Protein Phosphorylation Affects Binding of the *Escherichia coli* Transcription Activator UhpA to the *uhpT* Promoter

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Expression of the *Escherichia coli* sugar phosphate transporter UhpT is induced by extracellular glucose 6-phosphate through a transmembrane signaling process dependent on the sensor kinase UhpB and the UhpT homolog, UhpC. These proteins are thought to regulate the phosphorylation of the transcription activator, UhpA. To examine the effect of protein phosphorylation on the binding of UhpA to target sequences in the *uhpT* promoter region, the UhpA protein was overexpressed and purified. Purified UhpA was phosphorylated by acetyl phosphate in a reaction that was dependent on Mg$^{2+}$ and on the presence of aspartate 54, the site of phosphorylation in homologous response regulators. Gel electrophoretic mobility shift and DNase I and hydroxyl radical protection assays showed that UhpA bound specifically to the region of the *uhpT* promoter extending from −80 to −50 bp, relative to the transcription start site. At higher concentrations of UhpA, binding was extended to the −32 region. Binding to the −64 element exhibited positive cooperativity and was stimulated severalfold by phosphorylation of UhpA, whereas extension to the downstream region was more strongly affected by phosphorylation. The consensus sequences for the high affinity UhpA-binding sites in the −64 element and for the downstream, low affinity sites are proposed. The pattern of *in vitro* binding by UhpA agreed with the *in vivo* observations that phosphorylation-independent assembly of the transcription initiation complex can occur at elevated concentrations of UhpA.

Expression of the *uhpT* gene allows growth of *Escherichia coli* on various phosphorylated sugars. The UhpT anion-exchanging transporter mediates the uptake of many organophosphate compounds, but its synthesis is absolutely dependent on UhpA and is also subject to catabolite repression through the action of the catabolite gene activator protein, CAP.

Sequence comparisons group UhpA into a family of response regulator proteins that includes NarL from *E. coli*, DegU from *Bacillus subtilis*, and FixJ from *Rhizobium* species (7). Members of this family share a common modular organization with an N-terminal phosphorylation module linked through a highly variable, flexible linker to a C-terminal output module (8). The N-terminal domain contains a highly conserved aspartyl residue (Asp-54 in UhpA), which is the site of phosphorylation in homologous proteins, CheY and NtrC (9, 10). This aspartyl residue is part of an acidic pocket with bound Mg$^{2+}$ necessary for structure and catalytic activity (11, 12). Response regulators are normally phosphorylated by their cognate sensor kinases, but many can be phosphorylated by acetyl phosphate or other low molecular weight phospho-donors (13). The C-terminal domains of the UhpA family of response regulators contain a highly conserved segment predicted to form a helix-turn-helix motif that is similar to the DNA-binding regions of some transcription factors that are not regulated by protein phosphorylation, such as MalT from *E. coli* and LuxR from *Vibrio fischeri* (14). The existence of these predicted structural motifs has been verified by the recent description of the structure of NarL at 2.4-Å resolution (15).

When the *uhpA* gene is overexpressed from multicopy plasmids, high level constitutive expression from the *uhpT* promoter occurs even in the absence of UhpB and UhpC function (16). The D54N variant of UhpA, in which aspartate 54 at the putative site of phosphorylation is replaced with asparagine, is totally inactive for Uhp expression when in single gene copy, but is as active as the wild-type protein when overexpressed (17). It appears, therefore, that both protein concentration or relative stoichiometry and phosphorylation are important parameters for UhpA activity, and that the requirement for phosphorylation of UhpA is lost when it is overexpressed.

Previous genetic analyses identified four regulatory elements in the *uhpT* promoter: a −10 region typical of σ$^{70}$-dependent promoters, a 10-bp inverted repeat centered at −32, a 31-bp hyphenated inverted repeat centered at −64, and a CAP-binding sequence centered at −103.5 (all nucleotide coordinates are relative to the transcription start site) (18). Multiplex plasmids carrying portions of the *uhpT* promoter were used in *in vitro* titration experiments to identify a binding site for UhpA by their ability to compete with the chromosomal *uhpT* promoter for limiting amounts of UhpA. Titration of UhpA was seen only if the multiplex plasmid carried the −64 element. Either half of the −64 element conferred reduced *in vivo* titration activity (18), suggesting that each half of the −64 element

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1 The abbreviations used are: CAP, catabolite gene activator protein; PAGE, polyacrylamide gel electrophoresis; bp, base pair(s); PCR, polymerase chain reaction; IPTG, isopropyl-β-D-thiogalactopyranoside; DTT, dithiothreitol; HPLC, high performance liquid chromatography; Tricine, N-tris(hydroxymethyl)methylglycine.

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Promoter*

| Promoter | Element 1 | Element 2 | Relative Activity |
|----------|-----------|-----------|------------------|
| *uhpT*   | −64       | −32       | 1.0              |
|          | −64       | −32       | 0.5              |

This paper is available on line at [http://www-jbc.stanford.edu/jbc/]
DNA Site for UhpA Binding

can bind UhpA, but not as well as the intact element. Disruption of the -32 element with a 6-bp linker substitution eliminated promoter activity, but the -32 element did not compete for UhpA binding in vivo and its function is yet unknown. Although UhpA protein has been implicated genetically as an essential activator of uhpT expression, the biochemical demonstration of its role in transcription activation has been lacking. Here we report that phosphorylation of UhpA by acetyl phosphatase occurs on Asp-54, that UhpA binds specifically to target sequences in the uhpT promoter, and that phosphorylation strongly affects the ability of UhpA to bind to the -64 element and a downstream region.

