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Dual Transcriptional Regulation of the *Escherichia coli* Phosphate-Starvation-Inducible *psiE* Gene of the Phosphate Regulon by PhoB and the Cyclic AMP (cAMP)-cAMP Receptor Protein Complex

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We have shown that the *Escherichia coli* phosphate-starvation-inducible *psiE* gene is regulated by both phosphate and the carbon source by using both *lacZ* and chloramphenicol acetyltransferase gene (*cat*) fusions. Yet, under all conditions tested, a single transcriptional start site lying 7 bp downstream of a predicted −10 region was revealed by primer extension analysis. DNase I footprinting showed that the PhoB transcriptional-activator protein protects two predicted *pho* boxes lying upstream of and near the −35 promoter region. Similar analysis showed that the cyclic AMP (cAMP)-cAMP receptor protein (cAMP-CRP) complex binds a region that overlaps with the downstream *pho* box. These results, together with measurements of the in vivo *psiE* promoter activity under various conditions, show that expression of the *psiE* gene is under direct positive and negative control by PhoB and cAMP-CRP, respectively.

The phosphate regulon (*pho*) of *Escherichia coli* includes at least 31 genes whose expression is induced under conditions of phosphate starvation and whose products have roles in the transport or degradation of various phosphorus sources. These genes are under the transcriptional control of the PhoB and PhoR proteins, which belong to a large family of two-component regulatory systems that respond to a variety of environmental stimuli (15, 26). PhoB is the transcriptional activator, which acts by binding *pho* box sites immediately upstream of all known *pho* regulon promoters (16, 17). These *pho* boxes comprise two well-conserved 7-bp direct repeats, which are separated by poorly conserved 4-bp spacer sequences, suggesting that PhoB molecules always bind on the same face of the DNA helix (20). PhoR is the histidine protein kinase that activates PhoB by phosphorylation and (presumably) inactivates phosphorylated PhoB (phospho-PhoB) by dephosphorylation in response to the extracellular phosphate concentration (14, 17, 22). Transcriptional activation by phospho-PhoB involves protein-protein interaction with the σ^{70} subunit of RNA polymerase holoenzyme (13).

By using λp*lac*Mu53 phage (3), we had previously identified several *pho* regulon genes, including ones in the *phn* (*psiD*) operon involved in phosphonate utilization (18); the *phoH* (*psiH*) gene, encoding an ATP binding protein with a helicase motif (9, 12); and a gene called *psiE*, the subject of this study. These phosphate-starvation-inducible (*psi*) genes had also been previously found using the Mu d1 transposon (24, 25, 27, 28). More recently, it was shown that the *psiE* gene of *Salmonella enterica* serovar Typhimurium is induced during infection of macrophages (23).

Wanner and coworkers (24, 28) had previously reported that expression of the *psiE′-′lacZ* gene in a *cya* or *crp* mutant was greater than that in the wild-type strain, indicating that *psiE* expression is negatively regulated by the cyclic AMP (cAMP)-cAMP receptor protein (cAMP-CRP) complex. They had also indicated that transcription from the promoter was also positively regulated by PhoB. However, these studies do not answer the following questions. How many promoters are there? How many transcription initiation sites are there? Where do PhoB and cAMP-CRP bind? How does RNA polymerase interact with the promoter(s) in the presence or absence of PhoB and/or cAMP-CRP?

Here we report more-detailed molecular studies on the regulation of the *psiE* gene, including identification of its transcription start site and DNA binding sites for PhoB and cAMP-CRP at the promoter region. Our results show that these proteins directly regulate transcription from only one mRNA initiation site of the *psiE* promoter. Based on the results, we discuss the possible mechanism of transcriptional regulation of the *psiE* gene.

Cloning of the *psiE* promoter region. The *psiE* fusion strain SE5031 used in this study was found among a collection of *E. coli* SE5000 mutants made with λp*lac*Mu53 in an earlier study (9, 18). The strain displayed a strong Lac^+ (dark blue) color on Tris-glucose low-phosphate (LP) agar and a weak Lac^+ (pale blue) color on Tris-glucose high-phosphate (HP) agar containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and kanamycin. β-Galactosidase activities were induced by phosphate starvation of strain SE5031 grown in both Tris-glucose (667 U for LP medium; 90 U for HP medium) and Tris-glycerol (245 U for LP medium; 47 U for HP medium) media. However, the basal level (HP) and the phosphate-starvation-induced level (LP) were 1.9-fold and 2.7-fold higher, respectively, in Tris-glucose

