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Analysis of *bvgR* Expression in *Bordetella pertussis*

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***Bordetella pertussis*, the causative agent of whooping cough, produces a wide array of factors that are associated with its ability to cause disease. The expression and regulation of these virulence factors are dependent upon the *bvg* locus, which encodes three proteins: BvgA, a 23-kDa cytoplasmic protein; BvgS, a 135-kDa transmembrane protein; and BvgR, a 32-kDa protein. It is hypothesized that BvgS responds to environmental signals and interacts with BvgA, a transcriptional regulator, which upon modification by BvgS binds to specific promoters and activates transcription. An additional class of genes is repressed by the products of the *bvg* locus. The repression of these genes is dependent upon the third gene, *bvgR*. Expression of *bvgR* is dependent upon the function of BvgA and BvgS. This led to the hypothesis that the binding of phosphorylated BvgA to the *bvgR* promoter activates the expression of *bvgR*. We undertook an analysis of the transcriptional activation of *bvgR* expression. We identified the *bvgR* transcript by Northern blot analysis and identified the start site of transcription by primer extension. We determined that transcriptional activation of the *bvgR* promoter in an in vitro transcription system requires the addition of phosphorylated BvgA. Additionally, we have identified *cis*-acting regions that are required for BvgA activation of the *bvgR* promoter by in vitro footprinting and in vivo deletion and linker scanning analyses. A model of BvgA binding to the *bvgR* promoter is presented.**

Whooping cough is an acute respiratory disease caused by the small, gram-negative bacterium *Bordetella pertussis*. *B. pertussis* expresses several factors that contribute to its ability to cause disease (12–15, 23, 30–34, 46). Several of these factors, including filamentous hemagglutinin (FHA), pertactin, fimbriae, and tracheal colonization factor, contribute to the interaction between the bacterium and host cells. Other virulence factors are exotoxins that impair the function of immune cells or are capable of causing damage to host tissues. These factors include pertussis toxin, adenylate cyclase toxin, dermonecrotic toxin, and tracheal cytotoxin. The expression of these virulence factors, with the exception of tracheal cytotoxin, is activated at the level of transcription by a single locus, referred to as the *bvg* locus (3, 35, 40, 41, 43).

The *bvg* locus encodes a two-component regulatory system consisting of a sensor protein, BvgS, and a transcriptional activator, BvgA. Although the relevant signals for regulation of the *bvg* locus in vivo are unknown, the activity of the *bvg* locus is repressed when cells are grown in the presence of $MgSO_4$ or nicotinic acid or when the cells are grown at reduced temperature in vitro (22). This *bvg*-mediated change in the patterns of transcription in response to environmental signals is referred to as phenotypic modulation. Under nonmodulating conditions, autophosphorylation of BvgS on a conserved histidine residue is followed by two intramolecular phosphotransfer reactions and the transfer of the phosphate moiety to a conserved aspartate residue on BvgA (42, 44, 45). Upon phosphorylation by BvgS, BvgA binds to *cis*-acting sequences in the promoter regions of the *bvg*-activated genes and activates transcription of those genes (1, 6–8, 10, 18, 21, 35, 47).

Several of the *bvg*-activated virulence operons are regulated in a differential manner. For example, after a shift in temperature from 25°C to 37°C, the *fha* operon is expressed within 10 min, while the *ptx* and *cya* operons are not expressed for several hours (36). Similarly, under steady-state conditions, the expression of the *ptx* locus is shut off at lower concentrations of $MgSO_4$ than is the expression of the *fha* locus (39). The *fha* promoter contains a high-affinity BvgA binding site consisting of nearly perfect inverted heptanucleotide repeats that are centered at position −88.5 relative to the start of transcription (35). Binding of a BvgA dimer to this site, followed by cooperative binding of two additional BvgA dimers 3′ to the high-affinity site, leads to the activation of *fha* transcription (4, 5, 7–9).

The *ptx* promoter also contains a site of initial BvgA binding which extends from approximately positions −167 to −123 relative to the start of transcription. Binding of two BvgA dimers to this site, followed by cooperative binding of multiple BvgA dimers downstream, leads to the activation of *ptx* transcription (8, 25). These observations are consistent with the hypothesis that differential regulation of the *fha* and *ptx* promoters is the result of differences in the sensitivity of each promoter to intracellular concentrations of phosphorylated BvgA (36). The requirement for a higher concentration of phosphorylated BvgA at the *ptx* promoter can be attributed to the lower affinity of the primary binding site of BvgA and the requirement for the cooperative binding of a larger number of BvgA dimers (8, 25). According to this model, when the temperature is shifted to induce *bvgAS* expression, the concentration of phosphorylated BvgA increases with time and, under steady-state conditions, the concentration of phosphorylated

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant features	Source or reference
E. coli K-12		
DH5α	High-efficiency transformation	Invitrogen
S17	Tra functions of IncP plasmids integrated into chromosome	37
B. pertussis		
Tohama I	Patient isolate	20
BP338	Tohama I, Nal^r Str^r	46
TM1316	TohamaI, *bvgR-phoA* Nal^r Str^r	26
TM1316::Δ*Xho*I	TohamaI, *bvgR-phoA*, 4-kb deletion of *bvgR* upstream region, Nal^r Str^r	This study
Plasmids		
pSS2809	Ap^r Gm^r *oriT, B. pertussis* chromosomal DNA, *E. coli lac* operon	9
pTM025	pSS2000, *bvg* sequences 4711–5991 (*bvgR*)	27
pTM093	*bvgR-phoA*	27
pTM193	pBBRKAN with multiple cloning site inserted	27
pTE-BVGR	pTE103, P_{bvgR}	This study

BvgA is inversely related to the $MgSO_4$ concentration (22, 36, 39).

The function(s) of the *bvg*-repressed genes is unknown. The fact that these genes are repressed by the locus responsible for the regulation of known virulence genes suggests that they may play a role in the pathogenesis of the bacterium, perhaps by contributing to the late stages in the infectious cycle. Alternatively, inappropriate expression of the *bvg*-repressed genes may interfere with the bacterium's ability to cause disease (28). The regulation of the *bvg*-repressed genes has been shown to be dependent upon *bvgR* (27). The *bvgR* locus lies immediately downstream of *bvgAS* and is convergently transcribed relative to *bvgAS* (26). Expression of *bvgR* was determined to be dependent upon an intact *bvgAS* locus and shown to be responsive to modulators of BvgA activity (26). Taken together, these results suggest that *bvgR* expression is activated by the binding of BvgA to the *bvgR* promoter. In this study we examined the BvgA-regulated expression of *bvgR* and BvgA binding to the *bvgR* promoter.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are presented in Table 1. *Escherichia coli* strains were grown on L-agar or in L-broth supplemented with antibiotics when appropriate (29). *B. pertussis* strains were grown on Bordet-Gengou (BG) agar (Difco, Detroit, Mich.) containing 1% proteose peptone (Difco) and 15% defibrinated sheep blood. Unless otherwise noted, the concentrations of antibiotics used were: gentamicin sulfate, 10 μg/ml; kanamycin sulfate, 10 μg/ml; and streptomycin sulfate, 100 μg/ml. *E. coli* strain DH5α was obtained from Bethesda Research Laboratories (Bethesda, Md.).