EXPERIMENTAL PROCEDURES

Strains and Plasmids—Strain BL21(DE3) was obtained from Novaga, Inc. and bears a λ lysogen with the phage T7 gene 1 (19). Two oligonucleotides (i, 5′-GGGCGGTAAAGCGGAAGACTGTAGTACCG-GTGGCCCTTATA-3′; and ii, 5′-GGGTAATATAGGCTAGAGAAGGT-C-3′) were used as primers in a polymerase chain reaction (PCR) (20) with plasmids pRJK10 (21) or pAlter uhpA (DS4N) (17) as DNA template. Primer i generates a BspHI site at the initiation codon of uhpA, and primer ii generates an Xhol site downstream of the uhpA coding sequence. PCR products were purified using Wizard spin columns (Promega, Madison, WI) and labeled at the 5′ end by incubation with T4 polynucleotide kinase (Life Technologies, Inc.) and [γ-32P]ATP (3,000 Ci/mmol; DuPont NEN). Nucleotide precursors were removed by gel filtration through G-50 Spin columns, and the labeled DNA was suspended in buffer B (25 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 1 mM EDTA, 0.5 mM EDTA) and quantified by ethidium bromide fluorescence using the saran wrap method (20). DNA binding reactions (10 µl) contained buffer C (50 mM Tris-HCl, pH 7.5, 6 mM MgCl2, 1 mM EDTA, 1 mM DTT, 5% (v/v) glycerol), 120 ng of poly(dI-dC) (Boehringer Mannheim), 5.4–7 nM 32P-labeled DNA fragment, and differing amounts of UhpA in buffer B. The reaction mixtures were incubated at 25 °C for 20 min. Unlabeled competitor DNA was added in H2O, dried by lyophilization in the reaction tube, and dissolved in binding assay components before addition of UhpA. Gels were pre-electrophoresed to constant current before samples were loaded with addition of 1.1 µl of 50% glycerol with 0.25% xylene cyanol and 0.25% bromophenol blue in buffer B. Samples were subjected to electrophoresis in 1.5-mm-thick 10% polyacrylamide gels (acrylamide: bisacrylamide, 38:1) with gel and electrode buffer of 96 mM Tris, pH 8.6, 90 mM borate, 3 mM EDTA. Electrophoresis was performed at 560 V for 1 h. DNA gels were dried under vacuum, and the positions of radioactive fragments were visualized on a phosphor storage screen, which was analyzed on a Molecular Dynamics PhosphorImager, running the ImageQuant program.

DNase I Footprinting—PCR primers A and 1 were labeled with T4 polynucleotide kinase and [γ-32P]ATP and purified with Sephadex G-25 Spin columns. The labeled primers were used in separate PCR reactions with or without UhpA. Reaction products were purified using Wizard spin columns (Promega, Madison, WI), digested with the KpnI linker (Novagen, Inc.) to form plasmids pBW6 and pJLD1, respectively, in which the uhpA gene is expressed under the control of the IPTG-inducible T7 promoter (24), connected to an Applied Biosystems model 470A Sequenator with Applied Biosystems model 470A Sequenator on-line model 120A phenylthiohydantoin analyzer, using the manufacturer's suggested reaction cycles.

Phosphorylation of UhpA—UhpA was phosphorylated by incubation at 37 °C in a mixture containing 13 µM UhpA and 10 mM acetyl phosphate in buffer D (50 mM Tris-HCl, pH 7.5, 6 mM MgCl2, 1 mM DTT). Acetyl [32P]phosphate was prepared as described (13). For the experiments shown in Fig. 3, proteins (2.5–10 µM) were incubated at 37 °C in a 27-µl reaction volume containing buffer D and 20 mM acetyl [32P]phosphate. The half-life of phospho-UhpA (P-UhpA) was determined by labeling UhpA with acetyl [32P]phosphate for 60 min and then removing excess acetyl phosphate by passing the reaction mixture through a Sephadex G-50 spin column (25). The filtrate was then incubated at 37 °C, and portions were pipetted at various times onto nitrocellulose filters (0.05 µm pore size, 25 mm diameter, presoaked in buffer D) on a porous plastic filter support. Each 24-µl sample was filtered under gentle vacuum and washed immediately with 0.5 ml of buffer D. Filters were dried and exposed to a phosphorimager plate. Background binding by buffer D without UhpA was less than 0.01% of filter-bound [32P]-P-UhpA at time 0.

Electrophoretic Mobility Shift Assay—DNA fragments used for gel mobility shift assays were generated with PCR reactions. PCR primers that annealed to the coding strand of the uhp locus were as follows: A, 5′-CCCTTTTGAATCCGGACAGAC-3′; B, 5′-CGGCGAATAAAGTTTATACGAAATTTCTCAGGTTCG-3′; and C, 5′-GCTATATCGACCGCCTGATTTGCTG-3′. PCR primers used to anneal to the noncoding strand of the uhp locus were as follows: 1, 5′-GGTGGTGGCCTGGCACTG-3′; 2, 5′-GATAACGTCCAGGAAA-3′; and 3, 5′-TAT-GAGTGAAGGTTAG-3′. The DNA templates were plasmid pRJK10 or plasmid pRS415-P. Rsal derivatives containing 6-bp Ncol linker substitutions at selected sites throughout the uhpT promoter (18). Plasmid pET-15b DNA products were purified using Wizard spin columns (Promega, Madison, WI) and labeled at the 5′ end by incubation with T4 polynucleotide kinase (Life Technologies, Inc.) and [γ-32P]ATP (3,000 Ci/mmol; DuPont NEN). Nucleotide precursors were removed by gel filtration through G-50 Spin columns, and the labeled DNA was suspended in buffer B (25 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 1 mM EDTA, 0.5 mM EDTA) and quantified by ethidium bromide fluorescence using the saran wrap method (20). DNA binding reactions (10 µl) contained buffer C (50 mM Tris-HCl, pH 7.5, 6 mM MgCl2, 1 mM EDTA, 1 mM DTT, 5% (v/v) glycerol), 120 ng of poly(dI-dC) (Boehringer Mannheim), 5.4–7 nM 32P-labeled DNA fragment, and differing amounts of UhpA in buffer B. The reaction mixtures were incubated at 25 °C for 20 min. Unlabeled competitor DNA was added in H2O, dried by lyophilization in the reaction tube, and dissolved in binding assay components before addition of UhpA. Gels were pre-electrophoresed to constant current before samples were loaded with addition of 1.1 µl of 50% glycerol with 0.25% xylene cyanol and 0.25% bromophenol blue in buffer B. Samples were subjected to electrophoresis in 1.5-mm-thick 10% polyacrylamide gels (acrylamide: bisacrylamide, 38:1) with gel and electrode buffer of 96 mM Tris, pH 8.6, 90 mM borate, 3 mM EDTA. Electrophoresis was performed at 560 V for 1 h. DNA gels were dried under vacuum, and the positions of radioactive fragments were visualized on a phosphor storage screen, which was analyzed on a Molecular Dynamics PhosphorImager, running the ImageQuant program.

