Mode of Action of the *Bordetella* BvgA Protein: Transcriptional Activation and Repression of the *Bordetella bronchiseptica* *bipA* Promoter

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The *Bordetella* BvgAS signal transduction system controls the transition among at least three known phenotypic phases (Bvg⁺, Bvgⁱ, and Bvg[−]) and the expression of a number of genes which have distinct phase-specific expression profiles. This complex regulation of gene expression along the Bvg signaling continuum is best exemplified by the gene *bipA*, which is expressed at a low level in the Bvg⁺ phase, at a maximal level in the Bvgⁱ phase, and at undetectable levels in the Bvg[−] phase. The *bipA* promoter has multiple BvgA binding sites which play distinct regulatory roles. We had previously speculated that the expression profile of *bipA* is a consequence of the differential occupancy of the various BvgA binding sites as a result of variation in the levels of phosphorylated BvgA (BvgA-P) inside the cell. In this report, we provide in vitro evidence for this model and show that *bipA* expression is activated at low concentrations of BvgA-P and is repressed at high concentrations. By using independent DNA binding assays, we demonstrate that under activating conditions there is a synergistic effect on the binding of BvgA and RNA polymerase (RNAP), leading to the formation of open complexes at the promoter. We further show that, under in vitro conditions, when *bipA* transcription is minimal, there is competition between the binding of RNAP and BvgA-P to the *bipA* promoter. Our results show that the BvgA binding site IR2 plays a central role in mediating this repression.

Bordetella establishes respiratory tract infections by coordinately regulating the expression of several virulence factors including adhesins and toxins (21, 22). Similar to other bacterial systems, this regulation is mediated by initiating a signal transduction cascade in response to environmental fluctuations and is controlled by a two-component system encoded by the BvgAS locus (22, 31). BvgS functions as the sensor histidine kinase, which autophosphorylates in the presence of ATP in vitro and then transfers the phosphoryl group to BvgA, the cognate response regulator (4, 30). Genetic and biochemical evidence suggests that phosphorylation of BvgA leads to the alteration of its DNA binding affinity for target promoters, resulting in transcriptional activation or repression of Bvg regulon genes (3, 6, 11, 13, 19, 20).

One of the striking features of the BvgAS signal transduction system is its ability to control at least three known (Bvg⁺, Bvg[−], and Bvgⁱ) and potentially multiple phenotypic states, as opposed to mediating a biphasic transition in response to environmental cues (22). The switch among different phenotypic phases is a direct consequence of differential expression of a distinct set of gene products. For example, when BvgAS is active, *Bordetella* cells are in the Bvg⁺ phase, which is characterized by maximal expression of Bvg-activated factors like adhesins and toxins and lack of expression of Bvg-repressed genes. Inactivation of BvgAS by modulating signals (sulfate anion, nicotinic acid, or growth at low temperature) results in the switch to the Bvg[−] phase, which is characterized by expres-

sion of Bvg-repressed factors (e.g., flagella in *Bordetella bronchiseptica* and outer membrane proteins of unknown function in *Bordetella pertussis*) and the repression of Bvg-activated genes (22). The Bvgⁱ phase is expressed either as a result of specific genetic mutations in BvgS or by growth of wild-type *Bordetella* strains in the presence of semimodulating concentrations of chemical signals (9). The Bvgⁱ phase is principally characterized by maximal expression of a set of antigens of which BipA is the first to be identified at the molecular level (13, 27).

Expression of *bipA* is low in the Bvg⁺ phase, peaks in the Bvgⁱ phase, and is at nearly background levels in the Bvg[−] phase (11, 13). Previously, we have shown that, depending on its phosphorylation state, BvgA binds with differential affinities to several sites located both upstream and downstream of the *bipA* transcription initiation site (Fig. 1) (11, 13). We demonstrated that, while the upstream site, IR1, is essential for transcriptional activation, the downstream sites IR2 and IR3 are involved in repression (Fig. 1) (11). Most importantly, we showed that the phase-specific expression of *bipA* can be altered by changes in the *bipA* promoter region, thereby providing direct evidence for the role of these sites in determining the expression profile of *bipA* (11, 12). Based on these results and other studies on Bvg-activated promoters, we proposed that, by adjusting the occupancy of various BvgA binding sites as a direct consequence of changes in BvgA-P levels, *Bordetella* cells display such variation in gene expression (8, 11, 12). The detailed mechanism of *bipA* regulation has not been elucidated, since the levels of BvgA-P inside the cell are unknown.

We are studying the mechanics of the phase-dependent expression profile of *bipA* as a model to understand how BvgAS is able to achieve and sustain such a precisely regulated pro-

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FIG. 1. Arrangement and boundaries of different BvgA binding sites relative to the transcription initiation site (+1) encompassing the *bipA* promoter. IR1, -2, and -3 represent the three inverted repeat motifs. HS1 and HS2 denote the two half-site binding sequences. The genetic structures of the mutant promoter derivatives utilized for in vitro transcription and DNA-protein interaction analysis are shown below. The region of IR2 deleted in the ΔIR2 promoter derivative is shown by gaps.

gram of gene expression. We believe that the *bipA* gene offers an excellent choice towards this end, since it displays a complex expression profile that involves interplay of transcriptional repression and activation. In this study, we have developed an in vitro transcription system that replicates the phase-dependent expression of *bipA* as a function of phosphorylated BvgA (BvgA-P) concentrations, providing direct in vitro evidence for the proposed model. We have previously invoked the possibility of the existence of a repressor that might act to efficiently repress *bipA* transcription in the Bvg⁺ phase (13). Our results using purified BvgA and RNA polymerase (RNAP) holoenzyme suggest that no other protein components are required for the dual activation-repression of *bipA* transcription.