Construction of promoter deletion fusions. Fusions of a nested set of *bvgR* promoter deletions to the *phoA* gene on a low-copy-number plasmid were constructed as follows. Oligonucleotide B1 (Table 2) was used in combination with oligonucleotides F1, F2, F3, F4, and F5 in a PCR with plasmid pTM093 as a template. Plasmid pTM093 (27) contains a transcriptional fusion of the gene encoding alkaline phosphatase (*phoA*) to the *bvgR* promoter. The products of these PCRs were double-stranded DNA fragments containing *phoA* fused to truncated *bvgR* promoters with terminal *Bgl*II sites. After digestion with *Bgl*II, these fragments were inserted into plasmid pTM193 (26) which had been digested with *Bgl*II.

Although the entire sequences of the PCR products were not determined, at least two independent PCRs were performed, and the products from each reac-

TABLE 2. Sequences of oligonucleotides used to generate promoter deletion and linker substitution mutants

Primer	Sequence
F1	5′-AGATCTCCTGCCGGTCCGCCACATCGAGCAGGGCTGCAAC-3′
F2	5′-AGATCTCTGGTGGTTTCGATGCGGCCGCTGAAGGCCGCGG-3′
F3	5′-AGATCTGCGACCCCGCATTGATCGGTATCGCCGACCTCGG-3′
F4	5′-AGATCTCCGGCCACATGCTGGTCACCGATCTGCTCAACAG-3′
F5	5′-AGATCTGTTCATTTCGCCAACGTCAGTACTGCTCCATGAT-3′
F6	5′-AGATCTACAGCCGGCTGGCCGCCTTCTAAGAGATGGGCGA-3′
F7	5′-AGATCTCGCCTTCTAAGAGATGGGCGAACGCGCCACGCAG-3′
F8	5′-AGATCTGAGATGGGCGAACGCGCCACGCAGTACGATTGGG-3′
F9	5′-AGATCTCGCAGTACGATTGGGAAAAGTACGGCGGACATGT-3′
F10	5′-AGATCTCGGCGGACATGTGAGTTGTATCCTATGGCTTGGAT-3′
F11	5′-AGATCTAGTTGTATCCTATGGCTTGGATGGCATCGGTCCA-3′
F12	5′-AGATCTGGATGGCATCGGTCCATTTCAATATGACGTTGTT-3′
B1	5′-AGATCTGGAATGAATTCATCGCGCTTGAGCGCGGCGCGCA-3′
Fs1	5′-GGCCGGCCTGGCTTGGATGGCATCGGTCCATTTCAATATG-3′
Fs2	5′-GGCCGGCCAACTCACATGTCCGCCGTACTTTTCCCAATCG-3′
Fs3	5′-GGCCGGCCAAAGTACGGCGGACATGTGAGTTGTATCCTAT-3′
Fs4	5′-GGCCGGCCGTACTGCGTGGCGCGTTCGCCCATCTCTTAGA-3′
Fs5	5′-GGCCGGCCTGGGCGAACGCGCCACGCAGTACGATTGGGAA-3′
Fs6	5′-GGCCGGCCAAGGCGGCCAGCCGGCTGTTGAGCAGATCGGT-3′

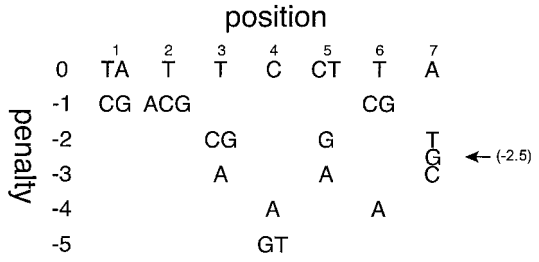


FIG. 1. Algorithm for the identification of BvgA binding sites. For each position in the consensus half-site, substitutions are assigned a penalty value depending on their effect on in vitro binding and/or in vivo effect on *fha* transcriptional activation (4).

tion gave the same result, indicating that differences in activity between the truncated DNA fragments generated by PCR and the full-length wild-type fragment are a result of the deletion of required sequences at the termini rather than results of misincorporation of nucleotides during the extension reactions. The resultant plasmids were transformed into *E. coli* strain S17 and subsequently transferred into *B. pertussis* strain BP338 by conjugation.

Fusions of a nested set of *bvgR* promoter deletions to the *lacZ* gene on the *B. pertussis* chromosome were constructed as follows. Oligonucleotide B1 (Table 2) was used in combination with oligonucleotides F1 through F12 in PCRs with plasmid pTM025 (27) as the template. Plasmid pTM025 contains the sequences encoding *bvgR* and the sequence extending 476 bp upstream of the *bvgR* open reading frame. The products of these PCRs were double-stranded DNA fragments containing truncated *bvgR* promoters with terminal *Bgl*II sites. After digestion with *Bgl*II, these fragments were inserted into plasmid pSS2809 (9) which had been linearized by digestion with *Bgl*II. The sequence of each PCR product was confirmed by sequence analysis. The resultant plasmids were transformed into *E. coli* strain S17 and subsequently transferred into *B. pertussis* strain BP338 by conjugation.

Use of an algorithm to identify BvgA binding sites. The DNA sequence of the *bvgR* promoter region was examined for the presence of putative BvgA binding sites through the use of an algorithm derived from the results of a mutational analysis of the high-affinity BvgA primary binding site in the *fha* promoter. As shown in Fig. 1, for each position in the 7-bp half-site, substitutions were assigned a penalty value depending on their effect on in vitro BvgA binding and/or on *fha* transcriptional activation in vivo (4). All heptad sequences on both strands of the *bvgR* promoter region were analyzed and assigned a value. For reference, both heptad sequences in the *fha* promoter primary binding site have a score of 0, while the most likely primary binding half-sites in the *ptx* promoter have scores of −4 or −5.

Construction of linker-substituted promoter fusions. The linker substitution mutations in the *bvgR* promoter were generated by a modification of the method of Ho et al. (17). Oligonucleotide F1 was used in combination with oligonucleotides Fs1, Fs3, and Fs5 in PCRs with plasmid pTM025 as the template. The products of these PCRs were double-stranded DNA fragments containing *bvgR* promoter fragments with a *Bgl*II site at the upstream end and an *Fse*I site at the downstream end. These fragments were designated BgFs1, BgFs3, and BgFs5, respectively.