Hydroxyl Radical Footprinting—Hydroxyl radical footprinting (25) was performed essentially as described by Craig et al. (26). 5′-32P-Labeled primer 1 was used in a PCR reaction with primer D, 5′-CTGACATCCGGACCGCCTGACGGA, and plasmid pRJK10 as template. The digest reaction volume contained 285-µl DNA fragment labeled with 32P. Labeled DNA was suspended in buffer B (prepared with HPLC-grade water) and incubated for 20 min at room temperature in a 40-µl volume with 480 ng of poly(dI-dC) and UhpA or P-UhpA at indicated concentrations. A volume of 4 µl of 14 mM sodium acetate and 2 µl of 3% hydrogen peroxide was pipetted onto the side of the microcentrifuge tube. A drop containing freshly prepared solution of 60 mM borate in 32 element did not compete for UhpA binding.
with its predicted molecular mass (data not shown). Finally, nitrogen flow. Spectra were acquired by scanning the first quadrupole to the position for 21 kDa on Tricine gels. Based on 4 residues was determined by automated Edman degradation provided by several tests. The sequence of the first 20 N-terminal residues was matched exactly that of the deduced UhpA polypeptide given in the previous section.

**Mass Spectrometry**—Protein molecular weight measurements were made by electrospray mass spectrometry using a Finnigan-MAT TSQ7000 mass spectrometer system, as described previously (27). Samples were introduced by either capillary column HPLC or infusion at 0.6 μl/min. Electrospray ionization was carried out at 4.5 kV with a sheath liquid flow of 70% methanol, 30% water at 1.2 μl/min and a co-axial nitrogen flow. Spectra were acquired by scanning the first quadrupole of the tandem quadrupole instrument and were deconvoluted using the algebraic method of Mann.

**RESULTS**

**Overexpression and Purification of UhpA Proteins**—UhpA and its D54N variant were overexpressed using the pET15b expression plasmid carrying inserts of PCR products that should yield the UhpA proteins with their natural termini. IPTG induction of synthesis of phage T7 RNA polymerase resulted in high level expression of a 24-kDa polypeptide, which was not seen in uninduced cells or in cells carrying the vector plasmid lacking an insert. Fig. 1 shows the SDS-PAGE display of the purification of the wild-type UhpA protein through the steps of polyethyleneimine precipitation, fractional precipitation between 10% and 45% saturation with ammonium sulfate, and salt gradient elution from a DEAE column. Similar results were obtained for the UhpA/D54N variant (data not shown). The size of the 24-kDa polypeptide is in reasonable agreement with the molecular mass of 20,889 Da deduced from the uhpA nucleotide sequence, and this polypeptide migrates at the position for 21 kDa on Tricine gels. Based on densitometric scanning of the electropherograms, the degree of purification was >90%.

Confirmation that the purified protein was UhpA was provided by several tests. The sequence of the first 20 N-terminal residues was determined by automated Edman degradation and matched exactly that of the deduced UhpA polypeptide (28), showing that the N-terminal methionine residue was retained in the product. Purified UhpA was subjected to analysis by electrospray mass spectrometry, and the major protein species had a molecular mass of 20,900 ± 4 Da, in close agreement with its predicted molecular mass (data not shown). Finally, rabbit polyclonal antisera raised to this polypeptide reacted in Western blot analysis with a 21-kDa polypeptide that was present only in uhpA+ cells and was present in amplified amounts in cells with a uhpA-bearing plasmid (17).

**Phosphorylation of UhpA by Acetyl Phosphate**—Several response regulators can be phosphorylated and activated by incubation with low molecular weight phosphate donors, such as acetyl phosphate (13, 29–31). The transfer of phosphate to purified UhpA from acetyl [32P]phosphate and the properties of the phosphorylated protein were investigated.

Fig. 2B demonstrates that [32P]phosphate was transferred from acetyl phosphate to UhpA in a reaction containing 5 mM MgCl2, 1 mM DTT, 50 mM Tris-HCl (pH 7.5), 4.8 μM UhpA, and 20 mM acetyl [32P]phosphate at 37°C (lane 2). Transfer of phosphate was completely blocked in the presence of 10 mM EDTA (lane 1) or when aspartate-54 of UhpA was replaced with asparagine (lane 3). The requirement for magnesium and for aspartate 54 is consistent with the proposed role of the conserved acidic pocket for binding the Mg2+ atom necessary for the phosphotransfer reaction (11, 12, 32, 33). There was no detectable difference in the electrophoretic mobilities of the phosphorylated and unphosphorylated forms of UhpA during SDS-PAGE (Fig. 2A).

The time course of phosphorylation was slow, and steady-state levels were reached only after incubation for 1 h (Fig. 3A). From the amount of radioactivity in the separated protein species, a rough estimate of the stoichiometry of labeling was 0.5–1 mol of phosphate/mol of protein. Labeling was competed by nonradioactive acetyl phosphate (Fig. 3B). Half-maximal inhibition was obtained at 20 mM acetyl phosphate, indicating that the phosphate donor was acetyl phosphate and not some other species that might have formed during the chemical synthesis of the labeled acetyl phosphate.

The stability of phospho-UhpA (P-UhpA) was determined by separating UhpA from acetyl [32P]phosphate by rapid gel filtration through a QuickSpin column after a 120-min incubation period to allow maximal protein labeling. At intervals, UhpA was separated from released inorganic phosphate by collection on nitrocellulose filters. The rate of loss of filter-bound radioactivity indicated a half-time for hydrolysis of UhpA + 1/2 of 50% formamide, and separated by electrophoresis as described in the previous section.

