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Transcriptional Control Mediated by the ArcA Two-Component Response Regulator Protein of *Escherichia coli*: Characterization of DNA Binding at Target Promoters

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ArcA protein bearing an amino-terminal, oligohistidine extension has been purified, and its DNA binding activity has been characterized with or without prior incubation with carbamoyl phosphate. Electrophoretic mobility shift assays and DNase I protection assays indicate that where the phosphorylated form of the ArcA protein (ArcA-P) is expected to act as a transcriptional repressor (e.g., of *lctPRD* and *gltA-sdhCDAB*), the effect is likely to be mediated by sequestration of *cis*-controlling transcriptional regulatory elements. In contrast, in the case of *cydAB*, for which ArcA-P is expected to function as a transcriptional activator, two discrete binding sites have been identified upstream of a known promoter, and activation from these sites is likely to be mediated by a mechanism typical of the type I class of prokaryotic transcriptional activators. An additional ArcA-P binding site has also been located downstream of the known promoter, and a distinct role for this site in the regulation of the *cydAB* operon during anoxic growth transitions is suggested. These results are discussed within the framework of an overall model of signaling by the Arc two-component signal transduction system in response to changes in aerobiosis.

A bacterial cell possesses numerous two-component signal transduction systems which facilitate adaptation to environmental changes. Such systems typically comprise a membrane-associated sensor kinase and a response regulator which controls gene expression (20). In *Escherichia coli*, adaptation to the respiratory conditions of growth is mediated by the Arc system, consisting of the *arcB*-encoded sensor kinase and the *arcA*-encoded response regulator (24, 26, 29). To date, some 30 operons which are regulated directly or indirectly by the Arc system have been identified (33, 34).

Although the expression of Arc-regulated genes varies in response to environmental O_2, this compound is not thought to be the direct signal detected by the ArcB kinase. Instead, ArcB probably senses the redox state of the cell through detection of an electron transport component in reduced form (25). The net activity of ArcB as a kinase for ArcA is expected to progressively increase during transition from aerobic to anaerobic growth, and the concentration of the phosphorylated form of the ArcA (ArcA-P) is therefore predicted to reach peak levels in anoxic cells. However, significant levels of ArcA-P are apparent in aerobic cells (26, 28, 34), and differential patterns of expression of the members of the Arc modulon can therefore be attributed, at least in part, to differences in the intrinsic affinity of ArcA-P for DNA binding sites located in the transcriptional regulatory regions of its target operons.

Working in concert with the Arc system is the Fnr protein, which acts as a transcriptional activator of genes of anaerobic function (including components of fermentation and anaerobic respiration pathways) and as a repressor of some genes encoding proteins of aerobic function (18, 33, 47). Although the mechanism by which the Fnr protein senses the anaerobic state has not been definitively established, it seems most likely that it senses the intracellular redox potential and becomes activated when the midpoint redox potential ($E^{\circ\prime}$) of the exogenous electron acceptors falls below about +0.44 V (18, 23, 47, 48). Hence, in contrast to the Arc system, which appears to be active over a much broader range of intracellular redox conditions, the Fnr protein appears well poised as a regulator of anaerobic respiration and fermentation pathways. These properties of the Fnr and Arc systems, in combination with input from the Nar system (10), provide *E. coli* with an integrative system for fine-tuning cellular metabolism to ensure maximal exploitation of energy sources (27, 28).

Data from genetic studies suggest that the Arc system is involved in anaerobic repression of operons encoding several tricarboxylic acid cycle enzymes (26), including *gltA* and *sdhCDAB*, which encode citrate synthase and the succinate dehydrogenase complex, respectively (9, 50, 51). These two operons are adjacent on the *E. coli* chromosome but are divergently transcribed. Although their patterns of expression in response to anaerobiosis and carbon supply are broadly similar (39, 40), a distinct difference is apparent in that a considerable basal level of *gltA* expression is maintained in anoxic cells, ensuring continued provision of citrate as a biosynthetic precursor (38, 39).

Of all the operons known to be regulated by the Arc system, the *lctPRD* operon shows the widest range of response, with 90- to 100-fold anaerobic repression apparent (23, 26). The operon encodes an L-lactate-specific permease (LctP) and flavin adenine dinucleotide-dependent dehydrogenase (LctD), and also a putative transcriptional regulator (LctR) of the GntR family of helix-turn-helix proteins (12, 41). LctR is expected to serve as a transcriptional repressor in cells grown in the absence of L-lactate.

Several observations implicate the *arcA* gene product as a transcriptional activator of the *cydAB* operon, encoding sub-

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TABLE 1. Strains and plasmids used

Strain or plasmid	Relevant characteristic or genotype	Reference or source
E. coli strains		
ECL525	F$^-$ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flb5301 deoC1 ptsF25 rbsR* Δ(*frd*)*101*	26
ECL589	F$^-$ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flb5301 deoC1 ptsF25 rbsR* Δ(*frd*)*101 sdh*$^+$ Φ(*sdh-lac*) *arcA2*	Laboratory collection
MC4100	F$^-$ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flb5301 deoC1 ptsF25 rbsR*	Laboratory collection
M15	F$^-$ *lac ara gal mtl*	Qiagen
SP314	Δ(*deoD-arcA*)*253*	26
Plasmids		
pBluescript KSII$^-$	Cloning vector	Stratagene
pACYC184lacI^q	pACYC184 derivative expressing *lacI*^{q} allele; confers tetracycline resistance	J. C. Wang
pMW2	pBR322 derivative containing the *arcA* gene	26
pQE30	Vector for overexpression of His_6-tagged proteins using an IPTG-inducible phage T5 promoter	Qiagen; 44
pREP4	pACYC derivative which constitutively expresses the *lacI* gene	Qiagen
pBTKSArcA	pBluescript KSII$^-$ derivative containing the *arcA* ORF	This study
pQE30ArcA	pQE30 derivative which expresses a His_6-tagged ArcA protein	This study
pBTKSgltA-sdh1	pBluescript KSII$^-$ derivative containing the complete *gltA-sdhCDAB* intergenic region	This study
pBTKSgltA-sdh2	pBluescript KSII$^-$ derivative containing a 384-bp portion of the *gltA-sdhCDAB* intergenic region	This study
pBTKSlct	pBluescript KSII$^-$ derivative containing 310 bp of the *lctP* upstream region	This study
pBTKScyd1	pBluescript KSII$^-$ derivative containing 1017 bp of the *cydAB* upstream region	This study
pBTKScyd2	pBluescript KSII$^-$ derivative containing 623 bp of the *cydAB* upstream region	This study
pBTKScyd3	pBluescript KSII$^-$ derivative containing 480 bp of the *cydAB* upstream region	This study
pBTKScyd4	pBluescript KSII$^-$ derivative containing 458 bp of the *cydAB* upstream region	This study
pBTKScyd5	pBluescript KSII$^-$ derivative containing 287 bp of the *cydAB* upstream region	This study

units of cytochrome *d* oxidase (7, 8, 17, 25, 46, 49). Cytochrome *d* oxidase levels are maximal under microaerobic conditions when the function of the enzyme becomes most useful to the cell (42), and the responses of strains bearing a Φ(*cyd-lac*) fusion suggest that a significant proportion of this regulation is effected at the transcriptional level, with the *fnr* and *arcA-arcB* gene products required for normal regulation (7, 8, 17, 25, 49). The results of a recent study indicate that the *arcA* gene product is most effective as a transcriptional activator of *cydAB* expression during microaerophilic growth and that Fnr acts as a transcriptional repressor in anoxic cells (46).

DNA binding activity of ArcA were first reported in studies of *sodA*, encoding the manganese-containing superoxide dismutase, which is repressed by the *arcA* gene product during anaerobic growth (5, 45). In experiments which used extracts prepared from cells overexpressing the ArcA protein, an ArcA-dependent DNase I footprint of approximately 65 bp encompassing the −35 and −10 elements of the *sodA* promoter was observed (45). Site-specific DNA binding of purified ArcA protein in the promoter region of the *pfl* gene (encoding pyruvate-formate lyase), as determined by both electrophoretic mobility shift (EMS) and DNase I footprinting assays, was also recently reported (13). The DNA binding activity of the purified ArcA protein was found to be enhanced approximately 10-fold by prior incubation with either carbamoyl phosphate or acetyl phosphate. Qualitatively identical footprints were reported for the ArcA and ArcA-P forms of the protein. In a 531-bp DNA fragment encompassing the P_6 and P_7 promoters of the *pfl* operon, four distinct sites of DNase I protection were identified. The site with highest affinity for ArcA (designated A3) extends over 94 bp located between the P_6 and P_7 promoters. Protection of the three remaining sites (A1, A2, and A4) from DNase I cleavage, ranging in size from 25 to 66 bp, was observed only at significantly higher ArcA concentrations (~0.1 mM).

We report here studies of the binding of phosphorylated and unphosphorylated forms of an oligohistidine-tagged ArcA protein to the transcriptional regulatory regions of the *lctPRD* operon, the *gltA-sdhCDAB* intergenic region, and the *cydAB* operon.

MATERIALS AND METHODS

Bacterial strains and plasmids. Relevant characteristics of the bacterial strains and plasmids used in this study, and their sources, are listed in Table 1. Oligonucleotides were obtained from Operon Technologies Inc.; DNA sequence analysis was done with Sequenase 2.0 (U.S. Biochemical Co.). Vent DNA polymerase (New England Biolabs) was used in PCR, with the exception of reactions used to generate templates for EMS or DNase I footprinting assays, in which case Amplitaq DNA polymerase (Perkin-Elmer) was used.

