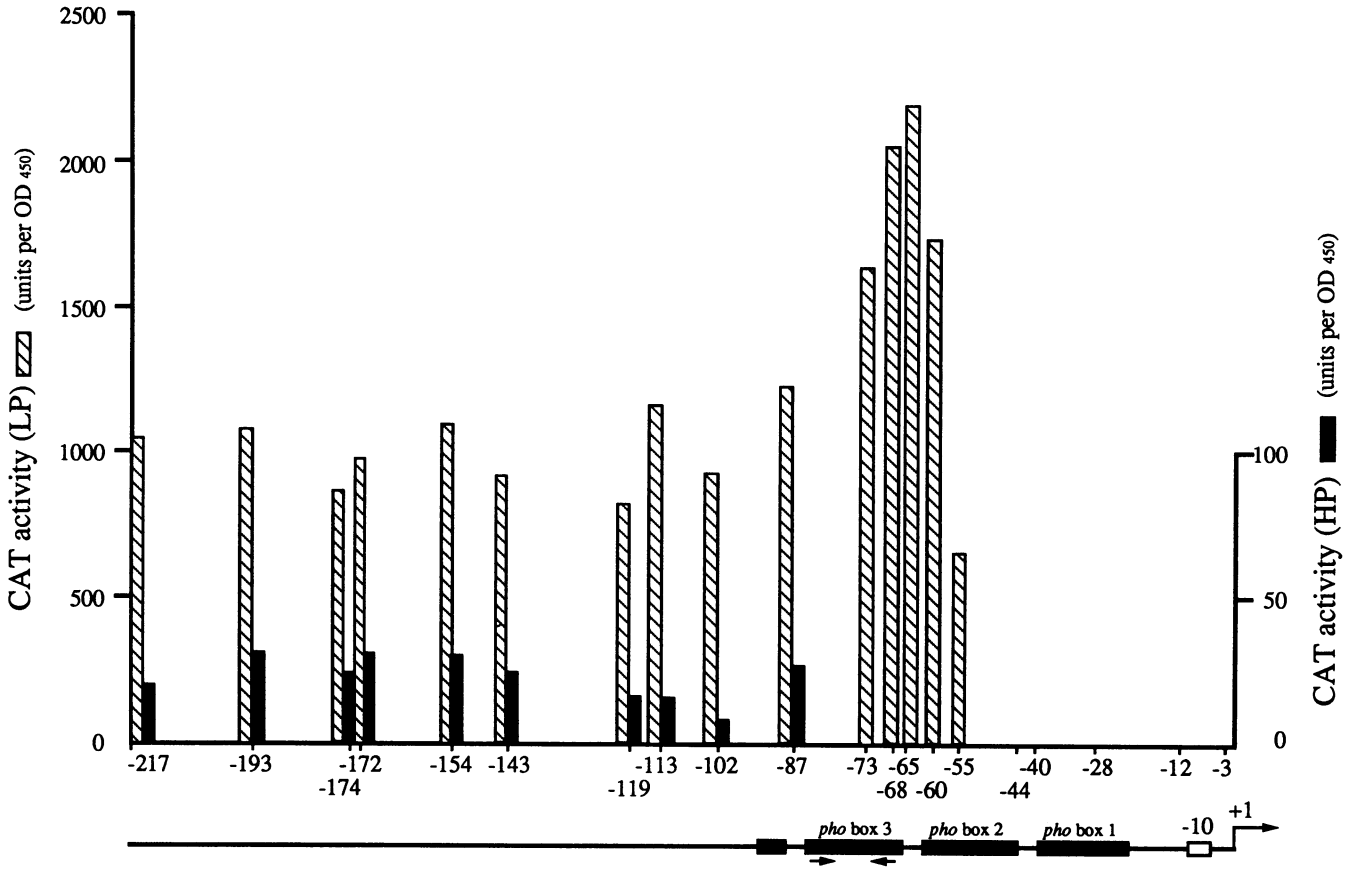

FIG. 3. Schematic representation of promoter activity of the *ugp* regulatory region with upstream deletions. The specific activities of CAT in the cells carrying the deletion plasmids with excess phosphate (HP) and limited phosphate (LP) are shown. The numbers indicate the nucleotide numbers from the start site of the *ugp* mRNA transcribed under the limiting phosphate conditions. At the bottom of the figure, the *pho* boxes (■) and the −10 region (□) are shown. Arrows indicate the inverted repeats.