**Protein molecular weight measurements** were performed by SDS-PAGE and stained with Coomassie Blue. Lane 1, molecular size standards; lane 2, French press lysate of uninduced cells; lane 3, French press lysate of IPTG-induced cells; lane 4, proteins precipitated between 10% and 45% saturation with ammonium sulfate; lane 5, fraction eluted from MacroPrep DEAE chromatography.

Fe(NH₄)₂(SO₄)₂ and 120 mM EDTA was pipetted as a second drop onto the side of the tube. The solutions were mixed by gentle vortex action. Reactions were allowed to proceed for 2 min and quenched by addition of 4 μl of a solution containing 143 mM thiourea and 26 mM EDTA. Following addition of 10 μg of yeast tRNA and 200 mM sodium acetate, the DNA was precipitated with ethanol, denatured by dissolution in 50% formamide, and separated by electrophoresis as described in the previous section.

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The stability of phospho-UhpA (P-UhpA) was determined by separating UhpA from acetyl [32P]phosphate by rapid gel filtration through a QuickSpin column after a 120-min incubation period to allow maximal protein labeling. At intervals, UhpA was separated from released inorganic phosphate by collection on nitrocellulose filters. The rate of loss of filter-bound radioactivity indicated a half-time for hydrolysis of about 60 min at pH 7.5 (Fig. 3C). The stability of P-UhpA is thus comparable to the t₁⁄₂ of ~1.5 h for P-OmpR (34) and P-PhoB (13), and is much higher than for P-ChrY and P-NtrC, whose half-lives are 6–15 s and 4–8 min, respectively (35, 36).

**Properties of P-UhpA**—The extent of incorporation of radioactivity, the kinetics of labeling, and the lack of phosphorylation of the D54N variant suggested that treatment with acetyl phosphate resulted in the phosphorylation of Asp-54 on the majority of the UhpA molecules. The stoichiometry of labeling was investigated in two ways. First, UhpA protein before and...
portions were removed and filtered through nitrocellulose filters (0.05 m). The protein was stopped by addition of 10 mM EDTA and was analyzed by SDS-PAGE, as above.

**Fig. 3. Kinetics of P-UhpA formation and hydrolysis.** A, UhpA (5 μM) was incubated with 20 mM acetyl [32P]phosphate at 37 °C for indicated times. Reaction was stopped with 10 mM EDTA, and samples were separated by SDS-PAGE. Transfer of radioactivity to UhpA was detected on a phosphor storage screen and was quantified using a PhosphorImager equipped with ImageQuant software (Molecular Dynamics, Sunnyvale, CA), and is plotted as pixel density units. B, indi- cated concentrations of unlabeled acetyl phosphate were used to com- pete with 20 mM acetyl [32P]phosphate for the phosphorylation of UhpA (2.5 μM). Samples were incubated at 37 °C for 1 h before the reactions were stopped by addition of 10 mM EDTA and were analyzed by SDS- PAGEdw as above. C, UhpA (10 μM) was incubated with 20 mM acetyl [32P]phosphate at 37 °C for 2 h. Excess acetyl [32P]phosphate was re- moved by passage through a Sephadex G-50 QuickSpin column. The filtrate was incubated at 37 °C for the indicated times, after which portions were removed and filtered through nitrocellulose filters (0.05 μm, pore size). Filters were washed and then exposed to a phosphor storage screen. The half-life of P-UhpA was calculated with a semilogarithmic plot.

**Fig. 4. Separation of UhpA and P-UhpA by nondenaturing gel electrophoresis.** UhpA protein was incubated with acetyl [32P]phosphate for 2 h at 37 °C in the presence (lane 1) or absence (lane 2) of 10 mM EDTA. The proteins were subjected to electrophoresis on a 10% polyacrylamide gel with a 4% stacking gel for 1.5 h. The gel and electrode buffer was 1 × TBE. A, Coomassie-stained protein; B, 32P-labeled protein detected by exposure to a phosphor storage screen. All Coomassie-stained and radiolabeled protein species are shown. Dashes indicate positions of radioactive dye markers used to align the two images.

images were aligned with the aid of radioactive dye marks. Fig. 4A shows that, after 2 h of electrophoresis in a water-cooled electrophoresis chamber, the phosphorylated form of UhpA (lane 2) migrated more rapidly than the native form (lane 1). The pattern of radioactivity shown in Fig. 4B confirmed that the radioactivity co-migrated with the Coomassie Blue-stained material in the phosphorylated sample (lane 2) and that phosphorylation was completely blocked by EDTA (lane 1). The majority (>90%) of the protein molecules incubated with acetyl phosphate exhibited this increased mobility and hence must be phosphorylated. The increased mobility of P-UhpA may be due to a change of conformation into a more compact structure, or to an increase in the net negative charge of the protein, or both. There was no indication for the formation of higher oligomers of UhpA as a result of phosphorylation.

**UhpA Binds to the −64 Element of the uhpT Promoter—**Merkel et al. (18) identified four regions of the uhpT promoter that are important for its function. The binding of UhpA to these DNA elements was investigated by electrophoretic mobility shift assays using DNA fragments, which were prepared by PCR and whose locations are indicated in Fig. 5. Fragment A1 carries the entire promoter region, extending from −142 to −68 bp. 32P-End-labeled fragment A1 was incubated at 25 °C for 20 min with increasing concentrations of UhpA, followed by electrophoresis in a 10% polyacrylamide gel. An unusual gel shift pattern was routinely observed (Fig. 6A), in which increasing concentrations of UhpA resulted in a progressive decrease in probe mobility until a protein-DNA complex of defined mobility was obtained at higher concentrations of UhpA. In the range of UhpA concentrations giving shifted complexes of intermediate mobility, these complexes migrated with a characteristic wavy pattern, whereas the unshifted and the maximally retarded species formed discrete, straight bands. Silver staining of the gels revealed that the bulk of the UhpA protein comigrated with the maximally shifted complex (data not shown). Immunoblot analysis of mobility shift gels using polyclonal anti-UhpA antisera (17) failed to detect any UhpA protein migrating with the intermediate shifted probe (data not shown).