To create pBTKSArcA, DNA corresponding to the *arcA* open reading frame (ORF) was amplified by PCR with primers ARCA1 (5′ CCCGGATCCCATAT GCAGACCCCGCACATTCTTATC) and ARCA2 (5′ CCCGGATCCTGCAG CCGATTAATCTTCCAGATCACC), using pMW2 (26) as a template. The PCR product was digested with *Bam*HI and cloned into the *Bam*HI site of pBluescriptII KS$^-$. The PCR-derived region of one of the resulting clones (designated pBTKSArcA) was sequenced and found to correspond to GenBank entry M1004.

To create pQE30ArcA, a 734-bp *Bam*HI-*Pst*I fragment of pBTKSArcA (including the complete ArcA coding sequence) was cloned between the corresponding sites of pQE30. The resulting vector expresses an ArcA fusion protein (composed of full-length ArcA with an amino-terminal Met-Arg-Gly-Ser-His-His-His-His-His-His-Gly-Ser-His- extension) under transcriptional control of an isopropylthiogalactopyranoside (IPTG)-regulated phage T5 promoter.

To create pBTKSgltA-sdh1, a 764-bp region of DNA from the *gltA-sdhC* intergenic region, corresponding to bases 346 to 1100 in GenBank entry X00980, was amplified by PCR with primers GLT1 (5′ CCCCAAGCTTCCCCGTTGA GGGTGAGTTTTGC) and SDH1 (5′ CCGAATTCTTATCATGAATAACGC CCACATGC), using *E. coli* ECL525 DNA as a template. The PCR product was digested with *Eco*RI and *Hin*dIII and cloned in the corresponding sites of pBluescriptII KS$^-$.

To create pBTKSgltA-sdh2, a 384-bp region of DNA from the *gltA-sdhC* intergenic region, corresponding to bases 536 to 920 in GenBank entry X00980, was amplified by PCR with primers GLT2 (5′ AGTGGATCCATTGATGACG AATAAATGG) and SDH2 (5′ ATCGAATTCGACACGTTACAACGCTGG GTGG), using *E. coli* ECL525 DNA as a template. The PCR product was digested with *Bam*HI and *Eco*RI and cloned in the corresponding sites of pBluescriptII KS$^-$. The PCR-derived region of one of the resulting clones (designated pBTKSgltA-sdh2) was sequenced and found to correspond to GenBank entry X00980.

To create pBTKSlct, a 310-bp region of DNA from the upstream region of the *lctP* gene (corresponding to bases 1424 to 1734 in GenBank entry L13970) was amplified by PCR with primers LCT1 (5′ AGTGGATCCTAAGGGCAATTCT CTGATGAGG) and LCT2 (5′ ATCGAATTCCCGGCGGGATCGTAGTTTT G), using pLCT3 (12) as a template. The PCR product was digested with *Bam*HI and *Eco*RI and cloned in the corresponding sites of pBluescriptII KS$^-$. Sequencing of the PCR-derived region of one of the resulting clones (designated pBTKSlct) revealed a single sequence difference with GenBank entry L13970, corresponding to an extra A residue present at position 1542 in GenBank entry L13970. This same change was found in the genomic sequence of *E. coli* MG1655 (see annotation to GenBank entry ECOUW76).

To create pBTKScyd1, a 1,017-bp region of DNA from the upstream region of the *cydAB* operon (corresponding to bases 1 to 1017 in GenBank entry J03939) was amplified by PCR with primers CYD1 (5′ GCGGATCCGAGGTGATTTC TTGCTCAGAAACG) and CYD2 (5′ GGGAATTCAAGGCAAACTGTAAG CGCGACAGTTCG), using *E. coli* MC4100 DNA as a template. The PCR product was digested with *Bam*HI and *Eco*RI and cloned in the corresponding sites of pBluescriptII KS$^-$.

To create pBTKScyd2, a 623-bp region of DNA from the upstream region of the *cydAB* operon (corresponding to bases 260 to 882 in GenBank entry J03939) was amplified by PCR with primers CYD3 (5′ AGTGGATCCGTAGACACTG GCAAGCGTAGCG) and CYD4 (5′ ATCGAATTCATCATACCATGTGAAT GCACAC), using *E. coli* MC4100 DNA as a template. The PCR product was digested with *Bam*HI and *Eco*RI and cloned in the corresponding sites of pBluescriptII KS$^-$.

To create pBTKScyd3, a 480-bp region of DNA from the upstream region of the *cydAB* operon (corresponding to bases 403 to 882 in GenBank entry J03939) was amplified by PCR with primers CYD5 (5′ AGTGGATCCTATTGGCGGG GTGGATTATGCC) and CYD4 (5′ ATCGAATTCATCATACCATGTGAA TGCACAC), using *E. coli* MC4100 DNA as a template. The PCR product was digested with *Bam*HI and *Eco*RI and cloned in the corresponding sites of pBluescriptII KS$^-$.

To create pBTKScyd4, a 458-bp region of DNA from the upstream region of the *cydAB* operon (corresponding to bases 260 to 717 in GenBank entry J03939) was amplified by PCR with primers CYD3 (5′ AGTGGATCCGTAGACACTG GCAAGCGTAGCG) and CYD6 (5′ CCGAATTCGCTAAATTACCGCCTTT CAGCC), using *E. coli* MC4100 DNA as a template. The PCR product was digested with *Bam*HI and *Eco*RI and cloned in the corresponding sites of pBluescriptII KS$^-$.

To create pBTKScyd5, a 287-bp region of DNA from the upstream region of the *cydAB* operon (corresponding to bases 731 to 1017 in GenBank entry J03939) was amplified by PCR with primers CYD7 (5′ CGGATCCGTCGAAAAATGC AAATTTGCTTC) and CYD2 (5′ GGGAATTCAAGGCAAACTGTAAGCG CGACAGTTCG), using *E. coli* MC4100 DNA as a template. The PCR product was digested with *Bam*HI and *Eco*RI and cloned in the corresponding sites of pBluescriptII KS$^-$.

DNA sequence analysis of plasmid pBTKScyd1 to -5, covering the region shown in Fig. 10, revealed a number of discrepancies between the cloned sequences and GenBank entry J03939. First, an A residue (position 6 in Fig. 10) was found instead of a C at the position corresponding to nucleotide 343 of GenBank entry J03939. Second, a GGG triplet (positions 466 to 468 in Fig. 10) was found instead of a CCC triplet at positions corresponding to 803 to 805 in GenBank entry J03939; in the sequence reported by Fang and Gennis (14), the corresponding triplet is GGG. As these sequence differences were found in a number of independent PCR clones, it is assumed that the sequence shown in Fig. 10 represents that of *E. coli* MC4100.

Purification of His_6-ArcA protein. The His-tagged ArcA protein from IPTG-induced *E. coli* M15 cells transformed with pREP4 and pQE30ArcA was purified by nickel chelate affinity chromatography using Ni-nitrilotriacetic acid resin (Qiagen). Protein purification was performed at room temperature under denaturing conditions, using the buffer system described in standard protocol 7 in the QIAexpressionist manual (available from Qiagen), which employs 6 M guanidium hydrochloride for cell lysis and 8 M urea throughout subsequent purification steps. Removal of the urea from the protein preparation was achieved by dialysis (at 4°C) initially against 0.1 M sodium phosphate (pH 8.0)–1 mM Na_2EDTA–150 mM NaCl–10 mM β-mercaptoethanol–10% glycerol–0.5 mM phenylmethylsulfonyl fluoride and then with the same buffer containing 25% glycerol. Following dialysis, the protein was concentrated to ~2.5 mg/ml in Centricon 10 units (Amicon) and stored at −20°C. On average, 15 mg of purified His_6-tagged ArcA was obtained from 1 g of cell paste. Protein concentrations were estimated by using the Coomassie Plus protein assay reagent (Pierce), using bovine serum albumin as a standard.

Phosphorylation of His_6-ArcA protein. For the preparation of His_6-ArcA-P for use in DNA binding studies, we used a standard phosphorylation reaction in which His_6-ArcA (60 μg/ml, final concentration) was incubated for 1 h at 30°C in a buffer containing 100 mM Tris-Cl (pH 7), 10 mM $MgCl_2$, 125 mM KCl, and 50 mM disodium carbamoyl phosphate (Sigma). In parallel, similar reactions lacking disodium carbamoyl phosphate were used to prepare His_6-ArcA for use in DNA binding studies. Where indicated, 50 mM dilithium acetyl phosphate (Sigma) was used in similar reactions. The concentrations of acetyl phosphate and carbamoyl phosphate and the times of incubation used were based on the results of a previous study (13).

Preparation of radioactively labeled DNA fragments. PCR-amplified products were purified from acrylamide gels, and the concentration of the purified DNA was determined spectrophotometrically (1). End labeling of the purified DNA fragments was performed with T4 polynucleotide kinase and [γ-^{32}P]ATP (3,000 Ci/mmol; New England Nuclear). End-labeled fragments for DNase I footprinting assays were generated by digestion of the labeled DNA fragments with a restriction endonuclease that cleaves (uniquely) close to either of the termini. Nonincorporated nucleotides and the short ^{32}P-labeled, double-stranded DNA fragments generated by restriction enzyme digestion were removed by sequential ethanol precipitation.