We have characterized the BvgA-RNAP interactions at the *bipA* promoter. Our results show that, at low concentrations of BvgA-P, conditions where transcription of *bipA* is activated in vitro, BvgA-P synergistically interacts with RNAP, leading to the stimulation of open complexes at the promoter. We further show that, at higher concentrations of BvgA-P, when *bipA* transcription is minimal, BvgA-P interferes with the binding of RNAP to the promoter. The results reported in this study describe the mechanism of BvgA in transcriptional repression of *bipA* and highlight the essential role of the downstream binding site IR2.

MATERIALS AND METHODS

Strains and plasmids. *Escherichia coli* DH5α strain (grown in Luria broth at 37°C) was used as the host for propagation of plasmids. Plasmids pRD555, pRD572, and pRD408, utilized to generate the wild-type (wt), the ΔIR2, and the ΔIR1 promoter fragments for electrophoretic mobility shift assays (EMSA) and DNase I footprinting assays have been described previously (11). For generating supercoiled templates for in vitro transcription assays, these plasmids were digested with EcoRI and HindIII and the resultant promoter fragments were cloned into similarly digested plasmid pMP7, a supercoiled vector whose multiple cloning site is flanked by T7 transcriptional terminators (17). This resulted in the creation of pRD565, pRD566, and pRD568, containing, respectively, the wt, the ΔIR2, and the ΔIR1 promoter fragments.

DNA manipulation. QIAGEN (Valencia, Calif.) plasmid kits were used for all plasmid purification. Prior to use for in vitro transcription assays, plasmid DNA was extracted twice with a mixture of phenol-chloroform-isoamyl alcohol to inactivate any residual RNase A. The plasmids were then purified by ethanol precipitation using standard methods.

Phosphorylation of BvgA. Phosphorylation was conducted as previously described by incubating BvgA in 1× binding buffer in the presence of 20 mM acetyl phosphate (4, 13).

EMSA. To generate DNA fragments for EMSA, plasmids pRD555 (wt) and pRD572 (ΔIR2) were digested with EcoRI and BamHI and the resulting DNA fragments were labeled by T4 polynucleotide kinase (New England Biolabs) and

[γ-³²P]ATP (Amersham Biosciences). Unincorporated nucleotides were removed by gel filtration through G-50 quick-spin columns (Amersham Biosciences). The standard binding reaction mixture contained (in a volume of 18 μl) various amounts of purified BvgA or BvgA-P and the radiolabeled promoter in 1× binding buffer [10 mM Tris-HCl, pH 7.8, 2 mM MgCl₂, 50 mM NaCl, 1 mM dithiothreitol, 0.5 μg of poly(dI-dC), 0.01% NP-40, 10% glycerol, and 100 ng of bovine serum albumin]. Reaction mixtures were incubated at room temperature for 15 min. One unit of *E. coli* RNAP (Epitentre) was added to the samples, whereas to the samples devoid of RNAP, 1 μl of the 1× binding buffer was added, followed by incubation at 37°C for 15 min. One unit of RNAP enzyme catalyzes the incorporation of 1 nanomole of ribonucleoside triphosphates into RNA in 10 min at 37°C. Samples were then treated with heparin (50 μg ml⁻¹) for 15 min at 37°C. To visualize the supershift of the gel complexes, samples were additionally incubated for 15 min at 37°C with 1 μl of a 1:100 dilution of the monoclonal antibody raised against the β' subunit of *E. coli* RNAP (Neoclone). Gels (4% polyacrylamide, 1× Tris-acetate-EDTA [TAE] containing 1% glycerol) were preelectrophoresed in 1× TAE buffer at a constant voltage of 150 V for 1 h at 4°C prior to the loading of the samples. Electrophoresis was performed at 150 V for 2 to 3 h at 4°C. The gels were dried under vacuum, and the DNA-protein complexes were visualized by direct autoradiography on phosphorimager screens.

Elution of proteins from polyacrylamide gels and immunoblot analysis. The protein content of different DNA-protein complexes formed on the wt and the ΔIR2 promoter derivatives was analyzed by a modification of the procedure described previously (16). DNA-protein complexes were formed as described above and loaded onto a 4% polyacrylamide-TAE gel. After electrophoresis the wet gel was exposed to an X-ray film. After overnight exposure the portion of the gels corresponding to the two complexes was excised. The gel pieces were then suspended in the elution buffer (0.1% sodium dodecyl sulfate [SDS], 0.05 M Tris-HCl [pH 7.8], 5 mM dithiothreitol, 0.1 mM EDTA, and 0.2 M NaCl) and crushed. Proteins were eluted for 12 to 14 h at room temperature with constant agitation. After elution, the mixture was centrifuged for 5 min at 13,000 rpm to pellet the crumbled gel. An equal volume of 2× SDS gel loading buffer was then added to the supernatant, and the samples were loaded onto an SDS-polyacrylamide gel. Proteins separated on SDS-polyacrylamide gels were transferred to a nitrocellulose membrane (Osmonics Inc.). For detection of RNAP, primary antibody NT73, raised against the β' subunit of *E. coli* RNAP (Neoclone) was used at a 1:2,000 dilution. For detecting BvgA, anti-BvgA antibody was used at a 1:1,000 dilution. Appropriate secondary antibody conjugated to horseradish peroxidase was used at a 1:12,000 dilution. The ECL Western blotting analysis system (Amersham Biosciences) was used for detection of proteins on the membrane.