The location of the UhpA-binding sites were investigated by examining the retardation of electrophoretic mobility of a series of 32P-labeled DNA fragments that carry different portions of the uhpT promoter and were incubated in the absence or presence of 5.4 μM UhpA. Fig. 6B shows that the mobility of fragments A1 (coordinates −142 to −68), B1 (−81 to −68), and A2 (−142 to −38) was substantially retarded by UhpA. The only region present on all three of these fragments contains the...
were no high affinity UhpA-binding sequences outside of the −10 region, short inverted repeat centered at position −32.5, a 30-bp hyphenated inverted repeat centered at −64, and the DNA-binding site for CAP (18). The PCR primers used to generate promoter fragments for UhpA-binding analysis (A, B, C, 1, 2, and 3) are indicated. Promoter fragments were named from the two primers used in each PCR reaction. The sizes of PCR products A1, B1, C1, A2, and A3 are 210, 149, 112, 105, and 60 bp, respectively.

The genetic elements, showing the −10 region, short inverted repeat centered at position −32.5, a 30-bp hyphenated inverted repeat centered at −64, and the DNA-binding site for CAP (18). The PCR primers used to generate promoter fragments for UhpA-binding analysis (A, B, C, 1, 2, and 3) are indicated. Promoter fragments were named from the two primers used in each PCR reaction. The sizes of PCR products A1, B1, C1, A2, and A3 are 210, 149, 112, 105, and 60 bp, respectively.

A second gel mobility shift assay to demonstrate UhpA binding specificity examined the ability of excess unlabeled DNA fragments to compete with full-length labeled fragment A1 (−142 to +68) for binding to UhpA. As shown in Fig. 6C, fragment B1 (−81 to +68), which carries the −64 element, competed with fragment A1. However, even a 250-fold molar excess of fragments A3 (−142 to −83) or C1 (−44 to +68) did not block binding of fragment A1 to UhpA, showing that there were no high affinity UhpA-binding sequences outside of the −64 element.

Binding of UhpA to uhpT Promoter Variants—The genetic relevance of the in vitro UhpA binding to linear uhpT promoter fragments was tested with a series of mutant promoters. Fig. 7A indicates the sites of seven linker substitutions (designated 2–8 in this figure), in which the sequence of individual 6-bp intervals was changed to the Ncol recognition sequence, CCATGG. Promoter activities under inducing conditions were determined previously from the level of expression of uhpT-lacZ reporter constructs carrying each substitution (18). PCR primers A and 1 were used to generate 32P-labeled DNA fragments carrying these changes, and binding of 4 μM UhpA was assayed by gel mobility shift (Fig. 7B). Only the DNA fragment with a 6-bp substitution in the downstream half of the −64 element (mutant 4, at −56 to −51) showed a significant change from the band shift pattern of wild-type uhpT promoter DNA. Fig. 7C shows the mobility shift behavior for several of the promoter variants at a range of UhpA concentrations. Mutant promoter 4 displayed a decreased affinity for UhpA in the formation of complexes of intermediate electrophoretic mobility, and it did not form the maximally retarded complex in the range of UhpA concentrations employed. The electrophoretic behavior of promoter mutant 4 was similar to that shown by DNA fragment C1, carrying only the sequences downstream of the −64 element. The mutations in promoters 3 and 4 are symmetrically related in opposite halves of the palindromic −64 element, but mutant 3 in the upstream half exhibits only a modest decrease in promoter function and a slight change in UhpA binding, as opposed to the substantial changes in mutant 4. These different effects on promoter activity and on UhpA binding indicates that the downstream half of the −64 element plays a more significant role in promoter function than does the upstream half.

Variant 7 (−35 to −30) contains a linker substitution in the −32 element and exhibits complete loss of promoter activity, but gave a normal pattern of UhpA binding in the in vitro titration assay (18) and in this gel shift assay (Fig. 7C).

Phosphorylation of UhpA Enhances Its DNA Binding Activity—The effect of phosphorylation by acetyl phosphate on the ability of UhpA to bind to the uhpT promoter region was examined in the electrophoretic mobility shift assay. As shown in Fig. 8A, addition of unmodified UhpA in the concentration range up to 3.4 μM resulted in the characteristic, progressive decrease in mobility of the A1 DNA fragment (coordinates −142 to +68) (lanes 2–6). The mobility of half of the DNA sample was shifted at 1.3 μM UhpA, but only a very small
of seven 6-bp linker substitutions, designated of these sequence elements in the uhpT promoter region, and the location carries the wild-type sequence. Each promoter variant was amplified by greatly increased DNA binding activity (the % of the presence of 0, 0.67, 1.35, 2.0, 2.7, 3.4, 4.0, 4.7, and 5.4 m was present in the maximally shifted complex at 2 μM UhpA. In contrast, P-UhpA displayed potentially all of the DNA fragment was shifted at 0.7 μM UhpA and was present in the maximally shifted complex at 2 μM P-UhpA. The presence of acetyl phosphate alone did not affect the mobility of the DNA fragment (lane 7).

Acetyl phosphate had no apparent effect upon the DNA binding activity of the UhpA[D54N] protein. As shown in Fig. 8B, the variation of DNA probe mobility as a function of concentration of UhpA[D54N] protein was roughly identical in the absence or presence of acetyl phosphate and was similar to that of unphosphorylated wild-type UhpA (compare to Fig. 8A, lanes 2–6). UhpA[D54N] was expected to bind the uhpT promoter, since overexpression of this protein results in high constitutive promoter activity (17). These data show that phosphorylation of UhpA strongly affects its ability to bind to its target DNA sequences, and are consistent with the conclusion that the requirement for phosphorylation can be circumvented by overexpression of UhpA.

**DNase I Protection by UhpA and P-UhpA**—The locations of the UhpA-binding sequences were shown by testing for the ability of UhpA protein species to protect against DNase I cleavage (37) of the labeled top (noncoding) (Fig. 9, A and C) or bottom (coding) strands (Fig. 9B) of the uhpT promoter. Unfortunately, few sites within the very A+T-rich −64 element were cleaved by DNase I. On both strands, the few susceptible sites within the −64 element showed decreased or enhanced cleavage upon addition of P-UhpA. At higher concentrations of P-UhpA, the protected region extended downstream from −50 to −32. No changes in DNase I sensitivity were seen at sequences upstream of −80 or downstream of −32. These results confirm the conclusions from the gel mobility shift analysis that the −64 element (−50 to −80) is the high affinity binding site for P-UhpA, and show that the sequences immediately downstream and overlapping the putative RNA polymerase-binding region are the low affinity binding region.