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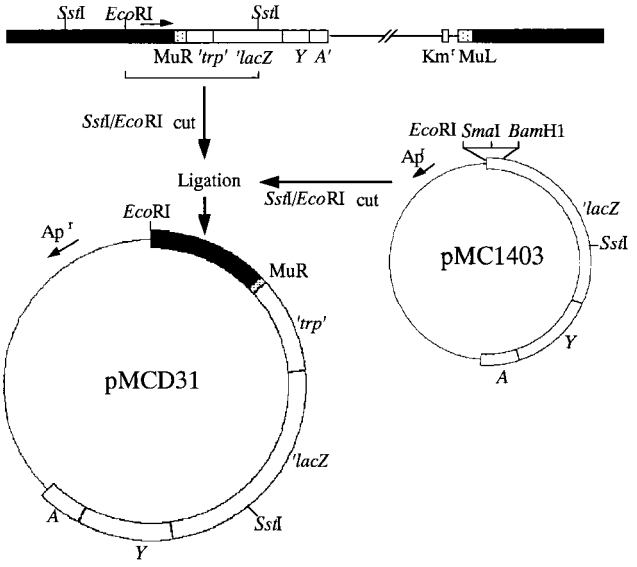

FIG. 1. Strategy for cloning the chromosomal *psiE'-'lacZ* fusion. Black boxes, *E. coli* chromosomal DNA; thin lines, lambda or vector plasmid DNA; dotted boxes, Mu sequences; open boxes, *'trp'-'lacZYA'*; arrow above the map, orientation of the *psiE* gene with respect to the λp*lac*Mu53 insertion. Ap^r, ampicillin resistance; Km^r, kanamycin resistance.

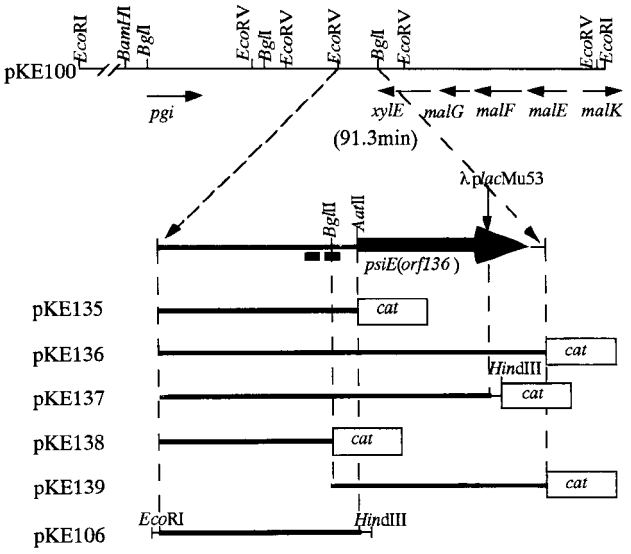

FIG. 2. Plasmids carrying the *psiE* regions. The restriction map and genes near the *psiE* gene in pKE100 are shown above. Vertical arrow, site of the λp*lac*Mu53 insertion; thick arrow and black boxes, *psiE* gene and *pho* boxes, respectively. Only relevant restriction enzyme sites are indicated. Boxes labeled *cat* show the gene encoding CAT.

medium than in Tris-glycerol medium. These results suggest that expression of the *psiE* gene is regulated by both phosphate and the carbon source. Since it is known that the amount of the cAMP-CRP complex increases under glucose starvation conditions such as Tris-glycerol medium, these results suggested that expression of *psiE* is negatively regulated by cAMP-CRP independent of the phosphate concentration.

In order to further study the *psiE* promoter, we cloned the chromosomal *psiE'-'lacZ* fusion. Southern hybridization using the *Bam*HI-*Sst*I *lacZ* fragment of pMC1403 (5) as a probe revealed a single 12-kb *Eco*RI-*Sst*I chromosomal fragment in SE5031 (Fig. 1). This fragment was gel purified, ligated with similarly digested pMC1403, and transformed into SE5000. A Lac^+ transformant showed higher β-galactosidase activity on Tris-glucose LP medium than on Tris-glucose HP medium. It carried recombinant plasmid pMCD31, which contains the 12-kb insert, suggesting that pMCD31 carries a functional *psiE* promoter.

