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A 23-Kilodalton Protein, Distinct from BvgA, Expressed by Virulent *Bordetella pertussis* Binds to the Promoter Region of *vir*-Regulated Toxin Genes

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Bordetella pertussis **coordinately regulates expression of its virulence factors in response to changing environmental conditions. These factors include pertussis toxin, adenylate cyclase toxin, and the filamentous hemagglutinin (FHA). The *vir* (or *bvg*) locus has been shown genetically to be required for this coordinate regulation. We have attempted to study the biochemical basis for coordinate regulation. DNA promoter deletion studies from other laboratories have shown that two tandem 20-bp repeats −157 to −117 bp upstream from the pertussis toxin promoter are essential for transcription. A similar 20-bp tandem repeat was found at the same site in the upstream region of the adenylate cyclase toxin promoter but is not present in the FHA or *vir* promoter region. Gel retardation revealed protein from virulent strains (able to express the virulence genes) but not from avirulent strains (unable to express the virulence genes) bound to the promoter region of the pertussis toxin gene, and this binding could be abolished by competition with an excess of oligonucleotides corresponding to either tandem repeat. The protein was determined to be 23 kDa by Southwestern (DNA-protein) analysis and could bind to either 20-bp oligonucleotide from the pertussis toxin promoter and either 20-bp oligonucleotide from the adenylate cyclase toxin promoter. BvgA, a 23-kDa protein encoded in the *vir* locus, has been reported to bind to a 14-bp inverted repeat in the FHA promoter which is not present in the pertussis toxin or adenylate cyclase promoter. We could not demonstrate binding of BvgA to the pertussis toxin promoter region. These data suggest that we have identified a second 23-kDa protein, distinct from BvgA but regulated by the *vir* operon, that binds to DNA sequences required for transcription of some, but not all, *vir*-regulated genes.**

Bordetella pertussis, the gram-negative bacterium that causes whooping cough, produces many virulence factors that are important for establishing the disease state. The virulence-associated characteristics include pertussis toxin, adenylate cyclase toxin, dermonecrotic toxin, filamentous hemagglutinin (FHA), a capsule, pertactin (a 69-kDa outer membrane protein), and other outer membrane proteins. A single chromosomal region called *vir* encodes the proteins which are essential for expression of the virulent-phase genes (1, 11, 25, 28). Arico et al. (1) sequenced the *vir* region, which they renamed *bvg*. It has two open reading frames, *bvgA* and *bvgS*, which could encode 23- and 135-kDa proteins, respectively (22). To avoid confusion, we will continue to use the established *vir* designation when referring to the locus and only use *bvg* when referring to the two genes encoded in the *vir* locus. DNA sequence analysis of *bvgA* and *bvgS* has revealed similarities between *vir* and several histidine-kinase response-regulator systems in bacteria (1, 24), formerly called two-component sensory processing systems (26), such as the *Escherichia coli* OmpR-EnvZ (9, 26, 27) genes, and suggests a model for *vir* activation. In this model, the transmembrane protein BvgS, which has homology to the transmembrane signalling protein EnvZ, senses the environment and under the appropriate conditions phosphorylates the BvgA protein, which is homologous to the DNA-binding protein OmpR. BvgA, like OmpR, would then bind the appropriate control sequences and activate transcription if phosphorylated (9, 26, 27). The BvgA protein has been shown to bind the FHA promoter and to be essential for transcription of the FHA gene when cloned into *E. coli* (16, 17, 19–22, 25). BvgA also autoregulates its own transcription (20, 22). A role for phosphorylation in this scheme has not yet been demonstrated experimentally.

While an intact *vir* locus is essential for expression of all of the virulence factors of *B. pertussis*, it may not be sufficient. Numerous studies have failed to demonstrate transcription from the pertussis toxin (16, 17, 19) or adenylate cyclase toxin (6, 13) promoter in the same *E. coli* constructs that permitted expression of FHA, suggesting that other components, in addition to the proteins encoded in the *vir* operon, are required for expression of these genes.

