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Nature of DNA Binding and RNA Polymerase Interaction of the *Bordetella pertussis* BvgA Transcriptional Activator at the *fha* Promoter

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The expression of virulence factor genes in *Bordetella pertussis* is mediated by the BvgA-BvgS two-component signal transduction system. The response regulator, BvgA, acts directly as a transcriptional activator at the loci encoding pertussis toxin (*ptx*) and filamentous hemagglutinin (*fha*). Previous studies have demonstrated that these two loci are differentially regulated by BvgA. As an initial step in gaining insight into the mechanism underlying this differential regulation, we initiated DNA binding and in vitro transcription analyses to examine the activities of BvgA and RNA polymerase (RNAP) purified from both *B. pertussis* and *Escherichia coli* at the *fha* promoter. We discovered that unphosphorylated BvgA binds to a single region (−100 to −70, relative to the start of transcription), whereas phosphorylated BvgA binds both this region and another, farther downstream, that extends to the −35 nucleotide. In the absence of BvgA, RNAP binds a region farther upstream than expected (−104 to −35). However, occupation of both sites by BvgA phosphate repositions RNAP to the site used in vivo. The binding of BvgA phosphate to the downstream site correlates with in vitro transcriptional activity at the *fha* promoter. As the DNA binding and transcription activities of the *E. coli*-derived RNAP are similar to those observed for the *B. pertussis* enzyme, we employed several mutant *E. coli* proteins in in vitro transcription analyses. We observed that polymerases carrying either a deletion of the C-terminal domain of the α subunit or substitution of alanine at either of two critical residues within this domain were severely impaired in the ability to mediate BvgA-activated transcription at *fha*.

The regulation of virulence potential is recognized as a common attribute of bacterial pathogens. In *Bordetella pertussis*, the causative agent of whooping cough, this regulation is mediated by the BvgA-BvgS two-component sensory transduction system (2, 34, 44; reviewed in references 36 and 42). Changes in the extracellular environment are monitored by the BvgS histidine kinase or sensor protein. BvgS undergoes a series of environmentally sensitive intramolecular phosphorylation events that eventually lead to the transfer of a phosphoryl group to the response regulator, BvgA (41). Thus activated, BvgA then converts this signal into changes in the pattern of virulence factor expression by directly interacting with specific DNA binding sites on target genes (27, 28). BvgA regulates both positively and negatively, two distinct subsets of genes referred to as *vag* (*vir*-activated) and *vrg* (*vir*-repressed) genes. Recent evidence suggests that BvgA regulation of *vrg* genes is indirect and involves the activity of a BvgA-regulated repressor (19). On the other hand, BvgA appears to directly activate expression of *vag* genes by directly binding to sites on target promoters (3, 4, 17). Among these *vag* genes are the genes encoding filamentous hemagglutinin (*fha*) and pertussis toxin (*ptx*).

Recent studies conducted in both our laboratory and others suggest that BvgA distinguishes between promoters by at least two very different mechanisms. Enhanced expression of *fha* almost immediately follows in vivo stimulation of BvgA, whereas levels of *ptx* mRNA are augmented only after several hours (30, 31). In addition, differential in vivo response of *fha* and *ptx* reporter gene constructs to various concentrations of BvgA-BvgS modulators has been observed (36a). Given these observations, Scarlato and coworkers proposed that activation of *ptx* requires a threshold concentration of intracellular BvgA phosphate higher than that required for *fha* promoter activation (30). Consistent with this proposal, recent DNA binding studies demonstrated that BvgA phosphate binds to the *ptx* promoter at multiple recognition sites that are far upstream of the RNA polymerase (RNAP) binding site (4). Thus, BvgA may differentiate between these two promoters by the need to occupy a multiplicity of lower-affinity recognition sites (relative to *fha*) on *ptx*.

The isolation and characterization of mutants that differentiate between these two promoters have suggested yet another level at which BvgA may distinguish between *fha* and *ptx*. Mutations in BvgA which abolished *ptx* expression but resulted in normal levels of *fha* expression were mapped to the very C-terminal region of the polypeptide (35). Carboxy-terminal deletion analyses of the LuxR activator revealed that this BvgA-homologous region may be important for transcriptional activation but not DNA binding activity (6). In addition, we have recently shown that mutations suppressing the phenotype conferred by short, C-terminal BvgA deletions map to the gene encoding the α subunit of RNAP in *B. pertussis* (36a). In both cases, these BvgA mutations lie downstream of the putative helix-turn-helix DNA binding motif. This result has led us to hypothesize that these mutations may identify a region in BvgA that directly interacts with RNA polymerase. Moreover, these observations suggest that BvgA-RNAP contacts may differ, depending on the promoter context.

Carbonetti et al. have recently shown that 1.5- to 2-fold

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overexpression of the α subunit of RNAP can result in a Ptx^- Fha^+ phenotype in *B. pertussis* (5). This observation was interpreted as a titration of BvgA from the intracellular pool by the α subunit to affect only *ptx* expression while leaving *fha* expression unaffected. Implicit in this model is a direct physical interaction between BvgA and the α subunit of RNAP. To examine this potential interaction in more molecular detail, we initiated in vitro DNA binding and transcription studies by using purified RNAP from both *B. pertussis* and *Escherichia coli*. We show that there are at least two regions in the *fha* promoter protected by BvgA phosphate in DNase I footprinting analyses and that in the absence of BvgA, an alternative upstream site is bound by polymerase. The binding of BvgA phosphate repositions RNAP to a site farther downstream and correlates with enhanced transcriptional stimulation of *fha*. In addition, studies utilizing *E. coli* polymerase mutants demonstrated that activation requires the C-terminal domain of the polymerase α subunit. Transcription analyses of several alanine-substituted mutants clustered in a single alpha helix within this domain strongly suggest that BvgA contacts the side chains of arginine 265 and asparagine 268 when both are bound to the *fha* promoter.

MATERIALS AND METHODS

Plasmid construction. To create plasmid p*fhaP*, a 202-bp DNA fragment containing 155 bp of the *fha* promoter sequence (−155 to +1) was synthesized by PCR with 5′ and 3′ oligonucleotides containing restriction enzyme sites for *Bam*HI and *Sal*I, respectively. The PCR fragment was digested with *Bam*HI and *Sal*I and cloned either into the pKS^+-Bluescript vector (Stratagene) to create p*fhaP* or into the pTE103 transcription vector (10) to yield plasmid pTE-FHA. The DNA sequence of the insert was confirmed by the dideoxyribonucleotide method of Sanger et al. (29). The DNA fragment containing the *lacUV5* promoter was created by amplifying a 300-bp region stretching from −225 to +75 relative to the transcription start site. Transcription from this promoter yields in vitro transcripts of 75 nucleotides (nt).