Oligonucleotide B1 was used in combination with oligonucleotides Fs2, Fs4, and Fs6 in PCRs with plasmid pTM025 as the template. The products of these PCRs were double-stranded DNA fragments containing *bvgR* promoter fragments with a *Bgl*II site at the downstream end and an *Fse*I site at the upstream end. These fragments were designated BgFs2, BgFs4, and BgFs6, respectively. After digestion with *Bgl*II and *Fse*I, fragments BgFs1 and BgFs2, BgFs3 and BgFs4, and BgFs5 and BgFs6 were combined and inserted into plasmid pSS2809 which had been linearized by digestion with *Bgl*II. Each of these three-part ligations resulted in a full-length *bvgR* promoter fragment containing a precise replacement of 8 bp within the promoter with the 8 bp *Fse*I recognition sequence. The sequence of each linker-substituted promoter was confirmed by sequence analysis. The resultant plasmids were transformed into *E. coli* strain S17 and subsequently transferred into *B. pertussis* strain BP338 by conjugation.

Quantitative alkaline phosphatase and β-galactosidase assays. Bacteria to be assayed were recovered by sterile swab into 3.5 ml of 1 M Tris-HCl, pH 8.0, and the absorbance at 600 nm was measured. For measurement of β-galactosidase activity, 50 μl of cell suspension was added to 1 ml of Z-buffer. Cells were permeabilized by the addition of 30 μl of 0.1% sodium dodecyl sulfate and 30 μl of chloroform, followed by vortexing. The remainder of the assay was performed as described by Miller (29). For measurement of alkaline phosphatase activity, 1 ml of cell suspension was added to 0.5 ml of 1 M Tris-HCl, pH 8.0, the cells were permeabilized as above, and the assay was completed as described by Brickman and Beckwith (16). Units in both cases were calculate by the equation $1{,}000 \times [(A_{420} - (1.75 \times A_{550})/(T \times V \times A_{600})]$, where T is the incubation time in minutes and V is the volume of permeabilized cells added to the assay in milliliters.

Bacterial conjugations. Prior to mating, *B. pertussis* strains were grown for 3 days and *E. coli* strains were grown overnight at 37°C. Matings between *E. coli* and *B. pertussis* strains were performed by swabbing bacteria from fresh plate cultures of each strain onto a BG agar plate supplemented with 10 mM $MgCl_2$. After 3 h of incubation at 37°C, bacteria were swabbed onto BG agar containing the appropriate antibiotics for the selection of exconjugants and incubated at 37°C in screw-top jars.

Northern blot analysis. Whole-cell RNA was prepared from *B. pertussis* strain BP338 grown in 1 liter of Stainer-Scholte medium (38) in the absence or presence of 50 mM $MgSO_4$ by the method described by Aiba et al. (2). Total cellular RNA (10 μg per lane) was electrophoresed on a 0.8% agarose gel containing 1.2% formaldehyde as described by Maniatis et al. (24) and transferred to a Nytran membrane (Schleicher and Schuell, Keene, N.H.) as described by the manufacturer. The 1,285-bp *Xho*I-*Sal*I restriction fragment from *bvgR* and a 1,158-bp PCR product corresponding to the *ompP* open reading frame were extracted from agarose gels and labeled with [^{32}P]ATP by nick translation (Lofstrand Laboratories, Gaithersburg, Md.). Labeled DNA fragments were used to probe the RNA on the Nytran membranes with QuikHyb hybridization solution (Stragene, La Jolla, Calif.), in accordance with the manufacturer's directions.

Primer extension analysis. Whole-cell RNA was prepared from *B. pertussis* strain BP338 grown in 1 liter of Stainer-Scholte medium (38) in the absence or presence of 50 mM $MgSO_4$ by the method described by Aiba et al. (2). Primer extension analysis was performed with a synthetic oligonucleotide that annealed to the *bvgR* transcript (5′-GTGCGCCGGGACGGACTGAG-3′). Total RNA (100 μg) and approximately 5 ng of the oligonucleotide were incubated together at 65°C for 20 min, followed by incubation at room temperature for 2 h. Power Script reverse transcriptase (BD Biosciences, Palo Alto, Calif.) was added, and the mixture was incubated at 42°C for 90 min. The samples were extracted with phenol-chloroform, precipitated with ethanol, resuspended in water, and resolved by electrophoresis on a 6% polyacrylamaide–7 M urea gel. A standard sequencing reaction was performed with the same oligonucleotide primer and pTM025 as the template. The resulting sequence ladder was electrophoresed next to the primer extension products in order to facilitate mapping of the transcription start site.

Footprinting and in vitro transcription. DNase I footprinting was conducted as described previously (7). DNA probes were obtained as *Bam*HI-*Sac*I fragments from the pBVGR plasmid. This vector was obtained by cloning an *Eco*RI-*Pst*I fragment containing the putative *bvgR* promoter (from −186 to + 57) into the pBluescript KS vector digested with *Eco*RI and *Pst*I. Promoter DNA fragments in which the nontemplate strand was radiolabeled were incubated with purified BvgA in the presence or absence of 20 mM acetylphosphate. In vitro transcription was conducted as described elsewhere (7). The transcription template pTE-BVGR was created by subcloning a *Bam*HI-*Hin*dIII fragment into the pTE-103 vector. σ^{70}-saturated *E. coli* RNA polymerase, when used, was added at 120 nM, while polymerase holoenzyme purified from *Bordetella bronchiseptica* (7), when used, was added at 700 nM.

RESULTS

Northern blot analysis of *bvgR* expression. Northern blot analysis (Fig. 2A) demonstrated that an 1,150-base band, corresponding to the *bvgR* transcript, was present in cells that were grown in the absence of $MgSO_4$. This transcript was undetectable in cells grown in the presence of 50 mM $MgSO_4$. Comparison of the intensities of the *bvgR* and *ompP* transcripts indicates that the *bvgR* transcript is either very poorly expressed or highly unstable. The oligonucleotide probes for *bvgR* and *ompP* were labeled with approximately the same specific activity, and the blot probed for *bvgR* transcript was exposed to film for a time period approximately 10 times longer than that of the blot probed for the *ompP* transcript.