Gross and Rappuoli (7) reported that two sequences, tandem 20-bp repeats 157 to 117 bp upstream from the start site of transcription, are necessary for pertussis toxin promoter activity and *vir*-regulated gene expression. We found two similar repeats at the same site upstream from the adenylate cyclase toxin gene but could not find them in the FHA or *vir* promoter region. In this study, we examined the ability of *B. pertussis* protein to specifically bind to the promoter regions of pertussis toxin and adenylate cyclase toxin genes by gel retardation and Southwestern (DNA-protein) analysis. In gel retardation assays (30), labeled DNA fragments incubated with unlabeled protein fractions are separated by electrophoresis. DNA fragments that bind proteins migrate more slowly than fragments that do not bind proteins. In Southwestern analysis (2), proteins are separated by electrophoresis and transferred to nitrocellulose, similar to Western blots (immunoblots). The blots are then

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TABLE 1. Bacterial strains and plasmids

Strain (plasmid)	Relevant genotype and/or phenotype[a]	Reference
B. pertussis		
BP338	Parental strain, virulent phase	28
BP347	*vir-1*::Tn*5*, avirulent phase	28
BP359	*vir-2*::Tn*5*, avirulent phase	28
E. coli		
DH5α	F$^-$ *recA1 gyrA96* Δ(*argF-lacZYA*)*U169* ϕ80d*lacZ*ΔM15 *endA1 hsdR17*(r_K^- m_K^+) *supE44 thi-1 relA1*	8
HB101	*mcrB mrr hsdS20*(r_B^- m_B^-) *recA13 supE44 ara-14 galK2 lacY1 proA2 rpsL20 xyl-5 leu mtl-1*	14
HB101(pUW972)	pHC79 *ptx*::Tn*5*	15
DH5α(pYJ900)	pUC18 containing *Eco*RI and *Ava*I from *ptx* promoter	This study
DH5α(pEB389)	pUC18 containing 2.4-kb *Bam*HI adenylate cyclase promoter	This study
HB101(pUW1004)	pACYC184 containing *Bam*HI *vir* and *fhaB*	25
DH5α(pCR436)	pMMB67HE with 1.1-kb *bvgA* fragment downstream of pTAC promoter	21

[a] *B. pertussis* genotype designations: *ptx*, pertussis toxin; *bvg*, *vir*; *fha*, filamentous hemagglutinin.

incubated with labeled DNA, similar to Southern blots. The advantage of Southwestern blots is that one can determine the size of the proteins that bind to specific DNA fragments. By using these two techniques, we have shown that the pertussis toxin and adenylate cyclase promoters both bound a 23-kDa protein and the protein was present in Vir$^+$ strains but not in Vir$^-$ strains or *E. coli* strains that overproduce the BvgA protein. These data suggest that we have identified a second 23-kDa protein, distinct from BvgA, that may be responsible for activating the toxin promoters of *B. pertussis*.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are described in Table 1. *B. pertussis* was grown on Bordet Gengou agar (29). X broth, Stainer-Scholte broth with 0.15% bovine serum albumin (BSA), was used for liquid cultures (29). *E. coli* was grown in L broth or on L-agar plates (14). Competent DH5α cells were purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md.

Labeling and isolation of DNA fragments. Labeling and isolation of DNA fragments were performed essentially as described by Maniatis et al. (14). DNA was digested with the appropriate restriction enzyme, and terminal phosphates were removed with calf intestinal alkaline phosphatase. The DNA was labeled at the 5′ end with [γ-^{32}P]ATP by T4 polynucleotide kinase, separated by electrophoresis on agarose gels, cut from the gels, and recovered by electroelution (10).

Production of synthetic oligonucleotides. Oligonucleotides were synthesized and purified by thin-layer chromatography as described previously (29). Oligonucleotides were end labeled with ^{32}P by the kinase exchange reaction (14). For double-stranded oligonucleotides, complementary strands were allowed to anneal by heating them together in annealing buffer (0.2 M Tris [pH 7.5], 0.1 M $MgCl_2$, 0.5 M NaCl, 0.01 M dithiothreitol [DTT]) at 90°C for 10 min and incubation at 42°C for 1 to 2 h.

Preparation of protein samples. One-liter cultures of *B. pertussis* were concentrated by centrifugation and washed in Tris buffer (50 mM Tris [pH 7.5], 1 mM DTT, 1 mM EDTA, 50 mM NaCl) containing 0.1 mM phenylmethylsulfonyl fluoride and suspended in 2.5 ml of the same buffer. The bacteria were lysed by two passages through a French pressure cell at 16,000 lb/in^2. The lysate was centrifuged at 20,000 rpm for 1 h in an SS34 rotor. The clear supernatant fraction was applied to a DEAE-cellulose column previously equilibrated with 10 mM Tris (pH 8.0), and proteins were eluted with a linear NaCl gradient from 50 to 500 mM at a flow rate of 20 ml/h. *E. coli* samples were prepared in a similar manner, with or without 1 mM IPTG (isopropyl β-D-thiogalactopyranoside) as an inducer.