Preparation of protein samples. Inclusion bodies containing wild-type BvgA proteins were prepared from 250 ml of isopropyl-β-D-thiogalactopyranoside (IPTG)-induced BL21 DE3 p*lys*S *E. coli* cells as previously described (4), with the following modifications. Inclusion bodies were washed twice in 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-NaOH (pH 7.4)–0.5 M NaCl–5 mM EDTA–1 mM dithiothreitol (DTT)–0.1% Triton X-100. The pellet was then washed once with 10 mM HEPES-NaOH (pH 7.4)–50 mM NaCl–5 mM EDTA–1 mM DTT. The pellet was resuspended in 4 ml of 50 mM imidazole-NaOH (pH 6.8)–8 M urea–1 mM DTT, and insoluble material was removed by centrifugation at 13,000 × *g* for 10 min. The sample was then loaded onto an 8-ml Q-Sepharose Fast Flow (Pharmacia) column pre-equilibrated with column buffer. The flowthrough fraction containing purified BvgA was collected and dialyzed against 10 mM HEPES-NaOH (pH 7.4)–5 mM EDTA–0.2 mM DTT. The white precipitate that formed was collected by centrifugation at 10,000 × *g* for 10 min. A portion of this precipitate was resuspended to a final total protein concentration of 0.15 mg/ml in 50 mM NaH_2PO_4 (pH 6.8)–6 M guanidinium-HCl–1 mM DTT. Insoluble material was again removed by centrifugation at 13,000 × *g* for 10 min. The sample was then dialyzed against several changes of dialysis buffer (20 mM HEPES-NaOH [pH 7.4], 10 mM $MgCl_2$, 50 mM KCL, 1 mM DTT, 50% glycerol) over 48 h at 4°C. BvgA remained soluble in this buffer at a maximal concentration of 150 μg/ml. Aliquots of this preparation were stored at −80°C.

RNAP purification. RNAP was purified from *B. pertussis* BP953 (Tohama I; *nal str fhaB-lacZ ptx-phoA*) by immunoaffinity chromatography according to a protocol developed by Thompson et al. (40) for the purification of *E. coli* RNAP. Polyol-sensitive monoclonal antibody 4RA2 recognizes an epitope on the α subunit of *E. coli* RNAP (39a). This antibody was conjugated to Sepharose, and the RNAP holoenzyme was purified from 10 g of cells as previously described, except that the enzyme was eluted from the affinity column with 30% 2,3-butanediol and 0.75 M ammonium sulfate in 50 mM Tris-HCl (pH 7.9)–0.1 mM EDTA. The eluted enzyme was dialyzed overnight at 4°C against 10 mM Tris-HCl (pH 7.9)–0.1 mM EDTA–0.2 mM DTT–100 mM NaCl–50% glycerol. Aliquots containing 4.2 mg of protein per ml were stored at −20°C. The specific activity of this preparation (80 U/mg) was determined by using a poly(dT)-poly(dA) template in the standard in vitro transcription protocol described below. One unit of enzyme activity is that amount which catalyzes the incorporation of 1 nmol of UTP into the acid-insoluble product in 10 min at 37°C. Purified σ^{70}-saturated *E. coli* polymerase (1,096 U/mg) was obtained from Pharmacia (Piscataway, N.J.). Both of the preparations of RNAP used were assessed to be at least 90% pure by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analyses.

Alanine-scanning mutagenesis of the C-terminal region of the *E. coli* α subunit was carried out as described elsewhere (21). Wild-type and mutant α-subunit proteins, as well as β and β′ subunits, were expressed, purified, and reconstituted into core enzymes as detailed previously (13). Prior to in vitro transcription analyses, reconstituted core enzymes were incubated with a fourfold molar excess of the purified σ^{70} subunit at 30°C for 30 min.

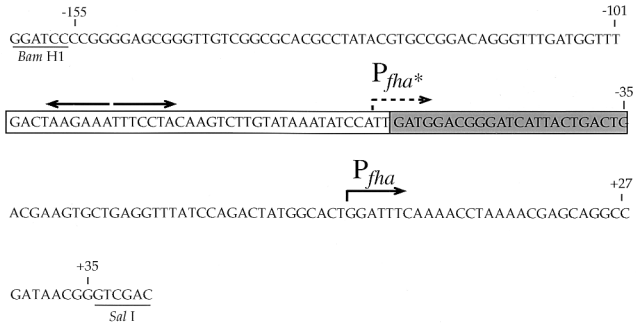

FIG. 1. Sequence of the 190-bp *fha* promoter used in these studies. The boxed sequences designate the BvgA primary (open box) and secondary (shaded box) binding sites. The BvgA-independent in vitro promoter (P_{fha}* start site) and BvgA-dependent in vivo promoter (P_{fha} start site) are shown. The dashed arrow indicates an approximation of the P_{fha}* start site based on the size of the transcript. The inverted repeats that serve as BvgA recognition half sites in the primary binding region are denoted by the divergent arrows.

DNase I footprinting. DNase I footprinting analysis of the *fha* promoter was conducted as described previously (4), except that the concentration of acetyl phosphate in the binding reactions was increased to 20 mM and 0.5 mM GTP and 0.05 mM ATP were included. Plasmid p*fhaP* was digested with either *Bam*HI and *Kpn*I (for the preparation of radiolabeled coding strands) or *Sac*I and *Sal*I (for radiolabeled template strands), and promoter DNA fragments were separated on agarose gels and purified by electroelution. These DNA footprinting probes were then radiolabeled to 130,000 cpm/ng as described previously (4), and where indicated, binding reactions contained a radiolabeled DNA fragment at 0.26 nM, various concentrations of purified BvgA, and either a commercially prepared RNAP holoenzyme (Pharmacia) or purified *B. pertussis* polymerase. Terminated reactions were electrophoresed on a 6% polyacrylamide-urea sequencing gel, and dried gels were exposed by using a PhosphorImager (Molecular Dynamics).