DNA binding assays. EMS assays were performed with both acrylamide and agarose gel matrices; in both instances, gels were run at 4°C in 1× Tris-borate-EDTA buffer (1). Protein samples were combined with ^{32}P end-labeled DNA substrates (~2 to 5 nM, final concentration; ~10,000 to 25,000 cpm) in 20- to 50-μl reaction volumes containing 100 mM Tris-Cl (pH 7.4), 100 mM KCl, 10 mM $MgCl_2$, 10% glycerol, and 2 mM dithiothreitol. After incubation for 30 min at 25°C, 1/5 volume of a 5× gel loading buffer (1) was added, and the mixtures were loaded directly onto prerun gels. Where indicated, sheared (100- to 600-bp) herring sperm DNA (Promega Corp.) was included as a nonspecific competitor at a concentration equivalent to a 500-fold molar excess over the labeled DNA binding substrate. The molar excess of competitor DNA was calculated on the basis of the number of fragments, using data provided by the suppliers of the herring sperm DNA. A high-ionic-strength buffer (50 mM Tris, 0.4 M glycine, 2 mM Na_2EDTA) was also used in some acrylamide EMS assays (1).

Analysis of DNA binding by nuclease protection assays was performed with RQ1 DNase I and reagents supplied in the Core Footprinting kit (Promega). Briefly, protein samples were combined with ^{32}P end-labeled DNA substrates (~2 nM, final concentration; ~10,000 cpm) in 100-μl reaction mixtures containing 50 mM Tris-Cl (pH 8.0), 100 mM KCl, 12.5 mM $MgCl_2$, 1 mM Na_2EDTA, 20% glycerol, and 1 mM dithiothreitol. The reaction mixtures were incubated for >10 min on ice, 100 μl of 5 mM $CaCl_2$–10 mM $MgCl_2$ was added, and the incubation was continued for 1 min at room temperature. Then 6 μl (0.3 U) of RQ1 RNase-free DNase I was added, and following a further minute at room temperature, the nucleolytic reaction was terminated by addition of 180 μl of 200 mM NaCl–30 mM Na_2EDTA–1% sodium dodecyl sulfate (SDS)–100 μg of yeast RNA per ml. Following phenol-chloroform extraction, DNA products were recovered by ethanol precipitation and resuspended in 0.1 M NaOH-formamide (1:2, vol/vol)–0.1% xylene cyanol–0.1% bromophenol blue. Reaction mixtures were heated at 95°C for 2 min prior to loading on 5% polyacrylamide sequencing gels.

RESULTS

Purification and characterization of His_6-ArcA. pQE30ArcA was constructed to facilitate simple purification of a version of the ArcA protein which bears an oligohistidine affinity tag (His_6) at its amino terminus. Similar fusions to a number of other response regulators of the two-component family have been found not to perturb in vivo function or in vitro activity (3, 11, 15, 20, 21, 30–32, 35, 43), and the results of two complementation studies suggest that the His_6-ArcA protein is functional in vivo. First, the toluidine blue sensitivity phenotype of an Δ(*arcA*) mutant strain (SP314) was found to be complemented by transformation with pREP4 and pQE30ArcA or with pACYC184lacI^q and pQE30ArcA. Second, the Φ(*sdh-lac*) fusion of ECL589 cells doubly transformed with pACYC184 lacI^q and pQE30ArcA became anaerobically repressed (data not shown). Since these observations were made in both the presence (5 μM) and the absence of IPTG, it appears that LacI-mediated repression of the phage T5 promoter of pQE30ArcA is incomplete in cells which also harbor either the pREP4 or pACYC184lacI^q plasmid (both of which constitutively express the *lacI* gene).

The His_6-tagged ArcA protein was purified from IPTG-treated *E. coli* M15 cells transformed with pREP4 and pQE30ArcA by nickel chelate affinity chromatography under denaturing conditions, and the protein was renatured by gradual removal of the denaturant (urea) by dialysis. SDS-polyacrylamide gel electrophoresis analysis revealed that the His_6-ArcA protein has an apparent molecular mass of 29 kDa (in good agreement with the theoretical molecular mass of 28.8 kDa) and that the preparation was essentially homogeneous. In addition, the purified His_6-ArcA protein was found to serve as a substrate for phosphorylation by purified ArcB kinase in vitro (34a), essentially as described previously for purified

ArcA protein (33). This finding suggests the His_6-ArcA protein had refolded appropriately during renaturation.

A number of response regulator proteins undergo autophosphorylation in vitro when incubated in the presence of various low-molecular-weight organo-phosphoryl compounds (3, 11, 15, 20, 21, 30–32, 35, 43), and purified ArcA has been reported to autophosphorylate in the presence of either acetyl phosphate or carbamoyl phosphate (13). Incubation of His_6-ArcA protein in buffers containing 10 to 50 mM acetyl phosphate or carbamoyl phosphate resulted in precipitation of the protein when the concentration of the protein exceeded 100 μg/ml (3.5 μM). Similar precipitation of the His_6-ArcA protein was also observed following in vitro phosphorylation by ArcB (34a). The precipitation may well result from a phosphorylation-mediated change in conformation, as has been suggested in the case of the homologous OmpR protein (30). In the DNA binding studies described below, all autophosphorylation reactions were carried out at 60 μg of protein per ml, and no apparent precipitation occurred. Protein treated in this way is hereafter referred to as His_6-ArcA-P.

Binding of His_6-ArcA and His_6-ArcA-P proteins to the transcriptional regulatory region of the *lctPRD* operon. In a preliminary search for specific DNA binding sites for His_6-ArcA and His_6-ArcA-P in the *lct* operon, EMS assays were performed with restriction fragments spanning a 6,633-bp region which includes the complete coding sequences of the *lctPRD* genes and 1,698 bp upstream of the initiation codon of LctP. However, electrophoretic mobility shifts were readily observed only when reaction mixtures included His_6-ArcA-P and DNA fragments that contain sequences located upstream of the *lctP* ORF; this region was therefore subjected to more detailed analysis.

The binding of His_6-ArcA and His_6-ArcA-P to a 325-bp DNA fragment (derived from pBTKSlct) which includes 275 bp of the region upstream of the initiation codon of LctP, as determined by an acrylamide gel EMS assay, is shown in Fig. 1A. The binding of His_6-ArcA to the fragment appears to be nonspecific since it is effectively counteracted by inclusion of a heterologous, nonlabeled competitor DNA (lanes 1 to 10). In contrast, the EMS patterns observed with His_6-ArcA-P (lanes 11 to 20) appear to be specific and are indicative of the possibility that multiple protein molecules are bound to the same probe fragment. Similar sets of DNA binding reactions were also analyzed by EMS assays which used agarose gels, and typical results are shown in Fig. 1B. Under this electrophoretic condition, mobility shifts are observed only with His_6-ArcA-P, presumably because the nonspecific complexes formed between the probe fragment and His_6-ArcA are not stable during electrophoresis. Finally, the notion that complexes formed by His_6-ArcA and this 325-bp fragment of *lct* are nonspecific is supported by the failure to observe DNA binding if the reactions are analyzed on polyacrylamide gels run with a high-ionic-strength buffer system (1). In contrast, use of these same gel conditions was found to have little effect on the nature of complexes observed with His_6-ArcA-P (data not shown).

The binding of His_6-ArcA and His_6-ArcA-P to the same 325-bp *lct* DNA fragment was also analyzed by the DNase I footprinting technique, in reactions in which the protein/DNA ratios were varied over a range comparable to those used in the EMS assays. Typical results are shown in Fig. 2. No discrete regions of protection from DNase I cleavage were observed in reactions with His_6-ArcA, whereas clear DNase I footprints were obtained with His_6-ArcA-P. The regions of the template DNA at which His_6-ArcA-P binding significantly alters DNase I reactivity are indicated in Fig. 3. In these and other, similar assays, minimal regions of DNase I protection of around 55 bp

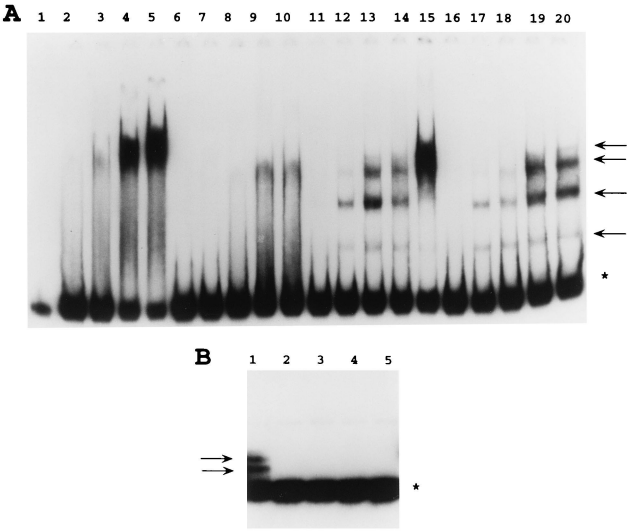

FIG. 1. (A) EMS assay of the binding of His_6-ArcA and His_6-ArcA-P to the *lctPRD* promoter region, using a 4% polyacrylamide gel. The ^{32}P-labeled, 325-bp DNA fragment (obtained by PCR amplification of the *lct* region of pBTKSlct with primers LCT1 and LCT2) was present at 5 nM in reaction mixtures which contained 0.75 μM His_6-ArcA (lanes 1 and 6), 1.5 μM His_6-ArcA (lanes 2 and 7), 2.25 μM His_6-ArcA (lanes 3 and 8), 3 μM His_6-ArcA (lanes 4 and 9), 3.75 μM His_6-ArcA (lanes 5 and 10), 0.75 μM His_6-ArcA-P (lanes 11 and 16), 1.5 μM His_6-ArcA-P (lanes 12 and 17), 2.25 μM His_6-ArcA-P (lanes 13 and 18), 3 μM His_6-ArcA-P (lanes 14 and 19), and 3.75 μM His_6-ArcA-P (lanes 15 and 20). A 500-fold molar excess (2.5 mM) of unlabeled competitor DNA was included in lanes 6 to 10 and 16 to 20. (B) EMS assay of the binding of His_6-ArcA and His_6-ArcA-P to the same 325-bp *lctPRD* fragment, using a 1.4% agarose gel. The DNA substrate was present at 2 nM in reaction mixtures which contained 1 μM His_6-ArcA-P (lane 1), 0.25 μM His_6-ArcA-P (lane 2), 1 μM His_6-ArcA (lane 3), 0.25 μM His_6-ArcA (lane 4), or no protein (lane 5). No unlabeled competitor was present in these reaction mixtures. Arrows indicate the positions of migration of discrete complexes formed between His_6-ArcA-P and the DNA substrate; asterisks indicate the position of migration of the unbound DNA fragment.