Gel retardation protein-DNA binding reactions. ^{32}P-labeled promoter DNA was incubated with 1 μg of protein in binding buffer (0.04 M Tris [pH 7.5], 0.1 M NaCl, 0.03 M $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT, 100 μg of BSA per ml, 5% glycerol) for 15 min at 37°C. Samples were separated in 0.5× Tris borate (14) on 5% polyacrylamide gels for 2.5 h at 20 mA. The gels were dried and exposed to film with intensifying screens. The quantities of DNA and protein were titrated to determine the optimal binding ratios (10). Salmon sperm DNA was used as a nonspecific inhibitor (23) at 100 μg/ml except where indicated.

Southwestern analysis. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (12), using 10% acrylamide, and transferred to nitrocellulose (2, 3). For most experiments, the protein samples were not boiled before electrophoresis; however, identical results were obtained when the samples were boiled first. The nitrocellulose strips were prehybridized with 5% nonfat dry milk in 10 mM Tris (pH 7.5), immersed for 30 min in binding buffer (1 mM EDTA, 10 mM Tris [pH 7.5], 0.02% BSA, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 50 mM NaCl), incubated with the DNA probe (10^5 cpm/ml) for 1 h at room temperature, and washed three times for 1 h in the binding buffer with or without salt. The blots were dried and autoradiographed. Molecular sizes were determined from prestained standards run on the same gel.

RESULTS

Gel retardation analysis of DNA-protein complexes. To determine whether the proteins involved in coordinate regulation of the virulence factors of *B. pertussis* bound to DNA, gel retardation studies were performed. Proteins that bind to a specific DNA sequence characteristically migrate as a single discrete band of a higher molecular mass (30), and this binding is not inhibited by the addition of nonspecific competitor DNA. In contrast, binding of nonspecific DNA-

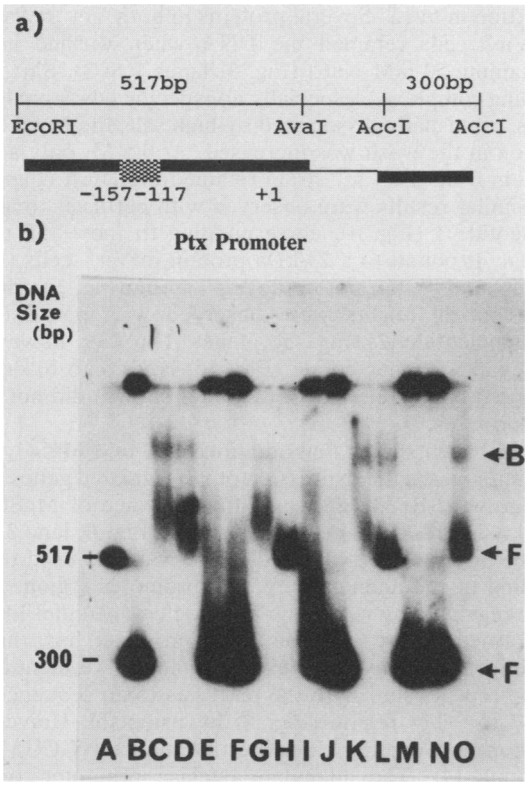

FIG. 1. Gel retardation to identify proteins that bind to the pertussis toxin promoter. (a) Restriction map of the pertussis toxin promoter region. +1, mRNA start site. The 517-bp *Eco*RI-*Ava*I fragment (from pYJ900) contains the −157 to −117 region essential for transcription of the pertussis toxin promoter. The 300-bp fragment (from pUW972) encodes part of the structural gene. (b) Analysis of DNA-protein complexes between the 517- or 300-bp fragment and partially purified protein from BP338. Lanes: A, 517-bp promoter fragment without protein; B, 300-bp structural gene without protein; C, 517-bp DNA and protein fraction eluting at 200 mM NaCl; D, same as lane C with salmon sperm DNA; E, 300-bp DNA and protein eluting at 200 mM NaCl; F, same as lane E with salmon sperm DNA; G, 517-bp DNA and protein eluting at 250 mM NaCl; H, same as lane G with salmon sperm DNA; I, 300-bp DNA and protein eluting at 250 mM NaCl; J, same as lane I with salmon sperm DNA; K, 517-bp DNA and protein eluting at 300 mM NaCl; L, same as lane K with salmon sperm DNA; M, 300-bp DNA and protein eluting at 300 mM NaCl; N, same as lane M with salmon sperm DNA; O, same as lane L with only 50 μg of salmon sperm DNA per ml. Arrows: B, DNA with bound protein; F, free DNA.

