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Repressor Binding to a Regulatory Site in the DNA Coding Sequence Is Sufficient To Confer Transcriptional Regulation of the *vir*-Repressed Genes (*vrg* Genes) in *Bordetella pertussis*

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Five Tn*phoA* fusions to *vir*-repressed genes (*vrg* genes) have been identified in the respiratory pathogen *Bordetella pertussis*. A comparison of *vrg* DNA sequences suggests a consensus DNA element within the coding regions of four of five *vrg* genes. To determine the role of this DNA sequence in *vrg* regulation, a nucleotide substitution mutation in the conserved region of *vrg-6* was isolated. This mutant showed constitutively high levels of expression in the absence of antigenic modulators, $MgSO_4$ and nicotinic acid, suggesting that this DNA element may be a control site for *vrg* repression. Moreover, Northern (RNA) analysis and transcriptional fusion analysis suggest that *vrg* genes are regulated at the transcriptional level. To determine whether sequences in the coding region were sufficient to respond to antigenic modulation, a *vrg-6*::Tn*phoA* promoter deletion plasmid that contained a heterologous promoter driving the expression of *vrg-6* coding sequences from the *vrg-6* translation start site to the Tn*phoA* fusion junction was constructed. This heterologous construct responded to modulators in a *vir*-dependent fashion, indicating that sequences upstream of the coding sequence are not required for antigenic modulation. Southwestern (DNA-protein) analysis and mutational studies suggest that the *vrg* consensus DNA sequence is specifically recognized by a 34-kDa *vir*-activated gene (*vag*) product, whose binding results in down-regulation of *vrg* transcript levels. We conclude, at least for the *vrg*::Tn*phoA* fusion strains, that a site on the DNA that corresponds to a consensus sequence located in the *vrg* coding region is sufficient to confer the transcriptional regulation (repression) of *vrg* genes when *B. pertussis* strains are grown under nonmodulating conditions.

Bordetella pertussis is the causative agent of a severe human respiratory disease known as whooping cough or pertussis. The ability of *B. pertussis* to successfully colonize and establish infection in the upper respiratory tract depends on the production of a wide array of virulence factors such as pertussis toxin, filamentous hemagglutinin, adenylate cyclase-hemolysin toxin, fimbriae, and other outer membrane proteins (8, 23, 38, 40). The expression of these factors in the laboratory requires specific growth conditions; i.e., their expression is highly reduced in the presence of certain modulating factors in the growth medium, such as nicotinic acid and $MgSO_4$, a process termed antigenic modulation (15, 17, 25).

Antigenic modulation is controlled by the *bvg* (*Bordetella* virulence gene) locus, also termed *vir*. This locus contains two genes, *bvgAS*, that are required for expression of the major virulence factors (1, 34). These genes encode products homologous to a number of proteins of the two-component regulatory systems found in both pathogenic and nonpathogenic bacteria (1, 28, 35). BvgA and BvgS coordinately regulate two distinct classes of genes in a reciprocal fashion: *vir*-activated genes (*vag* genes) are maximally expressed in the absence of antigenic modulators, whereas the expression of *vir*-repressed genes (*vrg* genes) is significantly reduced under these conditions (14, 39, 40). Mutation in the *bvgAS* locus results in a constitutively high level of expression of *vrg* genes accompanied by a concomitant reduction in *vag* expression (2, 14, 39). These data suggest that the products of the *bvg* locus directly or indirectly repress the expression of *vrg* genes and activate the expression of *vag* genes. The *vag* genes encode most of the known virulence factors, such as pertussis toxin and filamentous hemagglutinin (8); however, at least one of the *vrg* genes is required for virulence in a mouse model of respiratory infection (3).

BvgA and BvgS directly activate not only their own expression (29, 32) but also the expression of *fhaB* (a *vir*-activated gene) in *Escherichia coli* (21, 30). In contrast, several groups have shown that other virulence factors and coregulated genes cannot be activated or regulated in *E. coli* producing BvgA and BvgS, even though they require the *bvg* locus for expression in *B. pertussis*, suggesting that these genes require regulatory factors in addition to *bvgAS* (1a, 21).

In this study, we investigated both the *cis*- and the *trans*-acting elements involved in *vrg* regulation. We present evidence indicating that a consensus DNA sequence located in the *vrg* coding region is sufficient to confer *vrg* transcriptional regulation, and this site in the DNA is specifically recognized by a 34-kDa repressor of the *vir*-repressed genes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *E. coli* and *B. pertussis* strains and plasmids used in this study are listed in Table 1. All *B. pertussis* strains were grown on Bordet-Gengou (BG) agar (Difco Laboratories, Detroit, Mich.) supplemented with 10 ml of glycerol and 150 ml of defibrinated sheep blood per liter or in modified Stainer Scholte broth. Modulators and antibiotics were added by spreading stock solutions onto BG agar plates and drying to achieve

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TABLE 1. Strains, plasmids, and phage used in this study