(positions 74 to 128 in Fig. 3) were observed on both strands. The protected region extended to a maximal size of around 100 bp (positions 46 to 145 in Fig. 3) as the His_6-ArcA-P concentration was increased.

The locations of the two *lct* promoters shown in Fig. 3 were determined by primer extension analysis of mRNA transcripts originating from the *lctPRD* upstream region, using RNA derived from cells grown aerobically in the presence or absence of L-lactate (data not shown). The position of the *lct* P_2 promoter, which appeared to be active only in cells growing in the presence of L-lactate, is in accord with a prediction made from analysis of the genomic sequence of *E. coli* MG1655 (see annotation to GenBank entry ECOUW76) but is 11 bp downstream of the promoter proposed by Quail and Guest (41). As the *lct* P_2 promoter is within the minimal region protected from DNase I cleavage by His_6-ArcA-P, it seems most likely that binding of ArcA-P to this site in anaerobic cells would result in effective sequestration of the promoter from RNA polymerase and thereby mediate transcriptional repression. Since anaerobic expression of a chromosomal Φ(*lctD-lacZ*) fusion is elevated in cells which harbor pBTKSlct (bearing a 275-bp region upstream of the *lctP* ORF), it seems likely that LctR functions as a repressor.

Binding of His_6-ArcA and His_6-ArcA-P proteins to the *gltA-sdhCDAB* intergenic region. In a preliminary search for specific DNA binding sites for His_6-ArcA and His_6-ArcA-P in the *glt-sdh* intergenic region by EMS assays, a 769-bp DNA fragment (derived from pBTKSgltA-sdh1), which includes all of

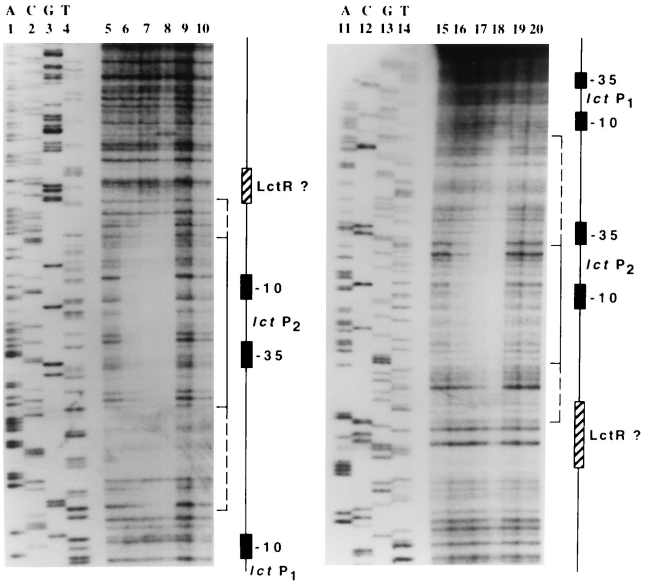

FIG. 2. DNase I protection assays of the binding of His_6-ArcA and His_6-ArcA-P to the *lctPRD* promoter region. A 325-bp DNA fragment (obtained by PCR amplification of the *lct* region of pBTKSlct with primers LCT1 and LCT2) was end labeled with ^{32}P at its 5′ termini by treatment with T4 polynucleotide kinase, and uniquely labeled substrates for DNase I protection assays were generated by *Eco*RI digestion (lanes 5 to 10) or *Bam*HI digestion (lanes 15 to 20). The substrate DNA was present at 2 nM in reaction mixtures which also contained no added protein (lanes 5 and 15), 0.2 μM His_6-ArcA-P (lanes 6 and 16), 0.4 μM His_6-ArcA-P (lanes 7 and 17), 0.8 μM His_6-ArcA-P (lanes 8 and 18), 0.2 μM His_6-ArcA (lanes 9 and 19), and 0.8 μM His_6-ArcA (lanes 10 and 20). The sequencing ladders were generated by extension of ^{32}P end-labeled primer LCT1 (lanes 1 to 4) or LCT2 (lanes 11 to 14), using pBTKSlct as a template.

the three known promoters and overlaps the ORF's of both *gltA* and *sdhC* (9, 40, 50, 51), was used. Electrophoretic mobility shifts of this fragment, and shorter derivatives thereof, indicated the presence of multiple binding sites for His_6-ArcA-P in the central promoter region. Subsequent EMS assays used a 395-bp DNA fragment (derived from pBTKSgltA-sdh2) which includes the three promoters, and the results of a typical acrylamide gel EMS assay are shown in Fig. 4A. As in the case of the *lctP* upstream region, while the binding of His_6-ArcA to the *glt-sdh* fragment appeared to be nonspecific, the binding of His_6-ArcA-P appeared to be specific and resulted in the formation of multiple discrete complexes. The binding of His_6-ArcA to this fragment seems less affected by the inclusion of the heterologous competitor DNA than that observed with the *lct* fragment. This may be due to some intrinsic feature of the *glt-sdh* fragment, such as its overall high A+T content, which may facilitate DNA bending. However, complexes formed between His_6-ArcA and the 396-bp fragment are not observed if the DNA binding reactions are analyzed on polyacrylamide gels run with a high ionic-strength-buffer system (1). In contrast, use of these same conditions has only a minor effect on the nature of complexes formed with His_6-ArcA-P (data not shown). The results of agarose gel EMS assays (as shown in Fig. 4B) also support the notion that the His_6-ArcA complexes are non-sequence specific, since electrophoretic mobility shifts are observed only with His_6-ArcA-P.

The binding of His_6-ArcA and His_6-ArcA-P to the same 396-bp DNA fragment was also analyzed by the DNase I footprinting technique, in reactions in which the protein/DNA ratios was varied over a range comparable to those used in the EMS assays. Typical results are shown in Fig. 5. No discrete regions of protection from DNase I cleavage were observed in reactions with His_6-ArcA, while clear DNase I footprints were obtained with His_6-ArcA-P. The locations of the His_6-ArcA-P-specific sites of protection from DNase I cleavage are depicted in Fig. 6. Two minimal regions of protection were observed at relatively low protein/DNA ratios that extended over regions of 105 bp (positions 120 to 225 in Fig. 6) and 31 bp (positions 271 to 302 in Fig. 6). As the His_6-ArcA-P concentration was further increased, continuous protection of bases from DNase I cleavage was observed on both strands over a region which encompasses some 230 bp (positions 72 to 302 in Fig. 6).

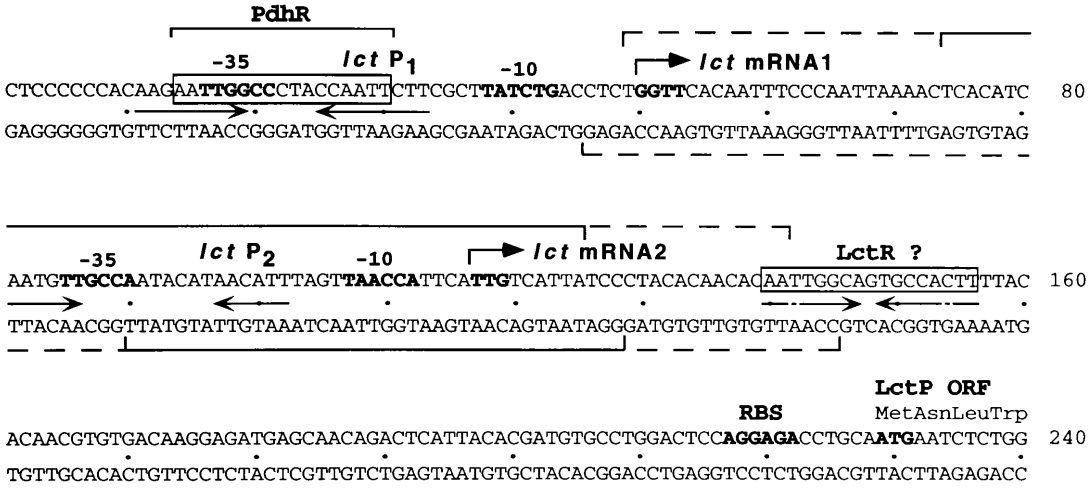

FIG. 3. Organization of transcriptional regulatory elements of the *lctPRD* operon. The *lct* P_1 and a *lct* P_2 promoters were identified by primer extension analysis of RNA preparations made from cells grown aerobically in the presence or absence of L-lactate; the *lct* P_2 promoter appears to be active only in cells growing in the presence of L-lactate. Also shown are the locations of potential binding sites for LctR and the pyruvate dehydrogenase regulator (PdhR) (see Discussion). In addition, regions of dyad symmetry in the DNA sequence are indicated by pairs of inverted arrows. The core and extended ArcA-P binding sites, as defined by the patterns of altered DNase I reactivity observed on both strands in the presence of His_6-ArcA-P, are indicated by continuous and dashed lines, respectively. RBS, ribosome binding site.