In vitro transcription. Transcription reactions (20-μl final volume) contained 20 nM supercoiled pTE-FHA template, 5 nM linear *lacUV5* promoter template, 10 U of RNAguard (Pharmacia), 0.10 to 0.39 μM BvgA, 20 mM acetyl phosphate, and either 0.15 μM σ^{70}-saturated *E. coli* RNA polymerase or 1.4 μM purified *B. pertussis* polymerase in transcription buffer (50 mM Tris-HCl [pH 8.0], 3 mM magnesium acetate, 0.1 mM EDTA, 0.1 mM DTT, 50 mM NaCl, 25 μg of nuclease-free bovine serum albumin [Pharmacia] per ml). Initiation complexes were allowed to form by incubation at 37°C for 15 min. A single round of transcription was initiated by addition of 2 μl of a solution containing ATP, GTP, and CTP at 5 mM each, 0.5 mM UTP, 2 μCi of [α-^{32}P]UTP (3,000 Ci/mmol), and 2 mg of heparin per ml. After incubation at 37°C for 10 min, reactions were stopped by addition of 20 μl of stop buffer (0.55 M sodium acetate [pH 7], 30 mM EDTA, 120 μg of tRNA carrier per ml). Samples were then extracted with phenol-chloroform, and nucleic acids were precipitated by addition of ethanol and subjected to electrophoresis on a 6% polyacrylamide sequencing gel. Dried gels were either exposed to film or analyzed by a PhosphorImager. The relative amount of each radiolabeled transcript was quantified by ImageQuant software (Molecular Dynamics). End-labeled, denatured 1-kb DNA ladder molecular weight markers (GIBCO Bethesda Research Laboratories) were run in parallel.

RESULTS

BvgA binds *fha* in both phosphorylated and unphosphorylated forms. Previous DNase I footprinting studies demonstrated that crude extracts derived from *E. coli* that overexpressed BvgA in the absence of BvgS protected a region between −78 and −101 (and a short region between −71 and −67) on the *fha* promoter (Fig. 1) (28). Although this observation suggested that BvgA, in its unphosphorylated form, specifically binds to a site within this region, binding by an *E. coli*-derived component or a protein that is regulated by BvgA remained a distinct possibility, as did phosphorylation of BvgA by either noncognate sensor proteins or intracellular acetyl phosphate. We therefore conducted DNase I footprinting