These results indicated that λp*lac*Mu53 had integrated into the *psiE* gene about 8.6 kb from a chromosomal *Eco*RI site (Fig. 1). Restriction analysis revealed that the *psiE*::λp*lac*Mu53 fusion in SE5031 lies near the carboxyl terminus-encoding region of *psiE*. By using the 12-kb *Eco*RI-*Sst*I fragment in pMCD31 as a probe, two phages in an *E. coli* genomic λ library (11) that carry chromosomal DNA near 91.3 min (λ632 and λ633) were found to hybridize. As expected, hybridization was also detected in phages containing *lacZ* or *trp* sequences as these are present within the fusion sequences. The 15-kb *Eco*RI fragment of λ633 was subcloned into pUC9 (9), yielding pKE100 (Fig. 2).

Two other *psiE'-'lacZ* fusions were previously shown by DNA sequence analysis to lie within the same gene (19), which corresponds to an open reading frame (*orf136*) of unknown function adjacent to the *xylE* gene (6). The *psiE* gene corresponds to the *yjbA* locus at 91.3 min (2). However, it was unknown whether the *psiE* gene was translated since the earlier study employed only transcriptional fusions (24, 28). Because the λp*lac*Mu53 phage generates *lacZ*-protein fusions, the above results show that the *psiE* gene is also translated. Based on its hydrophobic characteristics, the *psiE* gene product may be a membrane protein.

Deletion analysis of the *psiE* promoter. To define the functional promoter in vivo, we constructed five plasmids carrying different amounts of the *psiE* chromosomal region transcriptionally fused to *cat* (Fig. 2). These plasmids were introduced into ANCK10 (wild-type) and ANCH1 [Δ(*phoB-phoR*)] strains (8). The promoter activities were assessed by measuring chloramphenicol acetyltransferase (CAT) activities in transformants after growth in Tris-glucose LP and HP media (Table 1). Both wild-type and Δ*phoB* mutant cells carrying pKE135, pKE136, and pKE137 had high basal levels of CAT activity in comparison with cells carrying the vector control, pKK232-8 (4). The CAT activities of these wild-type transformants were induced 10-fold or more by phosphate starvation. This induction was not seen in Δ*phoB* mutant transformants. In contrast, transformants carrying pKE138 or pKE139, like transformants carrying the vector produced barely detectable levels of CAT. These data show that expression of *psiE* by phosphate starvation requires PhoB; however a substantial basal level is expressed indepen-

TABLE 1. *psiE* promoter activity

Plasmid	CAT activity in indicated medium for strain[a]:			
	ANCK10		ANCH1 [Δ(*phoB-phoR*)]	
	LP	HP	LP	HP
pKE135	310	25	24 (7)	28 (8)
pKE136	240	22	21 (9)	19 (7)
pKE137	320	23	25 (7)	24 (8)
pKE138	<1	<1	<1	<1
pKE139	<1	<1	<1	<1
pKK232-8	<1	<1	<1	<1

[a] Cells were grown overnight in Tris-glucose medium and were assayed. LP and HP, 0.1 and 2 mM phosphate concentrations, respectively. The activities of CAT are expressed in nanomoles of 5-thio-2-nitrobenzoate liberated per minute per optical density unit of the cell culture at 450 nm. CAT activities measured in cells grown in the presence of 2 mM cAMP are shown in parentheses.