experiments were done for both DNA strands. For the sense strand, the 600-bp *Pvu*II-*Eco*RI fragment labeled with ^{32}P at the 5′ end of *Eco*RI site was used (Fig. 1C, line a). For the antisense strand, the 240-bp *Eco*RI-*Pst*I fragment containing the *ugp* promoter region labeled with ^{32}P at the 5′ end of *Eco*RI site was used (Fig. 1C, line b). In the sense strand, PhoB protein protected the DNA segment spanning nucleotides −20 to −101 from DNase I digestion and G residues at positions −28, −44, −53, −57, −61, −66, −68, −72, −76, −79, −85, −88, and −90 from methylation, and it enhanced the methylation of G residues at −65, −84, and −87 (Fig. 6A). In the antisense strand, PhoB protein protected the DNA segment spanning −14 to −100 from the nuclease digestion, and G and A residues at positions −19, −26, −37, −48, −70, −81, −92, and −95 from methylation, and it enhanced the methylation of the G residue at position −86 (Fig. 6B). The results obtained from these two experiments suggest that PhoB protein interacts with the *ugp* promoter in the region spanning −14 to −101, which contains the three tandemly arranged *pho* boxes and a "half *pho* box" (Fig. 7).

Dual regulation of the *ugp* operon by the *pho* and *crp* promoters. Several lines of evidence described above show that there are two promoters for the *ugp* operon, one (P_{ugp1}) inducible by phosphate starvation, and another (P_{ugp2}) functional with excess phosphate and in T-broth medium. Inspection of the DNA sequence around the −35 region of the upstream promoter found an inverted repeat, TGTcAtctttc TgACA, which is similar to the consensus sequence of the promoter regulated by cAMP and CRP (cAMP receptor protein), TGTGAN^{6}TCACA (3, 20), and overlaps the *pho* box 3 (Fig. 2). Since *sn*-glycerol-3-phosphate can serve as the sole source of phosphate and carbon, it is rational that the *ugp* operon is inducible by phosphate and carbon starvation. To study the regulation of the two promoters individually, a set of operon fusion plasmids, each of which carries each one of the promoters, was constructed (Fig. 8). Plasmid pKD506 carries the entire regulatory region of *ugp*, covering both promoters, fused with the *cat* gene. Plasmid pKD5061 carries the upstream promoter only, fused with the *cat* gene, because the regulatory region carried on the plasmid lacks the −10 region and the initiation site of the downstream *pho* promoter P_{ugp1}. Plasmid pKD513 carries P_{ugp1} only, fused with the *cat* gene, because it has a deletion of the −35 region of the upstream promoter P_{ugp2}. Plasmid pKD5131 should carry neither promoter, since it lacks the −35 region of P_{ugp2} and the −10 region of P_{ugp1}.

To study the activity of the two promoter, we introduced these plasmids into the wild-type strain (ANCK10) and the *phoB-phoR* deletion strain (ANCHI) and measured the CAT activity in these strains grown in excess or limiting phosphate medium (Table 3). In the operon fusion with P_{ugp1} only