Protection against DNase I digestion by UhpA and P-UhpA was compared in Fig. 9C. Although there were too few DNase-susceptible sites for definitive conclusions, both forms of UhpA protected the −64 element effectively. Extension of the footprint to the low affinity site (−50 to −32) occurred with P-UhpA at 140 nM, but required much higher concentrations of unmodified UhpA (1.3 μM). Thus, phosphorylation of UhpA may enhance cooperativity between UhpA monomers at the uhpT promoter to enable formation of a maximally retarded gel complex and extension of the binding to the low affinity site.

**Hydroxyl Radical Footprinting with UhpA and P-UhpA**—High resolution analysis of the sites of contact of UhpA at the uhpT promoter was obtained by the technique of hydroxyl radical footprinting (25), using the DNA fragment D1 labeled at the 5’-end of the bottom strand. The results shown in Fig. 10 confirmed and extended the conclusions from the DNase I footprinting. P-UhpA protected five regions spaced at roughly 10-bp intervals. Three of the protected sites lie within the −64 element and share the consensus sequence RAAAAY, where R is either purine and Y is either pyrimidine base. The two protected sites downstream of the −64 element were centered at −43 and −33, and shared the consensus sequence ANGCY.

Unphosphorylated UhpA protected the three sites in the −64 element with only slightly lower affinity than did P-UhpA. However, its protection of the two downstream sites was markedly weaker, even at the highest concentration of UhpA tested.

**DISCUSSION**

The UhpA protein was overexpressed and purified to show its specific binding to DNA sequences in the uhpT promoter region and to compare its binding properties with those expected from the regulation of uhpT expression in the intact cell. Protein purification was necessary for this goal because we were unable to demonstrate a UhpA-specific electrophoretic
mobility shift using cell extracts. The DNA-binding properties of the purified protein agreed well with the expected behavior, and also provided additional information relevant to its mechanism of transcription activation.

Previous studies showing that disruption of the uhpA gene or deletion of portions of the −64 element completely block uhpT expression (16, 18) suggested that UhpA acts at the −64 element as a positive activator for Glu-6-P-induced uhpT expres-
of key promoter elements are indicated on the cleavage with hydroxyl radicals. Dideoxy sequencing ladders on the same template and uncut DNA samples are run in adjacent lanes. The locations of key promoter elements are indicated on the right. B, the intensity of radioactivity in several lanes were quantified with a Molecular Dynamics PhosphorImager. The figure displays the aligned pixel density for the reaction in the presence of 425 nM UhpA (black) in comparison to the reaction in the absence of added protein (top trace, gray) or, in the lower figure, in comparison to 425 nM P-UhpA (bottom trace, thin, gray).

![Figure 10](image)

**FIG. 10. Hydroxyl radical footprints of UhpA and P-UhpA complexes at the uhpT promoter.** A, the 285-bp PCR product D1 (40 nM) was labeled with 32P at the 5′ end of the bottom strand, incubated with indicated concentrations of UhpA and P-UhpA, and subjected to backbone cleavage with hydroxyl radicals. Dideoxy sequencing ladders on the same template and uncut DNA samples are run in adjacent lanes. The locations of key promoter elements are indicated on the right. B, the intensity of radioactivity in several lanes were quantified with a Molecular Dynamics PhosphorImager. The figure displays the aligned pixel density for the reaction in the presence of 425 nM UhpA (black) in comparison to the reaction in the absence of added protein (top trace, gray) or, in the lower figure, in comparison to 425 nM P-UhpA (bottom trace, thin, gray).

The work presented here confirms this hypothesis by demonstrating that UhpA binds preferentially and specifically to the −64 element. These results agreed with the results showing in vivo titration of UhpA activity by multicopy plasmids carrying portions of the uhpT promoter (18). No other high affinity binding sequence was present in the region from −142 to +68, which is consistent with results from our study of nested deletion sets showing that no specific sequences upstream of the CAP-binding site at −120 or downstream of the transcription start site are required for normal uhpT expression and regulation. Gel-mobility shift assays indicated the low affinity binding of UhpA to sequences downstream of the −64 element. These sequences were localized by DNase I and hydroxyl radical protection assays to the region immediately downstream of the −64 element and extending to the −32 element.

The number of UhpA molecules that bind to the promoter is not known, but the extent of the activator-binding regions in the uhpT promoter appear to be simpler than for other response regulators. For example, OmpR binds to four sites in the ompF promoter and to three sites in the divergently transcribed micF-ompC promoter (38–41). The best studied target of NarL action, the narG promoter, contains eight NarL consensus binding heptamers, although not all of them appear necessary for regulation (42).

Previous studies showed that both UhpBC function and UhpA aspartate–54 are required for uhpT expression (17, 28). Overexpression of UhpA resulted in high level, constitutive uhpT expression and loss of the normal requirements for inducer, UhpBC function, and the site of phosphorylation. One interpretation of these findings is that phosphorylation of UhpA might enhance its binding to its target sequences, but that DNA binding or formation of the active structure on the DNA could be achieved by unphosphorylated UhpA at elevated concentrations. Another possibility was that overexpressed UhpA is activated by covalent modification by another sensor kinase in some form of cross-talk. The observations obtained here favor the former hypothesis. Although phosphorylation of UhpA by soluble forms of UhpB has not been obtained yet, phosphate transfer from acetyl phosphate, a characteristic of many response regulators (13, 30, 31, 33, 43), was demonstrated. As seen in the other systems, phosphate transfer to UhpA was completely blocked by EDTA chelation and by the D54N substitution. Mass spectrometric analysis of the phosphorylated species showed an increase in molecular mass of 81 Da, indicating the covalent attachment of a single phosphate group, as found for P-OmpR (44). The shift of electrophoretic mobility of P-UhpA under nondenaturing conditions indicated that the majority of UhpA molecules (>90%) were modified. Furthermore, comparison of the electrophoretic mobility of P-UhpA in native gels of various polyacrylamide concentrations, in comparison to the migration of protein standards, indicated that P-UhpA was monomeric in solution under those conditions. The rates of phosphorylation and dephosphorylation of UhpA were comparable to those seen for OmpR and PhoB (13, 34) and were much slower than the corresponding processes in CheY and NtrC. This slow rate of spontaneous dephosphorylation of UhpA (i.e. around 1 h) implies that the putative co-phosphatase activity of UhpB must play an important role in uhpT regulation (28).