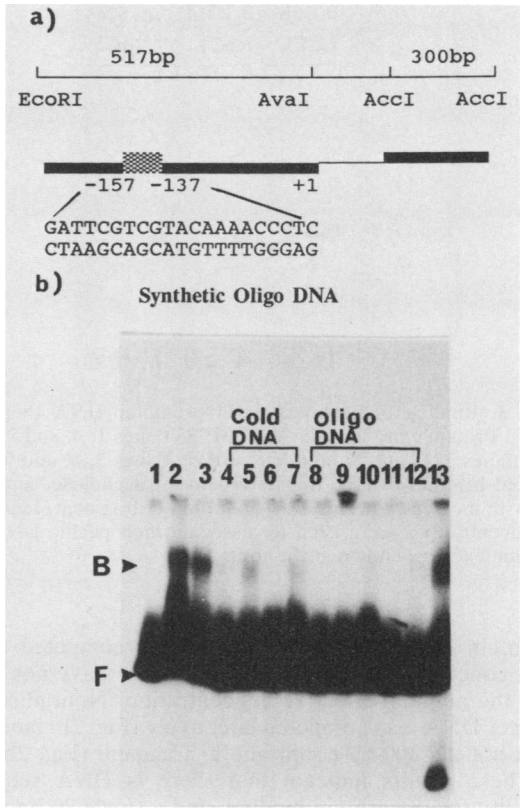

FIG. 2. Binding competition assays. (a) Restriction map indicating the 20-bp double-stranded oligonucleotide corresponding to the −157 to −137 region upstream from the pertussis toxin promoter. (b) Lanes: 1, 517-bp pertussis toxin promoter DNA; 2 and 3, promoter DNA with protein from BP338 eluting at 300 mM NaCl; 4 to 7, same as lanes 2 and 3, except the unlabeled 517-bp promoter fragment was added as a competitor at 10 (lane 4), 20 (lane 5), 50 (lane 6), and 30 (lane 7) ng/ml; 8 to 12, same as lanes 2 and 3, except the unlabeled double-stranded oligonucleotide was added as a competitor at 10 (lane 8), 20 (lane 9), 30 (lane 10), 40 (lane 11), and 50 (lane 12) ng/ml; 13, 517-bp pertussis toxin promoter DNA and labeled 300-bp DNA adjacent to the promoter. B, DNA with bound protein; F, free DNA.

binding proteins creates broad, smeared bands and the addition of nonspecific DNA inhibits this binding.

Protein samples from both virulent-phase (Vir^+) and avirulent-phase (Vir^-) strains were fractionated by ion-exchange chromatography and tested for the ability to bind to the pertussis toxin promoter (Fig. 1a). Protein from the Vir^+ strain, eluting in the 200 to 300 mM NaCl fractions from the DEAE-cellulose column, bound to the 517-bp promoter fragment, as shown by the appearance of a discrete band of a higher molecular mass and a second smeared band (Fig. 1b, lanes C, G, and K). To determine whether either band was caused by nonspecific DNA-binding proteins, salmon sperm DNA was added as an inhibitor (Fig. 1b, lanes D, H, and L). The lower band was lost, but the upper band was still present (Fig. 1b, lane L), suggesting that only the upper band was due to protein that bound DNA in a sequence-specific manner. In contrast, when a 300-bp *Acc*I fragment (Fig. 1a) which contains sequences for the structural gene but no promoter sequences was used, only smearing due to nonspecific DNA-binding proteins was observed (Fig. 1b, lanes E, F, I, J, M, and N). Similarly, when the Vir^- protein fractions were tested, only one fraction appeared to have activity, but it was inhibited with salmon sperm DNA (data not shown). Together these observations suggest that a protein from the Vir^+, but not the Vir^-, strain specifically binds to the pertussis toxin promoter region, not the adjacent DNA in the structural gene, and this binding could not be inhibited by nonspecific salmon sperm DNA.

