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Synergistic Binding of RNA Polymerase and BvgA Phosphate to the Pertussis Toxin Promoter of *Bordetella pertussis*

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Regulation of virulence factor expression in *Bordetella pertussis* is mediated by the BvgAS two-component regulatory system. Although previous studies have demonstrated that the transcriptional regulation of the filamentous hemagglutinin gene (*fhaB*) involves binding of the BvgA activator directly to the *fhaB* promoter region, the mechanism of pertussis toxin operon (*ptx*) regulation by BvgA has remained unclear. We demonstrate in vitro the specific binding of BvgA to a region upstream of the *ptx* promoter that encompasses a 20-bp directly repeated sequence (positions −157 to −117) previously shown to be critical for BvgA-dependent activation. This binding is strictly dependent on the phosphorylation of BvgA, which can be obtained by incubation of BvgA with acetyl phosphate. By DNase I protection studies, we demonstrate the synergistic binding of BvgA-phosphate and purified *Escherichia coli* RNA polymerase to the *ptx* promoter. In the presence of the polymerase holoenzyme, a greatly extended footprint encompassing the region between −163 and the putative polymerase binding site was observed. The implications of these observations for pertussis toxin expression and regulation are discussed.

Regulation of virulence factor expression in the respiratory pathogen *Bordetella pertussis* is mediated by the products of the *bvg* operon (previously *vir*) (34, 35, 40). The BvgS and BvgA proteins make up a sensor kinase and response regulator two-component signal transduction system that controls the expression of a variety of virulence factors that include the genes for filamentous hemagglutinin (*fhaB*), adenylate cyclase toxin-hemolysin (*cya*), and pertussis toxin (*ptx*). BvgS is an inner-membrane-spanning protein that responds to fluctuations of temperature or concentrations of nicotinic acid or magnesium sulfate by altering the phosphorylation level of the BvgA transcriptional activator in a process termed modulation (19). The BvgA transcriptional activator is placed within the large response regulator family by virtue of sequence homologies in the N-terminal, phosphate-accepting receiver domain shared by all family members (1, 26, 34). When stimulated by phosphorylation at the receiver domain, BvgA binds with a greatly enhanced affinity to target promoters via its C-terminal domain (2, 26). Similarities within this domain define a subfamily of bacterial regulatory proteins that contain a putative helix-turn-helix DNA binding motif and that includes LuxR, UhpA, RcsA, RscB, GerE, MalT, FixJ, and others (11, 17, 24, 37).

Several lines of evidence have suggested that the regulation of the *ptx* and *cya* operons may be mechanistically different from that of the *fha* operon. Investigators have to date been unable to provide in vivo or in vitro evidence for a functional interaction between BvgA and the *ptx* or *cya* promoters. Early reports (22, 36) demonstrated the reconstitution of Fha expression in response to the correct environmental signals when the *bvg* and *fha* loci are coexpressed in *Escherichia coli* cells. Similar efforts to reconstitute *ptx* and *cya* expression in this system have been unsuccessful (7, 22). A recent report suggests that under conditions of a specific plasmid context and DNA topology, the *ptx* promoter can be activated by the *bvg* locus in *E. coli* (29). However, as responsiveness to environmental signals was not observed in this instance, it was not clear whether the activation observed was due to the normal functioning of BvgAS. Furthermore, whereas biochemical evidence demonstrated a direct interaction between BvgA and the *fha* and *bvg* promoters (26), attempts to demonstrate in vitro binding of BvgA to the *ptx* promoter have thus far been unsuccessful (9, 26). Collectively, these observations have led to a hypothesis that regulation of *ptx* and *cya*, but not *fha*, requires the action of an accessory factor(s). This positive factor has been envisaged to act in concert with BvgA at the level of *ptx* and *cya* gene expression (i.e., as a regulatory cofactor) or to be responsive itself to *bvg* regulation (i.e., as a member of a regulatory cascade).

In an effort to characterize the mechanism of *ptx* regulation by BvgA, we propose that the binding of BvgA to the *ptx* promoter may be dependent on the phosphorylation state of the activator. Indeed, for the OmpR, PhoB, and FixJ response regulators, recognition of DNA target sites is strongly dependent on phosphorylation (5, 6, 21). Recent evidence demonstrated that acetyl phosphate can serve as a substrate for BvgA phosphorylation and that this modification results in a greatly enhanced affinity of BvgA for its binding site on the *fha* promoter (2). We have extended these observations and present data that demonstrate the ability of in vitro-phosphorylated BvgA to interact specifically with a region upstream of the *ptx* promoter. This binding occurs synergistically with the binding of RNA polymerase (RNAP) to this promoter. We propose a mechanism that incorporates these and earlier observations to explain how the *ptx* and perhaps *cya* loci are regulated by BvgAS.