DNase I footprinting assays. For preparation of DNA fragments, plasmids pRD555 (wt) and pRD572 (ΔIR2) were first digested with EcoRI and then end labeled with T4 polynucleotide kinase and [γ-³²P] ATP, followed by digestion with BamHI to release the promoter fragments. The radiolabeled DNA fragments were then separated on 4% polyacrylamide gels, excised from the gel, and purified by electroelution. Reaction mixtures for DNase I footprinting were identical to those used above for EMSA. Partial digestion of protein-bound DNA was initiated by the addition of DNase I (Promega Life Sciences) after dilution in 1× binding buffer containing 1 mM CaCl₂. DNase I activity was terminated after 2 min by the addition of stop solution (0.4 M sodium acetate, 0.2% SDS, 10 mM EDTA, 50 μg ml⁻¹ *Saccharomyces cerevisiae* tRNA, and 10 μg of proteinase K). After 10 min of incubation at 50°C, the mixtures were extracted

FIG. 2. Single-round in vitro transcription assays of the *bipA* promoter with *E. coli* RNAP as a function of BvgA or BvgA-P concentration. A. wt DNA fragment (lanes 1 to 11) and the Δ IR2 promoter derivative (lanes 12 to 22). Lanes 2 to 6 and 13 to 17 contain increasing amounts of BvgA, whereas lanes 7 to 11 and 18 to 22 contain BvgA-P. Lanes 1 and 12, RNAP only; lanes 2, 7, 13, and 18, 0.04 μ M BvgA or BvgA-P; lanes 3, 8, 14, and 19, 0.2 μ M BvgA or BvgA-P; lanes 4, 9, 15, and 20, 0.4 μ M BvgA or BvgA-P; lanes 5, 10, 16, and 21, 0.8 μ M BvgA or BvgA-P; lanes 6, 11, 17, and 22, 1.2 μ M BvgA or BvgA-P. The *Bordetella bipA* promoter-specific transcripts from the wt and the Δ IR2 promoter derivatives are shown at the left and right of the upper panel, whereas the BvgA-independent *E. coli* RNA-I promoter-specific transcript is shown at the left of the bottom panel. Amounts of the *bipA*- and the RNA-I-specific transcripts were determined by Phosphor-Imager quantitation, and the ratios are given at the bottom of each lane. B. Δ IR1 promoter derivative. Lane 1, RNAP alone; lanes 2, 3, 4, and 5, 0.04, 0.4, 0.8, and 1.2 μ M of BvgA, respectively; lanes 6 to 9, same as lanes 2 to 5 except that 20 mM acetyl phosphate was added to the reactions.

with phenol-chloroform and DNA in the aqueous phase was ethanol precipitated and analyzed on 6% polyacrylamide-7 M urea gels.

Purification of DNase I-treated complexes. To determine DNA-protein interactions specific to various gel complexes, these complexes were separated and purified after DNase I treatment. DNase I digestion was carried out as described above except that the reaction was terminated by addition of EDTA (pH 8.0) to a final concentration of 10 mM. The samples were then loaded onto 4% polyacrylamide gels and electrophoresed as described above for EMSA. After electrophoresis, the wet gels were exposed to X-ray film. Areas corresponding to individual complexes were excised, and radiolabeled DNA was electroeluted in 1 $\times$ TAE. Eluted DNA was ethanol precipitated, resuspended in 10 μ l of Tris-EDTA (pH 8.0) and formamide-dye solution, boiled for 3 min, loaded onto 7 M urea-6% polyacrylamide gel, and electrophoresed at 55 W.

In vitro transcription. Assays were performed in the presence of heparin as described previously with some modification (14, 15). Template DNA was first incubated in binding buffer as described above with either BvgA or BvgA in the presence of acetyl phosphate (BvgA-P) at 25°C for 15 min. One unit of *E. coli* RNA polymerase and 1 μ l of RNasin, an RNase inhibitor (Promega Life Sciences), were then added, followed by incubation at 37°C for 15 min. Transcription was then initiated by adding a mixture containing 0.25 mM ATP, 0.25 mM CTP, 0.25 mM GTP, 0.015 mM UTP, 10 μ Ci of [α - 32 P]UTP (ICN Biomedicals), and 50 μ g of heparin per ml. Reactions were terminated after 15 min at 37°C by the addition of stop solution (0.4 M sodium acetate, 0.2% SDS, 10 mM EDTA, and 50 μ g ml $^{-1}$ yeast tRNA). Nucleic acids were then extracted with a mixture of phenol-chloroform-isoamyl alcohol, and the nucleic acids in the aqueous phase were ethanol precipitated. RNA products were resuspended in 10 μ l of Tris-EDTA (pH 8.0) and formamide-dye solution, boiled for 3 min, loaded onto 8 M urea-6% polyacrylamide gel, and electrophoresed at 55 W.