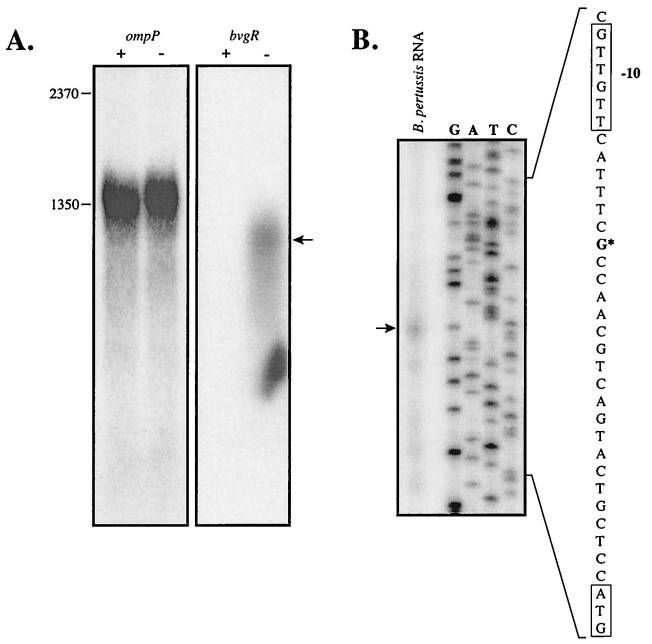


FIG. 2. Northern blot and primer extension analysis. (A) Total cellular RNA (10 μg per lane), prepared from *B. pertussis* strain BP338 grown in the absence (−) or presence (+) of 50 mM $MgSO_4$, was electrophoresed on an agarose-formaldehyde gel and transferred to a Nytran membrane. The blots were probed with [^{32}P]ATP-labeled probes specific for the *ompP* and *bvgR* transcripts. (B) A ^{32}P-labeled oligonucleotide primer, complementary to the *bvgR* coding strand, was annealed to 100 μg of total RNA from *B. pertussis* cells grown on BG agar. The primer was extended with reverse transcriptase, and the products were electrophoresed on a polyacrylamide-urea gel. A DNA sequencing ladder was prepared with the same primer on a recombinant pCR2.1-Topo:*bvgR* DNA template. The start site of *bvgR* transcription is indicated with an asterisk.

Nevertheless, the *bvgR* transcript band is much fainter than the porin transcript band, and the *bvgR* transcript shows clear signs of degradation, as evidenced by the presence of a smear of lower-molecular-weight cross-hybridizing RNAs.

Identification of the start site of *bvgR* transcription. Primer extension analysis of the *bvgR* transcript was undertaken in order to determine the start site of *bvgR* transcription (Fig. 2B). RNA was prepared from *B. pertussis* strain BP338 after growth on BG plates in the presence and absence of $MgSO_4$ and primer extension reactions were performed as described in Materials and Methods. The start site of the *bvgR* transcript is indicated in Fig. 2B. Two different primer extension experiments, performed on different days and with different RNA preparations, yielded the same start site. It should be noted that detection of the *bvgR* transcript by this method required exposures for up to 2 weeks.

Determination of the upstream boundary of the *bvgR* promoter. Strain TM1316 (26) bears a transcriptional fusion of *phoA* to *bvgR* on the chromosome. A chromosomal deletion of the 4-kb *Xho*I fragment located 476 bp upstream of the start of *bvgR* transcription was crossed onto the chromosome of strain TM1316 to generate strain TM1316::Δ*Xho*I. The replacement of the wild-type sequence with the 4-kb deletion of the *Xho*I fragment was confirmed by Southern blot analysis (data not shown). The expression and regulation of the *bvgR-phoA* transcriptional fusion were the same in strains TM1316 and TM1316::Δ*Xho*I, indicating that sequences upstream of the *Xho*I site at position −476 are not involved in the regulation of *bvgR* expression (data not shown).

Having determined that the sequences required for the regulated expression of *bvgR* lie downstream of position −476, we proceeded to generate a nested set of large 5′ deletions of the *bvgR* promoter fused to *phoA*. The fusions were transferred into the low-copy-number plasmid pTM193, and the resulting plasmids were transferred into *B. pertussis* strain BP338 by conjugation. The level of alkaline phosphatase was determined for each plasmid-bearing strain after growth in the absence and presence of 50 mM $MgSO_4$ (Fig. 3). The results demonstrated that the *bvgR* promoter deleted at the 5′ end as far as position −164 still retained wild-type levels of activity and normal induction. As expected, a deletion that removed all the 5′ sequences up to 9 bp upstream of the start of transcription eliminated expression of *bvgR*.

In vitro transcription of *bvgR*. To test the hypothesis that BvgA is directly involved as the transcriptional activator of *bvgR*, we cloned a 243-bp DNA fragment, containing *bvgR*-specific sequence from positions −186 to + 57, into plasmid pTE103 (11). The resulting clone, pTE-BVGR, was used for in vitro transcription assays. The presence of phosphorylated BvgA in the transcription reaction mix resulted in transcription from the *bvgR* promoter, as demonstrated by the presence of *bvgR* transcript (Fig. 4, lanes 5 to 7 and 10 to 12). No detectable transcript was present with either vector alone (Fig. 4, lane 2) or vector and unphosphorylated BvgA (Fig. 4, lanes 3 to 4 and 8 to 9). These results demonstrate that transcription from the *bvgR* promoter is dependent upon the presence of phosphorylated BvgA. The addition of increasing levels of phosphorylated BvgA was associated with increasing levels of *bvgR* transcription when either *E. coli* RNA polymerase (Fig. 4, lanes 5 to 7) or *B. bronchiseptica* RNA polymerase (Fig. 4, lanes 10 to 12) was used. Based on the migration of the *bvgR* transcript relative to an RNA ladder, the location of the transcription start site was determined to be at position −4 relative to the start site determined by primer extension. The error for this analysis is predicted to be ±3 bp

DNase I protection analyses of BvgA binding to the *bvgR* promoter. We proceeded to perform DNase I protection analyses in order to determine whether BvgA binds the *bvgR* promoter, and if so, whether that binding is dependent upon the phosphorylation of BvgA. The DNase I footprinting was performed on the same DNA fragments that were used in the in vitro transcription analysis. Protection of two regions, encompassing nucleotides −32 to −69 and nucleotides −88 to −134, was observed in the presence of unphosphorylated BvgA (Fig. 5, lane 2). In the absence of phosphorylation, BvgA failed to protect the region between these two binding sites. Upon the addition of phosphorylated BvgA, the entire region between positions −40 and −128 was protected, and three prominent hypersensitive sites, at positions −63 and 64, −71 and 72, and −86, and a less prominent hypersensitive site at position −108 were identified (Fig. 5, lanes 3 and 4). Therefore, in the presence of phosphorylated BvgA, the pattern of protection changes to include protection of the region between the two strong BvgA binding sites, and the upstream- and downstream-