MATERIALS AND METHODS

Bacterial strains and plasmids. The plasmid pSS1744 was constructed as follows. A PCR fragment containing the *bvgA* gene was generated by using the oligonucleotide 5′-CGCGAATTCAAGGAGATATACATATGTACAACAAA GTCCTCATCATCG-3′ as the upstream primer, 5′-CGCGGATCCTTAGGCG AGATTGTTGCGTTTGGCGAGG-3′ as the downstream primer, and the To-

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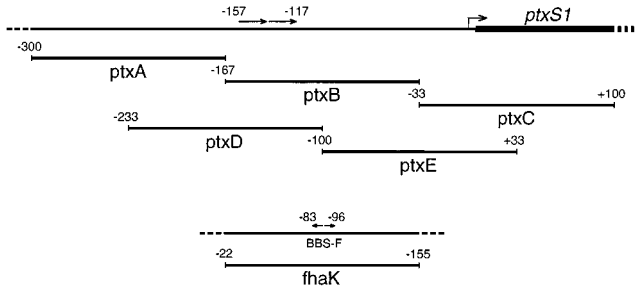

FIG. 1. Schematic of *ptx* promoter and DNA fragments used in DNA binding assays. The 20-bp direct repeats are shown by arrows and span the region between −157 and −117. Fragments 133 bp in length spanning 400 bp from the *ptx* promoter region were created by PCR. As a positive control, a 133-bp fragment derived from the *fhaB* promoter containing the BvgA binding site (BBS-F) was also created. The arrows above each promoter region designate the boundaries of the repeated sequences. *ptxS1*, coding region of the S1 subunit of pertussis toxin downstream of the transcription start site (vertical arrow).

hama I *bvgA* locus as a template. The resulting fragment contains an upstream *Eco*RI site together with a strong ribosome binding site and a downstream *Bam*HI site. This fragment was cloned between the *Eco*RI and *Bam*HI sites of the pT7-5 expression vector, which provides strong transcription by the phage T7 polymerase and a T7 promoter. The resulting plasmid, pSS1744, was transformed into the *E. coli* strain, BL21 DE3 p*lysS*, to allow the regulated expression of the cloned *bvgA* gene.

Preparation of protein samples. BL21 DE3 p*lysS* (pSS1744) cells were grown to an optical density of 0.4 to 0.5 (at 600 nm), at which point IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a concentration of 1 mM. After continuing growth for 3 h, the cells were pelleted, washed once with ice-cold phosphate-buffered saline, and resuspended in lysis buffer (20 mM Tris-HCl [pH 7.8], 50 mM KCl, 5 mM dithiothreitol, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride). The cell suspension was subjected to two passes through a French pressure cell at 16,000 lb/in^2. The sample was then centrifuged for 30 min at 12,000 × *g*, and the supernatant was discarded. The insoluble pellet containing the bulk of the BvgA protein was solubilized to a final protein concentration of 100 μg/ml in 6 M guanidinium HCl in buffer D (20 mM Tris-HCl [pH 7.8], 10 mM $MgCl_2$, 5 mM dithiothreitol). Renaturation of a 3-ml sample of denatured BvgA was initially carried out by dialysis against 250 ml of 3 M guanidinium HCl in buffer D and then by a slow gradient exchange of the dialysis buffer with 2 liters of buffer D alone over 24 h at 4°C. Dialysis was continued for 16 h against 1 liter of fresh buffer D. The dialysate was then passed through a Sephadex G-25 column (Pharmacia) equilibrated with 40 mM Tris-HCl (pH 7.8)–100 mM KCl–1 mM dithiothreitol–10 mM $MgCl_2$. The sample was concentrated in a Centricon-10 centrifugal concentrator (Millipore), glycerol was added to 20%, and the samples were stored at −80°C. The molar concentration of BvgA is stated as the nominal monomer concentration.

