Promoter Elements Required for Positive Control of Transcription of the *Escherichia coli uhpT* Gene

TOD J. MERKEL, DIANE M. NELSON, CHRISTINE L. BRAUER,† AND ROBERT J. KADNER*

*Department of Microbiology, School of Medicine, and Molecular Biology Institute,
University of Virginia, Charlottesville, Virginia 22908*

The *uhpABCT* locus of *Escherichia coli* encodes the transport system which allows the cell to accumulate a variety of sugar phosphates in unaltered form. The expression of *uhpT*, the gene encoding the transport protein, is regulated by the *uhpABC* gene products. The UhpA protein is required for expression; its deduced amino acid sequence shows that it belongs to a subfamily of bacterial transcription regulators including NarL, DegU, and FixJ. Members of this subfamily have an amino-terminal phosphorylation domain characteristic of so-called two-component regulators, such as OmpR, CheY, PhoB, and NtrC, and a carboxyl-terminal domain conserved among many transcriptional activators, including LuxR and MalT. The major sequence elements in the *uhpT* promoter that are needed for *uhpT* expression were investigated. Northern (RNA) hybridization analysis showed that the *uhpT* transcript was only present in cells induced for UhpT transport activity. The start site of transcription was identified by primer extension. Comparison of the regions upstream of the *uhpT* transcription start site in *E. coli* and *Salmonella typhimurium* suggested the presence of four sequence elements that might be involved in promoter function: a typical −10 region, a short inverted repeat centered at −32, a long inverted repeat centered at −64, and a cyclic AMP receptor protein-binding sequence centered at −103. Deletion and linker substitution mutations in the promoter demonstrated that the presence of the cyclic AMP receptor protein-binding site resulted in about an eightfold increase in promoter activity and that the −64, −32, and −10 elements were essential for promoter function. In vivo titration of transcriptional activator UhpA by the intact or mutant promoters on multicopy plasmids identified the −64 element as the UhpA-binding site. The two halves of the −64 inverted repeat did not contribute equally to promoter function and did not have to be intact for UhpA titration. The sequence recognized by UhpA is predicted to be 5′-GGCAAAACNNNGAAA.

The growth of *Escherichia coli* on various phosphorylated sugars requires the active transport system encoded by the *uhpT* gene (3, 7, 17). Although the UhpT transport system mediates the entry of a number of different sugar phosphates, the synthesis of the transporter is induced only by exogenous glucose 6-phosphate (Glu6P) or 2-deoxyglucose 6-phosphate (21, 42). The large pool of Glu6P normally present inside a cell does not trigger induction. The expression of *uhpT* is dependent on the function of the *uhpABC* regulatory genes and the cyclic AMP receptor protein (CAP) (6, 36, 40, 41). Genetic studies have shown that UhpA is absolutely required for the expression of UhpT (29, 41). The overproduction of UhpA from multicopy plasmids results in high-level, constitutive expression of UhpT even in the absence of UhpB and UhpC functions, consistent with the hypothesis that UhpA is a transcriptional activator (29, 40, 41). In the haploid state, UhpB and UhpC are required for the regulated expression of *uhpT*. Previous studies have supported a model in which membrane-localized UhpC binds external Glu6P and transmits a signal to UhpB, which subsequently converts UhpA to a form capable of activating transcription at the *uhpT* promoter (41).

The proposed mechanism of action of the Uhp regulatory proteins is supported by their sequence similarities to other regulatory factors. The UhpA and UhpB proteins are members of families of two-component regulatory proteins, which also include OmpR-EnvZ, CheY-CheA, PhoB-PhoR,

and NtrC-NtrB. Several members of these families have been shown to act through protein phosphotransfer reactions, by which the transfer of a phosphate moiety from a sensor protein kinase to its cognate receptor protein allows the latter protein to activate transcription from specific promoters (34). Analysis of their deduced amino acid sequences has indicated the existence of distinct subfamilies of these transcriptional activator proteins (25, 34). All of these proteins share a 100- to 120-residue amino-terminal domain which contains highly conserved aspartate residues at and near the site of phosphorylation and which is closely related in sequence and possibly in structure to chemotaxis protein CheY (33). Members of the NtrC subfamily contain, in addition to this phosphorylation domain, a large central activation domain containing consensus nucleotide-binding motifs and a carboxyl-terminal domain necessary for specific DNA-binding activity (34). NtrC appears to act as an enhancer-binding protein, since it activates transcription at σ^{54}-dependent promoters in a manner independent of the orientation or distance of its DNA-binding sites with respect to the transcription start site.

A second subfamily of transcriptional activators includes OmpR, PhoB, and VirG (34). These proteins consist of the amino-terminal phosphorylation domain and a DNA-binding domain, which mediates the binding of the phosphorylated forms of these proteins to sites in the activator sequences upstream of their σ^{70}-dependent promoters (34). A third subfamily of transcriptional activator proteins includes UhpA, NarL, DegU, and FixJ (34); they possess the amino-terminal phosphorylation domain, a short variable region, and a carboxyl-terminal region that contains a predicted helix-turn-helix motif. The carboxyl-terminal region is also

* Corresponding author.
† Present address: Food and Drug Administration, Rockville, MD 20850.

TABLE 1. Bacterial strains, plasmids, and phages used in this study

Strain, plasmid, or phage	Relevant characteristics	Reference or source
Strains		
JM101	F' *traD36 lacI*^{q} Δ(*lacZ*) *M15 proAB*$^+$ *supE thi* Δ(*lac-proAB*)	4
JM109	F' *traD36 lacI*^{q} Δ(*lacZ*)*M15 proAB*$^+$/*recA1 endA1 gyrA96 thi hsdR17* (r_K^- m_K^+) *supE44 relA1* Δ(*lac-proAB*)	4
XL-1 Blue	F' *zzf::*Tn*10 proAB*$^+$ *lacI*^{q} Δ(*lacZ*)*M15*/*recA1 endA1 gyrA96 thi hsdR17* (r_K^- m_K^+) *supE44 relA1 lac*	30
SE5000	*araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR recA*	43
Plasmids		
pKS$^+$	Bluescript Ap cloning vector	Stratagene
pKS$^+$:P$_T$*Rsa*I	*Rsa*I fragment of *uhpT* promoter in pKS$^+$	This work
pKS$^+$:P$_T$*Alu*I	*Alu*I fragment of *uhpT* promoter in pKS$^+$	This work
pRS415	Ap *lacZ* fusion plasmid	31
pRS415:P$_T$*Rsa*I	*uhpT* promoter-*lacZ* operon fusion plasmid	This work
pRS415:P$_T$*Alu*I	*uhpT* promoter-*lacZ* operon fusion plasmid	This work
Phages		
λRZ5	Ap *lacZ* fusion vector	24
λRZ5-P$_T$*Rsa*I	*uhpT-lacZ* operon fusion phage	This work

conserved in transcriptional activators that are not subject to phosphorylation control, such as LuxR and MalT (12).