To determine whether this protein bound to the 40-bp region shown by Gross and Rappuoli (7) to be essential for pertussis toxin expression, a double-stranded oligonucleotide corresponding to one of the 20-bp repeats upstream from the pertussis toxin promoter (Fig. 2a) was used as a competitor in gel retardation assays. The discrete, slow-migrating band was not observed when the oligonucleotide was added (Fig. 2b, lanes 8 to 12), suggesting that a 20-bp oligonucleotide could compete with the entire 517-bp promoter fragment

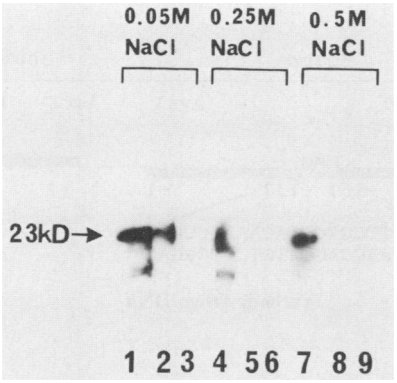

FIG. 3. Binding of pertussis toxin promoter DNA to protein extracts. Protein extracts from Vir$^+$ BP338 (lanes 1, 4, and 7), Vir$^-$ BP347 (lanes 2, 5, and 8), and Vir$^-$ BP359 (lanes 3, 6, and 9) were separated by SDS-PAGE, transferred to nitrocellulose, and incubated with the 517-bp pertussis toxin promoter fragment. Increasing salt concentrations were used to dissociate nonspecific DNA-protein complexes, as shown at the top.

for protein binding. The oligonucleotide competed at the lowest concentration tested, 10 ng, and we have not determined the minimal effective concentration. Nonradioactive promoter DNA also competed effectively (Fig. 2b, lanes 4 to 7), but not the 300-bp nonpromoter fragment (Fig. 2b, lane 13). These results indicate that there is DNA sequence specificity for the protein binding, and a single 20-bp repeat sequence is sufficient to compete for binding of the protein to the promoter fragment.

The partially purified fraction eluting at 300 mM NaCl contained several protein bands, as determined by SDS-PAGE (data not shown). Further purification of the DNA-binding protein was hampered because very little activity could be isolated from the bacteria and the DNA-binding activity was unstable in vitro.

Protein-DNA binding studies by Southwestern analysis. To determine the size of the protein which specifically bound to the promoter region, total protein from Vir$^+$ (BP338) and Vir$^-$ (BP347 and BP357) strains was separated by SDS-PAGE, transferred to nitrocellulose, and incubated with the labeled 517-bp pertussis toxin promoter fragment. BP359 contains a Tn*5* insertion in *bvgA*, and BP347 contains a Tn*5* insertion in *bvgS*. Several proteins in both Vir$^+$ cells as well as Vir$^-$ cells retained the DNA when washed in buffer containing 50 mM NaCl (Fig. 3, lanes 1 to 3). Since DNA-binding complexes, especially nonspecific DNA-binding proteins, are usually dissociated by high salt, the NaCl concentration in the wash was increased. At 0.5 M, only a 23-kDa protein from the Vir$^+$ strain retained the DNA (Fig. 3, lane 7). Similar results were observed with pertussis toxin oligonucleotide 1 (Fig. 4), corresponding to the −157 to −137 repeat. It bound to a 23-kDa protein in Vir$^+$ cells (Fig. 5a, lanes 7 and 8), but not in the Vir$^-$ strain (Fig. 5a, lanes 4 to 6). Identical results were observed with pertussis toxin oligonucleotide 2 (Fig. 5c, lanes 1 to 3); however, an irrelevant oligonucleotide (Fig. 5c, lanes 4 to 6) or single-stranded oligonucleotide 1 (data not shown) did not bind to any proteins.

It has been shown that addition of 20 mM $MgSO_4$ reversibly suppresses the expression of *vir*-regulated genes (15) but not growth. BP338 grown in the presence of $MgSO_4$ only weakly expressed the 23-kDa protein (Fig. 6, lane 3).

The Southwestern blot technique was also used to detect binding to the adenylate cyclase promoter region, another gene regulated by *vir*. While DNA deletion studies like those performed on the pertussis toxin promoter have not been done for the adenylate cyclase promoter, we identified two 20-bp repeats similar to the pertussis toxin sequence in the −157 to −114 region (Fig. 4) by using the University of Wisconsin Genetics Computer Group (UWGCG) BestFit program (4). The adenylate cyclase promoter-containing fragment also bound to a 23-kDa protein (Fig. 7, lanes A to C). Oligonucleotides 3 and 4 (Fig. 4), corresponding to the 20-bp repeats in the adenylate cyclase toxin promoter, displayed binding similar to that observed with oligonucleotides 1 and 2 (Fig. 5), that is they bound strongly to protein from the Vir$^+$ but not the Vir$^-$ strain (data not shown). In addition, the adenylate cyclase promoter DNA also bound to a 23-kDa protein present in the 300 mM NaCl fraction, which was isolated on the basis of its activity in the gel retardation assay by using the pertussis toxin promoter (Fig. 7, lane G). No bands were observed with Vir$^-$ cells (Fig. 7, lanes D to F).