Gel shift assays. Five 133-bp DNA fragments encompassing the *ptx* promoter region (−300 to +100, relative to the transcription start site) were created by PCR with the Tohama I *ptx* locus as a template. Primers containing *Bam*HI and *Sal*I recognition sequences and complementary to regions bordering positions −300, −233, −167, −100, −33, +33, and +100 were synthesized and used to generate PCR fragments ptxA, -B, -C, -D, and -E (Fig. 1). In addition, a positive control fragment of the same size containing the BvgA binding site in the *fhaB* promoter (−22 to −155) was also constructed. Each purified PCR product was digested with *Bam*HI and *Sal*I restriction enzymes and then cloned into the pBluescript-KS vector (Stratagene) and propagated in *E. coli* XL1-Blue cells. Each plasmid insert was sequenced to ensure that no mutations had occurred during the PCRs. Purified plasmids were then prepared and digested with *Bam*HI and *Sal*I. Gel-purified 133-bp DNA fragments were labeled with [α-^{32}P]dGTP and [α-^{32}P]dTTP as detailed elsewhere (27). The specific activity of each fragment was approximately 10^5 cpm/ng of DNA.

Binding reaction mixtures contained various concentrations of renatured BvgA and 25,000 cpm of labeled DNA probe (0.25 ng) in 10 μl of 1× binding buffer (10 mM Tris-HCl [pH 7.8], 2 mM $MgCl_2$, 0.01% Nonidet P-40, 50 mM KCl). Where indicated, 10 mM acetyl phosphate and/or 100 ng of competitive DNA was added to the binding reaction mixture. Binding was allowed to reach equilibrium for 25 min at 24°C, and then the samples were loaded onto a 6% polyacrylamide–Tris-borate-EDTA native gel. Electrophoresis was conducted at 4°C for 1 h, and the gel was dried and exposed onto a PhosphorImager screen (Molecular Dynamics) for 2 h.

DNase I protection assays. A 267-bp DNA fragment comprising fragments ptxD and -E was created by using the ptxD 5′ primer and the ptxE 3′ primer. The purified fragment was cloned between the *Bam*HI-*Sal*I restriction enzyme sites of pBluescript-KS and digested with a combination of restriction enzymes, such that only one end contained a 4-nucleotide 5′ overhang. The fragments were labeled in a manner identical to that performed for the fragments used in gel shift assays.

Binding reactions were conducted as described above. Where indicated, purified *E. coli* RNAP holoenzyme (Pharmacia) was added to the binding reaction mixture at a final concentration of 300 nM. The nucleolytic reactions were initiated by the addition of 10 μl of digestion buffer (10 mM Tris-HCl [pH 8.0], 5 mM $CaCl_2$, 10 mM $MgCl_2$) containing 0.09 U of DNase I (Promega). After 1 min, digestions were terminated by the addition of 20 μl of stop buffer (0.2 M NaCl, 30 mM EDTA, 1% sodium dodecyl sulfate [SDS], 200 μg of yeast tRNA per ml), and the samples were extracted once with phenol. The DNA was then ethanol precipitated, and the samples were run on a 6% polyacrylamide–urea sequencing gel. Dideoxynucleotide sequencing reactions were also conducted in parallel and electrophoresed on the same gel. After drying, the gels were exposed onto a PhosphorImager screen overnight.

RESULTS

Partial purification of recombinant BvgA. Expression of the wild-type BvgA protein was directed by the T7 phage promoter in plasmid pSS1744. Upon induction of *E. coli* cells with IPTG, the recombinant protein is expressed at high levels and is sequestered within inclusion bodies (Fig. 2). We have noticed that approximately 90% of the total protein isolated from inclusion bodies is recombinant BvgA. The distribution of recombinant proteins within insoluble complexes is often exploited as a step in purification schemes, and the insoluble fraction of disrupted cells was thus subjected to denaturation in guanidinium-HCl and subsequent renaturation by using a slow dialysis exchange protocol. During the renaturation process, a significant portion of the preparation is lost as a precipitate, although 15 to 20% remains soluble. The electrophoretic profile of the renatured sample on SDS-polyacrylamide gels appeared identical to that of the isolated inclusion bodies (data not shown). By gel shift analyses, the renatured BvgA retains a strong capacity to bind an oligonucleotide target site located in the *fhaB* promoter (data not shown).