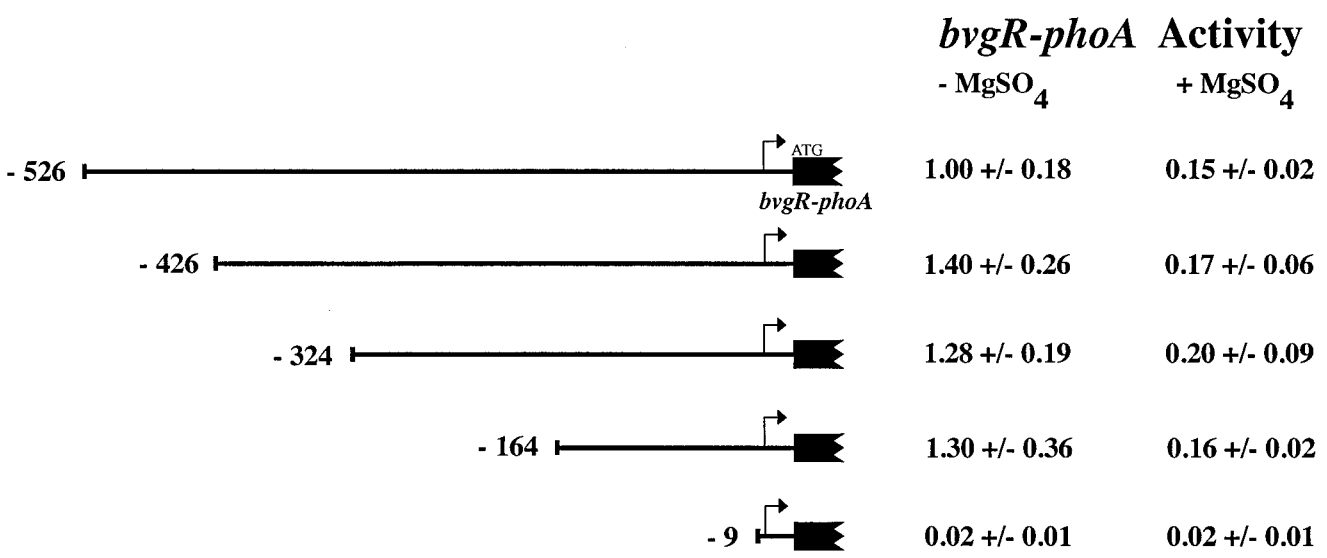


FIG. 3. P_{bvgR}-*phoA* reporter fusions. The alkaline phosphatase activity of strain BP338 carrying derivatives of plasmid pTM193 with increasing lengths of the *bvgR* upstream sequence fused to the *E. coli phoA* gene was determined. The upstream boundary of each promoter deletion, relative to the start of transcription, is indicated. Alkaline phosphatase activities are reported relative to strain BP338, bearing the plasmid with the *bvgR* sequence extending upstream to the *Xho*I site (−526) grown in the presence of 50 mM $MgSO_4$ (183 units). All values reported are the averages of at least four independent assays ± the standard deviation.

most boundaries of protection are shifted slightly towards the center of the BvgA binding region.

Deletion and linker-scanning analysis of the *bvgR* promoter. DNase I footprinting of BvgA binding to the *bvgR* promoter identified three regions of BvgA binding: −32 to −69, −69 to −88, and −88 to −134. Examination of the sequences within these regions, with an algorithm developed by the mutational analysis of the primary BvgA binding site found in the *fha* promoter, revealed the presence of at least one strong BvgA binding half-site consensus sequence centered within each region (Fig. 1 and 6A). In order to assess the contribution of these BvgA binding sites to the activity of the *bvgR* promoter, we undertook a deletion and linker-scanning analysis. A nested set of deleted fragments of the *bvgR* promoter was constructed, and three linker substitutions were independently introduced into the *bvgR* promoter. In order to determine the activities of these promoter derivatives in vivo, they were inserted into promoter assay vector pSS2809 (9). This vector has a cloning site for promoter fragments upstream of a promoterless *lac* operon. It also has multiple tandem transcription terminators upstream of the cloning sites to minimize transcriptional readthrough. A 2-kb fragment of *B. pertussis* genomic DNA enables insertion of the plasmid via homologous recombination into the *B. pertussis* chromosome at a site distant from the *bvg* locus.

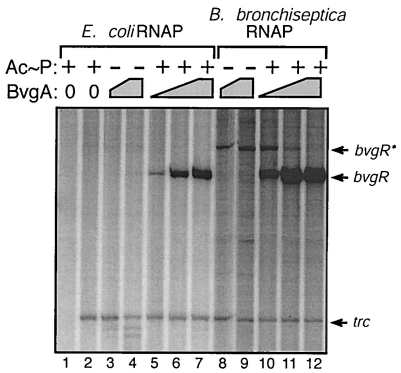


FIG. 4. In vitro transcription of the *bvgR* promoter. A single round of transcription was initiated from 20 nM template DNA in reactions containing purified BvgA (lanes 3 to 12) and RNA polymerase (RNAP) holoenzyme purified from either *E. coli* (lanes 1 to 7) or *B. bronchiseptica* (lanes 8 to 12). BvgA concentrations were 98 nM (lanes 5 and 10), 195 nM (lanes 3, 6, 8, and 11), and 390 nM (lanes 4, 7, 9, and 12). Additionally, BvgA was incubated either in presence of acetylphosphate (Ac≈P, lanes 1, 2, 5 to 7, and 10 to 12) or in its absence (lanes 3 to 4 and 8 to 9). The expected *bvgR* transcript, a larger *bvgR* transcript (*bvgR*∗), and the control *trc* transcripts are denoted by arrows.

This analysis demonstrated that deletion of all of the sequences upstream of position −135 does not affect the regulated expression from the *bvgR* promoter (Fig. 6, constructs Δ1 and Δ2). This position corresponds to the upstream boundary of the region protected by unphosphorylated BvgA. Promoter deletions Δ3, Δ4, and Δ5 removed progressively larger fragments of the most promoter-distal BvgA binding site. These truncated promoter constructs had a level of activity that was 65 to 83% of that observed with the full-length promoter fragment (fragment Δ1). Expression from each of these promoter constructs was not observed upon growth in the presence of $MgSO_4$. Deletion of the sequences up to position −71 resulted in a complete loss of *bvgR* promoter activity, indicating that sequences between −93 and −71 are essential for promoter activity (Fig. 6, constructs Δ6 to Δ9).

Three linker substitutions within the *bvgR* promoter were constructed. Each substitution replaced eight nucleotides within the identified BvgA binding site consensus sequences with the sequence recognized by the restriction enzyme *Fse*I.

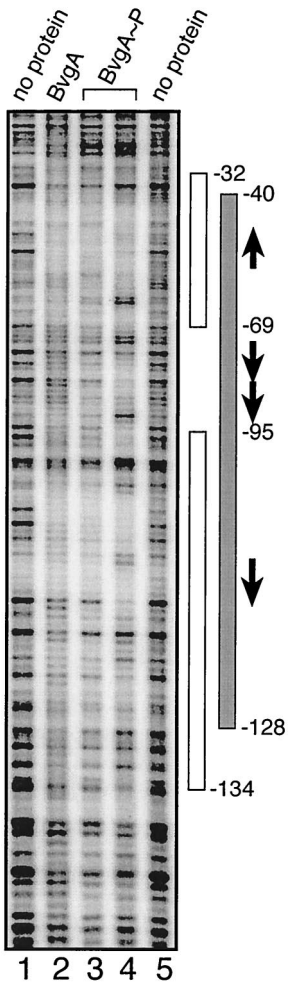


FIG. 5. DNase I footprinting of the *bvgR* promoter. Radiolabeled promoter fragment (0.25 nM) was incubated in the absence of protein (lanes 1 and 5), in the presence of 390 nM unphosphorylated BvgA (lane 2) or 195 nM phosphorylated BvgA (lane 3), and in the presence of 390 nM phosphorylated BvgA (lane 4; BvgA≈P). Regions of protection affected by binding of unphosphorylated BvgA are designated by open rectangles. Protection afforded by BvgA≈P is shown by solid rectangles. Arrows designate putative BvgA binding site heptamers (see text).