Catabolite repression also affects *uhpT* expression, which is reduced in the absence of the *cya* and *crp* gene products or in the presence of repressing effectors, such as glucose or Glu6P (2, 10). Both positive regulation and negative regulation of *uhpT* expression appear to operate at the level of transcription, since UhpT transport activity generally correlates with the levels of β-galactosidase encoded by *uhpT-lacZ* transcriptional fusions (28, 29).

The locations of the major sequence elements affecting *uhpT* promoter activity are identified here by mutational analysis. Deletions that enter the promoter region from either end and linker replacements that substitute a restriction site linker for various 6-bp segments of the wild-type promoter were constructed, and their effect on promoter activity was determined. In vivo assays for UhpA binding to the promoter region led to a presumptive identification of the region of the promoter necessary for this activity. The regulation of *uhpT* transcription by UhpA and CAP requires their binding to specific sites in the *uhpT* promoter region. The functional elements in this promoter differ from those in other positively controlled systems, particularly with respect to the spacing of the promoter elements relative to the start of transcription. The significance of these differences is discussed below.

MATERIALS AND METHODS

Strains and recombinant DNA techniques. The bacterial strains and plasmids used in this study are listed in Table 1. All DNA manipulations were performed by standard genetic and molecular techniques (17, 19, 30). DNA sequencing was performed on single-stranded templates derived from Bluescript plasmid pKS$^+$ (Stratagene, La Jolla, Calif.) by use of the Sequenase 2.0 kit from United States Biochemical Co. (Cleveland, Ohio).

DNA-modifying enzymes and kits were purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.), Boehringer Mannheim Biochemicals (Indianapolis, Ind.), and New England BioLabs, Inc. (Beverly, Mass.). The GeneAmp polymerase chain reaction (PCR) reagent kit was purchased from Perkin-Elmer Corp. (Norwalk, Conn.). All enzymes and kits were used in accordance with the recom-

mendations of the suppliers. [α-^{32}P]dCTP, [γ-^{32}P]dCTP, and [α-^{32}P]UTP were purchased from Amersham Corp. (Arlington Heights, Ill.). Oligonucleotides were purchased from Synthecell Corp. (Rockville, Md.). Chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Northern (RNA) hybridization analysis. Whole-cell RNA was prepared from *E. coli* as described by Aiba et al. (1). Total cellular RNA (10 μg per lane) and a [^{32}P]UTP-labelled RNA ladder were electrophoresed on a 0.8% agarose gel containing 1.2% formaldehyde as described by Maniatis et al. (17) and transferred to a Nytran membrane (Schleicher and Schuell, Keene, N.H.) as described by the manufacturer. The 1,092-bp *Pvu*II-*Eco*RV restriction fragment from *uhpT* and the 1,200-bp *Pst*I-*Eco*RV restriction fragment from *ompA* were extracted from agarose gels and labelled by nick translation with the Bethesda Research Laboratories nick translation kit. The labelled restriction fragments were used to probe the Nytran membranes in accordance with the manufacturer's directions.

Primer extension analysis. Primer extension analysis was performed with a synthetic oligonucleotide which anneals to the *uhpT* transcript between residues 3742 and 3771. Total RNA (100 μg) and about 5 ng of oligonucleotide were mixed and incubated at 65°C for 20 min and then at room temperature for 2 h. Avian myeloblastosis virus reverse transcriptase (20 U) was added, and the mixture was incubated at 42°C for 90 min. The samples were extracted with phenol-chloroform, precipitated with ethanol, resuspended in water, and resolved by electrophoresis on a 6% polyacrylamide-7 M urea gel. A standard sequencing reaction was performed with the same oligonucleotide as primer on a single-stranded DNA template containing the relevant portion of *uhpT*, and the resulting sequencing ladder was electrophoresed next to the primer extension products.

Generation of promoter deletions. The 377-bp *Rsa*I and the 629-bp *Alu*I fragments bearing the *E. coli uhpT* promoter were subcloned into the *Sma*I site of pKS$^+$. The 5' and 3' deletions were generated by linearizing the plasmids with *Eco*RI and *Bam*HI, respectively, and digesting them with *Bal*31 or ExoIII nuclease. After treatment with the Klenow fragment of DNA polymerase I to generate blunt ends, the plasmids were ligated in the presence of an *Nco*I-*Eco*RI-*Nco*I linker (5'-CCATGGAATTCCATGG) for the 5' dele-
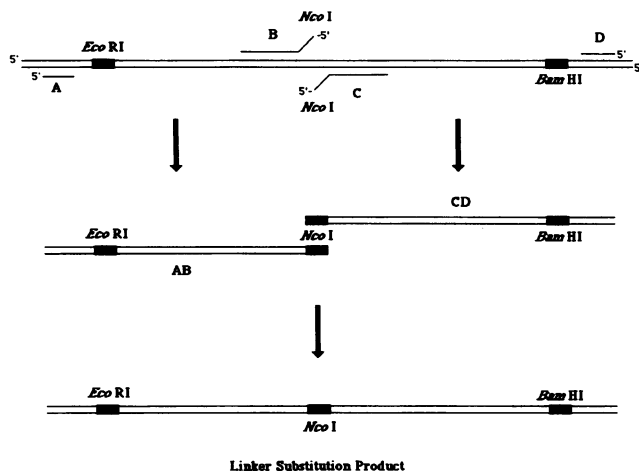
FIG. 1. Construction of linker substitutions by PCR. NcoI linker substitutions were constructed by a modification of the method of Ho et al. (13). Oligonucleotides B and C carry at their 5' ends the NcoI restriction sequence, which is followed by a sequence which anneals to the opposite strands of the uhpT promoter flanking the 6 bp to be replaced. Oligonucleotides A and D anneal to vector pKS⁺ sequences beyond the EcoRI and BamHI sites, respectively, and prime replication towards the uhpT promoter insert. The 5' half of each linker substitution was generated by PCR with pKS⁺:P_TRsaI as a template and oligonucleotides A and B as primers. The 3' half of each linker substitution was generated by PCR with the same template and oligonucleotides C and D as primers. The linker substitutions were constructed by ligating the EcoRI-NcoI-digested AB products to the NcoI-BamHI-digested CD products.

tions or an NcoI-BamHI-NcoI linker (5'-CCATGGATC CATGG) for the 3' deletions.