Gel shift analyses of the *ptx* promoter. To analyze the ability of BvgA to interact with DNA derived from the *ptx* promoter and flanking regions, PCR was used to generate three 133-bp fragments spanning −300 to +100 (ptxA, -B, and -C; Fig. 1) as well as two equally sized fragments encompassing the border sequences separating the other fragments (ptxD and -E). As depicted in Fig. 3, BvgA retards the migration of the positive control fhaK probe that harbors a defined BvgA binding site (lanes 2 and 3). This interaction is both specific (Fig. 3, com-

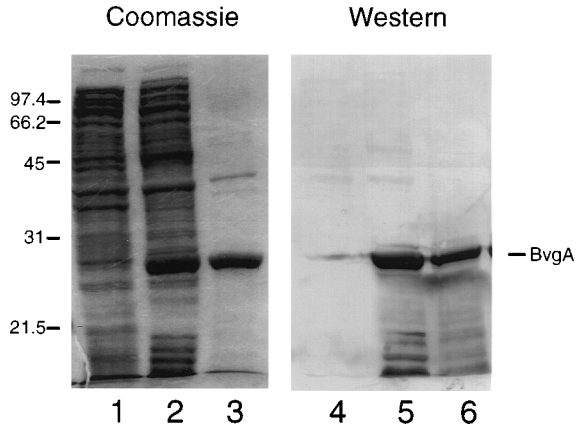

FIG. 2. Expression of recombinant BvgA in *E. coli* cells. (Left) Coomassie-stained polyacrylamide gel. (Right) The corresponding immunoblot (Western blot). Lanes 1 and 4, uninduced whole-cell lysate; lanes 2 and 5, induced whole-cell lysate; lanes 3 and 6, insoluble inclusion body pellet. Molecular weight markers are given on the left.

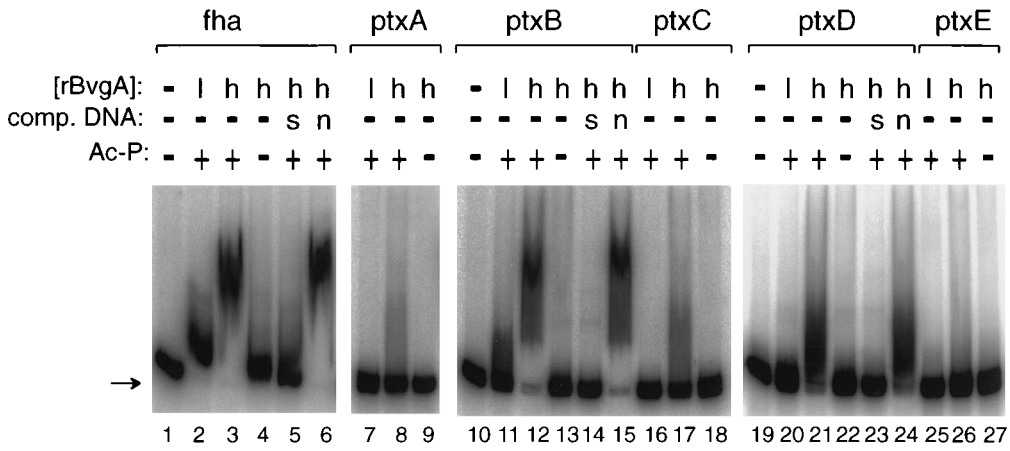

FIG. 3. DNA binding activity of BvgA by gel shift analyses. Radiolabeled DNA fragments were incubated with partially purified BvgA, and the complexes formed were resolved on a 6% polyacrylamide–Tris-borate-EDTA native gel. Binding reactions were conducted in the presence of 32 nM (lanes 1) or 65 nM (lanes h) BvgA and in the absence (−) or in the presence (+) of 10 mM acetyl phosphate (Ac-P). Excess unlabeled 24-bp oligonucleotide representing the BvgA binding in the *fhaB* promoter(s) (2) or a random 24-bp oligonucleotide (n) was added to some reaction mixtures. Lanes 1, 10, and 19 show the migration of the free probe (arrow). comp. DNA, competitive DNA.

pare lanes 5 and 6) and dependent on in vitro phosphorylation by acetyl phosphate (Fig. 3, lane 4). When the *ptx* fragments were subjected to analyses, only fragments ptxB and -D showed binding activity (Fig. 3, lanes 12 and 21). The affinity appears to be less than that observed for the fhaK probe, since no significant shift is observed at the low concentration (Fig. 3, lanes 11 and 20). Although the magnitude of the shift of the ptxB fragment is greater than that observed for the ptxD fragment, the proportions of labeled probe bound were approximately equivalent in the two cases. As before, the interactions are specific and require phosphorylation.