The two linker substitutions that affected the BvgA binding sites proximal to the start of transcription eliminated *bvgR* promoter activity (Fig. 6, constructs L1 and L2). The linker substitution in the predicted BvgA binding site most distal to the start of transcription had no effect on *bvgR* promoter activity (Fig. 6, construct L3).

Titration of *bvgR* promoter activity. In order to determine the placement of *bvgR* in the hierarchy of the differentially regulated BvgA-dependent genes, we examined the level of steady-state expression from the *bvgR* and *fha* promoters upon growth in the presence of different concentrations of $MgSO_4$ (Fig. 7). The results indicated that the *bvgR* promoter is induced under the same conditions as the *fha* promoter. However, the *bvgR* promoter is significantly stronger than the *fha* promoter, as demonstrated by the higher levels β-galactosidase activity (Fig. 7). Although both the *fha* and *bvgR* promoters were cloned into the same reporter plasmid and mated into the same bacterial strain, the strain bearing the *bvgR-lacZ* fusion had higher levels of β-galactosidase activity than the strain bearing the *fha-lacZ* fusion under all of the conditions examined. Both the *bvgR-lacZ* and *fha-lacZ* fusion strains were rederived and assayed, and the results were the same for both derivatives.

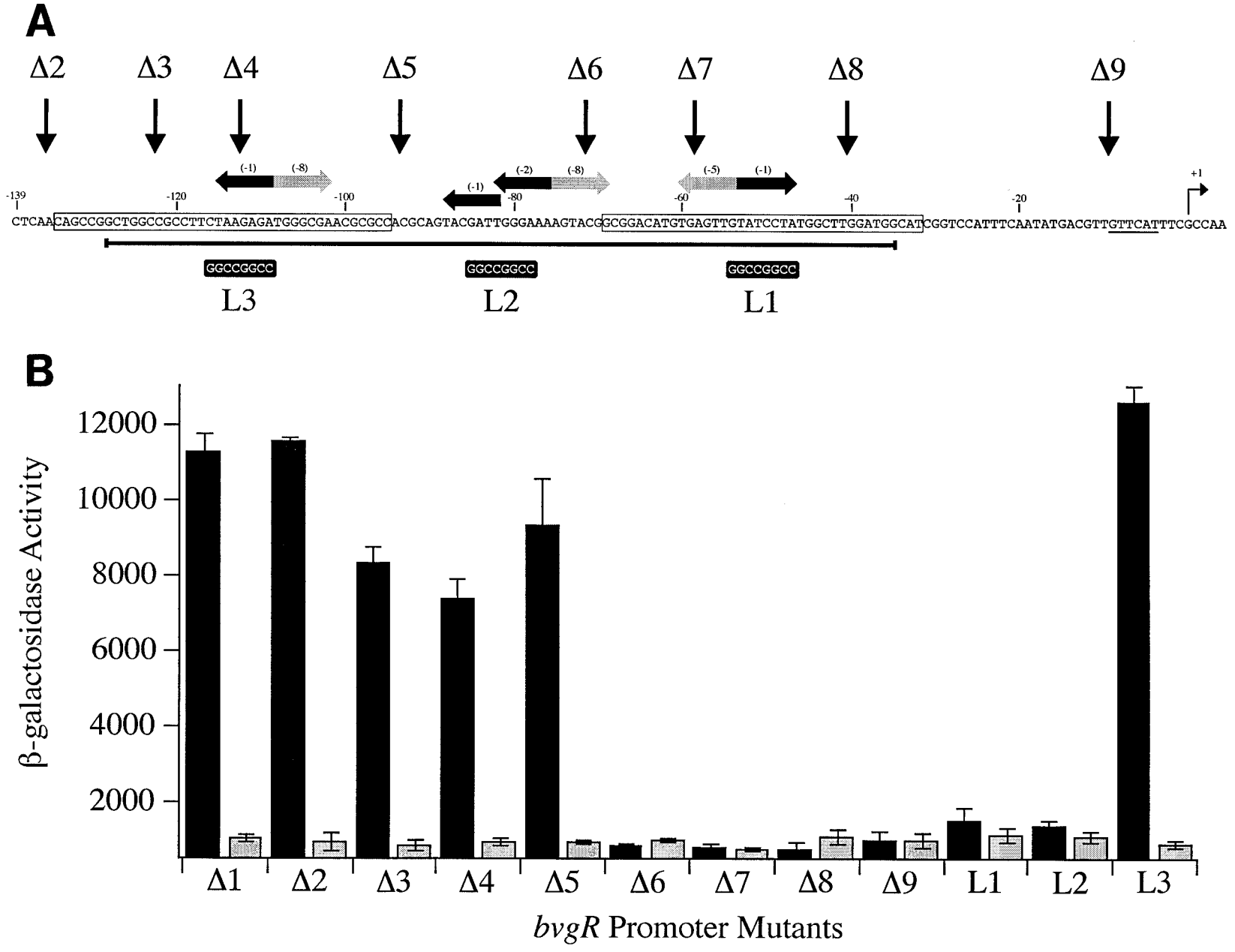
DISCUSSION

In vitro DNase I protection studies revealed the existence of a region of BvgA binding in the *bvgR* promoter extending from approximately position −32 to position −134 (Fig. 5 and 6). Significantly, the bases between positions −32 to −69 and between positions −88 to −134 were protected upon the addition of unphosphorylated BvgA. The entire region between positions −35 and −128 was protected when phosphorylated BvgR was added. These results indicate the presence of three BvgA binding sites in the *bvgR* promoter, located at positions −32 to −69, at positions −69 to −88, and at positions −88 to −134. We have designated these putative BvgA binding sites the P (primary), S (secondary), and U (upstream) BvgA binding sites, respectively (Fig. 8).

Examination of a number of BvgA-activated promoters resulted in the identification of a BvgA binding site consensus sequence (4, 18, 19, 25, 35). The consensus binding site consists of an inverted repeat of seven nucleotides with the sequence 5′-T/A-T-T-C-C/T-T-A-3′. An algorithm developed through the mutational analysis of the primary BvgA binding site in the *fha* promoter facilitates the identification of putative BvgA binding sites. The application of this algorithm to the *bvgR* promoter sequence revealed the presence of matches to the BvgA binding site consensus in each of the three regions protected by BvgA in the DNase I footprint studies. Interestingly, each of these predicted BvgA binding sites consists of a very high scoring half-site in association with a low-scoring half-site. The observation that sites U and P were bound by unphosphorylated BvgA suggests that these sites bind BvgA with high affinity. Binding of site S occurs only in the presence of high levels of phosphorylated BvgA, suggesting that BvgA binds this site with lower affinity.