Construction of linker substitution mutations. The linker substitutions replacing residues −46 to −51 and −51 to −56 were constructed by ligating the EcoRI-NcoI restriction fragments from the 5' deletions to −45 and −51 (see Fig. 3) with the NcoI-BamHI restriction fragments from the 3' deletions to −52 and −57, respectively, into EcoRI-BamHI-digested pKS⁺. The remaining linker substitutions were generated by PCR-mediated mutagenesis. Sharf et al. (26) and Erlich (9) described a procedure for attaching specific restriction site sequences to the ends of PCR products by adding these sequences to the 5' end of the oligonucleotide used as a primer; Ho et al. (13) described a method for introducing specific insertions or deletions into PCR products. Combining these methods allowed the generation of specific linker substitutions in the uhpT promoter (Fig. 1).

Generation of uhpT promoter-lacZ fusions. The RsaI and AluI restriction fragments bearing the wild-type uhpT promoter, the uhpT promoter deletions, or the linker substitutions were subcloned as EcoRI-BamHI restriction fragments from pKS⁺ into EcoRI-BamHI-digested pRS415 (31) to generate transcriptional fusions to lacZ. The fusions were transferred to phage λRZ5 as described by Roland et al. (24), and limiting amounts of the recombinant phage were used to lysogenize strain SE5000. The β-galactosidase activities of at least six separate lysogens were measured, and the isolates with the lowest activity were chosen to represent single lysogens.

β-Galactosidase assays. β-Galactosidase assays were performed as described by Miller (19) with the following modifications. Several independent isolates of strain SE5000 lysogenized with each λuhpT-lac phage were cultured in 200 μl of minimal medium supplemented with glycerol in a

96-well microtiter plate at 37°C to an optical density at 415 nm (OD_{415}) of 0.10 in a thermostat-controlled Thermomax microplate reader (Molecular Devices, Menlo Park, Calif.), with frequent agitation. Under these conditions, the cultures exhibited logarithmic growth with a doubling time of about 40 min. Glu6P was added to 0.25 mM for 40 min before the cells were treated with chloroform-sodium dodecyl sulfate. The permeable cells were mixed with Z buffer (19) and 2 mM o-nitrophenyl-β-D-galactopyranoside, and the change in the OD_{415} at 37°C was measured over a 5-min period on the Thermomax 96-well microplate reader. The rate of enzyme reaction was normalized for cell density.

UhpA titration assays. To measure sensitivity to fosfomycin, we grew cells of strain SE5000 or XL-1 harboring pKS⁺ derivatives with uhpT promoter mutations to early log phase in minimal medium supplemented with glycerol. When the cultures reached an OD_{650} of 0.05, 100 μl of each culture was plated in F-top agar (19) on minimal glycerol plates and three antibiotic disks with 12 μl of fosfomycin (2.5 mg/ml) were placed on each plate. After overnight incubation at 37°C, the diameters of the clear zones of cell killing around each antibiotic disk were measured.

To measure growth on fructose 6-phosphate (Fru6P), we streaked cells of SE5000 or XL-1 with pKS⁺ derivatives bearing uhpT promoter mutations on minimal agar plates with Fru6P (2 mg/ml) as the sole carbon source and scored growth after 3 days at 37°C.

RESULTS

Regulation and 5' end of the uhpT transcript. The uhpT-specific transcripts were detected by Northern hybridization analysis, using as a probe, the 1,092-bp PvuII-EcoRV fragment that hybridizes between residues 3870 and 4961 (nucleotide coordinates are as listed in reference 14). A major RNA transcript of approximately 1,550 nucleotides was present in Glu6P-induced cells (Fig. 2B). The size of this transcript corresponds to the length of the uhpT coding region up to the typical rho-independent terminator sequence past the gene (nucleotides 5086 to 5110). A minor species of about 4,300 nucleotides was present in induced cells. This longer species may have resulted from transcriptional readthrough beyond the uhpT terminator because it did not hybridize to probes from uhpA, uhpB, or uhpC (data not shown). No hybridization was detected to RNA from uninduced cells, although hybridization to an ompA-specific probe demonstrated that equivalent amounts of RNA were present.

The 5' end of the uhpT transcript was identified by primer extension with, as a primer, a synthetic oligonucleotide that hybridizes within the uhpT coding sequence between 70 and 99 nucleotides from the start of translation. The major transcript started at nucleotide 3637, 31 nucleotides upstream of the start of translation (Fig. 2A). This transcript was present in Glu6P-induced cells but not in uninduced cells. Cells carrying multicopy uhpA exhibited constitutive expression of uhpT and contained a uhpT transcript with the same start site as the transcript in Glu6P-induced cells. Minor bands with 5' ends at nucleotides 3652, 3653, and 3657 were also seen.

Sequence of the uhpT promoter. The region upstream of the uhpT transcription start site contains several sequence elements that might play a role in promoter function (Fig. 3). A typical −10 sequence (5'-TACAATG), which matches the consensus sequence for this region in σ⁷⁰-dependent promoters at six of seven positions, occurs 6 nucleotides upstream of the transcription start site. In place of a consensus −35 hexamer, a palindromic sequence, 5'-TCAGGCCTGA, is
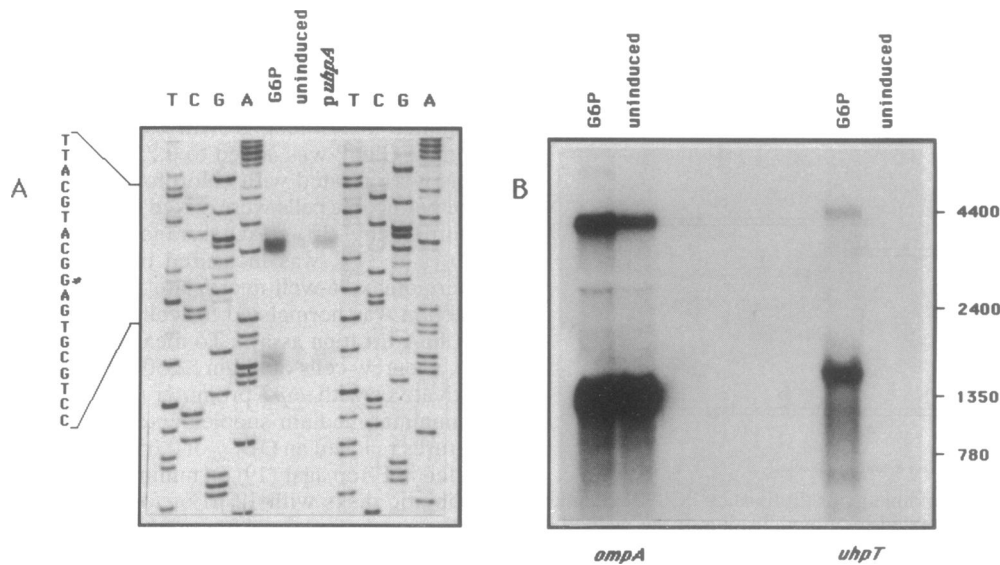
FIG. 2. Northern hybridization analysis and determination of the 5' end of the *uhpT* transcript. (A) A ^{32}P-labelled oligonucleotide primer complementary to the *uhpT* coding strand was annealed to 100 µg of total RNA from uninduced and Glu6P-induced cells and from cells bearing *uhpA* on a multicopy plasmid (p*uhpA*). The primer was extended with avian myeloblastosis virus reverse transcriptase, and the products were electrophoresed on a polyacrylamide-urea gel. DNA sequencing ladders were prepared by use of the same primer on a recombinant pKS$^+$ DNA template. The start site of transcription is indicated with an asterisk. The sequence represents the noncoding strand. (B) Equal amounts of total RNA (10 µg per lane) from uninduced and Glu6P-induced cells were electrophoresed on a formaldehyde-agarose gel and transferred to a Nytran membrane. The RNA was probed with ^{32}P-labelled restriction fragments internal to *ompA* or *uhpT*. The sizes of the transcripts were determined by comparison with ^{32}P-labelled RNA size markers, which were electrophoresed next to the cellular RNAs (data not shown). Sizes are given on the right in base pairs.

