JOURNAL OF BACTERIOLOGY, July 1989, p. 4026–4030
0021-9193/89/074026-05$02.00/0
Copyright © 1989, American Society for Microbiology
Vol. 171, No. 7

Pertussis Toxin Promoter Sequences Involved in Modulation

ROY GROSS AND RINO RAPPUOLI*
Sclavo Research Center, Via Fiorentina 1, 53100 Siena, Italy

Received 16 September 1988/Accepted 13 April 1989

Previous analysis of the pertussis toxin (PT) promoter has shown that expression of PT requires a *trans*-activating factor encoded by the *vir* locus and a 170-base-pair DNA sequence upstream from the transcription start site containing a 21-base-pair direct repeated sequence crucial for *trans*-activation (R. Gross and R. Rappuoli, Proc. Natl. Acad. Sci. USA 85:3913–3917, 1988). In this paper we extend the analysis to the modulative response to environmental stimuli. We show that modulation acts at the transcriptional level and occurs only in phase I bacteria. Modulation also requires a functional *vir* locus and the same promoter region of 170 base pairs. We show that, in addition to the previously identified direct repeat, even the sequences downstream from position −117 are required for *trans*-activation and modulation and that the deletion of four cytosine residues at position −31 causes the inactivation of the promoter. The kinetics of the change in transcription show that the PT promoter can be shut off very rapidly by adding 50 mM $MgSO_4$ to the medium, whereas resumption of transcription after removal of the modulative agents from the medium is slow.

The expression of the *Bordetella* virulence factors (e.g., pertussis toxin [PT], filamentous hemagglutinin, adenylate cyclase, hemolysin, fimbriae, etc. [20]) is regulated coordinately. Two regulatory mechanisms have been described elsewhere: (i) variation from the virulent phase I to an avirulent phase III, which occurs at a high frequency (10^{-3} to 10^{-6} per cell per generation) and is rarely reversible (23), and (ii) modulation, which is a response to culture conditions and regulates the virulence factors in a reversible manner (13).

Weiss and Falkow (23) have proposed a model for the coordinate regulation of the *Bordetella pertussis* virulence factors in which *trans*-acting elements encoded by a chromosomal locus (*vir*) activate the promoters of the virulence-associated genes. Inactivation of the *vir* locus results in a change from phase I to phase III of *Bordetella* strains (23). We have recently shown that the *vir* locus of phase I but not of phase III bacteria can *trans*-activate the PT promoter and that the *vir* systems of *B. pertussis*, *Bordetella parapertussis*, and *Bordetella bronchiseptica* are functionally equivalent (6). Furthermore, 170 base pairs upstream from the transcriptional start site of the pertussis toxin operon are required for *trans*-activation by the *vir* system. This region contains a 21-base-pair sequence which is repeated twice and is essential for *trans*-activation by the *vir* system (6). In this study we analyzed the modulative response of the PT promoter to environmental stimuli, showing that the *vir* locus and the same 170-base-pair region are involved in the modulation. In addition to the previously identified direct repeats, our results show that the entire 170-base-pair-long promoter region is essential for *trans*-activation and modulation.

MATERIALS AND METHODS

Strains and media. *B. bronchiseptica* CCUG BB7865 was obtained from the Culture Collection of the University of Göteborg, Göteborg, Sweden (1). *B. bronchiseptica* BB7866 is a spontaneous phase III derivative of this strain (6). These strains were usually grown on Bordet-Gengou plates (2) or in Stainer-Scholte liquid medium (SS medium) (22). The plasmid-containing strains were grown in the presence of 10 μg of tetracycline per ml.