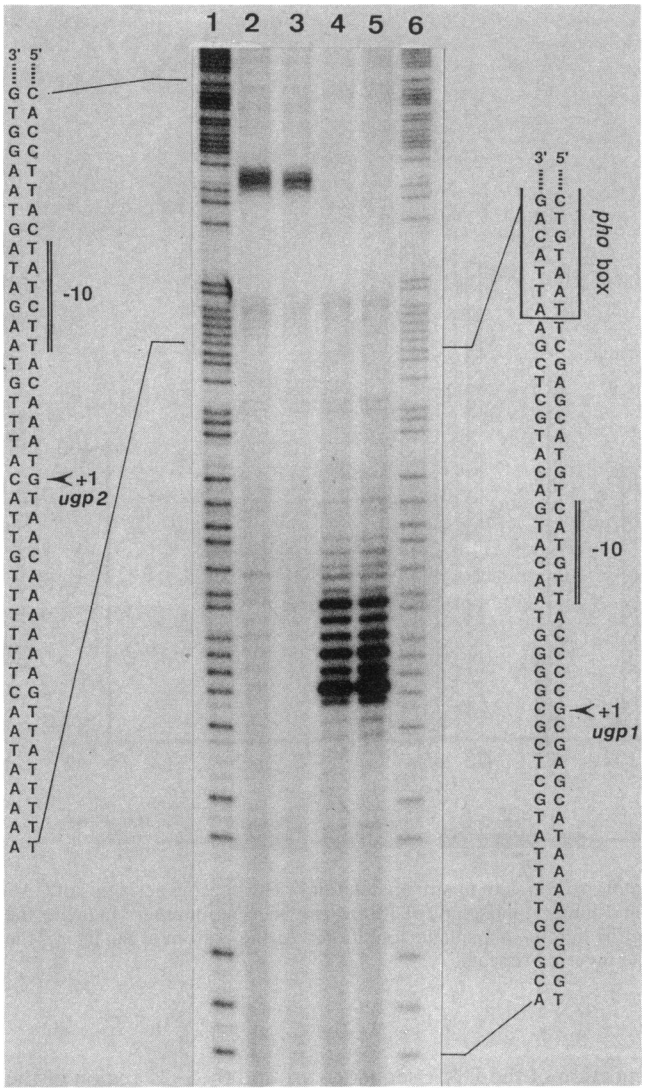

FIG. 4. S1 nuclease mapping for location of the transcription initiation site of the *ugp* operon in vivo. The end-labeled probe (0.5 pmol) shown in line c of Fig. 1C was hybridized to mRNA (50 μg) extracted from wild-type ANCK10(pKD507) cells (lanes 2 and 3) and ANCC75(pKD507) cells (lanes 4 and 5) grown in T broth. Lanes 1 and 6 show G+A ladders of Maxam-Gilbert sequencing of the probe DNA. The hybridized samples were treated with S1 nuclease for 5 (lanes 2 and 4) or 10 (lanes 3 and 5) min. The 5′ ends of the *ugp* transcripts identified as the major bands by S1 mapping are shown by arrowheads. The boxed sequence indicates the *pho* box. The double lines indicate the −10 regions of the promoters.

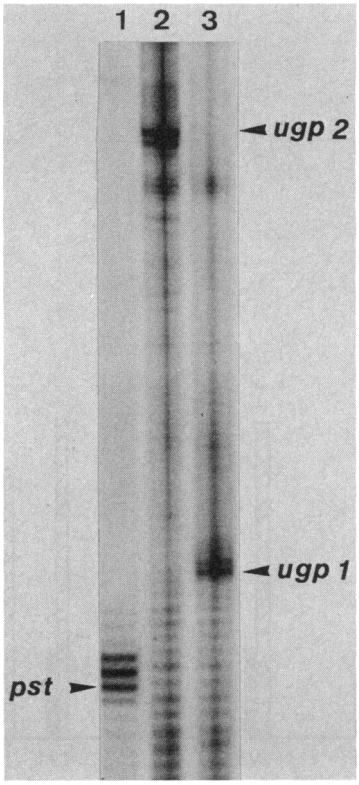

FIG. 5. In vitro transcription of the *ugp* operon. RNAs were directly labeled with [α-^{32}P]UTP, precipitated with ethanol, and analyzed by electrophoresis in 8% polyacrylamide gel containing 8 M urea. The *Eco*RI-*Hin*dIII fragment (Fig. 1C, line d) containing the *ugp* promoter region was incubated with RNA polymerase in the presence (lane 3) or absence (lane 2) of the phosphorylated PhoB protein. In lane 1, product of the in vitro *pstS* transcript (41 bp) was analyzed (13). The arrowheads indicate the position of the start points of the *ugp* transcripts (*ugp1* and *ugp2*). These points were identical to those of the in vivo transcripts.

(pKD513), the activity was induced about 20,000-fold by phosphate starvation, which was the highest among the fusions examined under our experimental conditions. No promoter activity was detected in this operon fusion in the *phoB-phoR* deletion strain, suggesting that P_{ugp1} has a strict requirement for the *phoB* function. The upstream promoter P_{ugp2} carried by pKD5061 in the wild-type strain had five-fold-higher activity under the excess phosphate conditions than under the limiting phosphate conditions; in the *phoB-phoR* deletion strain the P_{ugp2} activity varied less with the phosphate levels in the medium than in the wild-type strain. The promoter activity manifested by the plasmid pKD506 containing the two promoters appeared to be the combined activity of the two promoters. It was induced by phosphate starvation, but it showed residual activities in the *phoB-phoR* deletion strain and in the wild-type strain with excess phosphate.