centered at −32.5 relative to the major start site of transcription. A large region of imperfect dyad symmetry extends from −82 to −45 and is centered at −64. A region that matches the consensus sequence for a CAP-binding site at 11 of 14 positions extends from −114 to −94 and is centered at −103.5. The 5' half of this putative CAP-binding site overlaps the end of the *uhpC* coding sequence.

A comparison of the nucleotide sequences of the *uhpT* promoter region in *E. coli* and the homologous region in *Salmonella typhimurium* revealed that strongly conserved sequences are interspersed with regions of considerable variability (14). The −10 element is conserved intact. Although 6 of the 10 positions in the −32 element are maintained, the four substitutions in the *S. typhimurium* promoter reduce the region of dyad symmetry from 10 bp in *E. coli* to only 4 bp in *S. typhimurium*. The −64 element is strongly conserved, with only one substitution between −79 and −45. That substitution is not in a symmetry-related position. There are two substitutions at nonconserved positions in the CAP-binding site. The regions between the CAP-binding site and the start of the −64 element at residue −80 and between −30 and −18 show extensive variability. The strong conservation of the promoter sequence elements between *S. typhimurium* and *E. coli* suggests their functional importance.

Deletions in the *uhpT* promoter. To examine the role of these elements in promoter function, we constructed deletion mutations that removed portions of the *uhpT* promoter from either side. Two promoter-bearing fragments were used to ensure that all relevant sequences were included; one was the 377-bp *RsaI* fragment from −218 to +159, and the other was the 629-bp *AluI* fragment from −399 to +230. As described in Materials and Methods, the deletions were generated by digestion with exonuclease *Bal*31 or ExoIII, and the deletion junctions were ligated with an oligonucleotide that contained *NcoI-EcoRI-NcoI* sites for the upstream

deletions or *NcoI-BamHI-NcoI* sites for the downstream deletions. The use of these linkers allowed the subcloning of both upstream (5') and downstream (3') deletions as *EcoRI-BamHI* fragments into similarly digested pRS415 to generate *uhpT-lacZ* transcriptional fusions. The incorporation of the *NcoI* sites at each deletion junction was designed to allow linker-scanning mutagenesis by combining matching upstream and downstream deletions at their common *NcoI* sites. A total of 27 deletions from the upstream direction and 17 deletions from the downstream direction were obtained with unique endpoints between −120 and +1 (Fig. 3). Numerous deletions with endpoints outside this region were also obtained.

Promoter activity of deletions. *uhpT* promoter activity was determined by measuring the β-galactosidase activities of *uhpT-lacZ* operon fusions driven by the deletion variants. The fusions were made single copy by homologous recombination between the plasmids and phage λRZ5. This phage contains the distal portions of *lacZ* and *bla* oriented so that two homologous crossover events with plasmid pRS415 derivatives result in the formation of phage with a *bla*$^+$ gene and an intact *lacZ* gene driven by the *uhpT* promoter present on the plasmid (24). The recombinant phage were used to obtain single lysogens in a *uhp*$^+$ Δ*lac* strain, SE5000. Multiple lysogens were identified by the fact that their enzyme activities were two or three times higher than those of other isolates. Levels of β-galactosidase were determined after growth of several independent isolates in minimal medium with glycerol as a carbon source in the absence or presence of inducing levels of Glu6P.

All of the deletions that removed only sequences outside the *uhpT* promoter region displayed the same level and regulation of β-galactosidase activity as did the intact *RsaI* or *AluI* fragments, showing that sequences upstream of −120 or downstream of +1 do not contribute substantially to

```
        -100          -80          -60          -40          -20          +1
St  AAGCGTGATACACCTCACCTTTTGCGCTGAATGGGGCAAAACTAAGAAATTTTCCCGGTTTTGCCTGGA-GCTGTCGCAGGCCACTTTTCCTGTGGATTTTTACAATGC-TGCC
Ec  AAGCGTGATGCATCTCACCTTTTCACTTCATATCCGGCAAAACTAAGAAATTTTCCAGGTTTTGCCTGGACGCTATCTCAGGCCTGATTTGCTGCTGATTTTTACAATGCATGCC

CAP-BINDING SITE                    -64           -32          -10
```

Columns: **A** Promoter Activity (% of wild-type) — **B** Growth on Fru6P — **C** Fosfomycin Sensitivity

A. 5' Deletions

Endpoint	A	B	C
-115	100	-	0
-105	11	-	0
-94	15	-	0
-79	13	-	0
-72	16	-	10
-71	0	+	8
-70	0	++	10
-69	0	++	11
-58	0	++	10
-51	0	++	10
-45	0	++	12
-34	0	++	10
-2	0	++	10

B. 3' Deletions

Endpoint	A	B	C
-1	93	-	0
-5	91	-	3
-18	0	-	0
-29	0	-	0
-31	0	-	0
-36	0	-	0
-42	0	-	6
-46	0	-	4
-49	0	+	5
-52	0	+	10
-57	0	+	9
-59	0	+	10
-62	0	++	9
-68	0	++	9