Strain or plasmid	Relevant feature(s)	Source or reference
Strains		
B. pertussis 18323 derivatives		
18323	Wild type	ATCC 9797
SK6	18323 *vrg-6*::Tn*phoA*	14
SK18	18323 *vrg-18*::Tn*phoA*	14
SK24	18323 *vrg-24*::Tn*phoA*	14
SK34	18323 *vag-34*::Tn*phoA*	14
SK53	18323 *vrg-53*::Tn*phoA*	14
SK73	18323 *vrg-73*::Tn*phoA*	14
SK6SKCAT	*vrg-6*::Tn*phoA*::pSKCAT	This work
B. pertussis Tohama I derivatives		
347	338 *vir-1*::Tn*5*	39
348	338 *hly-1*::Tn*5*	39
357	338 *ptx-1*::Tn*5*	39
E. coli derivatives		
DH5α	*endA1 hsdR17* (r_K^- m_K^+) *recA1 relA1 supE44* λ^- *thi-1 gyrA* φ80 *lacZ*ΔM15Δ(*lacZYA-argF*)U169	10
SM10 λpir	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu λpirR6K	33
INVαF′	*endA1 hsdR17* (r_K^- m_K^+) *recA1 relA1 supE44* λ^- *thi-1 gyrA* φ80 *lacZ*ΔM15Δ(*lacZYA-argF*)U169	Invitrogen
ES942	*mutD5 metE lacZ trpA*	K. Strauch
Plasmids		
pRK2013	IncP1 *tra oriE1* Km^r	7
pUC19	Ap^r	41
pLAFR2	IncP1 *cos* Tc^r	9
pSKCAT	Promoterless *cat* gene	14
pUCP1	pUC19 *vrg-6*::Tn*phoA*	This work
pLM6	pLAFR2 *vrg-6*::Tn*phoA*	14
pLP1Δ8	pLAFR2 *vrg-6*::Tn*phoA* promoter deletion containing only 102 bp upstream of coding sequence	2
pSK18	pUC19 *vrg-18*::Tn*phoA*	2
pLSK18	pLAFR2 *vrg-18*::Tn*phoA*	2
pMJM4	pLAFR2 *vrg-24*::Tn*phoA*	This work
pMJM6	pLAFR2 *vrg-53*::Tn*phoA*	This work
pMJM8	pLAFR2 *vrg-73*::Tn*phoA*	This work
pCR1000	Cloning vector for polymerase chain reaction; Km^r	Invitrogen
pCRV1	pCR1000 with *vrg-6*::Tn*phoA* promoter deletion	This work
pUCP1ΔP	pUCP1 with entire promoter deletion	This work
pUCP1*asd*P	pUCP1ΔP with *asd* promoter	This work
pLUCP1*asd*P	Replicon fusion of pUCP1*asd*P	This work

the following final concentrations: 20 mM $MgSO_4$, 5 mM nicotinic acid, 50 μg of ampicillin per ml, 30 μg of kanamycin per ml, 100 μg of streptomycin per ml, and 12.5 μg of tetracycline per ml. *E. coli* strains were grown in Luria-Bertani (LB) broth or agar at 37°C, with the same drug concentrations.

Recombinant DNA methods. Standard methods were used for purification of plasmid and chromosomal DNA, transformation of competent cells, restriction enzyme digestion, agarose gel electrophoresis, band purification, and ligation (18). Restriction endonucleases and T4 DNA ligase were purchased from either New England Biolabs or Bethesda Research Laboratories. *Taq* polymerase was purchased from Perkin-Elmer Cetus. Oligonucleotides were synthesized in an Applied Biosystems DNA synthesizer.

Cloning of *vrg*::Tn*phoA* fusions. *Bam*HI-digested chromosomal DNA from SK18, SK24, SK53, and SK73 and pUC19 plasmid DNA were ligated overnight at 15°C and transformed into $CaCl_2$-competent *E. coli*. The transformants were then plated on LB agar containing ampicillin and kanamycin to select for both the *bla* gene of pUC19 and the *neo* gene of Tn*phoA*. Cloning of the *vrg-6*::Tn*phoA* fusion was reported previously by Knapp and Mekalanos (14).

Screen for repressor-resistant mutants of *vrg-6*::Tn*phoA*. *vrg-6*::Tn*phoA* deletion plasmid pLP1Δ8 is a pLAFR2 derivative which contains 168 bp of *vrg-6* DNA, 102 bp immediately 5′ to the *vrg-6* translational start site and the 66 bp of coding sequence prior to the *vrg-6*::Tn*phoA* fusion junction (2). This plasmid was transformed into *E. coli* ES942 (*mutD5*), which was then grown overnight at 37°C. An alkaline lysis plasmid preparation was made from this culture, and the entire pool of plasmids was transformed into *E. coli* SM10 *λpir*. This pool was then mated into *B. pertussis* 357 and the exconjugates were plated for single colonies on BG plates without modulators. The colonies were then screened for alkaline phosphatase activity. Nitrocellulose filters were used to lift colonies, which were subsequently placed onto filter paper soaked with 0.15 M NaCl to wash away the heme (red color) in the blood agar. The filters were then developed by being placed on filter paper soaked with 160-μg/ml 5-bromo-4-chloro-3-indolyl-phosphate (XP; Bachem). This yielded colonies that were light blue (wild

type), white (*phoA* coding sequence or *vrg-6* promoter mutants), and dark blue (putative repressor-resistant mutants). Putative repressor-resistant mutants were then purified on BG plates. To determine whether these mutants displayed unregulated levels of enzyme activity, alkaline phosphatase assays (see below) were performed in the presence and absence of modulators. Two such mutants, designated pLP1Δ8*mut12* and pLP1Δ8*mut13*, were isolated and sequenced as described previously (2).

Construction of a heterologous promoter for *vrg-6*::Tn*phoA*. A specific deletion of the 5′-untranslated region of the *vrg-6*::Tn*phoA* gene fusion was constructed by using the polymerase chain reaction. The following oligonucleotides were used: *vrg-6*ΔP-up, which hybridizes to the first 27 nucleotides of the *vrg-6* coding region and contains an 8-nucleotide tail on the 5′ end conferring an *Xba*I site, and *vrg-6*ΔP-down, which hybridizes to the opposite strand of Tn*phoA* from positions 390 to 413 (22 bp downstream from the *Hin*cII site, where bp 1 is the first base of transposon sequences). Polymerase chain reaction was performed according to the manufacturer's protocol, and the reaction product was used without purification in a ligation reaction with plasmid pCR1000 (Invitrogen). The ligation reaction was used to transform *E. coli* INVαF′ (Invitrogen), with kanamycin resistance selected for on LB agar with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal; Bachem). White colonies were picked for plasmid purification, and one containing both *Xba*I and *Hin*cII sites was chosen for further work.