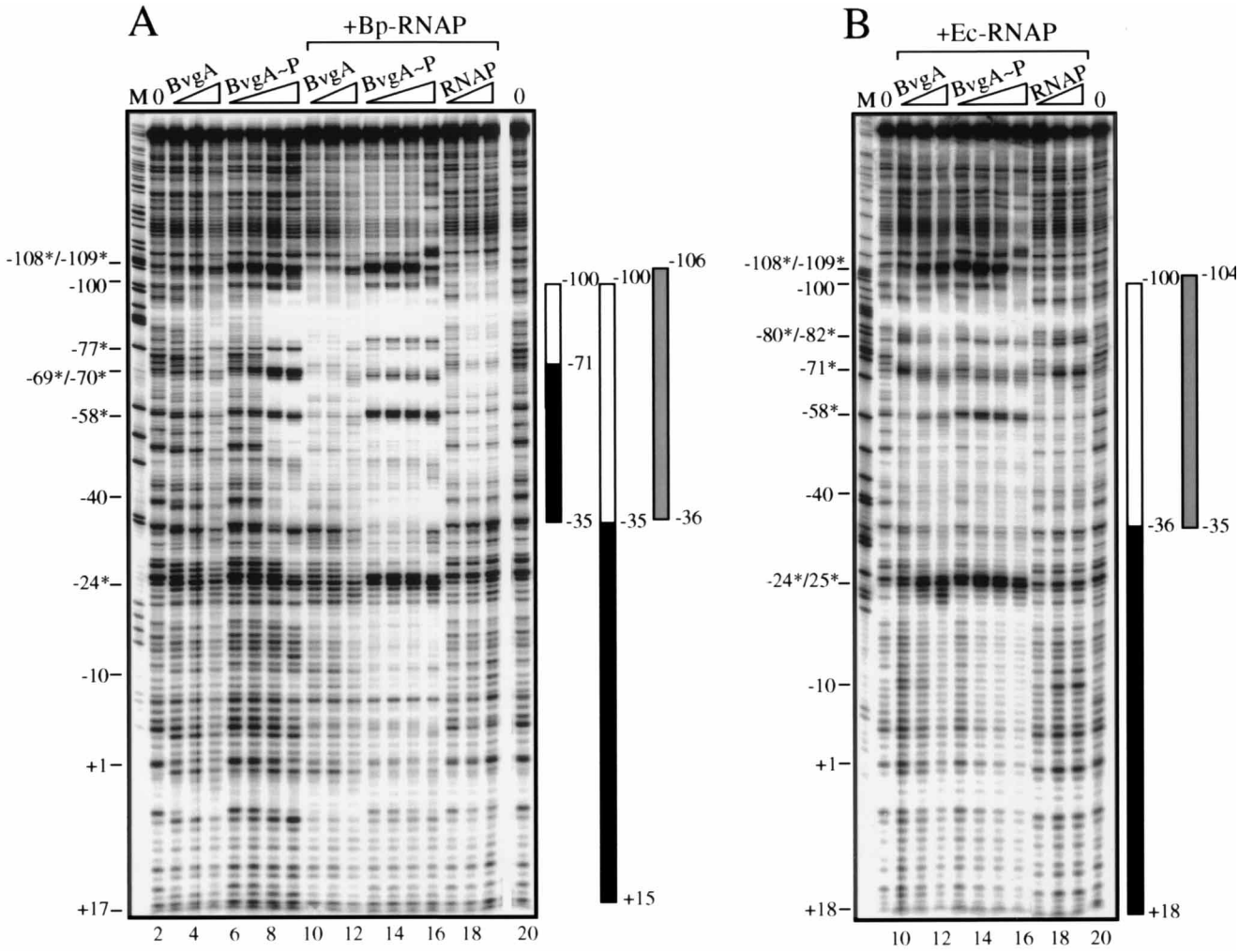

FIG. 2. DNase I protection analyses of the *fha* promoter by purified BvgA and RNAP. Binding reaction mixtures contained 0.26 nM radiolabeled template strand and 0.12, 0.23, or 0.47 μM unphosphorylated BvgA (BvgA lanes) or 0.06, 0.12, 0.23, or 0.47 μM BvgA phosphorylated in vitro with acetyl phosphate (BvgA~P lanes). Molecular weight sequencing ladders were in lanes M, and control reaction mixtures containing no added proteins were in lanes 0. Nucleotide positions at which hypersensitive sites were observed are marked with asterisks. (A) Binding reactions contained either BvgA alone (lanes 3 to 5) or BvgA phosphate alone (lanes 6 to 9). Digestion was also conducted with samples containing 0.93 μM purified *B. pertussis* (Bp) RNAP (lanes 10 to 16) in addition to BvgA or with samples containing 0.23, 0.47, or 0.93 μM polymerase alone (lanes 17 to 19, respectively). Rectangles to the right of panel A denote the extent of protection afforded by (from left to right) BvgA or BvgA phosphate, RNAP in the presence of either BvgA or BvgA phosphate, and RNAP alone. The open portion of each rectangle denotes protection by BvgA, while the black portion signifies the additional protection observed in the presence of BvgA phosphate. (B) Digestions were conducted in the presence of BvgA and 0.15 μM purified *E. coli* (Ec) RNAP (lanes 10 to 16) or 0.075, 0.15, and 0.3 μM polymerase alone (lanes 17 to 19, respectively). The rectangles to the right are equivalent to the last two beside panel A.

analyses with purified BvgA and *fha* promoter DNA fragments. BvgA was purified from an overexpressing strain of *E. coli* by a purification procedure that resulted in a preparation that was greater than 90% pure (data not shown).

DNase I footprinting analyses were initiated by using DNA fragments encompassing a 190-bp *fha*-specific promoter region (Fig. 1). Clear protection of a region encompassing nucleotides −100 to −71 relative to the transcription start site on the template strand (−99 to −70 on the coding strand; see Fig. 3, lane 3) was observed. This region includes the binding site identified earlier (28), and nuclease protection was observed only at the highest concentration of unphosphorylated BvgA used (0.47 μM; Fig. 2A, lane 5). The binding of BvgA phosphorylated in vitro with acetyl phosphate to this region resulted in a quantitatively and qualitatively different pattern of DNase I protection. The −100 to −71 region was clearly protected at half the concentration (0.23 μM; Fig. 2, lane 8) required for protection by unphosphorylated BvgA. Protection at this site by BvgA phosphate was also consistently stronger and gave rise to novel nuclease-hypersensitive sites at positions −108, −109, −77, −70, −69, −58, and −24 on the template strand and at −76, −75, −52, −51, and −35 on the coding strand. In addition, a second site spanning positions −71 to −35 on the template strand (−70 to −36 on the coding strand) was observed in the presence of BvgA phosphate. This region is resistant to nuclease attack only after the upstream, primary site is saturated with BvgA and represents a secondary BvgA phosphate binding site. This unexpected finding confirms that the earlier study detected the activity of unphosphorylated BvgA. Examination of the secondary site reveals no obvious inverted repeat sequence homologous to that found in the primary site (Fig. 1).

In the absence of BvgA, or in the presence of a low concentration of unphosphorylated BvgA, partial protection by puri-

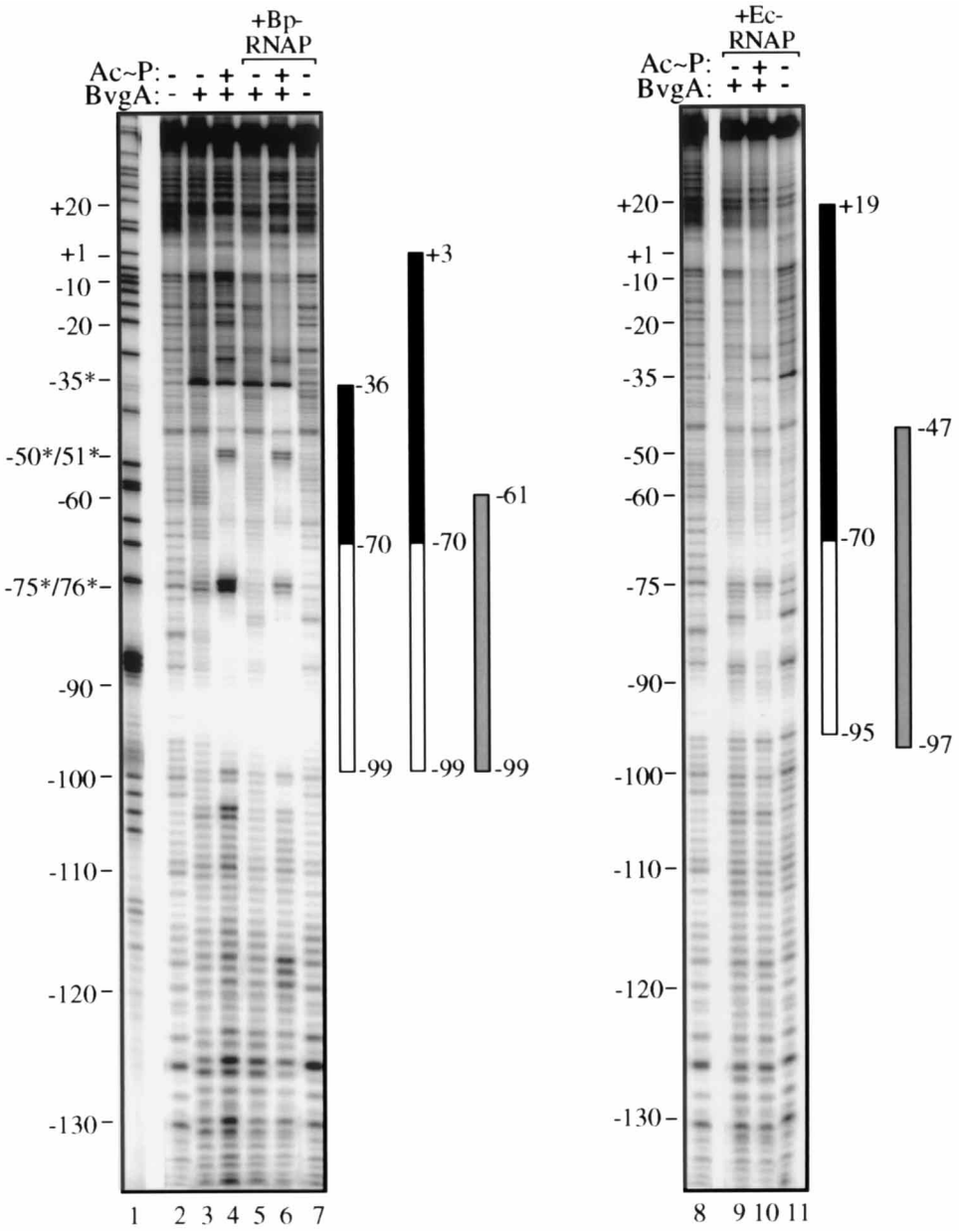

FIG. 3. DNase I protection analyses of the coding strand of the *fha* promoter. Binding reaction mixtures contained 0.23 μM BvgA (lanes 3, 5, and 9) or 0.23 μM acetyl phosphate-phosphorylated (Ac~P) BvgA phosphate (lanes 4, 6, and 10) alone or in combination with 0.93 μM *B. pertussis* (Bp) RNAP (lanes 5 and 6) or 0.15 μM *E. coli* (Ec) RNAP (lanes 9 and 10). Reaction mixtures containing polymerase alone were in lanes 7 and 11. A control sample containing no protein was in lanes 2 and 8. The rectangles are similar to those in Fig. 2.