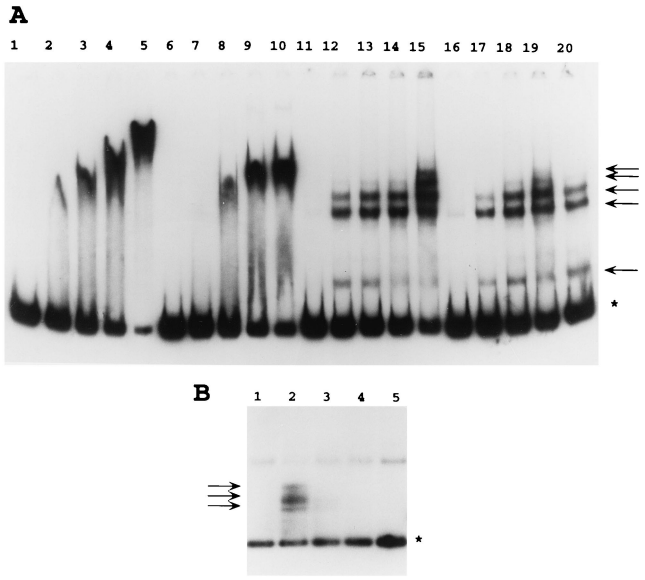

FIG. 4. (A) EMS assay of the binding of His_6-ArcA and His_6-ArcA-P to the *gltA-sdhC* intergenic region, using a 4% polyacrylamide gel. The ^{32}P-labeled, 395-bp DNA fragment (obtained by PCR amplification of the *glt-sdh* region of pBTKSglt-sdh2 with primers GLT2 and SDH2) was present at 5 nM in binding mixtures which contained 0.75 μM His_6-ArcA (lanes 1 and 6), 1.5 μM His_6-ArcA (lanes 2 and 7), 2.25 μM His_6-ArcA (lanes 3 and 8), 3 μM His_6-ArcA (lanes 4 and 9), 3.75 μM His_6-ArcA (lanes 5 and 10), 0.75 μM His_6-ArcA-P (lanes 11 and 16), 1.5 μM His_6-ArcA-P (lanes 12 and 17), 2.25 μM His_6-ArcA-P (lanes 13 and 18), 3 μM His_6-ArcA-P (lanes 14 and 19), and 3.75 μM His_6-ArcA-P (lanes 15 and 20). A 500-fold molar excess (2.5 mM) of unlabeled competitor DNA was included in lanes 6 to 10 and 16 to 20. (B) EMS assay of the binding of His_6-ArcA and His_6-ArcA-P to the same 395-bp *gltA-sdhC* fragment, using a 1.4% agarose gel. The ^{32}P-labeled, 395-bp DNA fragment was present at 2 nM in binding mixtures which contained no protein (lane 1), 1 μM His_6-ArcA-P (lane 2), 0.25 μM His_6-ArcA-P (lane 3), 1 μM His_6-ArcA (lane 4), and 0.25 μM His_6-ArcA (lane 5). Arrows indicate the positions of migration of discrete complexes formed between His_6-ArcA-P and the DNA substrate; asterisks indicate the position of migration of the unbound DNA fragment.

In considering the mechanism whereby ArcA-P effects transcriptional repression of the *sdhCDAB* operon during anoxic transition, it seems most likely that binding of the protein to the *sdh* promoter region results in effective sequestration of promoter elements required for both basal and activated transcription. Binding of integration host factor (IHF) or catabolite gene activator protein (CAP) to the sites indicated in Fig. 6 has not been demonstrated, but studies of the expression of an Φ(*sdh-lac*) fusion in mutants that lack either of these activities suggest that IHF, but not CAP, plays a role in the activation of *sdhCDAB* expression in aerobic cells (40). It is noted that in the presence of His_6-ArcA-P, a site of DNase hypersensitivity (indicated by arrows in Fig. 6) was observed on both template strands at a position corresponding to one end of the putative IHF site. The high A+T content of this region (77% over the extended ArcA-P footprint) may facilitate ArcA-P (or IHF)-mediated deformation of the DNA.

The complex pattern of expression of the *gltA* gene may be accounted for by these data in the following way. In cells growing with high aeration, the ArcA-P levels are expected to be low and both the *gltA* P_1 and *gltA* P_2 promoters are expected to be active. As anoxia approaches, ArcA-P levels should rise and occupancy of the ArcA-P site(s) which span the *sdh* P_1-*gltA* P_1 region is predicted to increase, resulting in repression of these promoters. Since complete repression of a Φ(*glt-lac*) fusion is not observed in fully anaerobic cells, and as even the extended DNase I footprint observed with His_6-ArcA-P in these studies does not overlap elements of the *gltA* P_2 promoter, it seems likely that the activity of this promoter is not regulated by ArcA-P. However, the location of a binding site for an additional putative regulator of the *gltA* operon (39) remains to be identified.

Binding of His_6-ArcA and His_6-ArcA-P proteins to the promoter region of the *cydAB* operon. In contrast to *lct* and *glt-sdh*, for which ArcA-P is expected to serve as a transcriptional repressor, the *cydAB* operon appears to be activated by ArcA-P (7, 8, 17, 25, 46, 49). EMS assays were initially used to locate specific DNA binding sites for His_6-ArcA and His_6-ArcA-P in the *cydAB* promoter region, using DNA fragments (derived from pBTKScyd1) which span a 1,017-bp region including 974 bp upstream of the initiation codon of CydA and therein the previously characterized *cydAB* promoter (14). Whereas His_6-ArcA was found to bind this fragment nonspecifically, His_6-ArcA-P was found to bind at two distinct, well-separated sites upstream of the known promoter (designated sites I and II) and also at a further site (III) located downstream of that promoter. Binding of His_6-ArcA and His_6-ArcA-P to a DNA fragment containing site III, as determined by acrylamide and agarose gel EMS assays, is shown in Fig. 7.

The locations of the three binding sites were more precisely determined by DNase I footprinting, using a variety of substrate DNA templates generated by PCR amplification of the inserts of plasmids pBTKScyd1 to -5. Typical results are shown in Fig. 8 and 9. The locations of the His_6-ArcA-P specific sites of protection from DNase I cleavage are depicted in Fig. 10. Analysis of templates which contained sites I and II indicated that His_6-ArcA-P can bind at both sites simultaneously; similar observations were made with templates that contained both sites II and III. In contrast to the previous results obtained with the *lct* and *glt-sdh* templates, the size of the protected region observed at these sites was not significantly increased as the protein/DNA ratio was increased. Whereas ArcA-P binding at site III was readily detected by EMS assays, the DNase I footprints obtained on both strands at this site were weaker than those observed at the upstream sites I and II.

In considering the mechanism(s) whereby ArcA-P effects transcriptional activation of the *cydAB* operon, it seems most likely that binding of the protein at the upstream sites (I and II) mediates transcriptional activation of the *cydAB* P_1 promoter. The location of the ArcA-P binding site II relative to the putative Fnr binding site is intriguing. If Fnr does serve directly as a transcriptional repressor, then binding of Fnr to the Fnr box might prevent ArcA binding at its site II and possibly also interfere with contacts made between RNA polymerase and ArcA-P bound at site I. Alternatively, Fnr binding may have no effects on ArcA-P occupancy of sites I and II but mediate transcriptional repression by blocking access of RNA polymerase to the *cydAB* P_1 promoter.

ArcA-P binding at site III may serve either of two physiological roles. First, promoter mapping studies in which the region containing the *cydAB* P_1 promoter was deleted (14) indicate the presence of a second promoter located between the *Fnu*4HI and *Sph*I sites shown in Fig. 10. Since this second promoter was not detected by analysis of RNA prepared from aerobically grown cells, it is presumed that the *cydAB* P_1 promoter is strongly preferentially used in aerobic cells. Within the *Fnu*4HI-*Sph*I region, a potential promoter is apparent (an 8/12 match to the σ^{70} promoter sequence consensus; *cydAB* P_2 in Fig. 10). Hence, we propose that ArcA-P bound at site III is responsible for activation of this promoter in anoxic cells and that this accounts for the significant levels of expression of the *cydAB* operon observed in such cells (46) when Fnr action