The effects of carbon starvation under the excess phosphate conditions on the two *ugp* promoters were studied in the wild type and *phoB-phoR* deletion strains carrying the operon fusion plasmids (Table 4). P_{ugp1} in pKD513 did not respond to carbon starvation, and it was not functional at all under the excess phosphate conditions. Only the operon fusions that contained P_{ugp2} as in pKD506 and pKD5061 showed any promoter activities and the activities were slightly induced by carbon starvation. PhoB protein appeared to slightly repress P_{ugp2}. No promoter activity was detected in the *cat* fusion carried on pKD5131 under our experimental conditions.

To examine the dependence of P_{ugp2} on cAMP and CRP, we introduced pKD5061 into the nearly isogenic strains with deletions in *cya* or *crp* and assayed the CAT activity in these strains grown in excess or limited carbon medium (Fig. 9). The results were more convincing than those obtained by the experiments done with the ANCK10 strain and its derivative

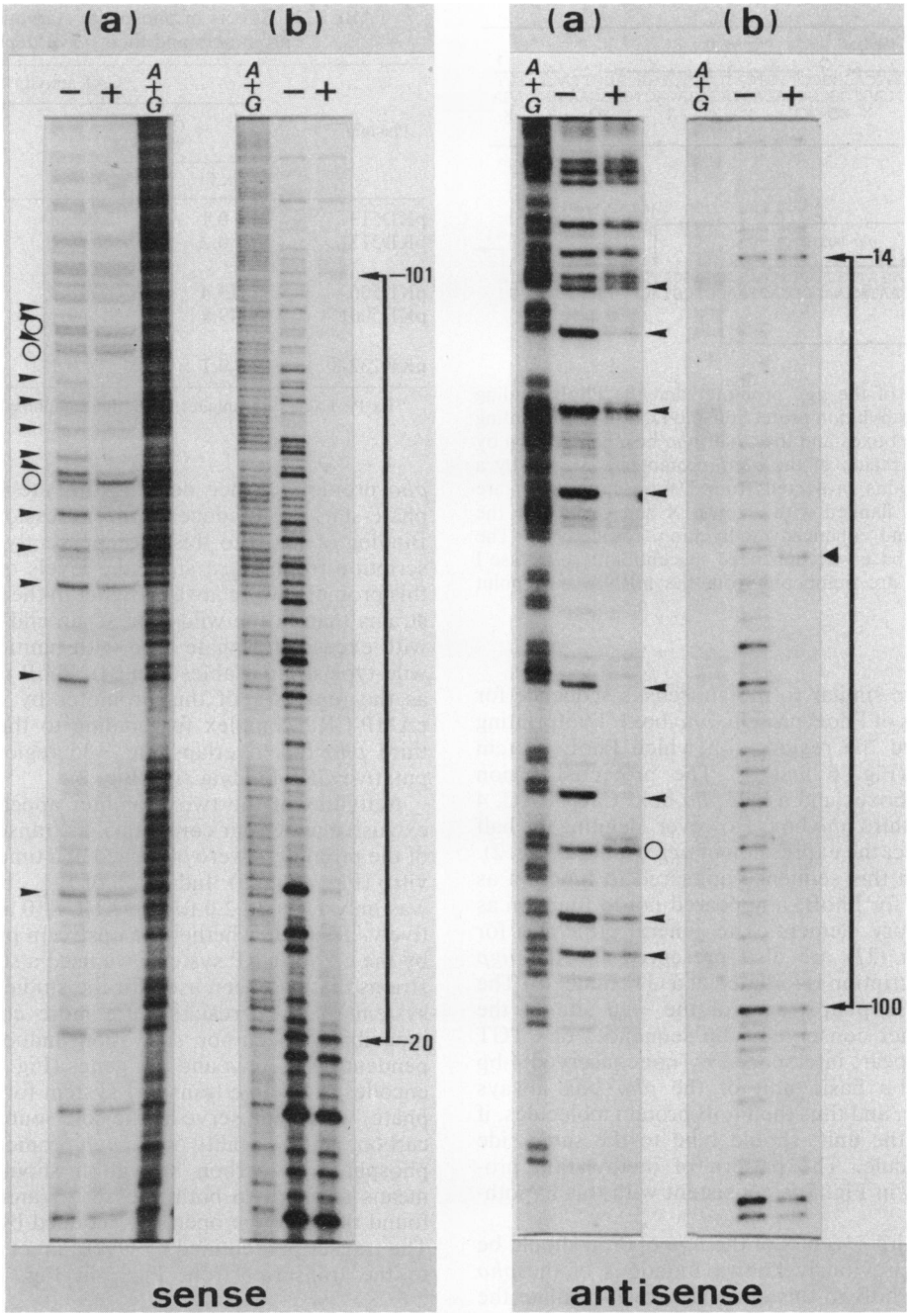

FIG. 6. DNase I and methylation protection of the *ugp* promoter region by PhoB protein. (a) Effects of PhoB protein on methylation of the *ugp* promoter region by dimethyl sulfate. (b) Protection of the *ugp* promoter region by PhoB protein from DNase I digestion. The sense strand probe was the *Pvu*II-*Eco*RI fragment (Fig. 1, line a), and the antisense strand probe was the *Eco*RI-*Pst*I fragment (Fig. 1C, line b). DNA fragments were methylated or digested in the presence (+) or in the absence (−) of PhoB protein. In the A+G lanes, the products of the Maxam-Gilbert A+G reactions of the probe DNAs were analyzed and used as size markers. The DNA bases protected from methylation and those with enhanced methylation are marked with arrowheads and open circles, respectively. The bracketed areas show the regions protected by PhoB protein. The solid triangle shows a base with enhanced susceptibility to DNase I digestion. The numbers are those counted from the start point of the mRNA transcribed under the limiting phosphate conditions.