The 429-bp *Xba*I-*Hin*cII fragment from one of these clones containing the *vrg-6*::Tn*phoA* junction was purified and used in a ligation reaction with pUCP1, which had been digested with *Xba*I and *Hin*cII. This ligation was transformed into *E. coli* DH5α, with ampicillin resistance selected for on LB agar with X-Gal. White colonies were picked for plasmid purification. Three that released a 429-bp fragment upon digestion with *Xba*I and *Hin*cII were subjected to DNA sequencing, which confirmed the deletion of the *vrg-6* promoter. One of these, designated pUCP1ΔP, was chosen for further work.

Plasmid pCP27 (C. Parsot) was digested with *Eco*RV and *Xba*I to release a 250-bp fragment containing the promoter of the *B. pertussis asd* gene. Plasmid pUCP1ΔP was digested with *Sma*I and *Xba*I and ligated with the *asd* promoter fragment. This ligation was used to transform *E. coli* DH5α. Transformant colonies were picked for plasmid purification and screened for those that released a 260-bp fragment upon digestion with *Xba*I and *Sac*I (a site in the polylinker 10 bp from the *Eco*RV-*Sma*I-undigestible hybrid site). A clone with the predicted pattern was designated pUCP1*asd*P and used for construction of a replicon fusion for return to *B. pertussis* 357.

Preparation of RNA. RNA was prepared from cultures of *B. pertussis* grown in the presence and absence of modulators. The protocol was a modified version of the hot phenol method (36).

Northern (RNA) blot analysis. RNA (5 to 10 μg) was subjected to electrophoresis in a denaturing agarose-formaldehyde gel and transferred to nitrocellulose as described previously (18). The 829-bp *Eco*RI fragment containing *vrg-6* was used as a probe. Hybridization was performed in 50% formamide–2× Denhardt's solution–0.1% sodium dodecyl sulfate (SDS)–5× standard saline-phosphate-EDTA (SSPE) (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7])–10% dextran sulfate–100-μg/ml salmon sperm DNA. The blot was washed three times in 2× SSPE–0.1% SDS at 25°C for 10 min and twice in 0.5× SSPE–0.1% SDS at 25°C for 10 min and autoradiographed for 48 h at −70°C with enhancing screens.

Primer extension analysis. The 5′ end of the *vrg-6* transcript was mapped by a modification of the primer extension method described previously (22). RNA (5 μg) prepared from strain SK6 was annealed with the Tn*phoA* primer (100 to 200 ng [2]) in annealing buffer consisting of 400 mM NaCl–10 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] at 60°C for 2 h and then precipitated in absolute ethanol at −70°C for 15 min. The pellet was dried and then resuspended in a mixture containing 2.5 μl of 10× reverse transcriptase buffer (500 mM Tris-HCl [pH 8.3], 400 mM KCl, 10 mM dithiothreitol, 60 mM $MgCl_2$), 1 μl of RNasin (Promega), 2.5 μl of bovine serum albumin (BSA; 1 mg/ml), 12.5 μl of deoxynucleoside triphosphate (dNTP) mix (200 μM each), 1 μl of ^{35}S-dATP (DuPont/NEN), and 2.5 μl of Moloney leukemia virus reverse transcriptase (2 U; Bethesda Research Laboratories). This reaction mixture was incubated at 42°C for 15 min, and 5 μl of dNTP chase (1 mM each) and 30 μl of stop buffer (U.S. Biochemical) were added. Samples were boiled and loaded onto a DNA sequencing gel with a standard double-strand Sequenase reaction mixture of pUCP1 and the Tn*phoA* primer used as a ladder. Gels were run as described above.

Alkaline phosphatase activity assays. Alkaline phosphatase activity expressed by Tn*phoA*-containing strains of *B. pertussis* was determined after 2 days of growth on BG plates at 35°C. Cells were harvested in 0.15 M NaCl, washed, and suspended in 1 M Tris hydrochloride, pH 8.0. Alkaline phosphatase activity expressed by strains of *E. coli* was determined after overnight growth in LB broth at 37°C. Cells were centrifuged, resuspended, and washed in 1 M Tris hydrochloride, pH 8.0. The alkaline phosphatase activity was determined as described previously (19) and expressed as units per optical density at 600 nm.

Protein gel electrophoresis. Bacterial cell pellets were resuspended in 100 to 250 μl of 2× loading buffer, boiled for 5 min, and centrifuged for 5 min. The supernatant was used for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with 15% slab gels as described elsewhere (16). Aliquots of 25 μl of supernatant were loaded in each gel lane.

Electrophoretic transfer of proteins. SDS-PAGE gels of bacterial proteins or lysates were run as described above. The gels were then subjected to electrophoretic transfer to nitrocellulose filters as described previously (18) for 1 to 3 h at 0.8 to 1 A in a submarine electrophoresis unit (Hoefer, San Francisco, Calif.). These filters were then used for Western (immunoblot) or Southwestern (DNA-protein) analysis.

Western blot analysis. Western blot analysis was performed as described previously (5). Briefly, nitrocellulose filters were incubated for 2 h at room temperature in TBS (20 mM Tris-HCl [pH 8.0], 0.5 M NaCl) containing 2% BSA. The filters were then incubated for 2 h at room temperature with a 1:1,000 dilution of rabbit antiserum in TBS containing 5% nonfat dry milk. The filters were washed three times in TBS and mixed for 2 h at room temperature with a 1:2,000 dilution of anti-immunoglobulin G-alkaline phosphatase (Miles Laboratories) in TBS containing 5% nonfat dry milk. After being washed with TBS, the filters were developed with 40 μg of XP (Bachem) per ml and 500 μg of Nitro Blue Tetrazolium (Sigma) per ml in 1 M Tris-HCl (pH 8.0).