Deletion analysis of the *bvgR* promoter demonstrated that all of the sequences required for regulated expression of *bvgR* were downstream of position −135 (Fig. 6). The three deletions that removed site U, either in part or in its entirety, reduced *bvgR* promoter activity only slightly. This result indicates that this binding site is not required for the regulated activity of the *bvgR* promoter but does contribute to promoter strength. The deletions that extended into or removed site S eliminated promoter activity, indicating either that the central binding site is essential for promoter activity or that a minimum of two BvgA binding sites are required to initiate transcription.

Linker substitutions that individually replaced each of the predicted BvgA binding sites demonstrated that both sites P and S are essential for promoter activity (Fig. 6). Replacement of the strong half-site at either of these binding sites completely eliminated promoter activity. The observation that site U does not contribute significantly to promoter activity may


A
Δ2
Δ3
Δ4
Δ5
Δ6
Δ7
Δ8
Δ9
(-1)
(-8)
(-2)
(-8)
(-1)
(-5)
(-1)
-139
-120
-100
-80
-60
-40
-20
+1
CTCAACAGCCGGCTGGCCGCCTTCTAAGAGATGGGCGAACGCGCCACGCAGTACGATTGGGAAAAGTACGGCGGACATGTGAGTTGTATCCTATGGCTTGGATGGCATCGGTCCATTTCAATATGACGTTGTTCATTTCGCCAA
GGCCGGCC
GGCCGGCC
GGCCGGCC
L3
L2
L1
B
β-galactosidase Activity
12000
10000
8000
6000
4000
2000
Δ1
Δ2
Δ3
Δ4
Δ5
Δ6
Δ7
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bvgR Promoter Mutants

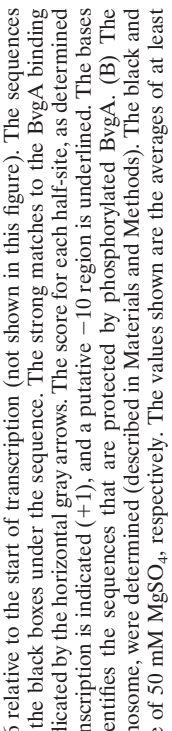

FIG. 6. Deletion and linker substitution mutations in the *bvgR* promoter. (A) The nucleotide sequence of the *bvgR* promoter is shown. The endpoints of each deletion of the *bvgR* promoter are indicated (Δ2 to Δ9). The endpoint of promoter deletion Δ1 is located at position −526 relative to the start of transcription (not shown in this figure). The sequences replaced with an *FseI* linker in each of the linker substitution mutations (L1 to L3) are indicated by the black boxes under the sequence. The strong matches to the BvgA binding half-site consensus sequence are indicated by the horizontal black arrows, and the weak matches are indicated by the horizontal gray arrows. The score for each half-site, as determined by the algorithm presented in Fig. 1, is indicated in parentheses above each arrow. The start site of transcription is indicated (+1), and a putative −10 region is underlined. The bases protected by unphosphorylated BvgA are boxed. The dark horizontal line under the sequence identifies the sequences that are protected by phosphorylated BvgA. (B) The β-galactosidase activities (in Miller units) of the *bvgR-lacZ* fusions, present in single copy on the chromosome, were determined (described in Materials and Methods). The black and gray bars indicate activity in the indicated strains grown in the absence of $MgSO_4$ and in the presence of 50 mM $MgSO_4$, respectively. The values shown are the averages of at least four experiments.

reflect an inability of BvgA bound at this site to engage in productive interactions with BvgA bound at sites S and P.

The examination of BvgA binding to the *fha* promoter indicated that BvgA binds to promoter DNA as a dimer that occupies 22 bp (34) along the DNA helix. On the *bvgR* promoter, site P is centered at position −53.5, site S is centered at −75.5, and site U is centered at −108.5. Sites P and S are centered 22 bp apart, which would facilitate contact between the BvgA dimers bound at those two sites. Sites S and U are centered 33 bp apart. A BvgA dimer bound at site U would therefore probably not form productive interactions with the BvgA dimer bound at site S. We conclude that the core *bvgR* promoter is contained within the sequences downstream of position −93 and consists of two BvgA binding sites (sites P and S) and the RNA polymerase binding site.

Binding of BvgA to the promoter-proximal BvgA binding site (site P) does not appear to be sufficient for the activation of transcription from the *bvgR* promoter. This conclusion is based on the observation that addition of unphosphorylated BvgA was not sufficient to induce transcription from the *bvgR* promoter in in vitro transcription assays (Fig. 4) and the observation that either deletion of or linker insertion into site S eliminated promoter activity (Fig. 6). The addition of phosphorylated BvgA resulted in occupation of site S and activation of *bvgR* transcription in vitro, indicating that formation of an active transcription complex at the *bvgR* promoter may require occupation of both site P and site S by phosphorylated BvgA.

The presence of multiple BvgA binding sites is a striking characteristic shared by all BvgA-activated promoters, including the *bvgR* promoter. The nature and organization of those binding sites in the *bvgR* promoter are, however, significantly different from those seen at previously characterized BvgA-activated promoters. For example, the BvgA-activated promoters at the *fha*, *ptx*, *prn*, *cya*, and *bip* loci all contain an upstream primary BvgA binding site and one or more secondary binding sites downstream of the primary site (1, 8, 10, 18, 21, 35, 47). We are defining primary binding as binding that does not require cooperative interactions with other BvgA dimers and secondary binding as binding that occurs only in the presence of BvgA dimers bound to adjacent sites on the DNA helix.

The model proposed for BvgA-dependent activation of these promoters proposes that BvgA first binds with high affinity to the primary binding site. Cooperative interactions drive the occupation of the secondary sites by BvgA, ultimately resulting in direct interaction between RNA polymerase and BvgA. According to this model, the relative strengths of the BvgA-activated promoters and the kinetics of their induction are determined primarily by a combination of the affinity of BvgA binding to the primary site and the number of secondary binding sites between the primary BvgA and RNA polymerase binding sites. In contrast to the promoters that have been described previously, the *bvgR* promoter appears to be characterized by the presence of a primary BvgA binding site adjacent to the RNA polymerase binding site (site P) and a secondary binding site upstream of the primary binding site (site S). Activation of transcription from the *bvgR* promoter requires occupation of both BvgA binding sites. These observations suggest that binding of a single BvgA dimer adjacent to the RNA polymerase binding site is not sufficient for transcriptional activation by BvgA.