These data suggest that only the Vir$^+$ strains produce a 23-kDa DNA-binding protein which binds specifically to the 20-bp repeated DNA sequence present upstream in the promoter region of the pertussis toxin and the adenylate cyclase

1. Pertussis toxin	-157	gATTCgTC-gTaCAaaAcCCtc	-137
2. Pertussis toxin	-136	gATTCtTCcgTaCA-t-cCCgc	-117
3. Adenylate cyclase	-157	tATTtaTCtcccttgaAgCCtt	-136
4. Adenylate cyclase	-135	gTTCtTCttTtCAttAgaaaga	-114
5. Irrelevant sequence		atgggggcaggtggctttg	

FIG. 4. Oligonucleotides used in this study. The sequences of the 20-bp repeats from the pertussis toxin promoter and the 20-bp repeats from the adenylate cyclase toxin promoter are aligned. The adenylate cyclase sequences (5) are numbered backwards from the start of transcription. We used the alignment containing gaps suggested by Gross and Rappuoli for the pertussis toxin repeats (7) and compared the adenylate cyclase repeats with that alignment. Other alignments gave similar results. Nucleotides that match in three or four of the sequences are shown in capital letters. Oligonucleotide 5 corresponds to an irrelevant sequence.

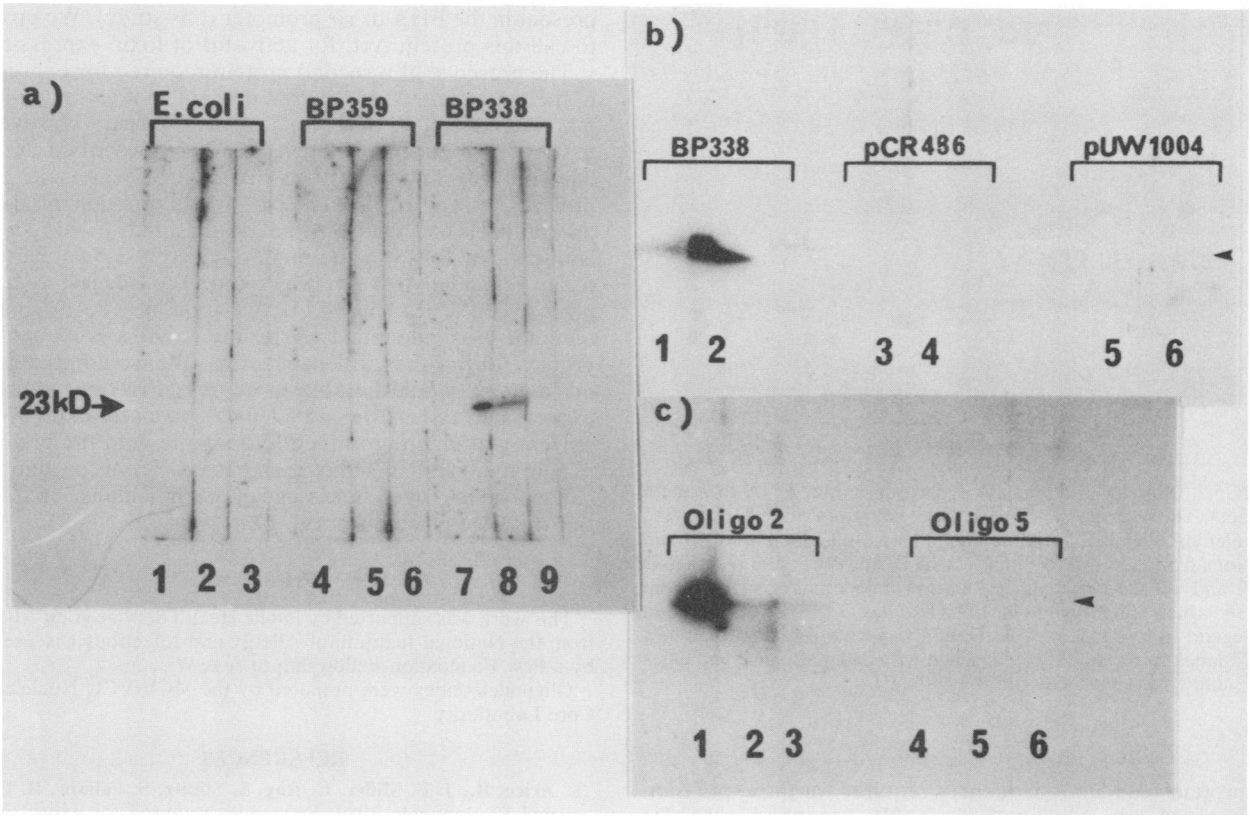

FIG. 5. Binding of the double-stranded oligonucleotides to protein extracts from *E. coli* and *B. pertussis*. (a) Protein extracts from *E. coli* DH5α (lanes 1 to 3) and *B. pertussis* Vir^- BP359 (lanes 4 to 6) and Vir^+ BP338 (lanes 7 to 9) were transferred to nitrocellulose (as described in the legend to Fig. 3) and incubated with labeled oligonucleotide 1 (Fig. 4) from the pertussis toxin promoter. Filters were washed in binding buffer containing 50 mM NaCl (lanes 1, 4, and 7), 250 mM NaCl (lanes 2, 5, and 8), and 500 mM NaCl (lanes 3, 6, and 9). (b) Binding of oligonucleotide 2 (Fig. 4) to protein from *B. pertussis* BP338 (lanes 1 and 2), *E. coli* DH5α(pCR436), which encodes *bvgA* only (lanes 3 and 4), and HB101(pUW1004), which encodes *bvgA* and *S* (lanes 5 