DNase I protection of the *ptx* promoter by BvgA. The 67-bp overlap region between the ptxB and -D fragments that were shifted by BvgA-phosphate contains two 20-bp repeats that were previously shown to be critical for BvgA dependent regulation (9, 10). We therefore proceeded to determine whether these repeats bind BvgA-phosphate directly in DNase I protection analyses (Fig. 4). Using a target that carries the ptxD and -E sequences (−233 to +33), we localized BvgA-phosphate binding sites spanning positions −163 to −93 on the *ptx* coding strand and −164 to −96 on the noncoding strand. This sequence encompasses the two 20-bp repeats (−157 to −117) plus short, flanking regions on either side (Fig. 4A). There is an additional region of weak protection spanning −90 to −65. We observed a similar protection when we examined the footprint derived from a target fragment labeled on the *ptx* noncoding strand (Fig. 4B). A DNase I hypersensitive site is observed on this strand at the junction between the two repeats (at position −136), and partial protection is also seen between positions −91 and −82. In this assay, the binding is again strongly dependent on phosphorylation by acetyl phosphate; partial protection by 32 nM phosphorylated BvgA is observed (Fig. 4, lanes 6), whereas no protection is observed even at 65 nM untreated BvgA (Fig. 4, lanes 4).

Synergistic binding of BvgA-phosphate and RNAP to the *ptx* promoter. The synergistic binding of other transcription factors and RNAP to DNA has been described (18, 20, 25, 32, 38). We therefore conducted DNase I protection assays on BvgA-phosphate in the absence (Fig. 5, lanes 3 to 7) or presence (lanes 8 to 12) of 300 nM *E. coli* RNAP. In the presence of RNAP, the protection in the region spanning −163 to −93 appears unchanged, except for the appearance of at least two novel DNase I hypersensitive sites immediately downstream from the 20-bp repeats at −117. However, clear protection is observed downstream of −93 only in BvgA binding reaction mixtures that contained RNAP. The weak protection at −90 to −65 seen for BvgA-phosphate alone is greatly enhanced with the inclusion of RNAP in the binding reaction mixture. In addition, protection is observed up to position −1 and is interrupted by short unprotected sequences that contain hypersensitive sites (between −80 and −77, −61 and −54, and −25 and −17).

In addition to the appearance of a novel protection pattern that covers the region separating the direct repeats and the putative RNAP binding site, we also observed the synergistic DNA binding of BvgA-phosphate and RNAP on the *ptx* promoter. Although BvgA-phosphate weakly protects the upstream binding sites at a concentration of 44 nM (Fig. 5, lane 7), in the presence of RNAP, one can notice partial protection at a concentration of 29 nM (Fig. 5, lane 10). In the absence of BvgA-phosphate, RNAP does not display any protection (Fig. 5, lane 13), but upon the addition of the phosphorylated activator, an extended footprint covering the putative RNAP binding sites is observed (i.e., the sequences centered at −10 and −35).

DISCUSSION

The emergence of a complete picture of how BvgA mediates the expression of multiple virulence factors has been hampered by apparent mechanistic differences of activation of different virulence loci. The differences between *fha* and *ptx* regulation are representative of these. The contrasting temporal expression of these genes has been previously noted. Although *fha* and *bvg* transcripts are detected minutes after a switch from modulating to nonmodulating conditions, *ptx* and *cya* transcripts are detected only after several hours (28, 30). These observations are consistent with the hypothesis that toxin expression requires a higher intracellular concentration of BvgA, because the appearance of toxin transcripts correlated with a high level of BvgA protein. However, the failure of earlier attempts to reconstitute the regulated expression of the *ptx* or *cya* operons by *bvg* in *E. coli* cells suggested that perhaps BvgA could not by itself activate toxin expression (7, 22). Indeed,