fied *B. pertussis* RNAP was observed between −106 and −36 on the template strand (Fig. 2A, lane 19) and between −99 and −61 on the coding strand (Fig. 3, lane 7). A similar pattern was observed when the *E. coli* polymerase was used (−104 to −35 on the template strand and −97 to −47 on the coding strand). Addition of even a low concentration of phosphorylated BvgA to the polymerase binding reaction mixtures resulted in a markedly different pattern of DNase I protection. The presence of RNAP, independent of its source, greatly facilitated the binding of BvgA phosphate to both sites on the *fha* promoter (Fig. 2A and B, lanes 13 to 16). In addition, the region of protection was markedly extended downstream of the second BvgA binding site (up to +15 on the template strand and +3 on the coding strand) in the presence of *B. pertussis* polymerase. In the presence of *E. coli* RNAP, protection extended to +18 on the template strand and +19 on the coding strand. At 0.12 and 0.23 μM unphosphorylated BvgA (Fig. 2A and B, lanes 10 and 11, respectively), the pattern of nuclease protection paralleled that observed in the presence of polymerase alone (Fig. 2A and B, lanes 19). Only at 0.47 μM unphosphorylated BvgA did the signature of BvgA binding become noticeable (i.e., the emergence of hypersensitive sites at −108, −109, −77, −70, −69, and −58).

The different patterns of nuclease protection appear to be dependent on the phosphorylation state of BvgA and presumably reveal the occupancy of both BvgA binding sites. This

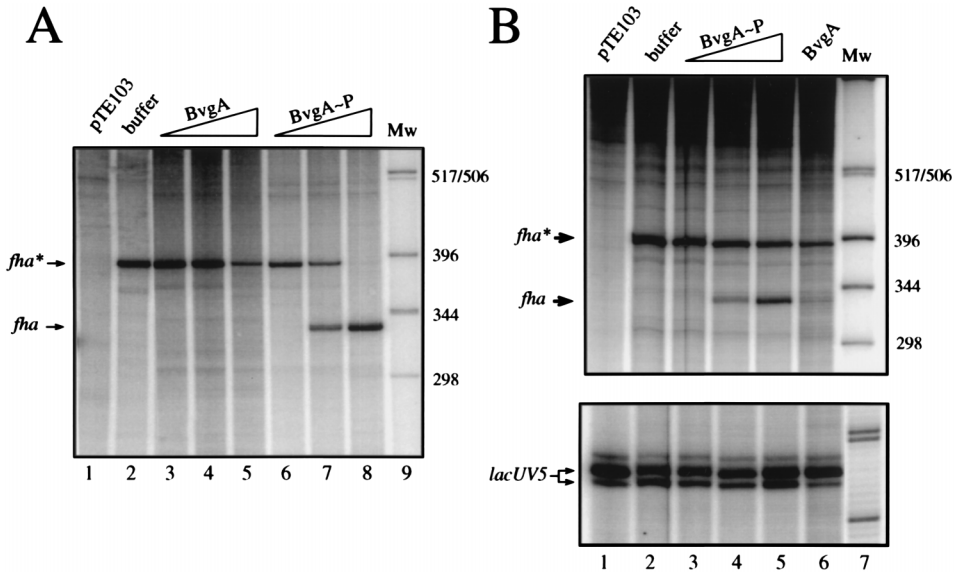

FIG. 4. In vitro transcription analyses of the *fha* promoter. A single round of transcription was initiated in reaction mixtures containing 20 nM template plasmid pTE-FHA, purified polymerase, and either BvgA or BvgA phosphate, and samples were run on a 6% sequencing gel. Samples were electrophoresed alongside denatured, single-stranded DNA molecular weight markers (lanes Mw); thus, the size predictions (in nucleotides) are only estimates. (A) Transcription by *B. pertussis* polymerase (1.4 μM) was tested with either 0.10, 0.20, or 0.39 μM BvgA (lanes 3 to 5, respectively) or the same concentrations of BvgA phosphate (lanes 6 to 8). The transcripts resulting from activation of the in vivo downstream *fha* promoter (*fha*) and that resulting from the upstream in vitro promoter (*fha**) are indicated. Control reaction mixtures containing the parental pTE103 vector or the dilution buffer alone are also shown. (B) Transcription was also conducted in the presence of *E. coli* RNAP (0.15 μM) in combination with 0.10, 0.20, or 0.39 μM BvgA phosphate (lanes 3 to 5) or 0.39 μM unphosphorylated BvgA (lane 6). As an internal control for polymerase activity at a BvgA-independent promoter, a DNA fragment containing the *lacUV5* promoter (5 nM) was included in each reaction mixture, and the transcripts are presented at the bottom.

observation suggests that in the absence of BvgA, or in the presence of a low concentration of unphosphorylated BvgA, RNAP occupies a site that overlaps the primary binding site for BvgA. This alternative *fha* promoter (P_{fha}*; Fig. 1) lies farther upstream than predicted by earlier primer extension studies (30, 31). However, competition for binding to this upstream site ensues in the presence of even a low concentration of BvgA phosphate, resulting in the redirection of polymerase to a site farther downstream. It is unlikely that BvgA itself is repositioned by RNAP to sites that extend into and beyond the P_{fha} transcription start site. BvgA phosphate did not protect this region in the absence of polymerase, and the hypersensitive sites and pattern of protection afforded by BvgA phosphate and RNAP between −100 and −35 (Fig. 2A, lane 15) more closely resembled those observed for BvgA phosphate alone (lane 8) than those observed for RNAP alone (lane 19).