Plasmids. The plasmids containing the PT promoter and its deletion derivatives fused to the chloramphenicol acetyltransferase (CAT) reporter gene have been described elsewhere (6). Figure 1 shows the sequences of the PT promoter present in these plasmids. Plasmid pBP4 contains a deletion of four cytosine residues at position −31 (Fig. 1). This plasmid was constructed by using the *Kpn*I-*Hin*dIII fragment of plasmid pBP17 (19), which contains the 5′ untranslated sequence of the PT operon (from the *Kpn*I site at position −62 to position +1) but which has a deletion of the four bases at position −31. This fragment was cloned together with the *Bam*HI-*Kpn*I fragment of plasmid pBP2, which contains the 5′ untranslated region of the PT operon between sequence positions −482 and −62 (Fig. 1), into the vector bluescript SK (Stratagene, San Diego, Calif.) digested with *Bam*HI-*Hin*dIII. From the resulting plasmid, the *Bam*HI-*Hin*dIII fragment was isolated and cloned together with the *Hin*dIII-*Xba*I fragment of pA10-CAT2 (4), which contains the promoterless CAT gene, into plasmid pLAFR2 (5) digested with *Bam*HI-*Xba*I. DNA manipulations were performed by standard procedures (16). The conjugations were carried out as previously described (6).

Viable counts. Earlier experiments have shown that the optical densities (ODs) of cell suspensions of phase I and phase III bacteria grown under different conditions do not reflect the same number of viable cells (11). To ensure that the experiments were performed with an equal number of living cells, viable counts of the cell suspensions were performed. The data reported in this paper were standardized with viable counts and not just with ODs of the cultures.

RNA procedures. For the primer extension experiments, the RNA was isolated as previously reported (19) from strains BB7865 and BB7866 containing plasmid pBP2 grown under the conditions described below. Strain BB7865, grown in normal SS medium (22) at 35°C, was used to inoculate four flasks, each containing 20 ml of normal SS medium (i) cultivated at 35°C, (ii) cultivated at 25°C, (iii) supplemented with $MgSO_4$ to a final concentration of 50 mM and cultivated at 35°C, or (iv) supplemented with 5 mM nicotinic acid and cultivated at 35°C. A fifth flask containing normal SS me-

* Corresponding author.

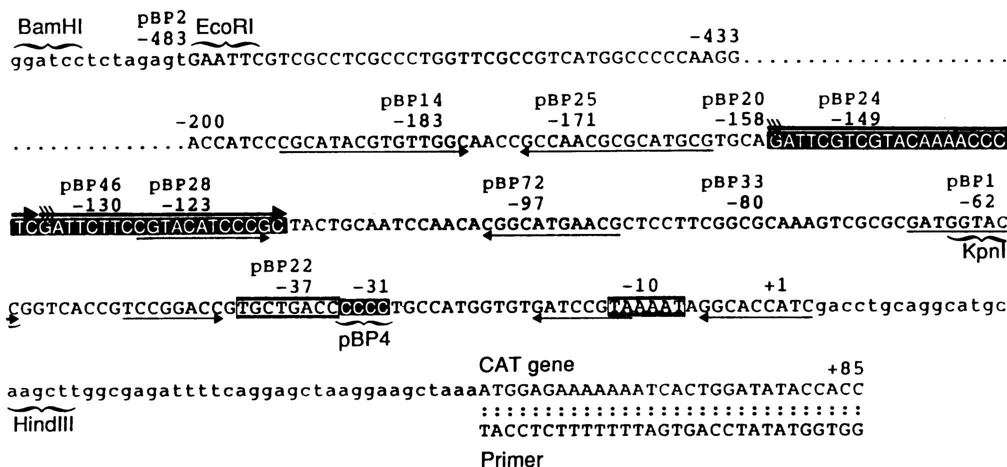

FIG. 1. Nucleotide sequence of the PT promoter region of *B. pertussis* 165 (18). The positions of the various deletion endpoints are shown at the top; the names of the deletion mutants (pBP series) are also shown. The nucleotides derived from polylinkers and from the upstream sequence of the CAT coding region from pA10-CAT2 (4) are indicated by lowercase letters. The −10 and −35 regions of the PT promoter are boxed. The primer complementary to the 30 nucleotides of the CAT coding region used for the primer extension analysis is shown. The black boxes show regions in the PT promoter which have been shown to be necessary for its *trans*-activation (i.e., the 21-base-pair direct repeat from positions −157 to −117 and the four cytosines at position −31). In plasmid pBP4, the four cytosine residues at position −31 have been deleted. The arrows above the sequence show the 21-base-pair direct repeat essential for promoter function (6); the arrows below the sequence show inverted repeats. Restriction sites used for cloning procedures are shown.

dium was inoculated with the BB7866 strain and incubated at 35°C.