C. Linker Substitutions

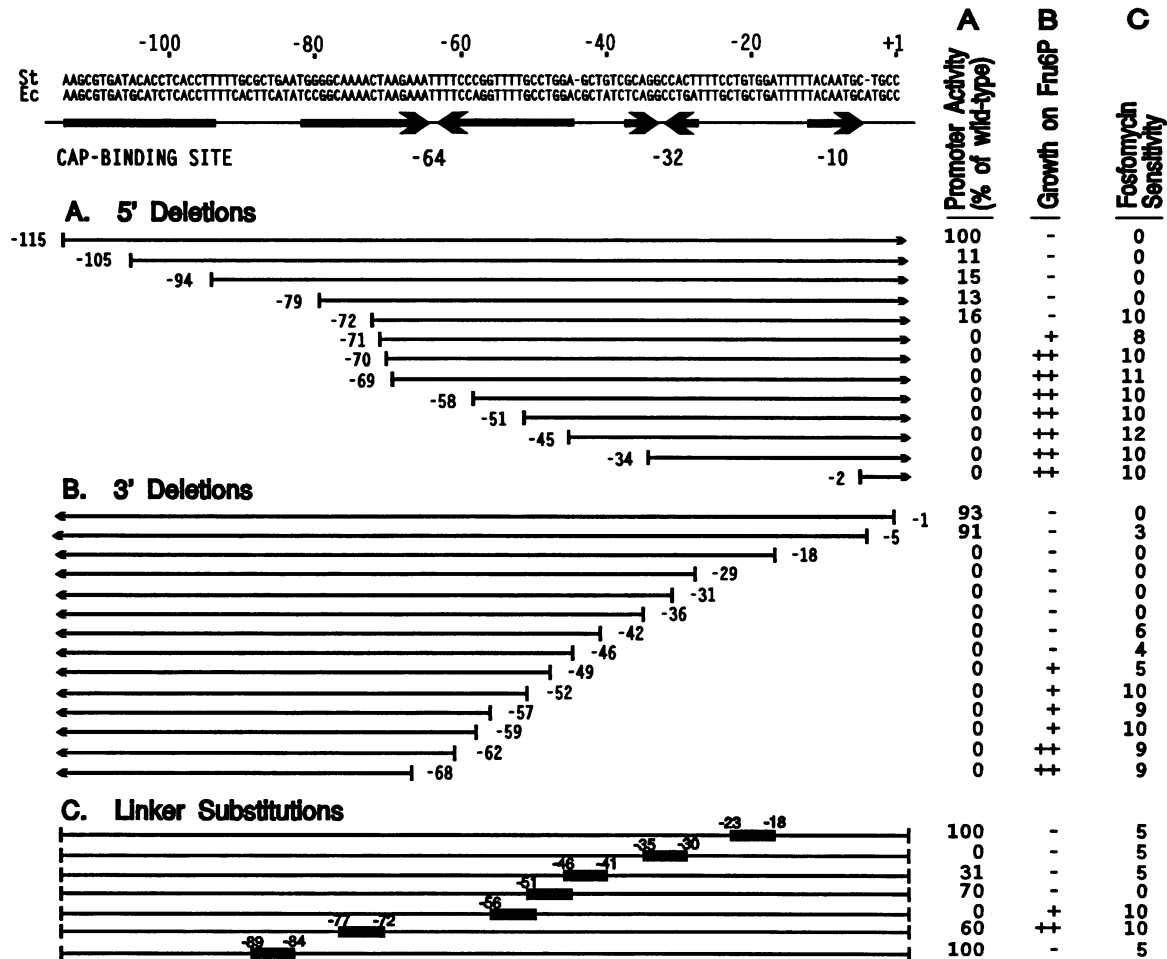| Substitution	A	B	C
-23 -18	100	-	5
-35 -30	0	-	5
-46 -41	31	-	5
-51	70	-	0
-56	0	+	10
-71 -72	60	++	10
-89 -84	100	-	5

FIG. 3. Deletion and linker substitution mutations in the *uhpT* promoter. The nucleotide sequences of the *uhpT* promoters from *S. typhimurium* (St) and *E. coli* (Ec) are shown on the top lines. The three lower sections define the extent of sequences retained in deletion mutations that remove portions of the *E. coli uhpT* promoter from the 5' or 3' direction and the locations of linker substitution mutations in which 6 bp of a wild-type sequence is replaced with NcoI sequence 5'-CCATGG. (Column A) Promoter activities. The β-galactosidase activities of the *uhpT-lacZ* fusions present in single-copy lysogens were determined as described in Materials and Methods. The values shown represent the averages of three to five experiments. All values are reported relative to wild-type levels. In all cases, the uninduced level of activity was negligible. (Column B) Titration of UhpA: growth on Fru6P. The titration of chromosomally encoded UhpA by the *uhpT* promoter on multicopy plasmids results in a greatly decreased ability to utilize Fru6P as the sole carbon source. The growth of cells carrying the indicated *uhpT* promoter mutations on Fru6P (2 mg/ml) is indicated as follows: ++, maximal growth, comparable to that of the vector-bearing strain; +, reduced growth; and -, no growth after 3 days, as occurs when the intact *uhpT* promoter region is present in the same plasmid vector. (Column C) Titration of UhpA: fosfomycin sensitivity. The Uhp⁻ phenotype resulting from the titration of UhpA, as measured in column B, is also manifested as resistance to fosfomycin. The sensitivity of cells carrying the indicated *uhpT* promoter mutations on multicopy plasmids to fosfomycin is determined as the diameter of the zone of growth inhibition, in millimeters, around the fosfomycin disk minus the diameter of the disk. Strains with the intact *uhpT* promoter showed a mean inhibition zone diameter of 10 mm.

promoter activity (data not shown). Even the removal of upstream *uhp* sequences to −115, the start of the CAP-binding site, had no effect on promoter activity under inducing conditions (Fig. 3, column A). Longer deletions, with endpoints at −105 within the CAP-binding site, at −94, removing the CAP-binding site, or at −79 and −72, removing about half of the upstream half of the −64 inverted repeat, displayed reduced promoter activity, ranging from 11 to 16% the wild-type level. All upstream deletions that extended beyond −71 were inactive. These results indicated that the −64 element is required for promoter function and that the CAP-binding sequence confers about eightfold stimulation of *uhpT* promoter activity.

Deletions from the downstream direction that ended within the transcribed segment or those that entered the promoter region with endpoints at +2 and −4 displayed wild-type levels of activity (Fig. 3, column A). The deletion to −18 and all deletions beyond that point were inactive, confirming the anticipated requirement for the −10 region for promoter function. None of the promoter deletions displayed any activity under noninducing conditions, suggesting that there are no sites for negatively acting transcription regulators.

Linker substitution mutagenesis. To examine the role of the various internal elements within the promoter, we constructed seven linker insertions either by matching upstream and downstream deletions at their NcoI sites or by using oligonucleotide-directed PCR. In either case, the linker substitution mutagenesis resulted in the precise replacement of 6 bp of wild-type sequence with the NcoI recognition

sequence, 5'-CCATGG, while maintaining proper spacing of the flanking elements. The activity of these mutant promoters was determined with the *uhpT-lacZ* reporter system described above.