RESULTS

Establishment of an in vitro transcription assay system that mimics the observed in vivo expression profile of *bipA*. Expression of *bipA* is low in the Bvg $^{+}$ phase (absence of modulators), maximal under the Bvg i phase (intermediate concentration of modulators), and at background levels in the Bvg $^{-}$ phase (high concentration of modulators) (13). Based on a combination of in vivo transcriptional assays and DNase I footprinting analy-

ses, we have previously speculated that at low BvgA-P concentrations BvgA preferentially occupies the upstream activation site IR1 (Fig. 1), resulting in maximal transcription in the Bvg i phase. At higher concentration of BvgA-P, the low-affinity downstream sites IR2 and IR3 (Fig. 1) are also occupied, leading to repression in the Bvg $^{+}$ phase (11, 12). To test the effect of concentration-dependent differential occupancy of BvgA on transcription of the *bipA* promoter, we utilized a single-round in vitro transcription assay in the presence of the polyanion heparin. Heparin inactivates unbound and nonspecifically bound holoenzyme but not promoter-bound enzyme (28). We previously showed that *bipA* is expressed in a BvgAS-dependent manner in *E. coli* (13). Additionally, studies utilizing other Bvg-regulated promoters have demonstrated the functional correspondence between *Bordetella* and *E. coli* RNAP (3, 5-7, 20). Thus, we used commercially available RNAP holoenzyme from *E. coli* for the studies reported here. The wild-type and mutant promoter derivatives (Fig. 1) were cloned in the supercoiled transcription vector pMP7, 345 bp upstream of the T7 early terminator, and in vitro transcription assays were performed using increasing amounts of highly pure recombinant BvgA and BvgA-P (phosphorylated in vitro with acetyl phosphate). It has been previously shown that BvgA is specifically labeled at an aspartic acid residue when incubated with acetyl- 32 P $_4$ (4).

wt promoter. Figure 2A shows that both BvgA and BvgA-P generate the expected 542-nucleotide transcript (Wt) from the

wt *bipA* promoter. This transcript was not observed either when the vector pMP7 lacking the promoter was used as the transcription template (data not shown) or in the absence of BvgA (Fig. 2A, lane 1), supporting our previous results that expression of *bipA* is dependent on BvgA (11, 13). Compared to nonphosphorylated BvgA, BvgA-P stimulated transcription at lower concentrations of the protein (Fig. 2A, compare lane 2 with 7 and 3 with 8). As the concentration of BvgA-P was increased in the transcription reactions, levels of the wt *bipA* transcript decreased in a reciprocal manner (Fig. 2A, lanes 7 to 11). This observed repression was specific for the *bipA* promoter and not due to a general inhibitory effect on transcription, since the concentration of the Bvg-independent *RNA-I* transcript, originating from the *RNA-I* promoter present on pMP7, was unaffected (Fig. 2A). Transcriptional repression also required acetyl phosphate, since no significant reduction in the intensity of *bipA* transcript was observed in the absence of acetyl phosphate (Fig. 2A, lanes 4 to 6).

Δ IR2. Previously by reporter assays, we showed that deletion of the BvgA binding site IR2 results in an enhancement of *bipA* promoter activity (11). Thus, if repression of the *bipA* promoter activity with increasing concentrations of BvgA is due to the binding of BvgA-P to IR2, we hypothesized that there would be increased transcription from a *bipA* promoter derivative lacking IR2. Similar to the wt promoter, no transcription was detected from the Δ IR2 template when RNAP alone was added to the reactions (Fig. 2A, lane 12). In the presence of RNAP and BvgA or BvgA-P, a transcript (Δ IR2; Fig. 2A) that is smaller than the wt transcript is detected. Note that the smaller size of the transcript is due to a deletion of 43 nucleotides in the Δ IR2 template spanning +17 to +59 (Fig. 1) of the *bipA* promoter. This result further highlights the specificity of the in vitro transcription assays.

In contrast to the wild-type template, the intensity of the *bipA* transcript generated from the Δ IR2 template increased gradually with increasing BvgA-P concentration (Fig. 2A, compare lanes 8 to 11 with 19 to 22). At the highest concentration of protein used, there was an $\approx$ 10-fold increase in transcript intensity as a result of deletion of IR2 (Fig. 2A, compare lane 11 with 22). In contrast, the intensity of the *bipA* transcript did not change significantly as a result of deletion of IR2, when nonphosphorylated BvgA (compare lane 6 with 17) was used. This supports our previous results that showed nonphosphorylated BvgA was unable to occupy IR2 (11).

Δ IR1. Compared to stimulation of *bipA* transcription from the wt promoter, a *bipA*-specific transcript was not detected from a promoter derivative that lacked the binding site IR1 (Fig. 2B). The only transcript detected from the Δ IR1 promoter derivative is that driven from the BvgA-independent promoter, *RNA-I* (Fig. 2B). Thus, these results corroborate our in vivo findings demonstrating the essential role of IR1 in transcriptional activation of the *bipA* promoter (11).

These in vitro results thus suggest that BvgA mediates both the activation and repression of the *bipA* promoter in a concentration-dependent manner and confirm the role of different BvgA binding sites in regulating *bipA* expression. In addition, our in vitro transcription assays conducted with highly purified BvgA and RNAP suggest that no additional cellular factors are required to mediate repression from the *bipA* promoter.