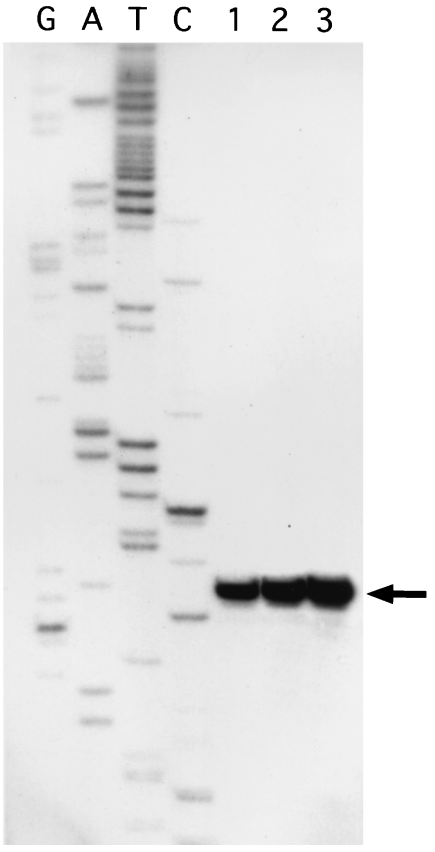

FIG. 3. Transcription initiation site of *psiE*. SE5000 carrying pKE106 (Fig. 2) was grown in Tris-glycerol HP medium or Tris-glucose HP or LP medium to early stationary phase, and RNA was isolated as described previously (1). The 23-base M13 forward sequencing primer (Gibco-BRL, Bethesda, Md.) was labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. The end-labeled primer was hybridized to RNA extracted from SE5000 cells carrying pKE106 grown in Tris-glycerol HP (lane 1), Tris-glucose HP (lane 2), or Tris-glucose LP medium (lane 3). Primer extension was done using rTth reverse transcriptase for 10 min at 60°C. Arrow, position of primer extension products. Lanes G, A, T, and C, sequence ladders of plasmid pKE106 obtained by using the same primer by the dideoxy chain termination method (21). The transcription start site on the DNA sequence is shown in Fig. 5.

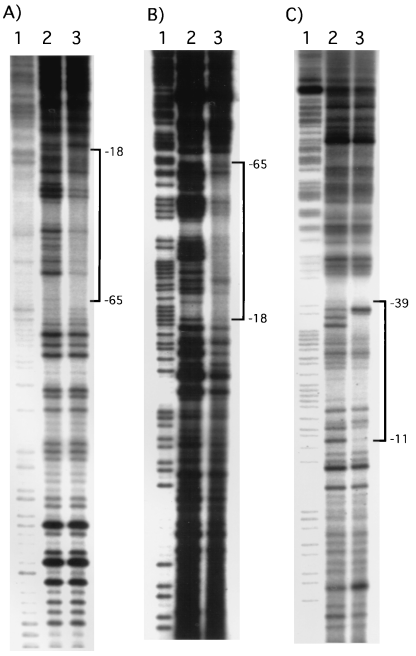

FIG. 4. DNase I footprinting of the *psiE* promoter region. The top and bottom strands were labeled at *Eco*RI and *Hin*dIII sites of pKE106 (Fig. 2) by [γ-^{32}P]ATP using T4 polynucleotide kinase. These fragments were then gel purified. (A) Footprinting on the top strand with the PhoB protein. PhoB and PhoR1084 proteins were purified and used to prepare phospho-PhoB protein as described previously (17). PhoR1084 (4 pmol) and PhoB (4 pmol) were used. (B) Footprinting on the bottom strand with the PhoB protein. PhoR1084 (4 pmol) and PhoB (4 pmol) were used. (C) Footprinting on the bottom strand with CRP (1 pmol) and cAMP (10 μM). Lanes 1, G>A ladders from Maxam-Gilbert sequencing of the probe DNA; lanes 2, products of DNase I digestion in the absence of proteins; lanes 3, products of DNase I digestion in the presence of proteins. The regions protected from DNase I digestion are indicated by brackets, with nucleotide positions labeled from the transcription initiation site (+1).

dently of the phosphate concentration. Moreover, these data show that sequences flanking the *Bgl*II site are required for both PhoB-dependent and -independent expression (Fig. 2; see Fig. 5).

As described in the previous section, expression of the chromosomal *psiE'-'lacZ* fusion gene occurred at higher levels in Tris-glucose medium than in Tris-glycerol medium, independent of the phosphate concentration. We therefore tested for involvement of the cAMP-CRP complex in the control of *psiE* expression. Expression of the *psiE'-'cat* fusions (pKE135, pKE136, and pKE137) was reduced in Δ*phoB* strain transformants when transformants were grown in the presence of cAMP in both Tris-glucose LP and HP media (Table 1), suggesting that expression of these constructs is also negatively regulated by cAMP-CRP. Previously, it was shown that expression of the *psiE* gene was negatively regulated by the *crp* and *cya* genes (24, 28).