The *Nco*I linker substitution for residues −18 to −23, between the −10 and −32 elements, resulted in wild-type levels of activity and normal regulation, whereas the linker substitution for positions −30 to −35 was completely inactive (Fig. 3). These results demonstrated the importance of the −32 element and the lack of recognition of at least some of the base pairs between the −32 and −10 elements. The linker substitution for positions −84 to −89, between the CAP-binding site and the −64 element, had no effect on promoter function. The linker substitution for positions −41 to −46 resulted in about 30% wild-type levels of activity, indicating that sequences at the downstream end of the −64 element or those in the region between the −32 and −64 elements contribute significantly to promoter activity.

The importance of the downstream half of the −64 element was shown by the complete loss of promoter function in the linker substitution for positions −51 to −56. The symmetrically related linker substitution for positions −72 to −77 in the upstream half of the −64 inverted repeat resulted in about 60% wild-type promoter activity. The linker substitution for positions −46 to −51 in the downstream half of the −64 element resulted in about 70% wild-type promoter activity; however, this substitution only affected two residues in converting the wild-type sequence GCCTGG to CCATGG.

In vivo titration of UhpA. We showed previously that the presence of the *uhpT* promoter region on a multicopy plasmid resulted in substantially reduced expression of the chromosomal copy of the *uhpT* gene (41). This phenomenon was manifested as poor growth on Fru6P when the chromosome carried the wild-type *uhp* locus or as reduced levels of β-galactosidase when the chromosome carried a *uhpT-lacZ* fusion. This decreased expression was attributed to the titration of a chromosomally encoded UhpA activator by the multiple copies of the UhpA-binding site in the plasmid-borne *uhpT* promoter, because it was overcome when *uhpA* was also present in multiple copies (41). To identify the *uhpT* promoter sequences responsible for this phenotype, we transferred plasmids carrying the various promoter deletion and linker substitution mutations into a *uhp*⁺ *recA* strain. The effect of these plasmids on *uhpT* expression was determined by measuring the growth of the strains on minimal agar plates with Fru6P as carbon source.

Transformants of strain XL-1 carrying the intact *uhpT* promoter-bearing *Rsa*I or *Alu*I fragments in plasmid pKS⁺ displayed substantially reduced growth on Fru6P relative to the strain with the plasmid vector. Plasmids with upstream deletions to −115, −105, −94, or −79 conferred the same strong Uhp⁻ phenotype as that seen with the intact promoter region, showing that this response did not require CAP binding (Fig. 3, column B). Deletions that removed part of the upstream half of the −64 element, with endpoints at −72 and −71, showed strong and weaker Uhp⁻ growth phenotypes, respectively. Further deletion to −70 or beyond resulted in a strong Uhp⁺ phenotype, indicating that these deletions failed to compete for the binding of UhpA.

Similar activities were seen with the deletions of the *uhpT* promoter from the downstream direction. Plasmids bearing deletions that removed downstream sequences up to position −46 strongly competed for UhpA, as indicated by their Uhp⁻ phenotype. Deletions that removed part of the 3' half of the −64 element, with endpoints at −49, −52, −57, or −59, demonstrated reduced but significant competition for

UhpA, whereas deletions with endpoints beyond −62 failed to compete for UhpA and had a Uhp⁺ phenotype. These results showed that competition for UhpA required only the sequence element centered at −64 and that substantial UhpA binding, as measured by this assay, was seen with either half of this element alone.

A similar dependence on the −64 element was seen when in vivo UhpA titration was assayed by the response to fosfomycin under conditions in which the entry of this antibiotic occurs through the UhpT transport system. In this assay, the entire −64 dyad symmetry region from −79 to −42 was required to confer the Uhp⁻ phenotype of fosfomycin resistance (Fig. 3, column C). This observation is somewhat different from the growth response.

The effect of the *Nco*I linker substitutions on UhpA titration was determined. The Uhp⁻ growth phenotype was conferred by all of the promoters with substitutions outside the −64 element: at −18 to −23, −30 to −35, −41 to −46, and −84 to −89. The ability to compete for UhpA was eliminated by the substitutions for −51 to −56 and −72 to −77, which are within the −64 element, confirming the importance of the −64 element for UhpA binding. The fosfomycin sensitivity assay gave intermediate results, indicating that several of the substitutions outside the −64 element reduced UhpA binding.

DISCUSSION

The sequence of the *uhpT* promoter region suggested the possible presence of several functional elements, which have been studied here by genetic analysis. The entire intergenic region between the end of *uhpC* and the start of transcription of *uhpT* contributes to promoter function. There appear to be four elements in the promoter: a typical −10 region, an element centered at −32 which could be involved in RNA polymerase binding, a large inverted repeat centered at −64 which appears to be the site of UhpA binding, and a CAP-binding sequence centered at −103. Deletions or linker insertions have shown that the CAP-binding site is needed for maximal expression and that the −64, −32, and −10 elements are each absolutely required for promoter function.

Most CAP-binding sites have the consensus sequence TGTGA in their upstream half but a weaker match to this consensus sequence in the downstream half. In *uhpT*, the GTGA sequence is present in both halves. The one difference from the consensus sequence is the T→C change at the first position. It is possible that this divergence from the consensus sequence is constrained by the fact that that position is part of the codon for the last amino acid in UhpC. Note that the TGA of the upstream half of the CAP-binding site is the termination codon for *uhpC*. Deletions that removed all *uhp* sequences upstream of the CAP-binding site had no effect on *uhpT* promoter function, indicating that the DNA bending induced by the CAP protein does not serve to bring specific upstream DNA sequences into the proximity of the RNA polymerase or UhpA. Deletion of the CAP-binding sequence reduced promoter function to 10 to 15% wild-type activity. The residual activity was not susceptible to catabolite repression elicited by the addition of glucose to the growth medium, indicating that the CAP-binding site plays a role in reducing *uhpT* expression in the presence of effective carbon sources (18). Since high levels of Glu6P elicit catabolite repression, it is likely that catabolite repression functions in the Uhp system to reduce the expression of the UhpT transporter rather than to allow preferential utilization of glucose as a carbon source. This feedback is

required to prevent the growth inhibition and lethality that result from high-level transport of sugar phosphates via UhpT (32).