(Table 4). The upstream promoter activity was induced about fivefold by carbon starvation and the induction required the functions of the *cya* and *crp* genes. Therefore, we conclude that P_{ugp2} is positively regulated by the cAMP-CRP system.

DISCUSSION

The *ugp* operon is known to be induced by phosphate starvation and regulated positively by PhoB protein. In the *ugp* regulatory region we could identify several nucleotide

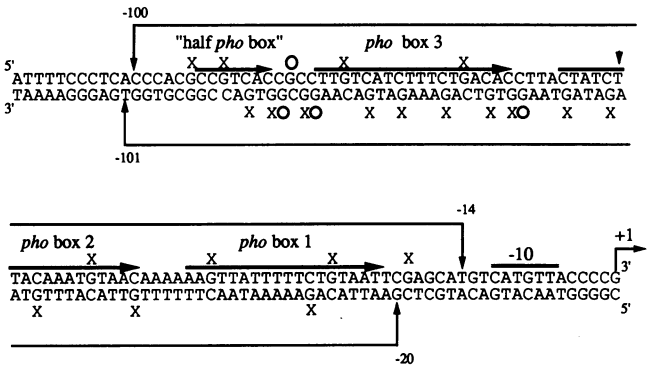

FIG. 7. *pho* boxes of the *ugp* promoter and the PhoB binding regions, as seen by methylation protection and DNase I footprinting experiments. The *pho* boxes and the "half *pho* box" are shown by arrows, and the −10 region of the *ugp1* promoter is shown by a double line. The regions protected from DNase I digestion are indicated by brackets flanked with arrows. X and O indicate the bases with reduced and enhanced methylation, respectively. The arrowhead shows the base with enhanced susceptibility to DNase I digestion. The bases are numbered from the mRNA start point of P_{ugp1}.

sequences that were similar to the consensus sequence for the recognition sites of PhoB protein (*pho* box). Footprinting experiments showed the region with which PhoB protein actually interacts (Fig. 6 and 7). The protected region includes three *pho* boxes and a half *pho* box, CcGTCAC, 4 bp upstream of the third *pho* box. However, deleting the half *pho* box did not affect the expression of *ugp* (Table 2; Fig. 2). Therefore, although this sequence appeared to function as the recognition site for PhoB, it appeared not to function as a *cis*-acting regulatory element. The general principles for the *pho* promoters (27) are also preserved for the *ugp* promoter. (i) Transcription is initiated at a G residue. (ii) The first *pho* box is 10 bp upstream of the −10 site of the promoter. (iii) Rather conserved 7-bp sequences of CTGT CAT repeatedly appear, interspaced by nonconserved 4-bp sequences. The 7-bp basic unit of the *pho* box always appears every 11 bp, and thus the PhoB protein molecules, if they interact with the unit, should bind to the same side of the DNA molecule. The pattern of methylation protection summarized in Fig. 7 is consistent with this hypothesis.