Determination of the *psiE* transcription initiation site. Primer extension analysis revealed a single band of the same size in all cells tested (Fig. 3). Each position of the gel corresponding to the bands was cut out, and the radioactivities were measured. When the radioactivity of the band derived from cells grown in Tris-glycerol HP medium was defined as 1 (Fig. 3, lane 1), the radioactivities of those derived from the cells grown in Tris-glucose HP and LP media were approximately 3 and 8, respectively (Fig. 3, lanes 2 and 3). These results are consistent with the promoter activities of the chromosomal *psiE'-'lacZ* gene as shown from in vivo data and indicate that transcription of the *psiE* gene initiates from the same start site under the various conditions examined. The data also suggest that transcription from this start site is positively regulated by PhoB and negatively regulated by cAMP-CRP. Seven base pairs upstream from the transcription initiation site, there is a DNA sequence (TATAca) similar to the consensus sequence for the −10 regions of *E. coli* promoters (see Fig. 5). Further-

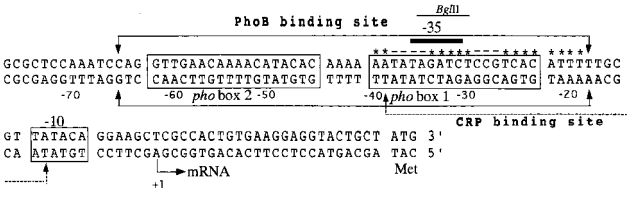

FIG. 5. Schematic presentation of the PhoB and cAMP-CRP binding sites in the *psiE* promoter. Boxes, *pho* boxes and the −10 region of *psiE*; brackets, regions protected from DNase I digestion in the presence of PhoB and cAMP-CRP; asterisks, positions identical with the consensus sequence for the cAMP-CRP binding site (7); thick line above *pho* box 1, putative −35 sequence. The *Bgl*II restriction enzyme site, the transcription initiation site, and a translation initiation codon (Met) are indicated.

more, 18 bp upstream from the −10 region, a putative −35 sequence (TaGAtc) exists. In general, PhoB-regulated promoters lack a consensus −35 sequence (15, 26).

Determination of PhoB and cAMP-CRP binding sites within the *psiE* regulatory region. To determine whether PhoB or cAMP-CRP directly binds the *psiE* promoter, we performed DNase I footprinting experiments as described previously (17). The PhoB protein protected the DNA segment spanning nucleotides −18 to −65 from DNase I digestion on both strands (Fig. 4A and B). This region includes two *pho* boxes (Fig. 5). The upstream one, *pho* box 2, agrees poorly with the consensus *pho* box, CTGTCATAA(T)AT(A)CTGTCAC(T). Tandemly arranged *pho* boxes in the *pstS* and *ugp* promoter regions have also been reported (8, 10). In the *ugp* promoter, the *pho* boxes agree poorly with the consensus *pho* box. These data suggest that multiple *pho* boxes might be required for the binding of PhoB to regions containing weakly conserved *pho* boxes such as the *psiE* promoter.

The binding of the cAMP-CRP complex to the bottom strand was assayed. The cAMP-CRP complex bound to the region from −11 to −39, which includes a sequence similar to the consensus for the cAMP-CRP binding site (Fig. 4C and 5). cAMP-CRP binding enhanced the DNase I digestion at positions −36A and −37T from the transcription initiation site. Interestingly, the cAMP-CRP binding site covers the putative −35 region and overlaps the downstream *pho* box (Fig. 5).

Based on these results, we propose that the amount of the *psiE* transcript, which is initiated from only one start site, is versatilely regulated by culture conditions. Under high-glucose and LP conditions, where the amount of PhoB is large and that of cAMP-CRP is small, PhoB activates transcription. Under high-glucose and HP conditions, transcription from the typical −35 and −10 promoter (Fig. 5) by RNA polymerase alone would be the predominant mode of expression, because of low concentrations of cAMP-CRP and PhoB. Transcription from the promoter would be blocked by cAMP-CRP under low-glucose and HP conditions, because of a high concentration of cAMP-CRP and low concentration of PhoB. Under low-glucose and LP conditions, where both proteins are abundant, PhoB and cAMP-CRP would compete for binding to the *psiE* promoter region, which contains overlapping sequences recognized by these proteins. The in vivo data using the chromosomal *psiE′-′lacZ* gene suggested that, under these conditions, PhoB binding to the *pho* boxes predominates over cAMP-CRP binding to its site, which overlaps the *pho* box (Fig. 5). There may be a possibility that RNA polymerase interacts synergistically with PhoB but not with cAMP-CRP. Our data strongly suggest that transcription initiation from the *psiE* gene is regulated by dual regulatory systems that respond to environmental stimuli.

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