Interaction of BvgA-P and RNAP at the *bipA* promoter. The role of BvgA-P in controlling RNAP interactions at the *bipA* promoter was addressed using nondenaturing EMSA to separate and identify protein-DNA complexes. For these assays, the wt (Fig. 3A) and Δ IR2 templates (Fig. 3B) were the same as those used for in vitro transcription assays above. EMSA were also performed in the presence of heparin so as to specifically detect only open complexes. Additionally, these reaction mixtures contain poly(dI-dC) as nonspecific competitor DNA.

Synergy between RNAP and BvgA-P binding under activating conditions (low concentrations of BvgA-P). Figure 3A and B, lanes 2 to 4, demonstrate that BvgA-P efficiently occupied both the wt and the Δ IR2 promoter derivatives, while RNAP alone poorly bound to these DNA fragments (Fig. 3A and B, lanes 5). This result is consistent with in vitro transcription assays where RNAP alone did not result in transcription from any of the promoter derivatives (Fig. 2A, lanes 1 and 12). In the presence of both BvgA-P and RNAP, a new complex (II) with slower mobility than the BvgA-P-DNA complexes (I) was observed for both the wt and Δ IR2 promoter fragments (Fig. 3A and B, compare lanes 6 and 7 with 2 to 4). Thus, it appeared to represent a ternary complex containing BvgA, RNAP, and DNA. Note that, for both the wt and the Δ IR2 promoter fragments, the band corresponding to the ternary complex was detected at concentrations of BvgA-P and RNAP together, which alone did not result in any significant DNA occupancy (Fig. 3A and B, compare lanes 2 and 5 with 6). This result suggests the existence of cooperativity between the two proteins. Additionally, at low concentrations of BvgA-P, the ratio of the intensity of the ternary complex to that of the respective radiolabeled promoter DNA was approximately threefold higher for the Δ IR2 promoter fragment than for the wt fragment, suggesting that deletion of IR2 leads to the enhancement of open complex formation at the *bipA* promoter (Fig. 3A and B, compare lanes 6).

Identification of protein components of DNA-protein complexes. To ascertain the identities of the different complexes, a monoclonal antibody specific to the β' subunit of the RNAP was added to the reaction mixture for EMSA. If the antibody reacted with RNAP bound to the probe, it was expected that the shifted bands either would have slower mobility (supershift) or would disappear as a result of dissociation of RNAP from the probe. With the addition of the RNAP antibody, the ternary complex band (II) that formed on both the wt and the Δ IR2 templates was shifted to a higher position (complex IV for wt and complex III for Δ IR2) on the gel (Fig. 3A and B, compare lanes 10 and 11 with 6 and 7), suggesting that RNAP was part of this complex. In contrast to the RNAP antibody, a BvgA-specific antibody did not result in a supershift of bands observed either with BvgA alone or BvgA with RNAP (data not shown). It is possible that the BvgA-specific epitope(s) recognized by this antibody is masked as a result of the binding of BvgA to the DNA.

To confirm that the proposed ternary complex (CII) for both the wt and the Δ IR2 template indeed contained BvgA, gel pieces corresponding to these complexes were separately excised and the proteins eluted as described in Materials and Methods. The protein mixtures were loaded on a denaturing

FIG. 3. Gel shift analysis and depiction of putative DNA-protein complexes formed in the presence of BvgA-P and RNAP at the *bipA* promoter. Approximately 500 cpm of the different *bipA* promoter fragments was incubated with BvgA-P, RNAP, and 50 μ g/ml of heparin as described in Materials and Methods. Lane 1, DNA alone; lanes 2 to 4, DNA plus BvgA-P. BvgA was phosphorylated in vitro with acetyl phosphate as described in Materials and Methods. Lanes 2 to 4, 0.04, 0.4, and 1.2 μ M of BvgA-P, respectively; lane 5, DNA plus RNAP; lanes 6 to 8, same as lanes 2 to 4 except that RNAP was added to the reactions; lanes 9 to 12, same as lanes 5 to 8 except that 1 μ l of a 1:100 dilution of anti- β' antibody was added. F represents free DNA, and the numbers represent the different DNA-protein complexes formed (see text for explanation). Diagrams representing the putative DNA-protein complexes are shown on the right of the gel picture. A. wt promoter. B. Δ IR2 derivative.

polyacrylamide gel and subjected to immunoblotting using antibodies specific for BvgA and RNAP. As seen from Fig. 4A, complex II contained both BvgA and RNAP. Thus, this suggests that the protein-DNA complex corresponding to band II is indeed a ternary complex.

Competition between RNAP and BvgA-P for binding to the *bipA* promoter under repressive conditions (high concentrations of BvgA-P). At the highest concentration of BvgA-P and in the presence of RNAP, two distinct DNA-protein complexes were observed for the wt promoter fragment (Fig. 3A, lane 8). The upper complex (III) had decreased mobility compared to that of complex II observed at lower BvgA-P concentrations (Fig. 3A, compare lane 8 with 6 and 7). In addition, a lower band ran at a mobility corresponding to that of the complex (I) observed with BvgA-P alone (Fig. 3A, compare lane 8 with 4). Whereas the RNAP antibody supershifted the upper complex (III) to a higher position (V), the mobility of the lower complex remained unaffected (compare lane 12 with 8). This result thus suggests that, while the upper complex (III) contains RNAP, the lower complex contains only BvgA. To confirm the pres-

ence of BvgA in these complexes, we excised these two complexes and carried out immunoblot analysis. As seen from Fig. 4B, the upper complex (III) contained both BvgA and RNAP whereas the lower complex (I) contained only BvgA.