Southwestern blot analysis. Southwestern blot analysis was performed as described by Huh and Weiss (11). Briefly, nitrocellulose filters were prehybridized overnight in 10 mM Tris-HCl (pH 7.4) containing 5% nonfat dry milk and then

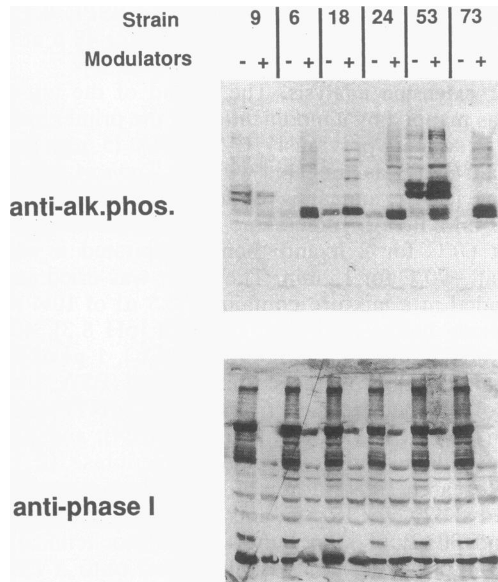

FIG. 1. Western blot of *B. pertussis* strains harboring Tn*phoA* insertions. Two identical SDS-polyacrylamide gels were run on whole-cell extracts of *B. pertussis* strains harboring Tn*phoA* insertions. The strains were grown on BG agar plates in the absence (−) and presence (+) of 5 mM nicotinic acid and 20 mM $MgSO_4$. The gels were then blotted to nitrocellulose and probed with either anti-alkaline phosphatase serum (top panel) or anti-*B. pertussis* phase I serum, which reacts to a number of the major virulence factors (bottom panel). Tn*phoA* fusion strains represented are SK9 (with a weakly *vir*-activated fusion) and SK6, SK18, SK24, SK53, and SK73 (all with *vir*-repressed fusions).

incubated for 30 min in binding buffer (10 mM Tris-HCl [pH 7.4], 1 mM EDTA, 1× Denhardt's solution, 50 mM NaCl). The DNA probe, containing the first 62 bp of *vrg-6* coding sequence, was added and incubation continued for 1 h, followed by three washes in binding buffer for 1 h each. The blot was then dried and autoradiographed overnight at −70°C with enhancing screens.

Nucleotide sequence accession numbers. The sequences reported here were filed with GenBank under accession numbers L06121 (*vrg-24*), L06122 (*vrg-53*), and L06123 (*vrg-73*).

RESULTS

Identification of *vir*-repressed genes. Beginning with a set of 99 Tn*phoA* fusions in *B. pertussis* 18323 that expressed alkaline phosphatase activity, Knapp and Mekalanos (14) identified five isolates with Tn*phoA* fusions that showed increased activity when grown on BG agar plates supplemented with an antigenic modulator, $MgSO_4$ or nicotinic acid. Under these conditions, colonies are nonhemolytic and produce negligible levels of the known virulence factors (12, 15, 25). Thus, these fusions identified genes regulated by the same environmental signals as the major virulence determinants but in a reciprocal fashion.

Western analysis, using anti-alkaline phosphatase serum, indicates that *vrg*::Tn*phoA* strains produce immunoreactive fusion proteins under modulating conditions (Fig. 1, top panel). The opposite pattern of expression is seen when a Western blot is probed with antiserum raised against whole-cell extracts of *B. pertussis* grown under nonmodulating conditions (anti-phase I serum), which reacts with a number of the major virulence determinants (Fig. 1, bottom panel).

TABLE 2. Antigenic modulation of *vrg*::Tn*phoA* fusions in *B. pertussis*[a]

Strain	Plasmid	Addition	Activity
348 (*bvgAS*$^+$)	pMJM4 (*vrg-24*::Tn*phoA*)	None	111
		Mg + NA	588
347 (*bvgAS*::Tn*5*)	pMJM4 (*vrg-24*::Tn*phoA*)	None	507
		Mg + NA	617
348 (*bvgAS*$^+$)	pMJM6 (*vrg-53*::Tn*phoA*)	None	550
		Mg + NA	1,758
347 (*bvgAS*::Tn*5*)	pMJM6 (*vrg-53*::Tn*phoA*)	None	2,472
		Mg + NA	2,797
348 (*bvgAS*$^+$)	pMJM8 (*vrg-73*::Tn*phoA*)	None	12
		Mg + NA	309
347 (*bvgAS*::Tn*5*)	pMJM8 (*vrg-73*::Tn*phoA*)	None	764
		Mg + NA	639

[a] Plasmids carrying a *vrg*::Tn*phoA* fusion were mated into *B. pertussis* 348 (*bvgAS*$^+$) or 347 (*bvgAS*::Tn*5*). Cultures of the exconjugates were grown on BG agar plates in the absence or in the presence of antigenic modulators, 20 mM $MgSO_4$ (Mg) and 5 mM nicotinic acid (NA).

In order to determine whether this class of genes is regulated by the products of the *vir* locus, Knapp and Mekalanos (14) cloned the chromosomal *vrg-6*::Tn*phoA* fusion in strain SK6 and returned it on a low-copy-number plasmid to *B. pertussis* Tohama I derivatives 347 and 348, which are strains with and without a Tn*5* insertion in *bvgS*, respectively (1, 39). Alkaline phosphatase activity was measured for these strains grown in the presence or absence of modulators. In strain 348, alkaline phosphatase activity was found to be regulated by modulators, similar to the chromosomal fusion; however, the activity in strain 347 was expressed at constitutively high levels. Similar analysis indicated that *vrg-18* from *B. pertussis* SK18 was regulated in an analogous fashion (2). On the basis of these data, the genes represented by the five Tn*phoA* fusions were designated *vir*-repressed genes (*vrg* genes).