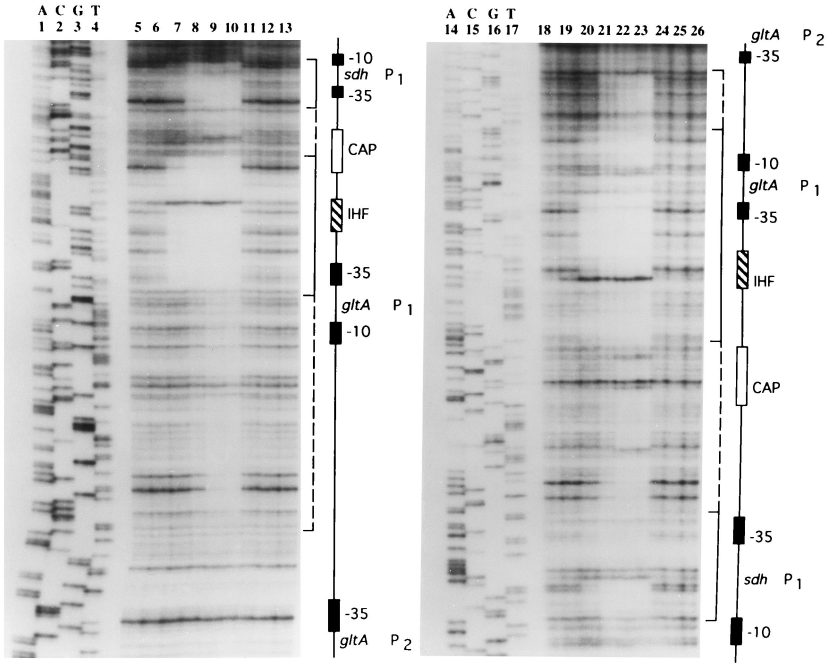

FIG. 5. DNase I protection assays of the binding of His_6-ArcA and His_6-ArcA-P to the *gltA-sdhC* intergenic region. A 395-bp DNA fragment (obtained by PCR amplification of the *glt-sdh* region of pBTKglt-sdh2 with primers GLT2 and SDH2) was end labeled with ^{32}P at its 5′ termini by treatment with T4 polynucleotide kinase, and uniquely labeled substrates for DNase I protection assays were generated by *Eco*RI digestion (lanes 5 to 13) or *Bam*HI digestion (lanes 18 to 26). The substrate DNA was present at 2 nM in reaction mixtures which also contained no added protein (lanes 5 and 18), 0.1 μM His_6-ArcA-P (lanes 6 and 19), 0.2 μM His_6-ArcA-P (lanes 7 and 20), 0.4 μM His_6-ArcA-P (lanes 8 and 21), 0.6 μM His_6-ArcA-P (lanes 9 and 22), 0.8 μM His_6-ArcA-P (lanes 10 and 23), 0.2 μM His_6-ArcA (lanes 11 and 24), 0.4 μM His_6-ArcA (lanes 12 and 25), or 0.8 μM His_6-ArcA (lanes 13 and 26). The sequencing ladders were generated by extension of ^{32}P end-labeled primer GLT2 (lanes 1 to 4) or SDH2 (lanes 14 to 17), using pBTKSglt-sdh2 as a template.

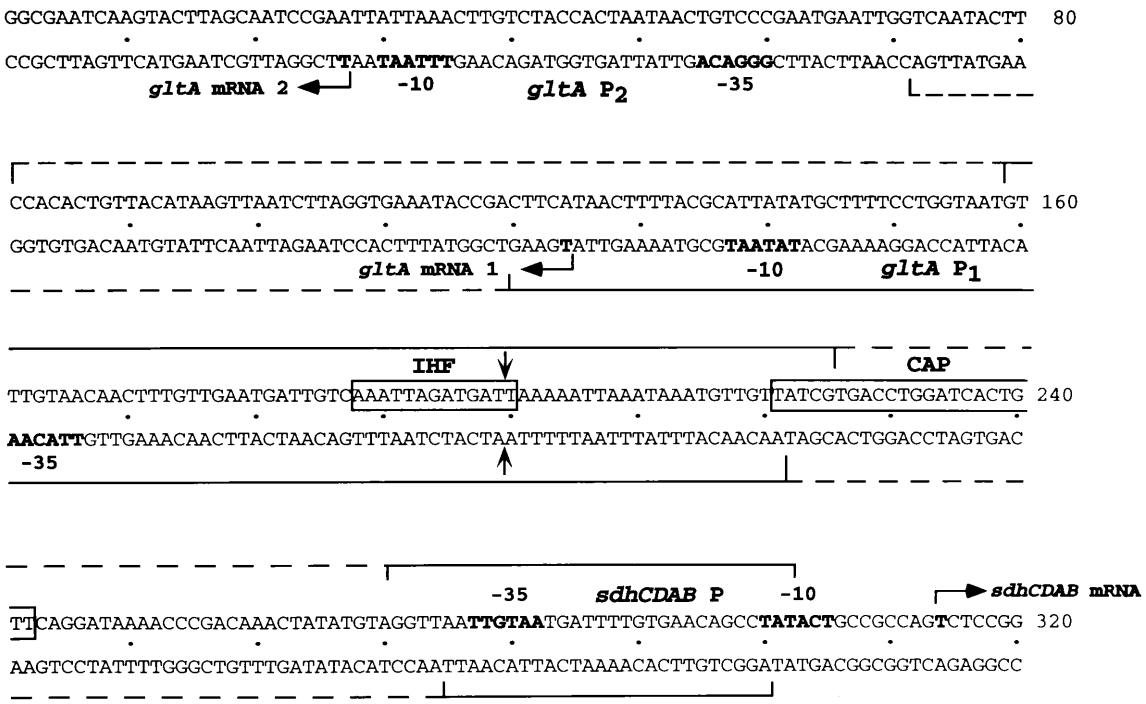

FIG. 6. Organization of transcriptional regulatory elements in the *gltA-sdhC* intergenic region. The position of a consensus CAP binding site is as identified by Busby and Kolb (4); the *sdhCDAB* P, *gltA* P_1, and *gltA* P_2 promoter locations are as described by Wood et al. (51). The location of a potential IHF binding site (37) is also shown. The core and extended ArcA-P binding sites, as defined by the patterns of altered DNase I reactivity observed on both strands in the presence of the His_6-ArcA-P protein, are indicated by continuous and dashed lines, respectively. The arrows indicate the position of a DNase I-hypersensitive site (see Results).

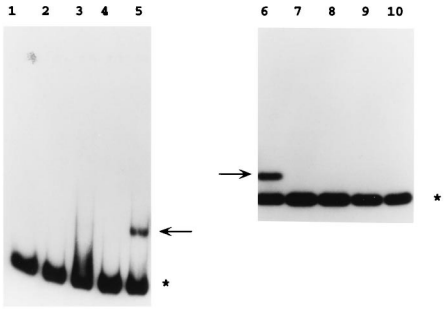

FIG. 7. EMS assay of the binding of His_6-ArcA and His_6-ArcA-P to a 298-bp DNA fragment derived from the *cydAB* upstream region containing ArcA-P binding site III, using a 4% polyacrylamide gel (lanes 1 to 5) or a 1.4% agarose gel (lanes 6 to 10). The ^{32}P-labeled, 298-bp DNA fragment (obtained by PCR amplification of the *cyd* region of pBTKScyd5 with primers CYD7 and CYD2) was present at 5 nM in binding mixtures which contained no added protein (lanes 1 and 10), 0.25 μM His_6-ArcA (lanes 2 and 9), 1 μM His_6-ArcA (lanes 3 and 8), or 0.25 μM His_6-ArcA-P (lanes 4 and 7), or 1 μM His_6-ArcA-P (lanes 5 and 6). A 500-fold molar excess (2.5 mM) of unlabeled competitor DNA was included in lanes 1 to 5. Arrows indicate the positions of migration of discrete complexes formed between His_6-ArcA-P and the DNA substrate; asterisks indicate the position of migration of the unbound DNA fragment.

prevents transcription from the upstream (*cydAB* P_1) promoter.

Alternatively, should the previously identified promoter (*cydAB* P_1) prove to be the sole promoter of the *cydAB* operon and Fnr found to serve as a direct repressor of that promoter, then ArcA-P may function as a corepressor of the operon by binding at site III and blocking progression of RNA polymerase in the elongation of transcripts emanating from *cydAB* P_1. In this case, it has to be assumed that the concerted actions of Fnr and ArcA-P fail to effect complete transcriptional repression of the *cydAB* operon in cells grown in the absence of oxygen. A priori to further experimentation, the former (dual promoter) model of transcriptional regulation seems more attractive.

DISCUSSION

The results obtained in this study are in broad agreement with those obtained with a number of other response regulator proteins of the two-component response regulator family (20). In particular, the results are in close accord with studies of the NarL protein, in which no sequence-specific DNase I footprints were obtained with the unphosphorylated form of the protein (31). However, our results are in apparent conflict with the results of a previous ArcA study, in which qualitatively similar DNase I footprints in the *pfl* promoter region were observed with or without prior incubation of the purified protein with carbamoyl phosphate or acetyl phosphate (13). As the DNase I footprints obtained with the untreated ArcA protein in the *pfl* study were observed only at protein/DNA ratios significantly higher than those used in our study, it is possible that the ArcA preparation used in the *pfl* study contained a significant fraction of protein in the phosphorylated form.