Binding of UhpA to its DNA target displayed unusual behavior in the electrophoretic mobility shift assay. As the UhpA concentration was increased, there was a progressive decrease in the mobility of the protein-DNA complex, associated with a wavy electrophoretic pattern, until a maximally retarded complex of defined mobility and typical sharp appearance was eventually achieved. The basis for this behavior is unresolved. Each DNA “step” may represent an increasing extent of protein oligomerization on the promoter or formation of complexes that differ in protein-DNA binding stability. We currently favor the view that the shifted species of intermediate mobility represent

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3 J. L. Dahl, B.-Y. Wei, and R. J. Kadner, unpublished data.
DNA molecules that have dissociated from their specific complex with UhpA during the course of electrophoresis. The progressive decrease in probe mobility with increasing UhpA concentration may result from the protein concentration-dependent rebinding of the probe to UhpA molecules. The maximally retarded complex could indicate the formation of a different type of UhpA-DNA complex that is more stable during electrophoresis, or that the UhpA concentration is high enough to trap any DNA molecules that dissociate. The latter view is favored by the co-migration of the maximally shifted complex with the bulk of the UhpA protein molecules. Their co-migration could be explained by the fortuitous balance between the retardation in mobility caused by the increased hydrodynamic volume of the UhpA-DNA complex and its increased mobility owing to the binding of the polyanionic DNA. Silver staining and immunoblot analysis did not reveal the presence of detectable amounts of protein migrating in association with the complexes of intermediate mobility, consistent with the release of the DNA during electrophoresis.

DNase I and hydroxyl radical protection footprinting extended the results of the gel shift experiment by confirming that the −64 element, from coordinates −80 to −50, was the site of high affinity binding by UhpA and showing that the extension of UhpA binding to downstream sequences occurred at higher concentrations. This extension to the vicinity of −32 could allow direct interaction with RNA polymerase. Binding of UhpA to the −64 element appears to be less strongly affected by its phosphorylation than was the ability to extend the binding complex to the downstream region. The 6-bp linker substitution at positions −51 to −56 in the downstream half of the −64 element eliminated uhpT promoter activity, increased the concentration of UhpA necessary to bind to DNA, and prevented the formation of the maximally retarded UhpA-DNA complex. The symmetrically related substitution at positions −72 to −77 in the upstream half of the −64 element only reduced promoter function by 40% and had no major effect on complex formation. The difference in the consequences of sequence changes in the two halves of the palindromic −64 element suggests that the downstream half is more important for promoter activity. Presumably, occupancy of the downstream half of the −64 element is a prerequisite for cooperative extension of complex formation. Alternatively, UhpA may not bind in a symmetrical manner to the −64 region, but segments in both halves of the −64 palindrome (−51 to −56 and −72 to −77) are contacted in an apparently symmetrical manner.

The five sites of P-UhpA binding are strongly conserved in E. coli and Salmonella typhimurium (28). The three high affinity sites are in the −64 element. They are centered at residues −74, −65, and −54 and share the consensus sequence RAAAAY. The low affinity sites centered at −43 and −33 have a different consensus sequence of ANGCY. This consensus could be a degenerate form of the high affinity site and occupancy of the low affinity sites may require cooperative extension of the protein complex from the upstream sites. There is evidence supporting the importance of the downstream region between the −64 and −32 elements for promoter activity. This region is much more strongly conserved in E. coli and S. typhimurium, in which 9 of the 11 nucleotides match, than is the region between the CAP-binding site and the −64 element, in which only 4 of the 13 nucleotides were conserved. In addition, the Ncol-linker substitution at residues −46 to −41 reduced promoter activity by 70%.

Studies are under way to identify the effect of phosphorylation on UhpA structure and activity. Phosphorylation does not appear to affect protein oligomerization in solution, as judged by PAGE and gel filtration chromatography, but it clearly affects the DNA-binding properties. Phosphorylation of other response regulators induces their formation of dimers or higher oligomers that is related to their transcriptional activity (45–47). The recent presentation of the structure of NarL, a close homolog of UhpA, reveals the presence of a DNA-binding helix-turn-helix motif in the C-terminal domain that is occluded by interaction with the N-terminal phosphorylation module (15). It is likely that phosphorylation decreases the interdomain interaction to render the DNA-binding surface accessible. This model is difficult to reconcile with our finding that unphosphorylated UhpA binds to specific targets in DNA, although with somewhat lower affinity than does P-UhpA. In the case of UhpA, the primary effect of phosphorylation seems to affect its ability to extend cooperatively along the DNA helix to the downstream sites. Perhaps the interdomain interaction in UhpA does not occlude the DNA-binding site, but the conformational change induced by phosphorylation might enhance the interaction on the DNA surface of UhpA monomers. The major difference in sequence between UhpA and NarL is that the flexible hinge in UhpA is 10 residues shorter than in NarL, which might rotate the C-terminal region relative to the phosphorylation module and thereby allow continuous exposure of the DNA-binding surface. This question can only be resolved through determination of the structure of UhpA.

The Uhp system offers several technical advantages for the study of transmembrane control of transcription activation by a two-component response regulator. There is a simple inducing signal, extracellular Glu-6-P in the μM concentration range. The output of this signal is the increase in transcription of a single gene, uhpT, from undetectable levels in the uninduced state. The entire transcription control region of the uhpT promoter is relatively small, at 120 bp, and the sites for UhpA binding are limited to the 50-bp region from −80 to −30. There are, however, multiple UhpA-binding sequences of different affinity within this region. Now that an active form of UhpA has been obtained, biochemical analysis of the interactions between UhpA, CAP, and RNA polymerase at the uhpT promoter are possible.