Cloning and sequence analysis of *vir*-repressed-gene fusions. In order to extend this analysis to other members of the *vir*-repressed genes, the same approach was used to characterize the *vrg-24*-, *vrg-53*-, and *vrg-73*::Tn*phoA* fusions carried in SK24, SK53, and SK73, respectively. *Bam*HI chromosomal digests of these fusion strains were cloned into pUC19, subsequently cloned into the broad-host-range plasmid pLAFR2, and mated into *B. pertussis* 347 (*bvgAS*::Tn*5*) and 348 (*bvgAS*$^+$) (Materials and Methods). Again, we observed regulated expression in strain 348 (elevated levels of expression in the presence of modulators) and high, constitutive levels of expression in 347 (Table 2). This indicates that all five of the cloned *vrg*::Tn*phoA* fusion strains tested are repressed directly or indirectly by the products of the *bvg* locus.

We have previously reported the DNA and deduced amino acid sequences of the *vrg-6*::Tn*phoA* and *vrg-18*::Tn*phoA* fusions (2). A comparison of these DNA sequences suggested a possible consensus element located within the coding region involved in *vrg* expression. In order to expand the list of genes analyzed and to further define the signals involved in *vrg* regulation, we sequenced (2) the coding regions preceding the fusion junctions in *vrg-24*, *vrg-53*, and *vrg-73*. Figure 2 shows that the sequences predict fusion proteins of molecular weights in agreement with Western blots of the *phoA* fusion proteins (Fig. 1). A sequence comparison shows considerable homology within four of the

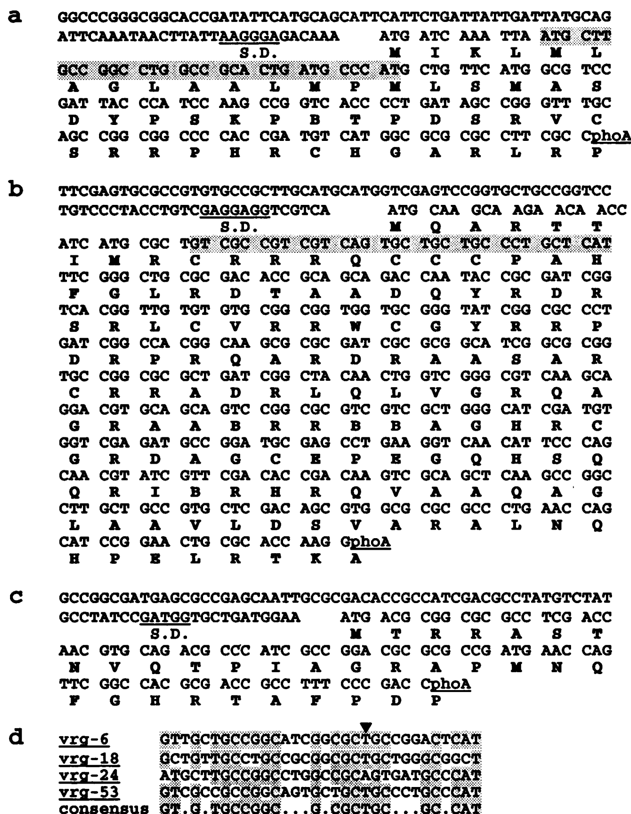

FIG. 2. DNA sequence comparison of the upstream coding region in *vrg-24*-, *vrg-53*-, and *vrg-73*::Tn*phoA* fusions. (a to c) DNA and deduced amino acid sequences. The predicted Shine-Dalgarno (S.D.) site and the site of the Tn*phoA* fusion junctions are indicated. Sequences conserved between *vrg* genes are shaded. (d) Alignment of four *vrg* coding sequences and a deduced consensus sequence. Identical bases are shaded, and bases appearing in three of four sequences are included in the consensus. The following bases (relative to the nucleotide of the initiation codon) are represented: *vrg-6*, 16 to 49; *vrg-18*, 18 to 51; *vrg-24*, 14 to 47; *vrg-53*, 29 to 60. The triangle indicates the base that was changed in the *vrg-6* mutants *mut12* and *mut13*.

five *vrg* structural genes tested. This homology is strictly at the DNA level, as no homology is observed in the deduced amino acid sequence of the proteins (Fig. 2d), suggesting that a site in the *vrg* DNA coding sequence is recognized by a regulatory element controlling *vrg* expression. The *vrg-73* gene appears to be an exception, as its coding region does not contain such homology.

Screen for repressor-resistant mutants of *vrg-6*::Tn*phoA*. In order to define the regulatory site involved in *vrg* regulation, a screen to allow the identification of repressor-resistant mutants was devised. The goal was to look for mutants that expressed alkaline phosphatase from a *vrg*::Tn*phoA* fusion in the absence of modulators. Along these lines, deletion plasmid pLP1Δ8 (2), which contains 168 bp of *vrg-6* DNA, 102 bp immediately 5′ to the *vrg-6* translational start site and the 66 bp of coding sequence prior to the *vrg-6*::Tn*phoA* fusion junction, was chosen as a parental construct as it had a low level of activity when repressed, thus allowing the identification of mutants showing increased activity.

pLP1Δ8 was grown in the *E. coli* mutator strain ES942 (*mutD5*), and the entire pool of mutagenized plasmids was mated into *B. pertussis* 357 (37). The exconjugates were plated for single colonies on BG plates without modulators and screened for alkaline phosphatase activity by making nitrocellulose filter lifts of the colonies and then developing the filters with XP. This yielded colonies that were light blue (equivalent to the parent fusion), white (likely *phoA* or *vrg-6* promoter mutants), and dark blue (putative repressor-resistant mutants). Putative repressor-resistant mutants were grown on BG plates with and without modulators. Alkaline phosphatase activity assays were performed to determine whether the mutants showed constitutively high (unregulated) levels of enzyme activity.

