Expression of virulence-associated genes in *Bordetella pertussis* is under the control of the pleiotropic regulator BvgA. Although previous studies have identified recognition sequences for BvgA in several promoter regions, their structures have not been clearly characterized. We show that the BvgA binding sites within the *bvgp* and *cyaA* promoters consist of inverted repeats and suggest that inverted-repeat motifs may represent the recognition elements for DNA-BvgA interaction.

In *Bordetella pertussis*, the causative agent of whooping cough, BvgA, the global transcriptional regulator and a member of the bacterial two-component signal transduction systems (1, 20), is responsible for the expression of virulence-associated genes encoding adenylate cyclase-hemolysin (*CyaA*), dermonecrotic toxin (*Dnt*), pertussis toxin (*Ptx*), filamentous hemagglutinin (*Fha*), fimbriae (*Fim*), and pertactin (*Prn*) (5, 8–10, 12, 14, 19). Recent studies have shown that a large region between *bvg* and *fhaB, ptx*, and *cyaA* has been evidenced by footprinting and electrophoretic mobility shift experiments (3, 7, 11, 21), and it was shown that binding of BvgA to individual promoter sequences and differential transcriptional activation of these promoters depend on the degree of BvgA phosphorylation (3, 4, 7, 17, 21). Nevertheless, the precise mechanism by which BvgA activates the expression of individual promoters is poorly understood. We present in this study a structural analysis of the BvgA binding sites within the upstream regions of *bvg*-regulated operons, *bvg* and *cya*, as the first step in the understanding of the BvgA activation mechanism.

Recently, we showed that a large region between −137 and −51 nucleotides from the transcriptional start site of *cyaA* is involved in BvgA binding (7). This region contains several heptamer variants of the BvgA binding consensus 5′-TTTC-CTA-3′, but it remains unclear which of these sequences are recognized by phosphorylated BvgA (BvgA-P). By constructing a set of mutated upstream regions of *cyaA* and *bvg* and analyzing them in electrophoretic mobility shift experiments, we show that the binding sites for BvgA within the upstream regions of *bvg* and *cyaA* are commonly organized as inverted repeats.

**Characterization of the BvgA binding site within the bvg upstream region.** The *bvg* locus of *B. pertussis* is subject to positive autoregulation (14, 15). DNase I footprinting studies showed that the BvgA protein protected sequences located at about −90 to −40 on the *bvg*-dependent *bvgp* promoter (11, 21). The protected region contains two direct repeats, 5′-TTT CCTA-3′ and 5′-TTTGGTA-3′, which are believed to be involved in BvgA interaction (11). We reexamined this region to identify sequences required for BvgA binding.

Electrophoretic mobility shift experiments were performed as described earlier (7), by using two 25-bp double-stranded *bvg* oligonucleotides shown in Fig. 1a. The *bvgDIR* oligonucleotide, derived from the −84 to −60 region of the *bvgp* promoter, contains two imperfect direct repeats, 5′-TTTCTCA-3′ and 5′-TTTTGTA-3′, suggested previously to be the BvgA binding site (11). The *bvgINV* oligonucleotide, derived from the −95 to −71 region of the *bvgp* promoter, contains a 5′-TTTTCTCA-3′ sequence and its imperfect inverted repeat 5′-CAGGAAT-3′, located 7 bp upstream. All DNA binding experiments were performed with purified BvgA, phosphorylated in vitro by acetyl phosphate (7). As shown in Fig. 2, the presence of BvgA-P did not alter the migration of the *bvgDIR* oligonucleotide (lanes 1 to 4) while BvgA-P could retard the mobility of the *bvgINV* oligonucleotide (lanes 5 to 8), indicating that it is the −95 to −71 region rather than the −84 to −60 region of the *bvgp* promoter sequence that corresponds to the BvgA-DNA interaction site. This result suggests that inverted repeats represent the essential target sequence for BvgA binding.

The BvgA binding site within the upstream region of *fhaB* is organized as a directly joined inverted repeat (11); hence, it was interesting to compare BvgA-P binding to the inverted repeats within the *bvg* and *fhaB* upstream regions. For the *fhaB* binding region we used a 24-bp *fhaB* oligonucleotide designed by Boucher et al. (4) (Fig. 1b). At low BvgA-P concentrations the major part of the *bvgINV* oligonucleotide migrated almost completely as unbound DNA (Fig. 2, lanes 6 to 8), we could barely detect the freely migrating *fhaB* oligonucleotide (lanes 10 to 12). This result is consistent with the earlier DNase I protection experiments of Roy and Falkow (11), who reported that higher concentrations of BvgA were necessary to protect the upstream region of *bvg* than for that of *fhaB*.

We then investigated BvgA-P binding to modified *bvgINV* oligonucleotides with either a 1-bp deletion (C at the position −85) or a 1-bp insertion (T between positions −84 and −85) between the repeats. No mobility shift could be observed when these derivatives were incubated with BvgA-P (data not shown). As a control, we used the derivatives of the *fhaB* oligonucleotide shown in Fig. 1b. We found that BvgA-P was able to retard the migration of the *fhaB* oligonucleotides with 3-bp (lanes 13 to 16) and 7-bp (lanes 17 to 20) insertions between the repeats, although its DNA binding activity was strongly reduced. These experiments indicate that the *fhaB* binding site is more tolerant to mutations that alter the spacing between the repeats than is the *bvg* binding site.