The role of the third *pho* box of the *ugp* operon should be different from the previously known functions of the *pho* boxes. Binding of PhoB to this site may down-regulate the

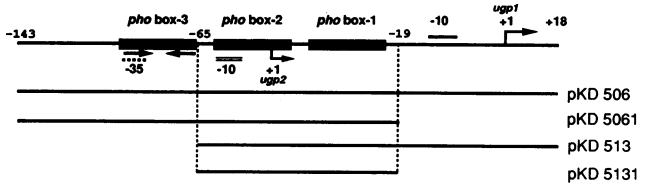

FIG. 8. Construction of pKD5131 and pKD5061. The *Sma*I-*Taq*I fragment of pKD513 and pKD506 (Table 2) were isolated, and the staggered ends were converted to blunt ends by T4 DNA polymerase. They were inserted into the *Sma*I site of pKK232-8, giving pKD5131 and pKD5061. These plasmids lack the −10 region of the *ugp1* (*pho*) promoter. The nucleotide sequence of this region is shown in Fig. 2.

TABLE 3. Effects of phosphate starvation on the two *ugp* promoters and their *pho*B dependence

Plasmid	CAT activity[a] (U/OD_{450})			
	ANCK10 (wild type)		ANCH1 [Δ(*phoB-phoR*)]	
	Ex Pi	Li Pi	Ex Pi	Li Pi
pKD513	0.1	2,009.8	0.2	0.1
pKD5131	0.2	<0.1	0.4	0.2
pKD506	13.4	718.4	21.8	6.8
pKD5061	49.8	9.1	57.1	26.5
pKK232-8	<0.1	<0.1	<0.1	<0.1

[a] Ex Pi, Excess phosphate; Li Pi, limiting phosphate.

pho promoter, since deleting the site increased the phosphate-starvation-induced *ugp* expression (Table 2; Fig. 3). Binding of PhoB to this site apparently repressed the transcription from P_{ugp2}, since the levels of transcription from this promoter were always higher in the *phoB-phoR* deletion strains than in the wild-type strain and were always higher with excess phosphate than with limiting phosphate in the wild-type strain (Tables 3 and 4). PhoB protein may function as the repressor of this promoter by competing with the cAMP-CRP complex for binding to the same region. The third *pho* box overlaps the −35 region of P_{ugp2} and the putative CRP-binding site (Fig. 2).

Activities of the two promoter appeared to be mutually exclusive under our conditions, as transcripts from only one of the promoters were observed at a time both in vivo and in vitro (Fig. 4 and 5). Induction of P_{ugp2} by carbon starvation was only 1.5- and 2.0-fold in ANCK10 and ANCHI, respectively. To settle whether the upstream promoter is regulated by the cAMP-CRP system, we used a set of nearly isogenic strains that had been used for the studies of the cAMP-CRP system (4). The results were more convincing as to the inducibility by carbon starvation; induction was totally dependent on the *cya* and *crp* genes (Fig. 9). The *ugp* operon encodes the active transport system for *sn*-glycerol-3-phosphate, which can serve as the sole source of phosphate and carbon, and the ability of the *ugp* promoter to be induced by phosphate or carbon starvation should provide the cell means to adapt to both stresses. Wanner (29) had already found that the *ugp* operon is induced by carbon starvation. The transcript identified by Overduin et al. (22) corresponds to the transcript from P_{ugp1} in Fig. 2. In addition, we

TABLE 4. Effects of carbon starvation on the *ugp* promoter

Plasmid	CAT activity[a] (U/OD_{450})			
	ANCK10 (wild type)		ANCHI [Δ(*phoB-phoR*)]	
	Ex Glc	Li Glc	Ex Glc	Li Glc
pKD513	0.1	0.1	0.2	0.7
pKD5131	0.2	0.2	0.4	0.3
pKD506	13.4	17.3	21.8	42.0
pKD5061	49.8	71.5	57.1	120.1
pKK232-8	0.1	0.1	0.1	0.1

[a] Ex Glc, Excess glucose; Li Glu, limiting glucose.