From approximately 3,000 exconjugates screened, two mutants that had a mutation in the *vrg-6* sequences on the plasmid, designated pLP1Δ8*mut12* and pLP1Δ8*mut13*, which showed constitutively high levels of *vrg-6* expression in the presence or absence of modulators, were isolated. DNA sequence analysis of *mut12* and *mut13* indicated that both had a single nucleotide substitution in the *vrg* consensus sequence at position 36 of the coding region (T→A). It is possible, on the basis of the nature of the screen, that these two mutants did not arise independently and are siblings. However, the position of the mutation suggests that the *vrg-6* consensus sequence may be the site of repressor binding.

Analysis of the *vrg-6* transcript. Knapp and Mekalanos (14) previously obtained evidence that the expression of a *vir*-activated gene, *vag-34*, was controlled at the transcriptional level by converting the *vrg-34*::Tn*phoA* fusion strain, SK34, into a transcriptional fusion strain, using the pSKCAT conversion vector, which contains a promoterless chloramphenicol acetyltransferase (*cat*) gene. Integration of this vector converts a Tn*phoA* translational fusion into a *cat* transcriptional fusion, whose level of resistance to chloramphenicol is under the control of the native promoter. We used this method to determine whether antigenic modulation of *vir*-repressed genes also occurred at the transcriptional level. Resistance to chloramphenicol (at 12.5 μg/ml) of strain SK6SKCAT, which contains a *vrg-6*::Tn*phoA*::pSKCAT transcriptional fusion, was dependent on the presence of modulators in the medium, suggesting that *vrg-6* is transcriptionally regulated.

To confirm this result, Northern analysis was performed to determine whether the level of *vrg-6* transcript increased in the presence of modulators. Figure 3 shows a Northern blot of RNA from two strains of *B. pertussis* that have been probed with a radiolabeled fragment of DNA containing the wild-type *vrg-6* gene (3). Lanes 1 and 2 contain RNA from *B. pertussis* 18323 grown under nonmodulating and modulating conditions, respectively, and lanes 3 and 4 contain RNA from *B. pertussis* 338 grown under nonmodulating and modulating conditions, respectively. The blot clearly indicates that the level of transcript greatly increases during modulation in both of these strains.

Sequences downstream of the *vrg-6* translational initiation codon are sufficient to confer modulation. Previous deletion analysis of the *vrg-6* and *vrg-18* promoters had indicated that sequences upstream of the transcription start site were not required for *vir* repression (2). However, these deletions did not clearly define the start site for transcription; here, it was determined by primer extension analysis of the *vrg-6*::Tn*phoA* transcript. RNA from SK6 grown under modulating conditions was used as a template for reverse transcription from the Tn*phoA* primer (Fig. 4). A standard sequencing reaction with the same primer provided a sequence ladder for reference (lanes ACGT). Lane 1 shows that no extension

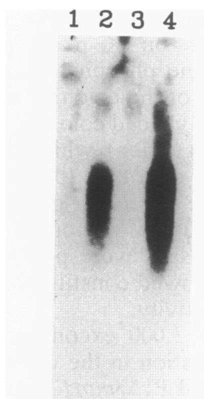

FIG. 3. Northern blot of *vrg-6* expression. RNA prepared from two *B. pertussis* strains, grown in the absence or presence of modulators (20 mM $MgSO_4$ and 5 mM nicotinic acid), was separated on an agarose-formaldehyde gel. The gel was transferred to nitrocellulose and probed with a radiolabeled 829-bp *Eco*RI fragment containing the *vrg-6* gene. Lanes 1 and 2, *B. pertussis* 338 with no additions and with modulators, respectively; lanes 3 and 4, *B. pertussis* 18323 with no additions and with modulators, respectively.

product is produced in the absence of primer, while lane 2 shows that the strongest primer extension stop occurs at a point 45 nucleotides upstream of the initiation codon. This is the most likely site of transcription initiation.

Sequence and mutation analysis suggests a site in the *vrg* coding region that is involved in transcriptional regulation. We tested this directly by constructing a complete deletion of the 5'-untranslated region of the *vrg-6*::Tn*phoA* gene fusion. An oligonucleotide that hybridizes to the first 27 nucleotides of the *vrg-6* coding region (and contains an 8-nucleotide tail on the 5' end conferring an *Xba*I site) was used in conjunction with an oligonucleotide well downstream in Tn*phoA* (bp 390 to 413; Materials and Methods). This allowed the amplification of the amino-terminal coding region of *vrg-6* devoid of any upstream sequences. The product was first cloned into plasmid pCR1000 (Invitrogen), which has 3' T-overhangs and takes advantage of the addition of single 5' A-overhangs added to all polymerase chain reaction products (4). The *Xba*I-*Hin*cII Δ*vrg-6*::Tn*phoA*' fragment from one these clones was purified. To place this promoter-deleted fusion fragment upstream (5') to a full-length *phoA* gene, pUCP1, which contains the original *vrg-6*::Tn*phoA* fusion, was digested with *Xba*I and *Hin*cII, removing all upstream *vrg-6* sequences from the *Xba*I site into the *Hin*cII site in Tn*phoA*. After ligation and transformation, the resulting Δ*vrg-6*::Tn*phoA* fusion clone, which contains *vrg-6* sequences from the translation start site to the Tn*phoA* fusion junction 66 bp downstream, was designated pUCP1ΔP.