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REFERENCES
1. Dietz, G. W. (1976) Adv. Enzymol. 44, 237–259
2. Maloney, P. C., Ambudkar, S. V., Anantharam, V., Sonna, L. A., and Varadachary, A. (1990) Microbiol. Rev. 54, 1–17
3. Pogell, B. M., Myati, B. R., Frimkin, S., and Shapiro, S. (1966) Arch. Biochem. Biophys. 116, 406–415
4. Kadner, R. J., Island, M. D., Dahl, J. L., and Webber, C. A. (1994) Res. Microbiol. 145, 381–387
5. Kadner, R. J. (1995) in Two-Component Signal Transduction (Hoch, J. A., and Silhavy, T. J., eds) pp. 263–274, ASM Press, Washington
6. Merkel, T. J., Dahl, J. L., Ehrlich, R. H., and Kadner, R. J. (1995) J. Bacteriol. 177, 1712–1718
7. Pao, G. M., Tam, R., Lipshtiz, L. S., and Saier, M. H., Jr. (1994) Res. Microbiol. 145, 356–362
8. Parkinson, J. S., and Kofoid, E. C. (1992) Annu. Rev. Genet. 26, 71–112
9. Sanders, D. A., Gillece-Castro, B. L., Stock, A. M., Burlingame, A. L., and Koshland, D. E., Jr. (1992) J. Bacteriol. 174, 5117–5122
10. Lukat, G. S., Stock, A. M., and Stock, J. B. (1990) Biochemistry 29, 5436–5442
11. Stock, A. M., Martinez-Hackert, E., Rasmussen, B. F., West, A. H., Stock, J. B., Ringe, D., and Petsko, G. A. (1990) Biochemistry 29, 13375–13380
12. McClure, W. R., and Stock, J. B. (1994) J. Biol. Chem. 269, 31567–31572
13. Henikoff, S., Wallace, J. C., and Brown, J. P. (1990) Methods Enzymol. 183, 111–132
14. Baikalov, I., Schroder, I., Kaczor-Grzeskowiak, M., Grzeskowiak, K., Gunsalus, R. P., and Dickerson, R. E. (1996) Biochemistry 35, 11053–11061
15. Weston, L. A., and Kadner, R. J. (1988) J. Bacteriol. 170, 3375–3383
16. Webber, C. A., and Kadner, R. J. (1993) Mol. Microbiol. 15, 881–893
17. Merkel, T. J., Nelson, D. M., Brauer, C. L., and Kadner, R. J. (1992) J. Bacteriol. 174, 2763–2770
18. Studier, P. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1990)
DNA Site for UhpA Binding

Methods Enzymol. 185, 60–89
20. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
21. Weston, L. A., and Kadner, R. J. (1987) J. Bacteriol. 169, 3546–3555
22. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
23. Laemmli, U. K. (1970) Nature 227, 680–685
24. Sheer, D. G., Yuen, S., Wong, J., Wassen, J., and Yuan, J. (1991) Biotechniques 11, 526–533
25. Tullius, T. D., Dombrowski, B. A., Churchill, M. E. A., and Kam, L. (1987) Methods Enzymol. 155, 537–558
26. Craig, M. L., Suh, W.-C., and Record, M. T., Jr. (1995) Biochemistry 34, 15624–15632
27. Covey, T. R., Bonner, R. F., Shusan, B. I., and Henion, J. (1988) Rapid Commun. Mass Spectr. 2, 249–255
28. Island, M. D., Wei, B.-Y., and Kadner, R. J. (1992) J. Bacteriol. 174, 2754–2762
29. Drapal, N., and Sawers, G. (1995) Mol. Microbiol. 16, 597–607
30. Feng, J., Atkinson, M. R., McCleary, W., Stock, J. B., Wanner, B. L., and Ninfa, A. J. (1992) J. Bacteriol. 174, 6061–6070
31. Schneider, I., Welin, C. D., Cavichiosi, R., and Gunsalus, R. P. (1994) J. Bacteriol. 176, 4985–4992
32. Lukat, G. S., Lee, B. H., Mottonen, J. M., Stock, A. M., and Stock, J. B. (1991) J. Biol. Chem. 266, 8348–8354
33. Lukat, G. S., McCleary, W. R., Stock, A. M., and Stock, J. B. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 718–722
34. Igo, M. M., Ninfa, A. J., Stock, J. B., and Silhavy, T. J. (1988) Genes Dev. 3, 1725–1734
35. Hess, J. F., Oosawa, K., Kaplan, N., and Simon, M. I. (1988) Cell 53, 79–87
36. Weiss, V., and Magasanik, B. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 8919–8923
37. Galas, D., and Schmitz, A. (1978) Nucleic Acids Res. 5, 3157–3170
38. Harlocker, S. L., Bergstrom, L., and Inouye, M. (1995) J. Biol. Chem. 270, 26849–26856
39. Huang, K. J., Schiebel, J. L., and Igo, M. M. (1994) J. Bacteriol. 176, 1309–1315
40. Maeda, S., and Mizuno, T. (1990) J. Bacteriol. 172, 501–503
41. Rampersaud, A., Harlocker, S. L., and Inouye, M. (1994) J. Biol. Chem. 269, 12559–12566
42. Li, S.-F., and DeMoss, J. A. (1988) J. Biol. Chem. 263, 13700–13705
43. Riggioni, M., and Dubnau, D. (1993) J. Bacteriol. 175, 3182–3187
44. Kenney, L. J., Bauer, M. D., and Silhavy, T. J. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8866–8870
45. Asayama, M., Yamamoto, A., and Kobayashi, Y. (1995) J. Mol. Biol. 250, 11–23
46. McCleary, W. R. (1996) Mol. Microbiol. 20, 1155–1163
47. Porter, S. C., North, A. K., Wedel, A. B., and Kustu, S. (1993) Genes Dev. 7, 2258–2273