**BvgA binding site within the cyaA upstream region.** We showed previously that BvgA-P could bind to the upstream region of *cyaA* and that a large region (>80 bp) is involved in BvgA interaction (7). To characterize the nucleotides within...
the 87-bp (-137 to -51) region of cyaA, crucial for BvgA-DNA interaction, we performed electrophoretic mobility shift experiments using a series of derivatives of the 87-bp fragment (Fig. 1c). Mutations were introduced into the upstream region of cyaA by the Kunkel method (13) using mutagenic oligonucleotides and a derivative of pTZ19 (Pharmacia) bearing the cyaA sequence corresponding to the -2180 to -1446 region. DNA fragments for electrophoretic mobility shift assays were then generated by PCR using each of the mutated DNA sequences as a template. We analyzed the effect of mutations within one of the putative BvgA binding sites previously identified and located between -2100 and -280 (7). This region contains an almost canonical BvgA binding consensus, 5'-TTTCCTG-3', and its imperfect inverted repeat 5'-TAGGATG-3', separated by 2 bp (Fig. 1c).

Figure 3 shows that, compared to the wild-type cyaA region (-137 to -51), BvgA-P bound poorly to the 87-bp cyaA fragments bearing single base changes at position -285 (T→G) (lanes 4 to 6) or -286 (T→G) (lanes 7 to 9) within the -87TTTCCTG-81 binding consensus. Moreover, when the 87-bp fragment containing a double mutation at positions -285 and -286 (TT→GG) was used in the electrophoretic mobility shift experiments, a DNA-protein complex could be observed only at a concentration of 2 μM BvgA-P (lanes 10 to 12). Single changes at positions -284 (C→A) and -281 (G→A) had no significant effect on DNA-protein interaction (data not shown).

Mutations which changed the spacing between the inverted repeats of the -2100 to -280 region had a more severe effect on DNA-protein interaction than any of the substitutions we analyzed. Indeed, BvgA-P failed to bind to the -137 to -51 fragment of cyaA, carrying an additional T between positions -87 and -88 (Fig. 3, lanes 13 to 15). As a control, we tested
the effect of 1-bp insertions upstream and downstream from the inverted repeats (between positions –96 and –97 and between positions –78 and –79). As expected, these mutations did not alter BvgA-P binding (data not shown). We also constructed an almost perfect directly joined inverted repeat, 5’-TAGGAATTTCTC-3’, by substituting tetranucleotide TGGAT for AA between the positions –91 and –88 (cydAINTV), using the PCR overlap extension method (6). This modification within the –137 to –51 fragment resulted in the strongest mobility shift: even at the lowest BvgA-P concentration (0.5 μM) (Fig. 3, lane 17) the majority of the fragments migrated as bound DNA. Complete disappearance of the free-migrating DNA was observed at 1 μM BvgA-P (lane 18).

The structure of BvgA binding sites within the upstream region of cyaA seems to be complex. Our previous results indicated that the cyaA region between –137 and –51 protected by BvgA-P might comprise several binding sites (7). Here we have demonstrated that the BvgA binding site located at positions –100 to –80 is the main target sequence for BvgA-DNA interaction. Nevertheless, our earlier data showed that this site by itself is not sufficient for efficient BvgA-P binding (7). Therefore, we suppose that binding to this centrally located site will trigger cooperative interactions of BvgA-P with the neighboring, presumably lower-affinity, binding sites. Many prokaryotic DNA binding sites consist of 5- to 10-bp inverted-repeat sequences, and this reflects the face-to-face oligomerization symmetry of DNA-binding proteins (16). Several studies indicate that BvgA has the capacity to dimerize (2, 4, 17), suggesting that each helix-turn-helix motif present in the C-terminal domain of BvgA interacts in a twofold symmetrical fashion with each of the halves of the DNA binding site. It is tempting to speculate that BvgA binding sites within virulence-associated promoters consist of inverted repeats. However, the repeat elements can be either directly joined or separated by several nucleotides. Different spacings might reflect specific binding properties of BvgA and its selective interactions with RNA polymerase.

The fhaB binding site is organized as a directly joined inverted repeat, centered at the position –89.5 relative to the transcriptional start site (11), and our present data show that BvgA binding sites within the upstream regions of bvg and cydA are also arranged as inverted repeats (Fig. 1). They are centered at positions –83 (bvg) and –88 (cydA) relative to the start point of transcription. The similar locations of these binding sites would suggest similar mechanisms of transcription activation at the fhaB, bvg, and cydA promoters. This would require direct protein-protein contact between BvgA-P and RNA polymerase, bound in close vicinity on the same face of the DNA helix. By acting as a class I activator, BvgA-P could directly contact the α subunit of RNA polymerase to yield a productive transcription initiation complex. However, recent results of Steffen et al. (18), showing that the RNA polymerase α subunit of B. pertussis confers enhanced expression of fhaB in Escherichia coli, suggest that BvgA-P-α subunit communications may not be exclusive. Sequence variations and the existence of multiple binding sites on certain promoters (3, 7) imply that the mechanism of transcription activation may be specific for each promoter, even though the recognition elements, inverted repeats, have similar structures.

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