Enhanced *fha* binding correlates with stimulation of transcription initiation. The cooperative binding between BvgA phosphate and RNAP to the *fha* promoter suggests that BvgA may activate transcription by recruiting the polymerase to an otherwise weak promoter or by displacing the polymerase from a relatively strong promoter to an adjacent weaker promoter by protein-protein interaction. To investigate this, we conducted in vitro transcription assays with purified BvgA and purified RNAP from both *E. coli* and *B. pertussis*. Our decision to investigate the activity of the former polymerase at *fha* was motivated by previous studies that clearly demonstrated that proper regulation of an *fha* transcriptional fusion by BvgA could be reconstituted in *E. coli* (20, 33). For these assays, we employed the promoterless transcription vector pTE103 containing a phage T7-derived terminator (10). A DNA fragment containing the same *fha*-specific sequences used in the previous DNase I footprinting assays was cloned into a site 302 bp upstream of the terminator yielding the pTE-FHA vector (Fig. 1).

Transcription driven from the *fha* promoter in pTE-FHA and in the presence of increasing amounts of phosphorylated BvgA and *B. pertussis* polymerase yielded the expected 337-nt transcript (Fig. 4A, lanes 7 and 8). This transcript was not observed when either the vector lacking the promoter (lane 1) or BvgA dilution buffer alone (lane 2) was used in the assay. In the absence of BvgA or in the presence of unphosphorylated BvgA, the 337-nt transcript was not evident. However, under these conditions, a transcript of approximately 390 nt was observed. The gradual disappearance of this transcript accompanied the gradual appearance of the 337-nt transcript as the concentration of BvgA increased. The approximate size of the larger transcript is consistent with the length expected for a polymerase initiating at the upstream P_{fha}* referred to earlier (Fig. 1). Although the transcription start site at this promoter was not mapped, it was of the expected size based on the earlier observations of Steffen et al. (32). These observations support our contention that as the full complement of BvgA binding sites become occupied, RNAP is progressively redirected to the downstream, in vivo site.

An almost identical pattern of transcription was observed when the *E. coli* polymerase was substituted for the *B. pertussis* enzyme (Fig. 4B). However, an approximately 10-fold lower concentration of the *E. coli* polymerase was used in these assays, as the specific activity of this enzyme was significantly higher. A barely detectable amount of the 337-nt transcript was observed in the presence of unphosphorylated BvgA when this polymerase was used. As an internal control for polymerase activity at a BvgA-independent promoter, a DNA fragment containing the *lacUV5* promoter was included in each reaction; the transcripts derived from this promoter are given in Fig. 4B.

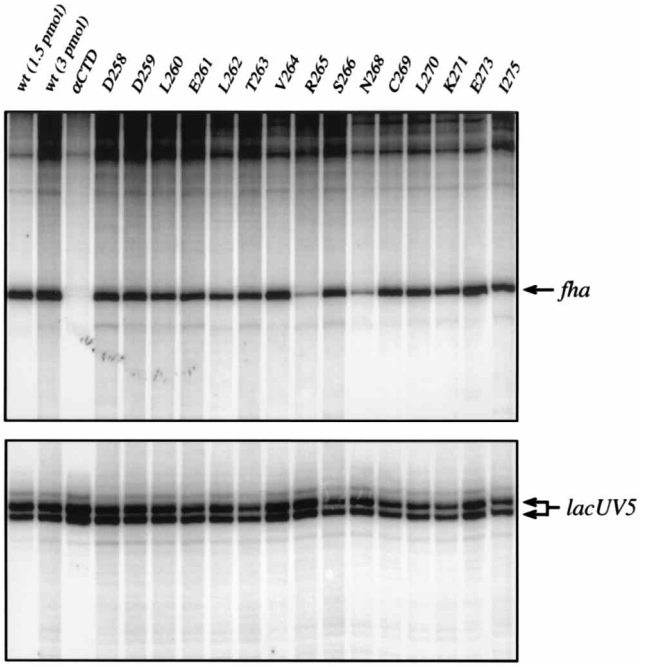

FIG. 5. In vitro transcription analyses of reconstituted *E. coli* RNAPs containing wild-type (wt) and mutant α subunits. σ^{70}-saturated wild-type (1.5 and 3.0 pmol) and mutant (3 pmol) holoenzymes were tested for the ability to support BvgA-dependent transcription at the *fha* promoter (top) and BvgA-independent transcription at the *lacUV5* promoter (bottom). Reaction mixtures contained 20 nM template plasmid pTE-FHA, 5 nM *lacUV5* DNA fragment, and 0.39 μM BvgA phosphate. Each alanine-scanning mutant α subunit name consists of the single-letter code for the amino acid residue that is substituted, followed by the location of that residue along the polypeptide.

From these observations, we concluded that BvgA phosphate stimulates transcription initiation by repositioning RNAP to its in vivo binding site by BvgA-polymerase interactions.

BvgA phosphate is a class I activator at the *fha* promoter and contacts residues in helix 1 of the RNAP α subunit. Our footprinting data suggested that RNAP and BvgA phosphate interact because the region protected by the regulator overlaps the polymerase −35 recognition hexamer. Once we had established that (i) enhanced binding of BvgA phosphate to the *fha* promoter correlated with stimulation of transcriptional activation and (ii) DNA binding and transcription activities of *E. coli*-derived RNAP did not differ significantly from those of the *B. pertussis* homolog, we felt justified in using mutant forms of the *E. coli* polymerase to examine BvgA-RNAP interactions. These mutant enzymes have been previously used to identify the structural domain and individual amino acid residues on the polymerase α subunit that contact transcriptional activators (21). The mutant proteins employed contained either a deletion of the C-terminal third of the RNAP α subunit or a substitution of alanine for one of several residues that lie within this C-terminal domain. The wild-type and mutant α subunits were overexpressed, purified, and reconstituted into $\alpha_2\beta\beta'$ core enzymes. All α derivatives were capable of supporting in vitro core enzyme assembly as efficiently as the wild-type subunit (data not shown). Prior to conducting the assay, we added a saturating quantity of the σ^{70} subunit to form the holoenzyme.