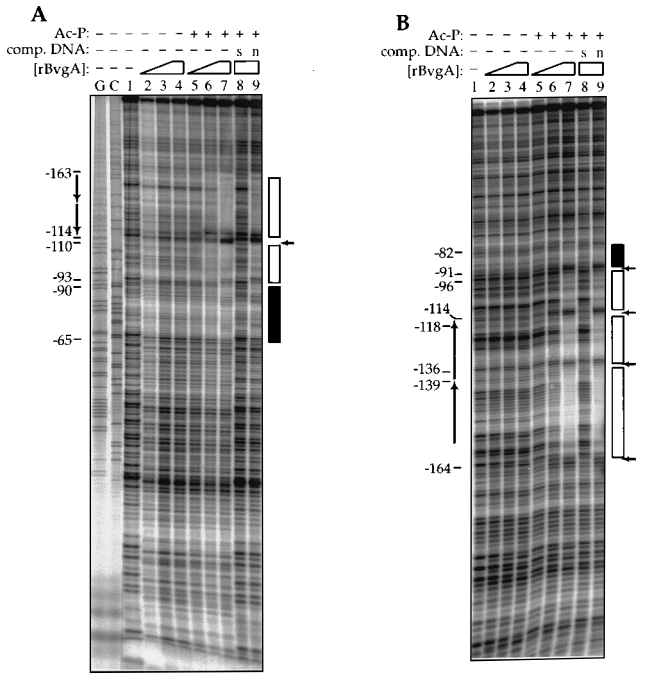

FIG. 4. DNA binding activity of BvgA by DNase I protection analyses. Radiolabeled 266-bp target DNA was incubated with 16 nM (lanes 2 and 5), 32 nM (lanes 3 and 6), or 65 nM (lanes 4, 7, 8, and 9) BvgA either in the absence (−) or in the presence (+) of 10 mM acetyl phosphate (Ac-P). An excess of unlabeled specific (s) or nonspecific (n) oligonucleotides was also added to some reaction mixtures. After binding reactions reached equilibrium, samples were treated with DNase I and reactions were terminated and loaded onto a 6% polyacrylamide–urea sequencing gel. Nucleotide positions that define the borders of protected regions are given at the left of each panel. The 20-bp direct repeats are shown as vertical arrows. Open boxes to the right of each panel depict protected regions, while the shaded rectangles depict regions of only weak protection. Short horizontal arrows point to DNase I hypersensitive sites. (A) *ptx* coding top strand labeled at the 5′ terminus is used as a target fragment. The G and C dideoxynucleotide sequencing reactions are depicted on the extreme left. (B) *ptx* noncoding bottom strand labeled at the 3′ terminus is used as a target fragment. comp. DNA, competitive DNA.

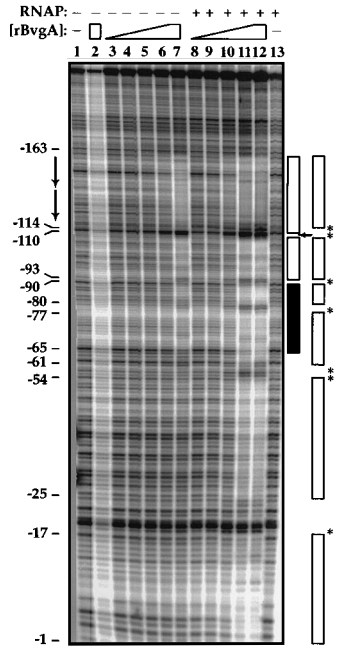

FIG. 5. DNase I protection assay of the *ptx* promoter (266-bp coding top strand) by BvgA-phosphate and RNAP. BvgA phosphorylated in vitro at 13 nM (lanes 3 and 8), 19 nM (lanes 4 and 9), 29 nM (lanes 5 and 10), 44 nM (lanes 6 and 11), or 65 nM (lanes 7 and 12) was incubated in the absence (−) or in the presence (+) of 300 nM RNAP before DNase I treatment. A binding reaction mixture containing 65 nM BvgA but lacking acetyl phosphate was loaded onto lane 2. The vertically aligned rectangles and asterisks on the right indicate the protected regions and DNase I hypersensitive sites, respectively, observed in the presence of both BvgA-phosphate and RNAP.

investigators had also failed to provide in vitro evidence for the binding of BvgA to promoters of either toxin (9, 26). To interpret these findings and explain the differential regulation exerted by BvgAS at these loci, some investigators have invoked the existence of accessory factors that are required for *ptx* and *cya* expression but are not necessary for *fha* expression.

In this report, we have examined the ability of in vitro-phosphorylated BvgA to specifically interact with the pertussis toxin promoter. A previous study revealed that the unphosphorylated form of BvgA retains a low level of binding to the target site in the *fha* promoter (2). The in vitro phosphorylation of BvgA by acetyl phosphate results in a greatly enhanced affinity of the regulator for the *fha* binding site. The absence of in vitro and in vivo activity of BvgA on the *ptx* promoter suggested the possibility that phosphorylation was an absolute requirement to detect specific binding by BvgA at this particular promoter. By gel shift analyses, we have shown this to indeed be the case for the *ptx* promoter. The affinity of binding to the *ptx* promoter is qualitatively less than that observed for the *fha* promoter. This observation is in agreement with the suggestion that *ptx* activation by BvgA may require a higher concentration of the activated regulator (28). The region of overlap between two fragments shifted in this assay contains two 20-bp direct repeats that are critical for BvgA-mediated regulation of *ptx* expression (9, 10). In addition, the extent of gel shifting of the ptxB fragment was greater than that of the ptxD fragment. One interpretation of this result is that the ptxB DNA sequence (−167 to −33) may harbor multiple BvgA binding sites.