A comparison of CAP-activated promoters reveals that the distance between the CAP-binding site and the start of transcription varies between promoters and, at some promoters, an additional protein is required for transcriptional activation (6). When acting alone, CAP can activate transcription from at least three different positions; i.e., when its binding site is centered at −41.5, as in the *galP1* promoter, at −61.5, as in the *lac* promoter, and at ca. −70.5, as in the *malT* promoter (11, 16, 37). There are no naturally occurring promoters in which the CAP-binding site is centered beyond −70.5, except for those which require additional activating proteins (6). The CAP-binding site is centered at −93 in the *araBAD* promoter and at −76 in the *malE* promoter. The activation of transcription at these promoters requires, in addition to CAP, the activating proteins AraC and MalT, respectively (5). The action of CAP bound at −103 in the *uhpT* promoter is thus expected to require the participation of UhpA as a transcriptional activator. Experiments are in progress to define further the mechanism of CAP action at the *uhpT* promoter.

The −64 element appears to be the only region required for in vivo UhpA binding. The two assays for measuring UhpA titration in vivo yielded a different minimum binding site, with respect to whether both halves of the inverted repeat were required or whether either half alone would suffice. This discrepancy may reflect the different levels of *uhpT* expression needed to yield a positive response in these assays. When the entire −64 element was intact on a multicopy plasmid, chromosomal *uhpT* expression was very low, resulting in poor growth on Fru6P and resistance to fosfomycin. When both halves of the −64 element were deleted, *uhpT* expression was fully inducible, resulting in full growth on Fru6P and sensitivity to fosfomycin. When either half of the −64 element was present on the plasmid, cells exhibited partially reduced growth on Fru6P but full sensitivity to fosfomycin. This phenotype is expected when the expression of the chromosomal *uhpT* gene is reduced to an intermediate level, because killing by fosfomycin requires the uptake of a lower amount of substrate than is needed for growth on Fru6P. This result suggests that either half of the −64 element can bind UhpA, but not as well as the intact element. On the basis of these results and comparisons of the sequences of the two halves of the inverted repeat in *E. coli* and *S. typhimurium*, it is likely that the sequence recognized by UhpA includes 5′-GGCAAAACNNNGAAA. Several of the deletion derivatives were completely inactive at promoter function but still showed full UhpA-binding activity, suggesting that the titration is not due to competition for other components of the transcription initiation complex. It also seems that the integrity of the downstream half of the −64 inverted repeat is more important for promoter activity than is that of the upstream half, although both probably contribute equally to UhpA binding. Analysis of the effect of individual point mutations throughout the promoter and of the locations of UhpA-DNA contacts is needed for a more precise definition of the extent and role of the UhpA-binding sites.

Sequence similarities within the amino-terminal domain place UhpA in the family of regulatory proteins which includes OmpR, CheY, PhoB, and NtrC (25, 34), whereas sequence relatedness in the carboxyl-terminal domain indicates that UhpA is a member of the UhpA-LuxR family of transcriptional activators (12, 35). Although homologies in

the carboxyl-terminal domains of the activator family of proteins allows the definition of at least three major subfamilies, one cannot conclude that all members of any subfamily show similar mechanisms of transcriptional activation. There is considerable variation in the locations of the probable binding sites for members of the UhpA-LuxR family of activators relative to the start site of transcription. This variability may suggest that they act in different manners. UhpA and LuxR bind immediately upstream of the RNA polymerase-binding site in *uhpT* and *luxICDABE* (*luxI-E*) promoters, respectively (27), while DegU binds 300 bp upstream of the start site of the *degQ* promoter (20). Some members of this family activate multiple promoters, and their putative binding sites are located at different distances from the transcription start site. For example, LuxR activates transcription of both *luxI-E* and *luxR* by binding to a single site approximately 40 bp upstream of the *luxI-E* transcription start site but 110 bp upstream of the *luxR* start site (27). Some activators have multiple binding sites within the same target promoter. There are two potential binding sites for NarL in the *narG* promoter, centered at −77.5 and −194.5, although it is not clear that the downstream gene is required for activation (15). MalT binds cooperatively to multiple sites in MalT-dependent promoters (22, 23). Several activators require additional proteins for full function. MalT requires the cyclic AMP-CAP complex to activate *malE* and *malK* (38); LuxR also requires the cyclic AMP-CAP complex to transcribe *luxR* (8); and NarL requires the FNR protein at the *narG* promoter (15). However, these same proteins can activate other promoters without the participation of additional factors. LuxR alone activates transcription of the *luxI-E* operon, and MalT alone activates the *pulC* and *pulA* promoters (27, 39). Members of the UhpA-LuxR family contain a predicted helix-turn-helix domain, and the specific binding of MalT to target sequences in promoter DNA has been demonstrated (22). It remains to be seen whether these structurally related transcription factors act similarly to stimulate RNA polymerase binding or action.

Sequences between the CAP-binding site and the −64 element and between the −32 and −10 elements appear to be nonessential, as shown by their evolutionary variability and their ability to tolerate nucleotide substitutions without a loss of function. These regions probably play an important role in maintaining the proper spacing between these elements, as changes in the lengths of these spacer regions resulted in the total loss of promoter activity (18).

The function of the −32 element is currently unknown. Although 6 of the 10 bp of this element are conserved between *E. coli* and *S. typhimurium*, the 10-bp length of dyad symmetry seen in *E. coli* is not conserved. Only 4 bp are in an inverted repeat orientation in *S. typhimurium*. However, this element is essential for promoter function, since replacement of this sequence with the *Nco*I linker completely eliminated promoter activity. Studies are under way to identify the sites of binding to the *uhpT* promoter of UhpA, RNA polymerase, and any other proteins present in cell extracts.