A more complete understanding of the regulation of the *lctPRD* operon will depend on knowledge of the DNA binding characteristics of the LctR protein, in the presence and absence of L-lactate. Recent studies of the pyruvate dehydrogenase regulator protein (PdhR) of *E. coli*, which is homologous to LctR in both the amino-terminal and carboxy-terminal domains, have led to the proposal of a potential LctR binding site located in the *lctPRD* promoter region (41). However, the location of this site seems incompatible with the functioning of

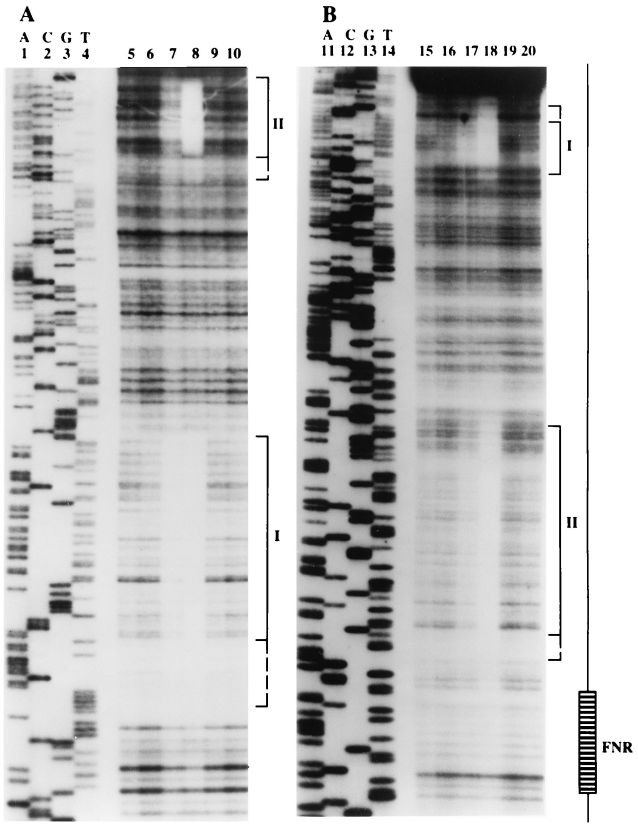

FIG. 8. DNase I protection assays of the binding of His_6-ArcA and His_6-ArcA-P to the *cydAB* upstream region containing ArcA-P binding sites I and II. A 475-bp DNA fragment (obtained by PCR amplification of the *cyd* region of pBTKcyd4 with primers CYD3 and CYD6) was end labeled with ^{32}P at its 5′ terminus by treatment with T4 polynucleotide kinase, and uniquely labeled substrates for DNase I protection assays were generated by *Eco*RI digestion (lanes 5 to 10) or *Bam*HI digestion (lanes 15 to 20). The substrate DNA was present at 2 nM in reaction mixtures which also contained no added protein (lanes 5 and 15), 0.2 μM His_6-ArcA-P (lanes 6 and 16), 0.4 μM His_6-ArcA-P (lanes 7 and 17), 0.8 μM His_6-ArcA-P (lanes 8 and 18), 0.2 μM His_6-ArcA (lanes 9 and 19), or 0.8 μM His_6-ArcA (lanes 10 and 20). The sequencing ladders were generated by extension of ^{32}P end-labeled primer CYD3 (lanes 1 to 4) or CYD6 (lanes 11 to 14), using pBTKScyd4 as a template.

LctR as a repressor of the *lct* P_2 promoter identified in this study and hence is labeled as a putative PdhR site in Fig. 3. A possible role for PdhR as an activator of the *lctPRD* operon, such as to enhance uptake and oxidation of L-lactate when glycolytic sources of pyruvate are exhausted, has been suggested (41). An alternate LctR binding site located downstream of the *lct* P_2 promoter seems more compatible with LctR function as a repressor of this promoter. One possibility regarding the function of the *lct* P_1 promoter is that it serves to maintain a basal level of the LctP protein in aerobic cells to facilitate the initial detection of L-lactate in the growth medium.

The results of the *glt-sdh* studies provide an elegant example of coordinated transcriptional regulation of genes encoding products of related metabolic function. Presumably, the physical coupling of promoters in this fashion affords the cell an efficient mechanism whereby coregulation of genes of related metabolic function can be achieved, in this case corepression of the *gltA* P_1 and *sdhCDAB* promoters (19). Numerous similar examples are already apparent in *E. coli* (2), and more can be expected with completion of the genome sequence.

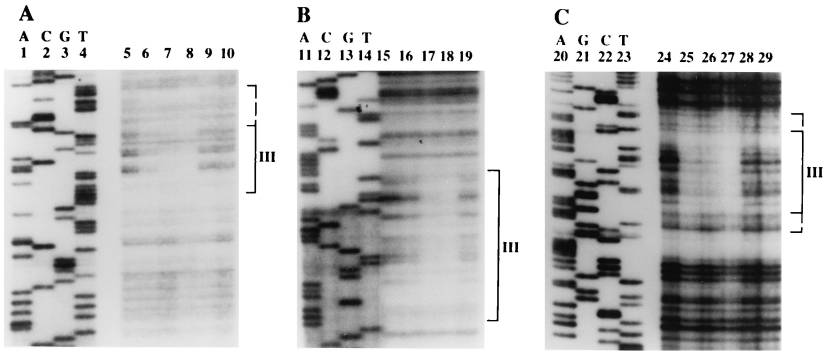

FIG. 9. DNase I protection assays of the binding of His_6-ArcA and His_6-ArcA-P to the *cydAB* upstream region containing ArcA-P binding site III. In panels A and C, a 298-bp DNA fragment (obtained by PCR amplification of the *cydAB* region of pBTKScyd5 with primers CYD7 and CYD2) was used; in panel B, a 495-bp DNA fragment (obtained by PCR amplification of the *cyd* region of pBTKScyd3 with primers CYD5 and CYD4) was used. Both templates were end labeled with ^{32}P at their 5′ termini by treatment with T4 polynucleotide kinase, and uniquely labeled substrates for DNase I protection assays were generated by *Eco*RI digestion (lanes 5 to 10) or *Bam*HI digestion (lanes 15 to 19 and 24 to 29). The substrate DNA was present at 2 nM in reaction mixtures which contained no added protein (lanes 5, 15, and 24), 0.2 μM His_6-ArcA-P (lanes 6, 16, and 25), 0.4 μM His_6-ArcA-P (lanes 7, 17, and 26), 0.8 μM His_6-ArcA-P (lanes 8, 18, and 27), 0.2 μM His_6-ArcA (lanes 9 and 28), or 0.8 μM His_6-ArcA (lanes 10, 19, and 29). In panels A and C, the sequencing ladders were generated by extension of ^{32}P end-labeled primer CYD7 (lanes 1 to 4) or CYD2 (lanes 20 to 23), using pBTKScyd5 as a template; in panel B, the sequencing ladder was generated by extension of ^{32}P end-labeled CYD4 primer, using pBTKScyd3 as a template.

Future studies of the transcriptional regulation of the *cydAB* operon should be aimed at determining whether Fnr serves in a direct manner as a transcriptional repressor, by binding at the consensus binding site located immediately adjacent to the ArcA-P site II. If it is found to do so, then it will be of interest to establish what effect(s) this has on ArcA-P function as an activator mediated by binding at sites I and II. Indeed, in vitro DNA binding studies using FNR* and ArcA-P proteins would appear to represent the simplest approach to resolving whether Fnr acts directly or indirectly in regulating the *cydAB* operon, as interpretation of in vivo studies is complicated by at least two effects that *fnr* mutations have on the Arc system. First, since Fnr is an activator of expression of the *arcA* gene (6), *fnr* mutants are expected to have significantly decreased anaerobic

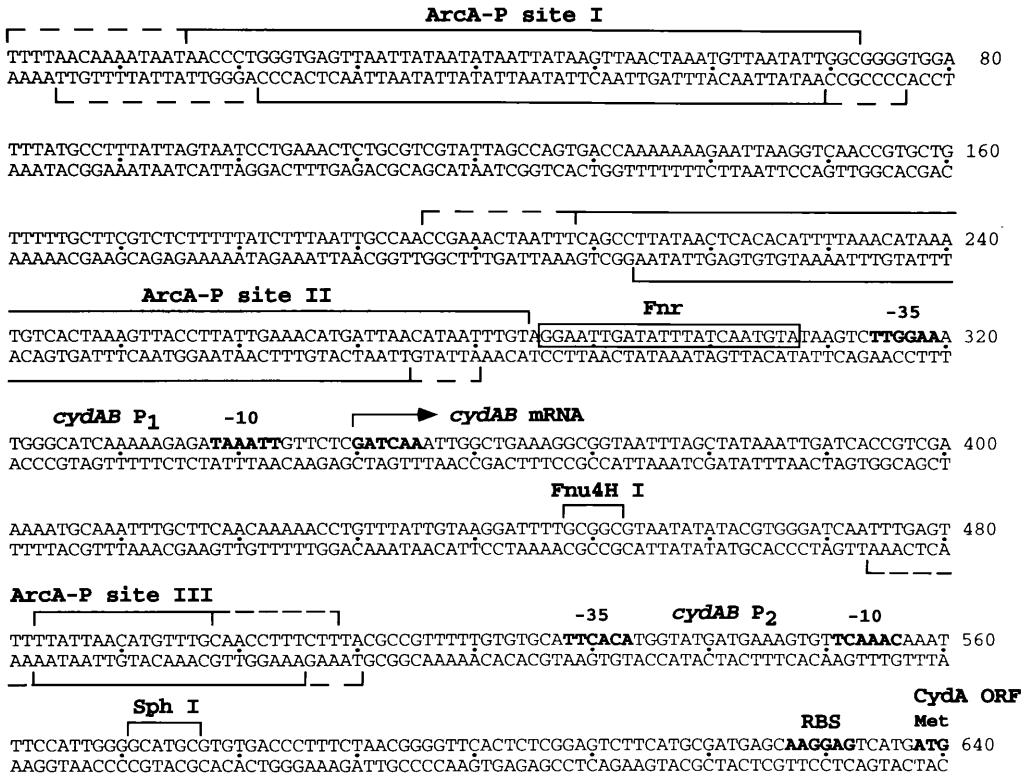

FIG. 10. Organization of transcriptional regulatory elements upstream of the *cydAB* operon. The positions of the Fnr binding site and the *cydAB* P_1 promoter are as described by Fang and Gennis (14); the location of a putative second promoter, labeled *cydAB* P_2, is also indicated (see text for details). The core and extended ArcA-P binding sites (labeled I, II, and III), as defined by the patterns of altered DNase I reactivity observed on both strands in the presence of the His_6-ArcA-P protein, are indicated by continuous and dashed lines, respectively.