By DNase I footprinting analyses, we were able to demonstrate the ability of phosphorylated BvgA to interact specifically with a region encompassing these direct repeats. Symmetric binding of BvgA-phosphate about the center of this motif is suggested by the induction of DNase I hypersensitive sites at its center. Upon close examination of the sequence of these repeats, two sites that share some homology to the consensus 5′-TTTCTTA-3′ BvgA binding site can be discerned (Fig. 6).

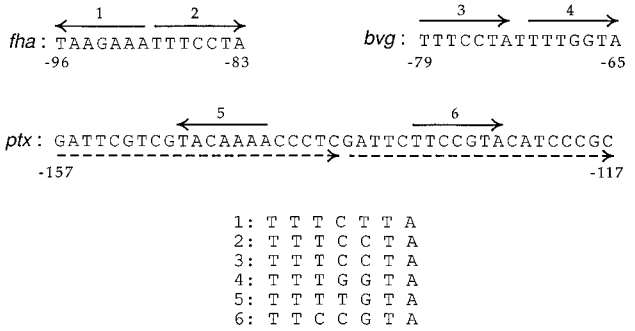

FIG. 6. *cis*-acting regulatory sequences controlling expression of *bvg*-regulated genes. The putative recognition sequences are presented for the *fha*, *bvg*, and *ptx* promoters. The BvgA target half-sites are indicated by solid arrows, and the 20-bp direct repeats are indicated by dashed arrows. The six half-site sequences are aligned below the promoter sequences.

Each half-site in this inverted repeat motif shares at least five of the seven nucleotides in the consensus. The half-sites in the *fha* promoter can accept single nucleotide changes without any significant changes in binding affinity in an in vivo operator titration assay (25a). However, whereas these sites are joined directly in the *fha* promoter, they are separated by one full turn of the DNA helix (10 bp) in the *ptx* promoter. These observations suggest that BvgA-phosphate dimers may cooperatively interact with the hyphenated half-sites within the 20-bp direct repeats, although the binding affinity may be reduced because of their physical separation.

It is clear from the variability of half-site orientation and spacing of recognition sequences that DNA recognition by BvgA is structurally complex. However, even at this early stage of our investigation, some semblance to other systems emerges. For example, the CysB activator binds to target sites that display various half-site arrangements with known stoichiometry. The binding affinity of this LysR-type activator is dictated by its interaction with an inducer molecule. Furthermore, the CysB regulator binds DNA as a tetramer (13, 14). Interestingly, CysB can interact with hyphenated half-sites separated by either 1 or 11 bp (12, 14, 23). Clearly, a more detailed understanding of BvgA-DNA interaction at promoters awaits studies that address the stoichiometry of DNA binding as well as base contacts made by BvgA. Nonetheless, it is tempting to speculate that BvgA, like CysB, may form higher-order complexes on the same face of the helix in order to bridge the gap that separates the two half-sites centered at −136.5.

At several bacterial promoters, the binding of activators to upstream sites is required for RNAP binding. For example, in the absence of the LuxR, OxyR, or PhoB activator, RNAP is unable to interact with the *luxI*, *katG*, or *phoA* promoter, respectively (20, 32, 38). We show here that the binding of BvgA-phosphate and *E. coli* RNAP to the *ptx* promoter is likewise reciprocally cooperative. Although we cannot rule out the possibility that the cooperative effect we observe is due to topological changes in the *ptx* promoter DNA induced by each protein, we suggest that this phenomenon is due to direct protein-protein interaction. Such an interaction is supported by the recognition that overexpression of the *rpoA* gene product in *B. pertussis* leads to the reduced transcription of the pertussis and adenylate cyclase toxin genes (4). The *rpoA* gene encodes the α subunit of RNAP, a target for direct protein-protein interaction by some transcriptional regulators (3, 15, 16). In the absence of RNAP, BvgA-phosphate strongly protects a region between −164 and −93 that encompasses the 20-bp direct repeats (−157 to −117). A weak protection from DNase I activity was also observed downstream of −93, but no protection was observed beyond position −65. However, upon the addition of purified RNAP holoenzyme to the binding reaction mixtures, clear protection was observed in a greatly extended region that spanned −163 to −54. Recent analyses suggest that both the spacing and sequence of the intervening region between the 20-bp direct repeats and the RNAP binding site may be critical for the *bvg* responsiveness of the *ptx* promoter (3a, 9). An examination of the sequence downstream of the direct repeats reveals no clear consensus BvgA binding sites, although several degenerate half-sites on the same face of the helix are discernable between positions −108 and −62. Although we cannot discriminate between the contributions of RNAP and BvgA to the protection pattern observed, earlier studies demonstrated that *E. coli* RNAP protects about 60 bp of promoter DNA between −45 and +15 (8).