In an initial experiment, we employed the reconstituted wild-type polymerase and a mutant polymerase lacking the C-terminal 94 amino acids from the α subunit (αCTD). The wild-type σ^{70}-saturated holoenzyme appeared to be functional in supporting BvgA-dependent transcription (Fig. 5). The reason for the absence of the *fha** transcript is unclear but may be related to the high purity of this polymerase preparation, being reconstituted from purified recombinant proteins. The reconstituted αCTD mutant polymerase remained fully functional relative to the wild-type holoenzyme when tested for the ability to stimulate BvgA-independent transcription at the *lacUV5* promoter in vitro (Fig. 5, bottom). However, this mutant polymerase was almost completely nonfunctional when tested for the ability to support BvgA-dependent transcription at the *fha* promoter (Fig. 5, top). Interpreted as the ratio between BvgA-dependent transcription (at *fha*) and BvgA-independent transcription (at *lacUV5*), activation at the *fha* promoter directed by the αCTD polymerase was less than 5% of the wild-type polymerase activity. Therefore, the BvgA activator appears to interact with the α subunit of RNAP at *fha*, thus demonstrating that BvgA is a class I transcription factor (14, 15).

To identify specific residues on α that contact BvgA phosphate bound to the *fha* promoter, we employed a bank of 15 alanine-scanning mutants clustered primarily at helix 1 of the α-subunit C-terminal domain (Fig. 6) (16). When tested for the ability to support BvgA-independent transcription at the *lacUV5* promoter, all of the mutant polymerases maintained approximately wild-type levels (Fig. 5, bottom). However, when BvgA-dependent activity was tested at the *fha* promoter, several mutants displayed reduced activities relative to the wild-type enzyme (Fig. 5, top). In particular, the mutants with alanine-substitutions at Arg-265 and Asn-268 on the α subunit displayed only 10 and 19% of wild-type activity at *fha*, respectively. We therefore conclude that the side chains beyond the β carbon of Arg-265 and Asn-268 are crucial for BvgA-dependent transcription and may form part or all of an activation target for BvgA phosphate at the *fha* promoter. Substitutions for leucine residues at positions 260 and 262 decreased the mutant polymerase activity to less than 50%. Although these residues may play a role in BvgA-dependent transcription, their contribution appears much less important. Indeed, a pre-

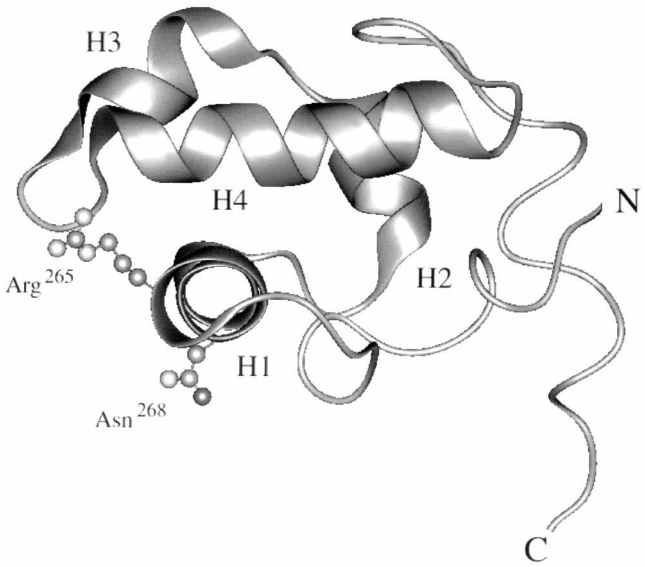

FIG. 6. Ribbon diagram representing the calculated mean structure folding of the α-subunit C-terminal domain determined by nuclear magnetic resonance spectroscopy (16) and produced by Quanta software. Each of the four alpha helices (H1 through H4) and the critical arginine and asparagine residues at positions 265 and 268, respectively, are shown.

vious study suggested that Leu-260 and Leu-262 may facilitate the proper folding of αCTD (21).

DISCUSSION

Recent studies have contributed to our understanding of how the BvgA-BvgS two-component signal transduction system coordinately regulates the expression of virulence factors in *B. pertussis*. However, until recently, very little was known about how the BvgA response regulator ultimately activates transcription of target promoters such as those of the *ptx* and *fha* operons. A concise model of BvgA activation is complicated by several observations that suggest that regulation of *ptx* and *fha* may occur by distinct mechanisms. In an ongoing effort to characterize the underlying molecular mechanism of BvgA activation, we have confirmed and extended earlier studies and shown that, at the *fha* promoter, BvgA phosphate (i) displays an enhanced affinity relative to the unphosphorylated form for a primary upstream binding site, (ii) binds an additional downstream site, and (iii) repositions RNAP in vitro from a site that overlaps both primary and secondary BvgA binding sites to its in vivo site farther downstream.

Our previous DNA binding analyses of the *ptx* promoter suggested that the phosphorylated form of BvgA may multimerize along the promoter template (4). This model proposed that BvgA initially binds to a relatively high-affinity site positioned far upstream of the transcription start site (centered at −136.5). This binding then forms a nucleation point from which weaker binding sites positioned on the same face of the DNA helix are progressively bound up to a site just adjacent to the RNAP recognition sequence. Thus, the proximal BvgA phosphate subunit may contribute an interface for BvgA-polymerase interaction. Remarkably, Karimova and coworkers discovered that BvgA phosphate binds in a similar manner to an extensive sequence spanning positions −137 to −51 on the *cya* promoter (17), a locus whose temporal BvgA regulation in vivo is similar to that of *ptx*.

When we applied the same approach in our analysis of BvgA regulation at the *fha* promoter, we found that although the unphosphorylated form binds a primary site between −100 and −71, only the phosphorylated form is capable of binding both the primary site and a secondary site that extends to −35 relative to the in vivo transcription start site (Fig. 1). This secondary site may therefore represent a lower-affinity BvgA binding site that alone cannot bind BvgA. Thus, the architecture of the *fha* promoter may parallel, in a broad sense, that of the *ptx* promoter. An examination of the secondary-site sequence reveals no obvious match to the BvgA consensus binding half site (5′-TTTC[C or T]TA-3′) (28). However, it should be noted that in the absence of any systematic examination of the optimal BvgA recognition sequence, identification of the half-site heptamers in the *fha* primary site as a consensus is premature. In in vitro transcription assays, we and others (32) were able to detect significant levels of the 337-nt *fha* transcript only in the presence of phosphorylated BvgA. We also observed that full occupancy of primary and secondary BvgA binding sites correlated with transcription activation.