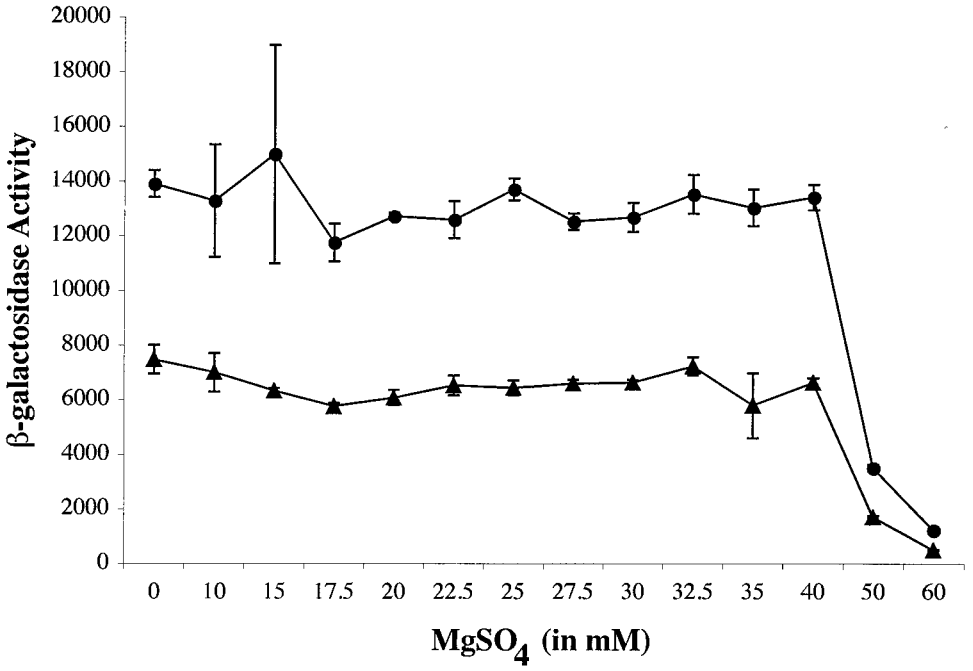

FIG. 7. Titration of BvgA activation of the *bvgR* and *fha* promoters. Fusions of the *bvgR* and *fha* promoters to *lacZ* on plasmid pSS2809 were crossed onto the chromosome of strain BP338, and the β-galactosidase activity of each strain was determined after growth in the presence of increasing amounts of $MgSO_4$. The absolute activity of the *bvgR* (circles) and *fha* (triangles) promoters (in Miller units) observed in cells grown at various concentrations of $MgSO_4$ was determined. Each point represents the average activity measured for three isolates grown at the indicated concentration of $MgSO_4$. The data shown are representative of three independent experiments.

We propose that the formation of an active initiation complex at Bvg-activated promoters requires the binding of two BvgA dimers immediately upstream of the RNA polymerase binding site. Recently, Boucher et al. reported that during the BvgA-dependent binding of RNA polymerase to the *fha* promoter, the carboxyl-terminal domains of the two RNA polymerase alpha subunits bind promoter sequences occupied by BvgA dimers. This simultaneous binding of the same sites on the DNA helix by BvgA and the alpha subunit is achieved through the binding of opposite faces of the DNA helix (5). It seems likely that the requirement for two BvgA dimers immediately upstream of the RNA polymerase binding site reflects the need to form productive interactions with the carboxyl-terminal domains of both alpha subunits of RNA polymerase.

Both the Northern blot and the primer extension analyses indicate that very low levels of *bvgR* transcript are present in the cell. This suggests that the *bvgR* transcript either is expressed at low levels or is very unstable. We addressed the question of *bvgR* promoter strength and the timing of induction of *bvgR* transcription by comparing the steady-state levels of *bvgR* and *fha* promoter activity when cells were grown on medium with various concentrations of $MgSO_4$ (Fig. 7). This analysis indicated that the *bvgR* promoter is significantly stronger than the *fha* promoter. Such a high level of expression is unusual for a transcriptional regulator. Taken together with the results of the Northern blot analysis, these results suggest that the *bvgR* transcript is very unstable and is rapidly degraded in the cell after synthesis.

We propose that the strength of the *bvgR* promoter reflects a need to maintain sufficient levels of BvgR protein in the cell to establish and sustain adequate repression of the *bvg*-repressed genes despite the rapid turnover of *bvgR* transcripts. The high level of *bvgR* transcription combined with the rapid degradation of the *bvgR* transcript would appear to be de-

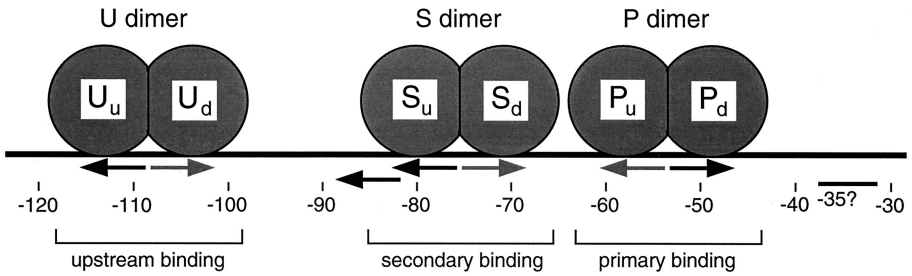

FIG. 8. Model of BvgA binding at the *bvgR* promoter. u, upstream monomer; d, downstream monomer.

signed to ensure repression of the *bvg*-repressed genes under repressing conditions while allowing rapid induction of expression of the *bvg*-repressed genes upon modulation of BvgAS activity. The comparison of *bvgR* and *fha* promoter activities also demonstrated that the timing of induction of the *bvgR* promoter is the same as that of the *fha* promoter (Fig. 7). It has been suggested that *fha* expression is induced early upon activation of BvgAS because of the important role of FHA in the initial colonization of the host. Based on this rationale, it could be argued that early expression of BvgR is required in order to repress the expression of a gene product(s) that interferes with colonization.

Our analysis of the activation of the *bvgR* promoter by BvgA revealed interesting differences between the *bvgR* promoter and previously described BvgA-activated promoters. First, the BvgA binding sites in the *bvgR* promoter are not symmetrical. All three BvgA binding sites on the *bvgR* promoter consist of a good match to only the consensus half-site. Second, the order of loading of BvgA dimers on the *bvgR* promoter is different from that observed for other BvgA-activated promoters. At previously characterized BvgA-activated promoters, BvgA first binds to the most upstream site and subsequently occupies the more downstream sites which are adjacent to the RNA polymerase. At the *bvgR* promoter, BvgA appears to occupy the site adjacent to the RNA polymerase binding site first and subsequently occupies the more upstream binding site.

A more detailed examination of the interactions of BvgA and RNA polymerase at the *bvgR* promoter and at other BvgA-activated promoters should lead to a better understanding of the mechanism of BvgA-mediated transcriptional activation. The analysis presented here has provided important insights into the dynamics of BvgR expression and will be the basis for future studies of the regulation of the *bvg*-repressed genes by BvgR.

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