The stimulation of transcription by activators can be either at the level of RNAP binding, open complex formation, promoter clearance, or effects on more than one of these steps. The reciprocal cooperativity of binding by RNAP and BvgA-phosphate suggests that at least one way BvgA stimulates *ptx* transcription is by increasing the affinity of RNAP for its promoter by protein-protein interaction. Many transcriptional activators have been tentatively grouped into two classes on the basis of the nature of their interactions with RNAP (15, 16): those that contact the DNA-binding, C-terminal domain of the alpha subunit (αCTD) of RNAP (class I activators) and those that contact the sigma subunit (class II activators). Class I activator binding sites are generally located upstream of −35, while class II activators bind to sites that overlap the −35 element. Based on current data, the BvgA activator may act as a class I activator at the *fha*, *bvg*, and *ptx* promoters. The location of the binding sites at the *fha* (−89.5) and *bvg* (−72) promoters would suggest that BvgA may contact the alpha subunit at these sites. At the *ptx* promoter, BvgA bound to the most promoter-proximal site could make favorable contacts with RNAP and may recruit αCTD to DNA upstream of −35. It has been proposed that the catabolite gene activator protein acts in the same manner to activate transcription at the −61.5 binding site on the *lac* promoter (3). Preliminary results presented here and elsewhere do not, however, exclude the distinct possibility that BvgA may contact an alternative RNAP subunit at some promoters. Indeed, a recent study reports the isolation of BvgA mutants carrying lesions at the extreme carboxy-terminal region which genetically separate its ability to transactivate *ptx* and *fha* (33). These mutations may define different points of contact for RNAP. The identification of BvgA binding sites in the *fha* and *bvg* promoters was achieved by the use of extracts that presumably contained predominantly unphosphorylated BvgA (26). It remains a possibility that phosphorylated BvgA protects a region that extends into the −35 element of these promoters. We are presently developing an in vitro transcription assay to examine the ability of BvgA and mutant forms of BvgA to stimulate transcription initiation at several promoters in the presence of either wild-type or mutant *E. coli* RNAP that carry lesions in either the α or σ^{70} subunits.

In the context of the interactions between BvgA and the *ptx* promoter demonstrated here, earlier discrepancies and differences observed between *fha* and *ptx* regulation are reconciled. The absence of binding of *ptx* promoter DNA by BvgA is resolved by its strict dependence on phosphorylation. Our studies have demonstrated that phosphorylated BvgA directly interacts with multiple sites on the *ptx* promoter and promotes binding by RNAP. The lack of *ptx* transcriptional activation by the *bvg* locus in *E. coli* cells can be interpreted in terms of the higher levels of activated BvgA required to stimulate transcription at *ptx* compared with *fha*. Mechanistically, this could be due to a lower binding affinity of BvgA at *ptx* or to the need for higher-order multimers of BvgA. A recent study demonstrates that under certain growth conditions, BvgAS is in fact sufficient for modulation-responsive activation of the *ptx* locus in *E. coli* cells (39). Uhl and Miller suggest that the relevant difference between the growth conditions promoting *ptx* expression and conditions previously used is a slower rate of growth with a concomitant increase in BvgA levels. Similarly, Marques and Carbonetti have shown that expression of *ptx* in *E. coli* cells is dependent on the copy number of the *bvgAS* locus supplied in *trans* and correlates with the amount of BvgA synthesized (21a). Finally, the delay of *ptx* expression following *bvgAS* induction, as the authors originally suggested, could be due to the additional time needed to reach a threshold level of activated BvgA required to activate *ptx* expression (28, 30, 31). Given the multifaceted nature of BvgA-mediated transcriptional activation, the *bvg* regulon may prove to be an excellent

model system for the study of the differential regulation of target promoters as a consequence of not only sequence variation of binding sites but their orientation and spacing as well.

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