Of particular interest was our observation that both *B. pertussis* and *E. coli* RNAP occupy sites that overlap the BvgA binding sites in the absence of BvgA or in the presence of low concentrations of unphosphorylated BvgA. The functional significance of this site in vitro has been confirmed by our own and others' observations of an alternative BvgA-independent *fha* transcript (32). Addition of the phosphorylated form led to footprint patterns that suggested that the polymerase had been shifted from its original site to one farther downstream and that there was a concomitant increase in the transcript corresponding to the in vivo start site. The repositioning of RNAP observed here is reminiscent of catabolite activator protein (CAP) regulation of the *lac* promoter (reviewed in reference 18). RNAP preferentially binds to an upstream promoter, but in the presence of CAP, the enzyme is shifted to a site farther downstream. However, the CAP-independent promoter does not appear to be active in vivo. Remarkably, a similar configuration is observed at the *bvgAS* promoter which is situated upstream and is oriented divergently with respect to the *fha* promoter (2, 34). In the absence of BvgA, we and others (32) have noticed an in vitro transcript attributable to initiation at the upstream, BvgA-independent promoter (P_{fha}*). Like the equivalent promoter in *lac*, the significance of the *fha** transcript is unclear because it was not detected in earlier primer extension experiments (30, 31). However, neither its existence nor its significance in vivo can be excluded, as it may possess a very short half-life and/or be transcribed only under certain physiological conditions.

In addition to the emergence of a secondary BvgA phosphate binding site, we also noted the appearance of several strong nuclease cleavage sites that lie on the same face of the promoter DNA (at −58, −69, and −80). A strikingly similar pattern of DNase I protection was observed on the *ptx* promoter (4) and suggests that the promoter DNA may wrap around a large BvgA phosphate-RNAP complex. The ability of transcriptional regulators to bend DNA at their recognition sites is rapidly being recognized as more of a rule than an exception (23). We are initiating studies that address the ability of BvgA to bend target promoter DNA. Nevertheless, three-dimensional characterization of protein-DNA complexes has justified a link between the appearance of DNase I-hypersensitive sites and the presence of regulator-induced DNA bends (1a, 25). Furthermore, patterns of alternating nuclease sensitivity and resistance displaying a 10.5-nt periodicity have been observed for proteins that curve DNA or induce wrapping of the DNA about themselves (9, 12). Therefore, occupancy of both primary and secondary sites on the *fha* promoter by BvgA phosphate may assemble polymerase into a large, compact nucleoprotein structure similar to those predicted for Lrp on the *ilvIH* promoter (43), MalT on the *malPQ* promoter (8), and the FIS regulator on the *tyrT* promoter (22). Such a structure would have important implications on BvgA-mediated transcription activation. First, the binding of BvgA phosphate to the proximal site on promoters could provide direct BvgA-RNAP contacts that stabilize an otherwise weak polymerase-promoter interaction. Second, the binding and bending of a distal site(s) could, in addition to possibly increasing the occupancy of the proximal site, provide stabilizing upstream contacts for RNAP (either protein or DNA mediated). Alternatively, it could provide elastic energy that may facilitate the activation of polymerase. Thus, although BvgA can clearly promote RNAP binding to *ptx* and *fha*, it may additionally increase the rate of isomerization from a closed to an open complex.

Implicit in our model of BvgA-mediated transcription activation is a direct interaction between BvgA and RNAP. This interaction has already been suggested by the characterization of a mutation in the α subunit of RNAP in *B. pertussis* that suppresses the phenotype conferred by short, C-terminal BvgA deletions (36a). These observations are supported by experiments that suggested a link between overexpression of the α subunit and inhibition of BvgA activity in vivo (5). To provide biochemical evidence for a BvgA-RNAP interaction, we used mutants of *E. coli* RNAP that have been useful in demonstrating polymerase-regulator interactions in other systems (14, 15).

We have provided evidence that *E. coli* RNAP behaves similarly to the *B. pertussis* protein by both DNA binding and in vitro transcription criteria. We found that mutants lacking the C-terminal third of the α subunit of RNAP, although fully functional in BvgA-independent transcription, were essentially devoid of activity at the *fha* promoter. Many transcriptional regulators have been tentatively grouped into two classes based on the type of contacts they make with RNAP (14, 15). Class I factors contact the C-terminal domain of the α subunit, while class II factors contact the C-terminal region of the σ^{70} subunit. BvgA, like several other activators, including the CAP protein, OxyR, and OmpR, is therefore considered a class I activator at the *fha* promoter.

To more finely map the activation target on the α subunit for BvgA phosphate, we employed a bank of alanine-scanning mutants of the α subunit that had previously been used to identify residues involved in DNA binding and CAP interaction (21). The use of alanine-substituted mutants offers several distinct advantages in analyzing BvgA-polymerase interactions (7, 37, 45). First, the introduction of alanine is less likely to disrupt secondary structure than is that of most other residues. Second, these mutant α subunits represent a chemically consistent set of substitutions. Finally, alanine substitution yields residues with all of the side chain atoms beyond the β carbon truncated, allowing assessment of the contributions made by these atoms to protein-protein interactions. Based on the ability of alanine substitution to hinder BvgA-dependent activation at *fha* while leaving BvgA-independent activation unaffected at *lacUV5*, we observed that the side chains beyond the β carbon of the arginine and asparagine residues at positions 265 and 268, respectively, were critical for *fha* transcription. These residues are located on the solvent face of helix 1 in the α-subunit C-terminal domain (Fig. 6) and are separated by the minimum distance possible for two residues within an alpha helix (16). Furthermore, Arg-265 is the target for ADP-ribosylation by the T4 phage (11). This lends further support to the notion that the Arg-265 and Asn-268 residues are readily accessible to other molecules that include BvgA phosphate. Intriguingly, these same residues have also been identified as important residues for activation mediated by CAP (21), OxyR (38, 39), and GalR (1). In addition to the two residues identified here, CAP and OxyR contact other sites on helix 1 that do not appear to be critical for BvgA-mediated transcriptional activation. Although it has previously been noted that Arg-265 and other residues in the C-terminal domain of the α subunit participate in the binding of an upstream sequence referred to as the UP element (generally localized between positions −40 and −60) (26), there is no evidence that such an element exists in the *fha* promoter. Indeed, this region in *fha* is not particularly AT rich (52%), a characteristic typical of well-studied UP element-containing promoters (24, 26).

Although BvgA-mediated regulation of the *fha* promoter appears to share some features with regulation at the *ptx* promoter, a variety of genetic evidence suggests that there are important differences. The nature of BvgA-polymerase contacts may therefore be quite different at the two promoters. Mutant BvgA proteins that genetically separate *fha* and *ptx* regulation and purified RNAP enzymes will provide valuable tools for the investigation of these mechanistic differences.

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