TABLE 2. The ArcA-P box, or primary sequence DNA binding site consensus for ArcA-P[a]

Operon (site)	DNA sequence (±10 bp)			Match to consensus	Sequence reference and site position
Consensus		T T AGTTAATTAA			
sodA	GGTTCATTAT	AGTTAATTAA	ATGATATTGA	10/10	920–891 in M94879
	GGCGGCCGAT	TGTTAATGCC	GCGTAAGCAG	8/10	952–923 in M94879
pfl (A1)	TATAATGCTT	TGTTAGTATC	TCGTCGCCGA	6/10	430–459 in M26413
pfl (A2)	GCAAATACGG	AGTAAATATT	TGATTATCCA	7/10	364–335 in M26413
pfl (A3)	CCGCTAAAAC	AGTTAATTAA	AAGGGAGCAT	10/10	281–252 in M26413
	CGATCGATAT	TGTTACTTTA	TTCGCCTGAT	8/10	224–253 in M26413
pfl (A4)	GATTAGTCTG	AGTTATATTA	CGGGGCGTTT	7/10	107–136 in M26413
cyd (I)	ACCCTGGGTG	AGTTAATTAT	AATATAATTA	10/10	17–46 in Fig. 10
	TATAATTATA	AGTTAACTAA	ATGTTAATAT	9/10	39–68 in Fig. 10
cyd (II)	CTACAAATTA	TGTTAATCAT	GTTTCAATAA	9/10	286–257 in Fig. 10
	ATGTCACTAA	AGTTACCTTA	TTGAAACATG	7/10	240–269 in Fig. 10
cyd (III)	GTTGCAAACA	TGTTAATAAA	AACTCAAATT	8/10	501–472 in Fig. 10
sdh P	CAGTATAGGC	TGTTCACAAA	ATCATTACAA	7/10	306–277 in Fig. 6
	ACTATATGTA	GGTTAATTGT	AATGATTTTG	8/10	261–290 in Fig. 6
gltA P_1	GTAACAACTT	TGTTGAATGA	TTGTCAAATT	7/10	163–192 in Fig. 6
	TCAACAAAGT	TGTTACAAAC	ATTACCAGGA	6/10	178–149 in Fig. 6
lct P_2	CATAACATTT	AGTTAACCAT	TCATTGTCAT	8/10	94–123 in Fig. 3
	GACAATGAAT	GGTTAACTAA	ATGTTATGTA	8/10	121–92 in Fig. 3
acnA	CAATTTGGGT	TGTTATCAAA	TCGTTACGCG	7/10	1132–1161 in X60293
aceA	ACTTCCGATG	AGTTAATTGA	TTTCCTGACC	9/10	8–37 in M22621
ald	TGATGATTGA	TGTTAATTAA	CAATGTATTC	10/10	136–165 in M64541
	GTGAATACAT	TGTTAATTAA	CATCAATCAT	10/10	167–138 in M64541
appY	GTTGGTTTTT	TGTTAATTGA	ATGGTTCTAA	9/10	543–572 in M24530
	AGTGAGTTTA	TGTTAATAAA	AAGCATAGTA	9/10	693–722 in M24530
arcA	ACGGTTGAGT	TGTTAAAAAA	TGCTACATAT	8/10	673–702 in L20873
betI	TCATCAGCGG	TGTTTATCTA	TTAAAGCGGT	7/10	3401–3372 in X52905
betT	GTGTCTTAAT	TGTTACGAAT	TTGATTTTAA	7/10	3316–3345 in X52905
cyoA–E	GATAATTATT	TGTTAAATAA	TTGTTTTATT	9/10	340–369 in M55258
dad	AAAAGCCGCA	TGTTGAATAA	TATTTTCAAC	8/10	1567–1596 in L02948
fadB	GTGTATTTTG	TGTTAAAAAT	ATGCAAACAA	8/10	329–300 in M74164
icd	AGCAATTTTT	TGTTAATGAT	TTGTAATTGG	9/10	206–177 in J02799
nuoA–N	ATGCTAATGG	TGTTGATATT	ATGTAAACTA	8/10	152–181 in X68301
mdh	AACATATCTT	AGTTTATCAA	TATAATAAGG	8/10	42–71 in Y00129
traY	GTTAATAAGG	TGTTAATAAA	ATATAGACTT	9/10	1395–1424 in K01147

[a] The 10-bp consensus (5′ [A/T]GTTAATTA[A/T] 3′) was derived from sequence alignments of regions in template DNAs most strongly protected from DNase I cleavage by ArcA-P (or His_6-ArcA-P) binding. For the larger sites of DNase I protection (i.e., all those shown in the top except the *pfl* A1, A2, and A4 sites), only the two best fits to the consensus sequence from within the protected regions are included. In the lower half are shown the best fits to the consensus sequence from the transcriptional regulatory regions of a number of other loci which are thought to be regulated by the Arc system or which are known to be regulated in response to oxygen. The FASTA and FINDPATTERNS computer programs (Genetics Computer Group, Madison, Wis.) were used in the identification of many of these sites and also revealed numerous other *E. coli* loci which may also therefore be expected to be regulated by the Arc system (data not shown). The sequence references correspond to GenBank entries.

levels of ArcA protein. Second, *fnr* mutations probably perturb the pools of certain metabolites which may modulate ArcB function in vivo (22, 23).

Future studies should also be aimed at establishing the specific role of the *cydAB* P_2 promoter in maintaining *cydAB* expression in anoxic cells and the dependence of this promoter on ArcA-P-mediated activation from binding site III. As the affinity of ArcA-P appears to be weakest for site III, occupancy of this site is expected to be low in microaerophilic cells. Thus, elongation of transcripts emanating from the *cydAB* P_1 promoter would not be hindered. However, proper determination of parameters to describe the absolute affinity of the ArcA-P protein for the three binding sites will require measurement of the extent of autophosphorylation obtained following incubation with carbamoyl phosphate and elucidation of the stoichiometry of the protein-DNA complexes formed at each site.

At sites of repression (*lctPRD* and *gltA-sdhCDAB*), relatively small (~50-bp) regions of altered reactivity to DNase I cleavage are apparent when His_6-ArcA-P is present at the lowest concentration used in the footprinting assays. As the concentration of His_6-ArcA-P is increased, the protected regions expand to a finite length, which in the case of *gltA-sdhCDAB* corresponds to some 230 bp. One possible explanation is that at sites of repression, the His_6-ArcA-P protein binds to a nucleation site of relatively high affinity. A protein molecule bound at such a site may then facilitate binding of additional molecules to adjacent, lower-affinity sites through cooperative interactions. As the nucleation sites appear to be somewhat larger than may be expected for a monomer or dimer of the His_6-ArcA protein, it seems likely that the protein binds DNA as a higher-order oligomer. DNA wrapping by the protein is also possible, although no characteristic periodic enhancements of DNase I cleavage are apparent in the footprints obtained.

In contrast, for the transcriptional activation target studied (*cydAB*), the small (20- to 50-bp) regions of DNase I protection observed at sites I to III do not expand with increasing concentrations of His_6-ArcA-P. Qualitatively similar observations were previously reported in studies of ArcA (or ArcA-P) binding in the promoter region of another transcriptional activation target, *pfl* (13). In light of the observations made at sites of transcriptional repression, it seems most likely that the se-

quence context of these activation sites prevents cooperative binding of the protein at adjacent sequences.

Two previous reports suggested primary DNA sequence motifs which may represent an ArcA box. The first motif (composed of an interrupted palindromic, A+T-rich element) was based on analysis of the sequence of promoters of oxygen-regulated genes (36), while the second motif (5′ TATTTaa) was based on comparisons of the *sodA* and *pfl* promoter sequences protected from DNase I cleavage by ArcA or ArcA-P (13). As neither of these motifs is found in all of the regions of His_6-ArcA-P-mediated DNase protection observed in our studies, we sought to define a new primary DNA sequence binding motif (or ArcA-P box) by computer alignment of the DNA sequences within such regions of protection, using all available data. The resulting consensus sequence (5′ [A/T]GT TAATTA[A/T] 3′), and the alignments on which it is based, are shown in Table 2. Also included in Table 2 are sequences derived from the transcriptional regulatory regions of a number of other loci which are thought to be regulated by the Arc system or are known to be regulated in response to oxygen. Validation of the ArcA-P box consensus will require corroboration from future studies involving higher-resolution DNA footprinting analysis and, more importantly, mutagenesis experiments directed toward establishing that the sequence is necessary for Arc-mediated regulation in vivo.

Two basic mechanisms whereby the phosphorylation-mediated switch in ArcA DNA binding activity could be effected can be envisaged. First, it is possible that in the unphosphorylated state, the amino-terminal receiver domain of ArcA acts as an inhibitory domain which blocks the carboxy-terminal DNA binding region from site specifically interacting with DNA. Alternatively, phosphorylation of the receiver domain of ArcA may result in a conformational change which facilitates oligomerization of the protein and consequent adoption of a site-specific DNA binding mode. Phosphorylation-mediated dimerization of the receiver domains of the NtrC and PhoB proteins has recently been reported and is proposed to result in their activation as transcriptional regulators in vivo (16). However, while chemical cross-linking studies indicate that phosphorylation of the His_6-ArcA protein stimulates dimerization of the protein (34a), the effect appears to be insufficient to explain quantitatively the large differences observed in the in vitro DNA binding properties of the His_6-ArcA and His_6-ArcA-P proteins. It is possible that phosphorylation of the ArcA protein also facilitates cooperative interactions of molecules bound to the DNA, as is apparent in the case of the NtrC protein (see chapters 5 and 9 in reference 20). Future studies will be directed toward understanding the nature of the phosphorylation-mediated switch in ArcA DNA binding activity.

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