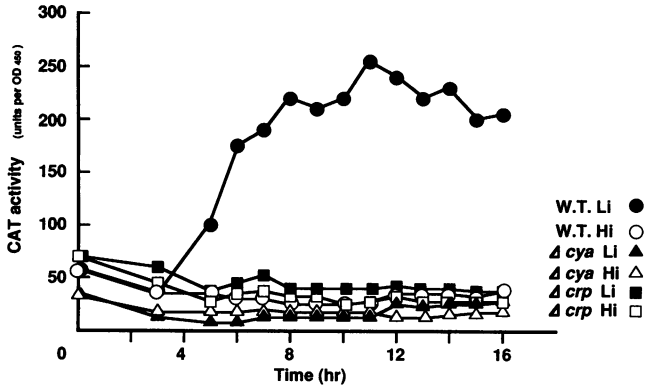

FIG. 9. Effects of the *cya* and *crp* mutations on *ugp2* expression. The activities of the *ugp2* promoter in TP7811 (wild type [W.T.]), TP7860 (Δ*cya*), and TP7839 (Δ*crp*) were measured by assaying the specific activities of CAT in these strains carrying pKD5061 grown in medium with excess or limited carbon at the indicated times after inoculation. Li; Limited carbon conditions, Hi; excess carbon conditions.

uncovered a second transcript from P_{ugp2}, whose expression may depend on the cAMP-CRP complex and be repressed by PhoB.

ACKNOWLEDGMENTS

We thank H. Aiba for the gift of the bacterial strains.

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture, Japan.