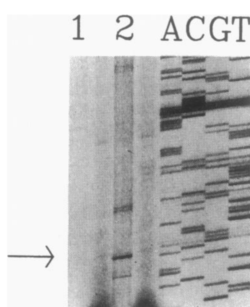

FIG. 4. Primer extension of the *vrg-6*::Tn*phoA* transcript. RNA prepared from SK6 grown under modulating conditions was used in a primer extension reaction without (lane 1) or with (lane 2) the Tn*phoA* primer (see Materials and Methods). A DNA sequence ladder of the *vrg-6* promoter was generated by double-strand sequencing of plasmid pUCP1 with the same primer. The primary primer extension product is marked with an arrow.

A constitutive *B. pertussis* heterologous promoter was cloned upstream of this new fusion in order to drive transcription. The promoter of the *B. pertussis asd* gene (encoding aspartate semialdehyde, an enzyme involved in diaminopimelic acid synthesis) was cloned out of plasmid pCP27 (gift of C. Parsot) and placed immediately 5' to the initiation codon of *vrg-6*::Tn*phoA* at the *Xba*I site, giving rise to plasmid pUCP1*asd*P. A replicon fusion of pUCP1*asd*P to pLAFR2 was isolated by methods described previously (2) and mated into *B. pertussis* 357. This heterologous reporter system demonstrated modulatable expression of alkaline phosphatase activity: 111 U under nonmodulating conditions versus 337 U in the presence of $MgSO_4$ and nicotinic acid. These data indicate that sequences in the coding region, from the start site of transcription to the Tn*phoA* fusion junction 66 bp downstream, are sufficient to confer antigenic modulation of the *vir*-repressed genes.

Southwestern blot analysis. Taken together, the experiments presented above suggest that the *vrg-6* consensus sequence in the coding region is somehow recognized in the process of *vir*-dependent repression. Southwestern analysis was performed to test whether this region is recognized by a DNA-binding protein (11). Whole-cell extracts of *B. pertussis* 18323 grown in the presence and absence of antigenic modulators were separated on a standard Laemmli protein gel. The gel was electrophoretically blotted to nitrocellulose and probed with a radioactively labeled fragment of DNA containing the first 62 bp of the *vrg-6* coding region. An autoradiograph of this blot (Fig. 5) shows that this region of *vrg-6* is recognized by a macromolecule, presumably a protein, of approximately 34 kDa present in whole-cell extracts. Moreover, a significant increase in the amount of this protein was observed in the absence of antigenic modulators, suggesting that the repressor is a *vir*-activated gene product.

A similar analysis was used to study whether the effects of the putative repressor-resistant mutation (*vrg-6mut13*), located in the consensus sequence of the *vrg-6* coding region, had any effect on repressor binding. A Southwestern blot of serial dilutions of *B. pertussis* cell extracts was probed with a DNA fragment containing the first 62 bp in the *vrg-6* coding sequence of either the wild-type or the *vrg-6mut13* allele. Specific activity was controlled by labeling equal molar ratios of the two fragments and adding an equal number of radioactive counts as determined by liquid scintillation. Autoradiographs and phosphoimager quantification of these blots indicated that the mutation results in a significant reduction in repressor binding, suggesting that the consensus sequence in the *vrg* DNA coding region is specifically recognized by a 34-kDa repressor in the absence of antigenic modulators (Fig. 6).

DISCUSSION

Many pathogenic bacteria possess genetic mechanisms for coordinately regulating expression of virulence factors such

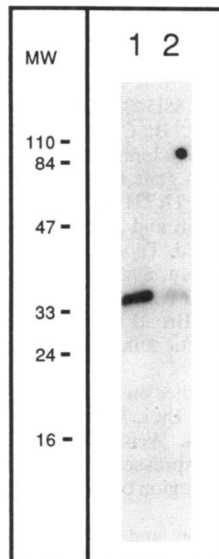

FIG. 5. Southwestern blot of *B. pertussis* extracts. Whole-cell protein extracts of *B. pertussis* 18323 grown in the absence (lane 1) and presence (lane 2) of modulators were separated on an SDS-polyacrylamide gel. The gel was transferred to nitrocellulose and probed with a radiolabeled fragment of DNA containing the first 62 bp of the *vrg-6* coding region (see Materials and Methods). Molecular weights (in thousands) are indicated.

as toxins, pili, and hemolysins. Such coordinate regulation is often at the transcriptional level, and examples include both positive and negative control mechanisms (20). In the respiratory pathogen *B. pertussis*, transposon mutagenesis has allowed the identification of five *vir*-repressed genes that are maximally expressed in modulated cultures, conditions under which expression of most known virulence factors is negligible. Sequence and deletion analysis define a consensus sequence in the *vrg* coding DNA that is sufficient to confer transcriptional regulation; no sequences in the native promoter region are required for repression in response to the absence of antigenic modulators. Furthermore, Southwestern and mutational analysis suggest that *vrg* transcription is repressed by the binding of a *vir*-activated gene product to the consensus DNA sequence located in the *vrg* coding region.

A DNA sequence comparison of four of five *vrg* genes showed considerable homology within the coding regions, suggesting a regulatory site whose sequence and placement are conserved (Fig. 2d). However, this homology is solely at the DNA level, as the predicted protein sequence shows no such consensus. A point mutation in the *vrg-6* DNA element abolished regulation, indicating a role of this consensus sequence in antigenic modulation. Three lines of evidence suggest that antigenic modulation of *vrg* genes is regulated at the transcriptional level. (i) A transcriptional fusion to *vrg-6* responds to antigenic modulation similarly to other *vir*-repressed genes. (ii) Northern analysis indicates that *vrg-6* transcript levels are specifically elevated in the presence of modulators. (iii) Southwestern analysis suggests that a consensus sequence in the *vrg-6* coding region is specifically recognized by a 34-kDa protein present in *B. pertussis* whole-cell extracts in the absence of modulators.