and 6). Lanes 1, 3, and 5 were washed with 500 mM NaCl, and lanes 2, 4, and 6 were washed with 50 mM NaCl. (c) Binding of other oligonucleotides to protein from BP338. Lanes 1 to 3 and 4 to 6 were incubated with labeled oligonucleotide 2 and the irrelevant oligonucleotide 5, respectively; lanes 1 and 4, 2 and 5, and 3 and 6 were washed with 50, 250, and 500 mM NaCl, respectively. Arrow, 23-kDa DNA-binding protein.

promoter region. This 23-kDa protein is also present in the partially purified fractions that displayed gel retardation.

The BvgA protein of *B. pertussis* has a predicted mass of 23 kDa, and we wanted to determine whether the 23-kDa protein we observed in the Southwestern analysis was in fact BvgA. Previous studies, however, have shown that the cloned *vir* region of *B. pertussis* could activate transcription of the cloned FHA gene in *E. coli* (16, 17, 19, 25), but not the pertussis toxin gene (16, 17, 19). We used plasmid pCR436, which contains the *bvgA* gene under the control of the pTAC promoter generated by Roy et al. (19), and pUW1004, which contains BvgA, BvgS, the FHA promoter, and part of the structural gene (25). Protein from *E. coli* containing pCR436 did not display gel retardation with the pertussis toxin promoter fragment, even when induced with IPTG (data not shown). A similar lack of activity was also observed when the Southwestern technique and the 20-bp oligonucleotides were used. No binding was observed with *E. coli* DH5α (Fig. 5a, lanes 1 to 3), DH5α(pCR436) containing the *bvgA* gene (Fig. 5b, lanes 3 and 4), or HB101(pUW1004) containing the *bvgA* and *S* genes (Fig. 5b, lanes 5 and 6).

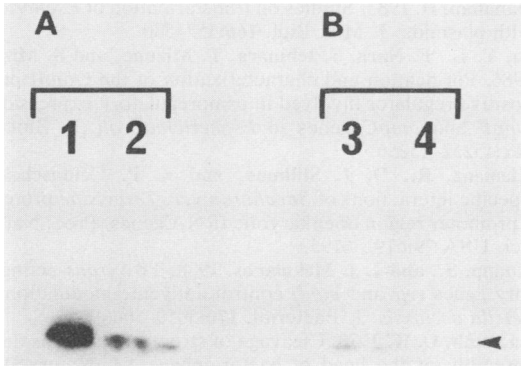

FIG. 6. Binding of protein from BP338 grown in the presence (B) or absence (A) of 20 mM $MgSO_4$ to double-stranded oligonucleotide 2 from the pertussis toxin promoter region. Lanes 1 and 3 and lanes 2 and 4 were washed with 50 and 0.5 mM NaCl, respectively.