REFERENCES

1. **Aiba, H., S. Adhya, and B. de Crombrugghe.** 1981. Evidence for two functional *gal* promoters in intact *Escherichia coli* cells. J. Biol. Chem. **256**:11905–11910.
2. **Amemura, M., H. Shinagawa, K. Makino, N. Otsuji, and A. Nakata.** 1982. Cloning of and complementation tests with alkaline phosphatase regulatory genes (*phoS* and *phoT*) of *Escherichia coli*. J. Bacteriol. **152**:692–701.
3. **Berg, O. G., and P. H. von Hippel.** 1988. Selection of DNA binding sites by regulatory proteins. II. The binding specificity of cyclic AMP receptor protein to recognition sites. J. Mol. Biol. **200**:709–723.
4. **Biville, F., B. Blazy, and N. Guiso.** 1983. Transcription termination factor rho of *Escherichia coli* K-12: some regulatory aspects of its expression and activity. Biochimie **65**:339–344.
5. **Brosius, J.** 1984. Plasmid vectors for the selection of promoters. Gene **27**:151–160.
6. **Brzoska, P., and W. Boos.** 1988. Characteristics of a *ugp*-encoded and *phoB*-dependent glycerophosphoryl diester phosphodiesterase which is physically dependent on the *ugp* transport system of *Escherichia coli*. J. Bacteriol. **170**:4125–4135.
7. **Craig, N. L., and H. A. Nash.** 1984. *E. coli* integration host factor binds to specific sites in DNA. Cell **39**:707–716.
8. **Hong, G. F.** 1982. A systematic DNA sequencing strategy. J. Mol. Biol. **158**:539–549.
9. **Johnson, A. D., B. J. Meyer, and M. Ptashne.** 1979. Interactions between DNA-bound repressors govern regulation by the λ phage repressor. 1979. Proc. Natl. Acad. Sci. USA **76**:5061–5065.
10. **Kasahara, M., K. Makino, M. Amemura, and A. Nakata.** 1989. Nucleotide sequence of the *ugpQ* gene encoding glycerophosphoryl diester phosphodiesterase of *Escherichia coli* K-12. Nucleic Acids Res. **17**:2854.
11. **Kimura, S., K. Makino, H. Shinagawa, M. Amemura, and A. Nakata.** 1989. Regulation of the phosphate regulon of *Escherichia coli*: characterization of the promoter of the *pstS* gene. Mol. Gen. Genet. **215**:374–380.
12. **Kohara, Y., K. Akiyama, and K. Isono.** 1987. The physical map of the whole *E. coli* chromosome: application of a new strategy for rapid analysis and sorting of a large genomic library. Cell **50**:495–508.
13. **Makino, K., H. Shinagawa, M. Amemura, T. Kawamoto, M. Yamada, and A. Nakata.** 1989. Signal transduction in the phosphate regulon of *Escherichia coli* involves phosphotransfer between PhoR and PhoB proteins. J. Mol. Biol. **210**:551–559.
14. **Makino, K., H. Shinagawa, M. Amemura, S. Kimura, A. Nakata, and A. Ishihama.** 1988. Regulation of the phosphate regulon of *Escherichia coli*: activation of *pstS* transcription by PhoB protein in vitro. J. Mol. Biol. **203**:85–95.
15. **Makino, K., H. Shinagawa, and A. Nakata.** 1984. Cloning and characterization of the alkaline phosphatase positive regulatory gene (*phoM*) of *Escherichia coli*. Mol. Gen. Genet. **195**:381–390.
16. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
17. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
18. **Messing, K., R. Crea, and P. H. Seeburg.** 1981. A system for shotgun DNA sequencing. Nucleic Acids Res. **9**:309–321.
19. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. **Morita, T., K. Shigesada, F. Kimizuka, and H. Aiba.** 1988. Regulatory effect of a synthetic CRP recognition sequence placed downstream of a promoter. Nucleic Acids Res. **16**:7315–7332.
21. **Nakata, A., M. Amemura, K. Makino, and H. Shinagawa.** 1987. Genetic and biochemical analysis of the phosphate-specific transport system in *Escherichia coli*, p. 150–155. *In* A. Torriani-Gorini, F. G. Rothman, S. Silver, A. Wright, and E. Yagil (ed.), Phosphate metabolism and cellular regulation in microorganisms. American Society for Microbiology, Washington, D.C.
22. **Overduin, P., W. Boos, and J. Tommassen.** 1988. Nucleotide sequence of the *ugp* genes of *Escherichia coli* K-12: homology to the maltose system. Mol. Microbiol. **2**:767–775.
23. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
24. **Schweizer, H., and W. Boos.** 1984. Characterization of the *ugp* region containing the genes for the *phoB* dependent *sn*-glycerol-3-phosphate transport system of *Escherichia coli*. Mol. Gen. Genet. **197**:161–168.
25. **Schweizer, H., T. Grussenmeyer, and W. Boos.** 1982. Mapping of two *ugp* genes coding for the *pho* regulon-dependent *sn*-glycerol-3-phosphate transport system of *Escherichia coli*. J. Bacteriol. **150**:1164–1171.
26. **Shaw, W. V.** 1975. Chloramphenicol acetyltransferase from chloramphenicol resistant bacteria. Methods Enzymol. **43**:737–743.
27. **Shinagawa, H., K. Makino, M. Amemura, and A. Nakata.** 1987. Structure and function of the regulatory genes for the phosphate regulon in *Escherichia coli*, p. 20–25. *In* A. Torriani-Gorini, F. G. Rothman, S. Silver, A. Wright, and E. Yagil (ed.), Phosphate metabolism and cellular regulation in microorganisms. American Society for Microbiology, Washington, D.C.
28. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19**:259–268.
29. **Wanner, B. L.** 1987. Phosphate regulation of gene expression in *Escherichia coli*, p. 1326–1333. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 2. American Society for

Microbiology, Washington, D.C.
30. **Wanner, B. L., and R. McSharry.** 1982. Phosphate-controlled gene expression in *Escherichia coli* K12 using Mu*dI*-directed *lacZ* fusions. J. Mol. Biol. **158**:347–363.
31. **Yamada, M., K. Makino, M. Amemura, H. Shinagawa, and A. Nakata.** 1989. Regulation of the phosphate regulon of *Escherichia coli*: analysis of mutant *phoB* and *phoR* genes causing different phenotypes. J. Bacteriol. **171**:5601–5606.
32. **Yamada, M., K. Makino, H. Shinagawa, and A. Nakata.** 1990. Regulation of the phosphate regulon of *Escherichia coli*: properties of *phoR* deletion mutants and subcellular localization of PhoR protein. Mol. Gen. Genet. **220**:366–372.