In contrast to the two complexes observed for the wt promoter at the highest concentration of BvgA-P, and in the presence of RNAP, only a single complex was apparent for the Δ IR2 fragment (compare lanes 8 of Fig. 3A and B). This complex was supershifted by the RNAP antibody to a higher position (III) (Fig. 3B, lane 12), suggesting that it corresponds to the ternary complex. For the wt promoter, the ratio of the intensities of the two bands observed at the highest concentrations of BvgA-P was approximately 1:1, while for the Δ IR2 promoter derivative almost all the detectable radioactivity at this concentration was observed to be associated with the ternary complex (Fig. 3A and B, compare lanes 8).

Thus, taken together these results suggest that the binding of BvgA-P to the wild-type *bipA* promoter interferes with the binding of RNAP and the deletion of IR2 results in enhanced formation of the open complex.

FIG. 4. Immunoblot analysis to determine the protein components of different complexes. The indicated DNA-protein complexes were isolated from a native 4% polyacrylamide gel, and the proteins were eluted as described in Materials and Methods. Protein samples were then loaded onto an SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and detected by using either an RNAP-specific antibody (upper panel) or a BvgA-specific antibody (lower panel). Lanes 1, purified proteins used as positive control. A. Lanes 2 and 3, CII complex corresponding to lanes 6 of Fig. 3A (wt) and B (ΔIR2), respectively. B. The upper (CIII) and lower (CI) DNA-protein complexes in Fig. 3A, lane 8.

DNase I footprinting of BvgA and BvgA-P with RNAP. To further elucidate the mechanism of dual activation and repression of the *bipA* promoter, we performed DNase I footprinting reactions. Footprinting was performed in the presence of heparin on wt and ΔIR2 promoter derivatives encompassing the same upstream regions (Fig. 1) as those used for in vitro transcription assays and EMSA. At low concentration of protein, BvgA-P protected, on the wt and the ΔIR2 promoter derivatives, the upstream region encompassing binding sites IR1 and HS2 (Fig. 5A and B, lanes 5). Consistent with our previously published results, efficient binding of nonphosphorylated BvgA to these regions was detectable only at higher concentrations (Fig. 5A and B, lanes 7 and 11). Higher concentrations of BvgA-P resulted in extended protection in the downstream region comprising the binding sites IR2 and IR3 on the wt promoter (Fig. 5A, lanes 9 and 13). In contrast, at the highest concentration, BvgA-P bound poorly to the downstream region encompassing IR3 on the ΔIR2 promoter derivative (Fig. 5B, lanes 9 and 13). This result is consistent with our previous finding that binding to IR3 occurs only in the presence of IR2 (11).

When either BvgA or BvgA-P was included along with RNAP in the reaction mixture, a strikingly different pattern of protection was seen than that observed with any of the proteins alone. In the presence of RNAP, the region of protection was extended both upstream and downstream of the transcriptional start site on both the wt and ΔIR2 promoter derivatives (Fig. 5A and B, lanes 6, 10, and 14). A similar extended protection encompassing both sides of the promoter was previously observed for the *fhaB* promoter (5).

At higher concentration of BvgA, for both the wt and the ΔIR2 promoter derivative, a decrease in the size of the probe DNA at the top of the gel is observed (Fig. 5A and B). We do not clearly understand the mechanism of this discrepancy in the sizes of the probe DNA. It is possible that conformational changes in the DNA at higher concentrations of BvgA result in greater susceptibility of the probe to DNase I.

With increasing concentrations of BvgA-P and in the presence of RNAP, a very strong protection of both the upstream and downstream regions was observed for the ΔIR2 promoter fragment compared to that for the wt promoter (Fig. 5A and B,

compare lanes 6, 10, and 14). At the highest concentration of BvgA-P, the regions between -55 and $+5$ (denoted with bars) and between -75 and -85 , for the wt promoter, are relatively less protected than that observed for the ΔIR2 promoter fragment (Fig. 5A and B, compare lanes 14). The diminished protection for the wt promoter is dependent on the phosphorylation state of BvgA since no significant concentration-dependent diminution in DNase I protection in this region was observed in the absence of acetyl phosphate (Fig. 5A, compare lanes 4, 8, and 12).

Analysis of BvgA-P and RNAP interaction by DNase I footprinting of purified complexes. Although the extent of protection of the -55 to $+5$ region with $1.2 \mu\text{M}$ BvgA-P and RNAP on the wt promoter is comparatively less than that observed for this region on the ΔIR2 promoter derivative, it is not as pronounced as one would expect based on the results of EMSA, where clearly two complexes (the ternary and the binary complexes) were observed at this concentration of BvgA-P (Fig. 3A, lane 8). We reasoned that, in order to study distinct complexes, it was necessary to purify protein-DNA complexes after DNase I footprinting and prior to analysis on denaturing polyacrylamide gels. We were particularly interested in characterizing protein-DNA interactions corresponding to the complexes represented by free DNA (F), BvgA-P-DNA (I), and BvgA-P-DNA-RNAP (III for wt and II for ΔIR2) detected in EMSA (Fig. 3A and B). After treatment with DNase I, we purified each of the complexes by native gel electrophoresis as described in Materials and Methods. The respective gel slices were then excised, electroeluted, and separated on denaturing urea-polyacrylamide gels.