DISCUSSION

The current working model of *vir* regulation in *B. pertussis* has been primarily inferred from the deduced amino acid sequence and not verified by direct experimentation. We used gel retardation and protein blotting analysis to identify

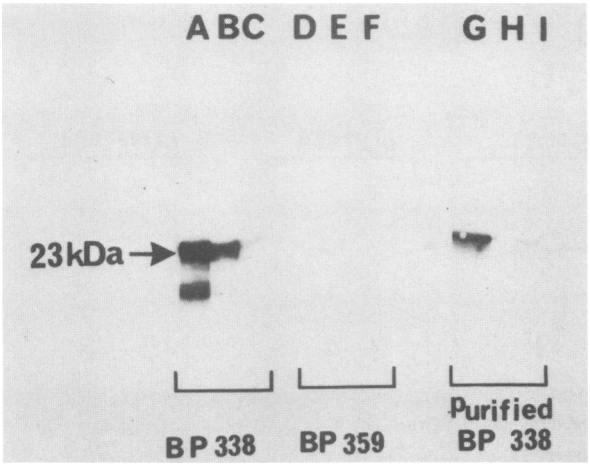

FIG. 7. Binding of adenylate cyclase promoter DNA to protein extracts. The 495-bp *Pst*I-*Bam*HI fragment (from pEB389) carrying the adenylate cyclase promoter region was incubated with protein fractions from Vir$^+$ BP338 (lanes A to C) and Vir$^-$ BP359 (lanes D to F) and with the partially purified protein fraction sample from BP338, which eluted in 300 mM NaCl (lanes G to I) as described in the legend to Fig. 3. Lanes A, D, and G were washed with 50 mM NaCl; lanes B, E, and H were washed with 250 mM NaCl; and lanes C, F, and I were washed with 0.5 M NaCl.

the proteins and DNA sequences required for the expression of the virulence-associated genes. We identified a 23-kDa protein produced by Vir$^+$ but not Vir$^-$ strains that binds to the −157 to −117 region containing two tandemly repeated DNA sequences present in both the pertussis toxin and adenylate cyclase toxin promoters. Deletion studies have shown that this region is essential for transcription of pertussis toxin (7). A double-stranded oligonucleotide corresponding to a single 20-bp repeat competed for protein binding. It is interesting that a single copy of the 20-bp sequence can inhibit binding, since in the study of Gross and Rappuoli (7) deletion of a single 20-bp copy of the repeat in the promoter region had a substantial negative effect on transcription, thus suggesting that a cooperative interaction between both DNA sequences occurs.

Several experiments suggest that this protein is distinct from the BvgA protein. First, in *E. coli*, the presence of the *vir* operon in *trans* induces expression of the FHA and *vir* genes (16, 17, 19, 25), but not the pertussis toxin (16, 17, 19) or adenylate cyclase toxin (6, 13) genes. Interestingly, the FHA gene appears to be regulated slightly differently from all of the other *vir*-regulated genes. The FHA gene seems to be less sensitive to modulation by sulfate ions (15). The FHA and *vir* structural genes map only 432 bp apart and are transcribed in opposite orientations from five different promoters (18, 20, 22). Transcription of the FHA promoter requires the presence of an active *vir* locus (20, 22, 25), and BvgA appears to bind to a specific sequence in this region, a 14-bp inverted repeat, specifically TAAGAAATTTCCTA (17, 21). This sequence is not present in the pertussis toxin or adenylate cyclase toxin promoter.

The protein we have purified from *B. pertussis* binds to the pertussis toxin and adenylate cyclase promoters and is present only in virulent-phase bacteria, suggesting that it is also regulated by the *vir* locus. The DNA consensus sequence recognized by the protein we have purified in the pertussis toxin and adenylate cyclase promoters is not present in the FHA or *vir* promoter (this study). We propose to call this protein Act, for activator of toxin expression.

The presence of a second protein required for expression of the toxin genes suggests that the model of *vir* regulation is more complicated than originally proposed; however, a cascade of regulatory proteins has been described in other bacterial regulatory systems (26). The BvgA and BvgS proteins appear to be sufficient for expression of the *vir* operon itself and for FHA, but studies with *E. coli* suggest additional factors are required for the expression of pertussis toxin and adenylate cyclase toxin. We suggest that this protein could be the 23-kDa Act protein. The original *vir* mutants were identified as bacteria that were unable to produce any of the virulence factors. We would predict that *act* mutants would be able to express FHA but unable to express pertussis toxin and adenylate cyclase toxin. We are currently attempting to identify mutants with the predicted phenotype. Cloning and overexpression of this protein in *E. coli* will help to overcome some of the difficulties in purifying this unstable protein from *B. pertussis*.

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