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. **Alper, M. D., and B. N. Ames.** 1978. Transport of antibiotics and metabolite analogs by systems under cyclic AMP control: positive selection of *Salmonella typhimurium cya* and *crp* mutants. J. Bacteriol. **133:**149–157.
3. **Biggin, M. D., T. J. Gibson, and G. F. Hong.** 1983. Buffer-gradient gels and ³⁵S label as an aid to rapid DNA sequence determination. Proc. Natl. Acad. Sci. USA **80:**3963–3965.
4. **Bullock, W. O., J. M. Fernandez, and J. M. Short.** 1987. XL-1 Blue: a high-efficiency plasmid transforming *recA Escherichia coli* strain with beta-galactosidase selection. BioTechniques **5:**376–379.
5. **Collado-Vides, J., B. Magasanik, and J. D. Gralla.** 1991. Control site location and transcriptional regulation in *Escherichia coli*. Microbiol. Rev. **55:**371–394.
6. **de Crombrugghe, B., S. Busby, and H. Buc.** 1984. Cyclic AMP receptor protein: role in transcription activation. Science **224:**831–838.
7. **Dietz, G. W.** 1976. The hexose phosphate transport system of *Escherichia coli*. Adv. Enzymol. **44:**237–259.
8. **Dunlap, P. V., and E. P. Greenberg.** 1988. Control of *Vibrio fischeri lux* gene transcription by a cyclic AMP receptor protein-LuxR protein regulatory circuit. J. Bacteriol. **170:**4040–4046.
9. **Erlich, H. E.** 1989. PCR technology: principles and applications for DNA amplification. Stockton Press, New York.
10. **Ferenci, T., H. L. Kornberg, and J. Smith.** 1971. Isolation and properties of a regulatory mutant in the hexose phosphate transport system of *Escherichia coli*. FEBS Lett. **13:**133–136.
11. **Gaston, K., A. Bell, A. Kolb, H. Buc, and S. Busby.** 1990. Stringent spacing requirements for transcription activation by CRP. Cell **62:**733–743.
12. **Henikoff, S., J. C. Wallace, and J. P. Brown.** 1990. Finding protein similarities with nucleotide sequence databases. Methods Enzymol. **183:**111–132.
13. **Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. Pease.** 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene **77:**51–59.
14. **Island, M. D., B.-Y. Wei, and R. J. Kadner.** 1992. Structure and function of the *uhp* genes for the sugar phosphate transport system in *Escherichia coli* and *Salmonella typhimurium*. J. Bacteriol. **174:**2754–2762.
15. **Li, S. F., and J. A. DeMoss.** 1988. Location of sequences in the *nar* promoter of *Escherichia coli* required for regulation by Fnr and NarL. J. Biol. Chem. **263:**13700–13705.
16. **Mandecki, W., and M. H. Caruthers.** 1984. Mutants of the *lac* promoter with large insertions and deletions between the CAP binding site and the −35 region. Gene **31:**263–267.
17. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. **Merkel, T. J., and R. J. Kadner.** Unpublished data.
19. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. **Msadek, T., F. Kunst, A. Klier, and G. Rapoport.** 1991. DegS-DegU and ComP-ComA modulator-effector pairs control expression of the *Bacillus subtilis* pleiotropic regulatory gene *degQ*. J. Bacteriol. **173:**2366–2377.
21. **Pogell, B. M., B. R. Maity, S. Frumkin, and S. Shapiro.** 1966. Induction of an active transport system for glucose-6-phosphate in *Escherichia coli*. Arch. Biochem. Biophys. **116:**406–415.
22. **Raibaud, O., D. Vidal-Ingigliardi, and E. Richet.** 1989. A complex nucleoprotein structure involved in activation of transcription of two divergent *Escherichia coli* promoters. J. Mol. Biol. **205:**471–485.
23. **Richet, E., D. Vidal-Ingigliardi, and O. Raibaud.** 1991. A new mechanism for coactivation of transcription initiation: reposi-

24. **Roland, K. L., C. Liu, and C. L. Turnbough.** 1988. Role of the ribosome in suppressing transcriptional termination at the *pyrBI* attenuator of *Escherichia coli* K-12. Proc. Natl. Acad. Sci. USA **85:**7149–7153.
25. **Ronson, C. W., B. T. Nixon, and F. M. Ausubel.** 1987. Conserved domains in bacterial regulatory proteins that respond to environmental stimuli. Cell **49:**579–581.
26. **Scharf, C. J., G. T. Horn, and H. E. Erlich.** 1986. Direct cloning and sequence analysis of enzymatically amplified genomic sequences. Science **223:**1076–1078.
27. **Shadel, G. S., and T. O. Baldwin.** 1991. The *Vibrio fischeri* LuxR protein is capable of bidirectional stimulation of transcription and both positive and negative regulation of the *luxR* gene. J. Bacteriol. **173:**568–574.
28. **Shattuck-Eidens, D. M., and R. J. Kadner.** 1981. Exogenous induction of the *Escherichia coli* hexose phosphate transport system defined by *uhp-lac* operon fusions. J. Bacteriol. **148:**203–209.
29. **Shattuck-Eidens, D. M., and R. J. Kadner.** 1983. Molecular cloning of the *uhp* region and evidence for a positive activator for expression of the hexose phosphate transport system of *Escherichia coli*. J. Bacteriol. **155:**1062–1070.
30. **Silhavy, T. J., M. L. Berman, and L. W. Enquist.** 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. **Simons, R. W., F. Houman, and N. Kleckner.** 1987. Improved single and multicopy *lac*-based cloning vectors for protein and operon fusions. Gene **53:**85–96.
32. **Stephens, C. M., P. Murphy, and R. J. Kadner.** Unpublished data.
33. **Stock, A. M., J. M. Mottonen, J. M. Stock, and C. E. Schutt.** 1989. Three-dimensional structure of CheY, the response regulator of bacterial chemotaxis. Nature (London) **337:**745–749.
34. **Stock, J. B., A. J. Ninfa, and A. M. Stock.** 1989. Protein phosphorylation and regulation of adaptive responses in bacteria. Microbiol. Rev. **53:**450–490.
35. **Stout, V., A. Torres-Cabassa, M. R. Maurizi, D. Gutnick, and S. Gottesman.** 1991. RcsA, an unstable positive regulator of capsular polysaccharide synthesis. J. Bacteriol. **173:**1738–1747.
36. **Ullmann, A., and A. Danchin.** 1983. Role of cyclic AMP in bacteria. Adv. Cyclic Nucleotide Res. **15:**1–53.
37. **Ushida, C., and H. Aiba.** 1990. Helical phase dependent action of CRP: effect of the distance between the CRP site and the −35 region on promoter activity. Nucleic Acids Res. **18:**6325–6330.
38. **Vidal-Ingigliardi, D., and O. Raibaud.** 1991. Three adjacent binding sites for cAMP receptor protein are involved in the activation of the divergent *malEp-malKp* promoters. Proc. Natl. Acad. Sci. USA **88:**229–233.
39. **Vidal-Ingigliardi, D., E. Richet, and O. Raibaud.** 1991. Two MalT binding sites in direct repeat. A structural motif involved in the activation of all the promoters of the maltose regulons in *Escherichia coli* and *Klebsiella pneumoniae*. J. Mol. Biol. **218:**323–334.
40. **Weston, L. A., and R. J. Kadner.** 1987. Identification of Uhp polypeptides and evidence for their role in exogenous induction of the sugar phosphate transport system of *Escherichia coli* K-12. J. Bacteriol. **169:**3546–3555.
41. **Weston, L. A., and R. J. Kadner.** 1988. Role of *uhp* genes in expression of the *Escherichia coli* sugar-phosphate transport system. J. Bacteriol. **170:**3375–3383.
42. **Winkler, H. H.** 1970. Compartmentation in the induction of the hexose-6-phosphate transport system of *Escherichia coli*. J. Bacteriol. **101:**470–475.
43. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.

tioning of an activator triggered by the binding of a second activator. Cell **66:**1185–1195.