Comparison of the DNase I digestion patterns of complex I (BvgA-P-DNA) to free-DNA control (F) shows that, as expected, BvgA-P occupied regions both upstream and downstream of the transcription initiation site on the wild-type promoter (Fig. 6A, compare lanes 2 and 3). Although the occupancy of BvgA-P to upstream binding regions (IR1 and HS1) remains unaffected as a result of deletion of IR2, the downstream regions comprising IR3 were only poorly bound (Fig. 6B, compare lanes 2 and 3). The complete absence of any bands in the lower half of the gels encompassing the binding sites IR1 and HS1 (Fig. 6A, lanes 1 and 3, and B, lanes 1 and

FIG. 5. DNase I protection assays of the *bipA* promoter by purified BvgA and RNAP. Binding reaction mixtures contained a radiolabeled DNA template strand. Lanes 3 to 6, 7 to 10, and 11 to 14 contained, respectively, 0.04 μ M, 0.8 μ M, and 1.2 μ M of BvgA or BvgA-P. Lane 1, control reactions with no added protein. One unit of RNAP was added where indicated. Molecular size markers are indicated on the left. Regions corresponding to binding sites HS1, IR1, HS2, IR2, and IR3 are indicated on the right. A. wt promoter. B. Δ IR2 derivative. The -55 to +5 region is denoted with bars. AC-P, acetyl phosphate.

2) is quite striking and is suggestive of very strong binding of the proteins in this region. Most importantly, DNase I footprinting with purified ternary complexes clearly shows markedly weaker protection in the region from -55 and +5 (indicated by asterisks, Fig. 6) on the wild-type promoter compared to that observed on the Δ IR2 promoter derivative (Fig. 6A and B, compare lanes 1).

DISCUSSION

In this study, we have addressed the nature of interactions between BvgA and RNAP at the *bipA* promoter with the principal objective of elucidating the dual mechanism of activation-repression at the *bipA* promoter. These studies offer an opportunity to enhance our understanding of BvgA-mediated activation and more importantly to understand the role of BvgA in transcriptional repression.

BvgA-dependent activation of *bipA* transcription. Previous studies have resulted in an understanding of how the response regulator BvgA positively controls the transcription of target Bvg-activated promoters like *fhaB*, *ptx*, and *prn* (3, 6, 20). For the *fhaB* and *ptx* promoters, transcriptional activation involves BvgA binding to an upstream high-affinity site followed by oligomerization of BvgA molecules to downstream sites. The binding of BvgA dimers to the promoter-proximal secondary site is believed to recruit RNAP. Our present study supports these previous observations and suggests the existence of co-

operativity of DNA binding between RNAP and BvgA-P. As evident by EMSA, a ternary complex (BvgA-P-DNA-RNAP) is formed at concentrations of RNAP and BvgA-P together, which failed to result in any significant DNA binding when added individually (Fig. 3). Similarly, clear protection from DNase I was observed in a large extended region when RNAP and BvgA are added together, and the protection is higher than that seen with either of these proteins alone (Fig. 5). In contrast, a much higher concentration of nonphosphorylated BvgA is required for efficient protection (Fig. 5). Concomitantly, compared to nonphosphorylated BvgA, BvgA-P stimulated higher levels of transcription (Fig. 2A). Thus, these results highlight the role of phosphorylation-induced BvgA binding to IR1 in efficient activation from the *bipA* promoter, since no transcription was detected from a template that had a deletion of this site (Fig. 2B).

BvgA-mediated repression of *bipA* expression. Based on our previous studies with the *bipA* promoter, we had proposed that phosphorylation-induced oligomerization of BvgA molecules to the downstream binding sites IR2 and IR3 results in repression of *bipA* expression in the Bvg⁺ phase (11). This model is based on the assumption that, under Bvg⁺ phase conditions, levels of BvgA-P are sufficiently high to bind to both low-affinity (IR2 and IR3) and high-affinity (IR1) binding sites. Although there appears to be indirect evidence for the existence of a gradient of BvgA-P along the Bvg signaling contin-

FIG. 6. DNase I footprinting with purified DNA-protein complexes. DNase I footprinting was performed as described for Fig. 5. The DNA-protein complexes were then purified on a native 4% gel and excised, and the digested DNA fragments were electroeluted. The samples were then loaded on 6 M urea denaturing polyacrylamide gels and electrophoresed at 55 W. Asterisks indicate the regions of the wt and Δ IR2 promoter fragments from -55 to $+5$ that are differentially protected. Regions corresponding to binding sites IR1, IR2, and IR3 are indicated on the left. A. wt promoter fragment. CI, lower DNA-protein complex from Fig. 3A, lane 8; F, radiolabeled free DNA from lane 1 of Fig. 3A; CIII, upper DNA-protein complex from Fig. 3A, lane 8. B. Δ IR2 promoter fragment. F, radiolabeled free DNA from lane 1 of Fig. 3B; CI, BvgA-DNA complex from lane 4 of Fig. 3B; CII, DNA-protein complex from Fig. 3B, lane 8.

uum, the levels of BvgA-P inside the cell have not been measured. If our model is correct, we would predict a dose-dependent repressive effect of BvgA on *bipA* transcription in vitro. Our results from in vitro transcription assays clearly show that, in presence of acetyl phosphate, as the levels of BvgA are increased, transcription from the *bipA* promoter is repressed (Fig. 2A). Additionally, we have shown that this repressive effect on transcription is not observed in the absence of either acetyl phosphate or the binding site IR2, further providing evidence for our model.