Previous studies of two *vir*-repressed genes, *vrg-6* and *vrg-18*, have suggested that sequences upstream of their signal sequences are not required for regulation (2). Deletion analysis presented here indicates that a *vrg-6*::Tn*phoA* deletion plasmid, which contains *vrg-6*::Tn*phoA* coding sequences from the translation start site to the Tn*phoA* fusion junction and a heterologous promoter for expression, still responds to antigenic modulation. These data suggest that, at least with *vrg*::Tn*phoA* fusions, sequences solely within the structural gene are sufficient to confer regulation of *vir*-repressed genes. Repressor binding sites within the protein-coding region have been established in several other bacterial systems, for example, *gal*, *lac*, and *pur* of *E. coli* (13, 24, 27). In each of these examples, however, an additional operator site outside the coding region is also required for repression; this appears not to be the case for *vrg* regulation. Certainly, regulatory sites outside the *vrg* consensus region may also contribute to the full range of antigenic modulation, since we have assayed *vrg*::Tn*phoA* fusions and not native *vrg* protein levels.

Clearly, both *vag* and *vrg* genes require an intact *bvg* locus for expression. However, unlike the *fhaB* gene and the *bvg* operon, other *vag* and *vrg* genes are unresponsive to *bvgAS* when present in *trans* in *E. coli* (1a, 21). This suggests that an additional *trans*-acting regulator element(s) is required in *vag* and *vrg* regulation. In the case of *vrg* genes, the *trans*-acting regulator may be a repressor since Southwestern and mutational analysis presented here suggest that a 34-kDa protein specifically binds to the *vrg* DNA consensus sequence, leading to modulation of *vrg* transcript levels. Moreover, the expression of this 34-kDa protein is maximal in the absence of modulators, suggesting that it is encoded by a *vir*-activated gene (Fig. 5). However, it is unlikely that the 34-kDa protein is encoded by the *bvgAS* locus because its apparent molecular mass is higher than the 23 kDa reported for the *bvgA* protein and much lower than 135 kDa reported for the *bvgS* protein (34). Taken together, these data suggest that a 34-kDa protein, distinct from BvgA and BvgS, binds to a consensus DNA sequence located in the *vrg* coding region, repressing the transcription of the *vir*-repressed genes. The details of the mechanism by which *vrg* repression occurs remain to be elucidated; i.e., repression could occur at the level of transcriptional initiation, elongation, or premature termination.

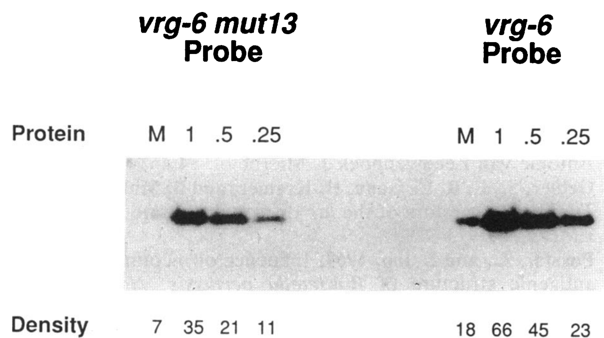

FIG. 6. Southwestern blots probed with *vrg-6* wild type and *vrg-6mut13*. Whole-cell protein extracts of *B. pertussis* 18323 grown in the absence (lanes 1, .5, and .25) and presence (lanes M) of 5 mM nicotinic acid and 20 mM $MgSO_4$ were separated on an SDS-polyacrylamide gel. The proteins were transferred to nitrocellulose and probed with a radiolabeled fragment of DNA containing either the first 62 bp of the *vrg-6* coding region (right panel) or the first 62 bp of the *vrg-6mut13* coding region. Samples from the nonmodulated cultures were loaded as serial two-fold dilutions (lanes 1, .5, and .25). Densitometry of the blots was determined by phosphoimaging, and the values are given below each lane.

Consistent with multiple regulatory elements implicated in *B. pertussis* virulence expression, there may be one or more regulatory elements involved in *vag* expression as well. Huh and Weiss (11) have identified a 23-kDa protein that binds to *cis*-acting regulatory elements in the promoter regions of *ptx* and *cya*; this protein is also *vir* regulated. However, the apparent molecular mass of this protein is significantly lower than that of the 34-kDa protein we have identified as a putative repressor of *vrg* expression, suggesting that at least two proteins distinct from BvgA and BvgS are required for regulation of the various virulence genes of *B. pertussis*.

Coordinate regulation of virulence gene expression by BvgA and BvgS appears to involve direct control at the *fhaB* and *bvgAS* promoters and indirect control of other promoters via a cascade of different *trans*-acting transcription factors. Such a cascade of virulence gene regulation has been demonstrated in *Vibrio cholerae* (6). In this system, the ToxR protein controls the expression of one class of genes directly, while others are controlled through the action of an additional regulatory protein, designated ToxT, whose expression is dependent on ToxR. Presumably, *B. pertussis* has adopted a similar regulatory cascade for the control of virulence determinants. That is, some *vir*-activated and *vir*-repressed genes appear to be controlled by additional regulatory components, which, in turn, are dependent on *bvgAS* for their own expression. Such a regulatory cascade may also explain the mechanism by which *vrg-73* responds to antigenic modulation in a *bvgAS*-dependent fashion, although it does not contain the *vrg* consensus sequence in the *vrg-73* coding region; i.e., *vrg-73* may be recognized by a regulatory element(s) distinct from the *vrg* repressor described here. The temporal expression of *B. pertussis* virulence factors also supports such a regulatory cascade. That is, upon a shift to medium lacking antigenic modulators, filamentous hemagglutinin and BvgA are expressed initially, followed much later by pertussis toxin and adenylate cyclase, whose expression coincides with increased levels of BvgA (26, 31). The delayed expression of these virulence factors may be due to the time required for the induction of additional regulatory component(s), whose expression is dependent on BvgA.

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