Our studies demonstrate that BvgA-mediated repression involves competition between BvgA and RNA polymerase in binding to the *bipA* promoter. It is apparent that, at higher concentrations, BvgA-P represses *bipA* transcription by physically competing with the RNAP in promoter binding. At in-

creased concentrations of BvgA-P, RNAP fails to bind efficiently to the wt *bipA* promoter, as evident by the formation of two different types of DNA-protein complexes, the ternary and the binary complexes seen by EMSA (Fig. 3A), and by reduced protection in DNase I footprinting assays (Fig. 5A and 6A).

While the manuscript was under revision, a recent study reported that purified BvgA-P and RNAP are sufficient to confer both activation and repression of the *B. pertussis bipA* promoter (32). In contrast to results from our present and previously published studies (11, 13), Williams et al. failed to detect binding of BvgA-P either to the upstream binding site HS1 or to the downstream sites IR2 and IR3 on the *B. pertussis bipA* promoter (32). The differences between the two studies could be attributed either to the utilization of different techniques to probe DNA-protein interactions or to the use of a

mutant BvgA protein in the study by Williams et al. Alternatively, the observed discrepancy between these two studies could be because of nucleotide differences in the sequences of the binding sites IR2 and IR3 and the regions spanning the BvgA binding sites (HS1, IR2, and IR3) between the *bipA* promoters of *B. bronchiseptica* and *B. pertussis* (13).

At which step of transcription initiation does BvgA act?

Transcription initiation is a sequential process involving multiple steps. Activation and repression of transcription by regulatory proteins could be mediated either at the level of closed complex (initial RNAP binding), open complex, initiated complex, or promoter clearance or by influencing a combination of these steps (10, 25). Prior studies addressing BvgA-RNAP interactions at Bvg-activated promoters have provided information only on the effect of BvgA on initial RNAP binding to the promoter (3, 5, 6, 20). Consequently, it has been speculated that BvgA can promote the rate of isomerization from a closed to an open complex (5). By studying the formation of heparin-resistant RNAP complexes, we have provided evidence for the rate-limiting step at which BvgA acts. Our studies show that BvgA mediates activation or repression of *bipA* transcription either during the transition from the closed to the open complex or at a step following open complex formation. Transcription activation at low concentrations of BvgA-P is mediated by stimulating the formation of open complexes at the *bipA* promoter. Repression at high concentrations of BvgA-P is primarily mediated by occupancy of IR2 and the subsequent occupancy of IR3. This results in reduced formation of open complexes at the wt promoter compared to those at the promoter derivative lacking IR2.

Although our studies show that there is significantly less binding of RNAP to the *B. bronchiseptica bipA* promoter under repressive conditions, the levels of *bipA*-specific transcript detected by in vitro transcription did not correlate with the amount of specific heparin-resistant ternary complexes formed under similar conditions. For example, at the highest concentration of BvgA-P, with the wt promoter DNA fragment, even though we detected ternary complexes by EMSA (Fig. 3A, lane 8), only a very low level of *bipA*-specific transcript was detected at this concentration by in vitro transcription (Fig. 2A). This suggests that, at high BvgA-P concentrations, the majority of the ternary complex is in a conformation that is unable to carry out productive RNA synthesis. It is possible that repression is mediated by blocking promoter clearance (18). Recently, it has been shown that the small *E. coli* protein H-NS inhibits transcription at the *rnnBP1* promoter by trapping the initiation complex into a conformation that is unable to extend RNA synthesis beyond template position +3 and not by interfering with the binding of the RNA polymerase (26). Similar nonproductive RNAP complexes exist at the *malT* promoter (29). More studies are needed to determine whether similar mechanisms are in effect at the *bipA* promoter.

By binding to auxiliary operators IR2 and IR3 to repress *bipA* promoter expression, BvgA shows some similarity to the repression mechanism of the other well-known repressors, LacI and GalR (reviewed in reference 24). Both LacI and GalR repress transcription of cognate promoters by binding to auxiliary operators (1, 23). In the case of the *gal* operon, the binding of GalR to the two operators in the presence of a chromatin-associated protein HU results in the formation of a

higher-order complex called the repressosome (1, 2). Although presently we cannot rule out the role of a yet-unidentified HU-like protein in *bipA* repression, our in vitro results strongly suggest that BvgA and RNAP are sufficient for *bipA* repression.

The expression profile of *bipA* in response to incremental changes in the signaling intensity represents the most intricate pattern of Bvg-mediated regulation identified to date in *Bordetella*. Based on our previous results and those reported here, it can be hypothesized that differential expression of *bipA* is mediated by altering BvgA-RNAP-promoter interactions, as a direct consequence of the variations in the BvgA-P concentrations and the affinities and positions of the various binding sites. This evolutionary attribute allows *Bordetella* to control a spectrum of distinct gene expression patterns and the resultant multiple phenotypic phases by a single regulatory protein, BvgA. Continued studies of the mechanistic features of *bipA* gene regulation will provide important insights into how bacterial pathogens fine-tune the expression of various genes in response to different and continuously changing microenvironments encountered during their infectious cycle in mammalian hosts.

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