The end labeling of the primer and the primer extension analysis were performed by using a primer complementary to the first 30 nucleotides of the CAT coding region, as described elsewhere (4) (Fig. 1).

RNA for the Northern (RNA) analysis was prepared by the method of Kornblum et al. (12). Strain BB7865 containing plasmid pBP2 was grown overnight at 35°C in normal SS medium or in SS medium containing 50 mM $MgSO_4$. Then the bacteria were inoculated in fresh medium to an OD at 580 nm (OD_{580}) of 0.05 under the same conditions. After an OD_{580} of 0.3 was reached, $MgSO_4$ was added to the culture in normal SS medium to achieve a final concentration of 50 mM. The bacteria growing in SS medium with 50 mM $MgSO_4$ were centrifuged, washed, and suspended in prewarmed normal SS medium. Samples (5×10^8 cells) were taken at different intervals to prepare the RNA for Northern analysis (12) and to perform the CAT assay. For detection of the CAT-specific mRNA on the Northern blot, the *Hin*dIII-*Xba*I fragment of the vector pA10-CAT2 (4) containing the CAT gene was isolated and radiolabeled (8). The relative intensity of the CAT-specific mRNA bands was quantitated by scanning the autoradiographies of the blots with an LKB Ultrascan XL.

CAT assay. Strains BB7865 and BB7866 containing the plasmids of the pBP series were grown in either normal SS medium or SS medium containing 50 mM $MgSO_4$ at 35°C. Usually, 8×10^8 cells were used for the CAT assay; 5×10^8 cells were used in the time course experiments. The CAT assay was carried out as described previously (6). The relative plasmid copy numbers under various growth conditions were estimated by laser scanning of photographs of agarose gels of plasmid minipreparations (16).

RESULTS AND DISCUSSION

Modulation causes rapid changes of transcription. Previously, the promoter of PT or derivatives of it were cloned in front of the CAT gene in the broad-host-range vector pLAFR2 to identify the sequences required in *cis* for the *trans*-activation of the PT promoter (6). Here we used the same plasmids for the analysis of the modulation response in *B. bronchiseptica*.

In order to verify whether modulation occurs at the transcriptional level, *B. bronchiseptica* BB7865 and BB7866 containing plasmid pBP2 (6) (Fig. 1) were grown under normal conditions, in the presence of modulative agents, such as 50 mM $MgSO_4$ or 5 mM nicotinic acid, or at low temperature (25°C). mRNA was prepared from each of these cultures, and primer extension experiments were performed. The CAT mRNA is present only in phase I bacteria grown under nonmodulative conditions and starts at the adenine residue, 8 base pairs downstream of the −10 box (Fig. 2), as in the case of PT (19) (Fig. 1). Plasmid copy numbers did not change significantly under the various growth conditions (data not shown). We therefore conclude that the presence of modulative agents results in the lack of transcription and that different agents, such as $MgSO_4$, nicotinic acid, or low temperature, very likely act by means of the same mechanism.

To analyze the kinetics of the change in transcription, strain BB7865 containing plasmid pBP2 was grown in normal medium or in the presence of 50 mM $MgSO_4$ to an OD_{580} of 0.3. Then the culture growing in the presence of $MgSO_4$ was centrifuged, washed, and suspended in normal medium, while $MgSO_4$ was added to the other culture to a final concentration of 50 mM. Samples were taken at intervals to determine the CAT-specific mRNA (by Northern blotting) (12) and the CAT activity. As early as 10 min after addition of $MgSO_4$, the CAT mRNA was no longer detected, whereas the CAT activity decreased slowly and reached background levels only when the culture reached the stationary phase (after approximately four to five cell divisions) (Fig. 3). This shows that the addition of modulative agents such as $MgSO_4$ causes immediate stopping of transcription of the PT genes, which results in the disappearance of the mRNA within 10

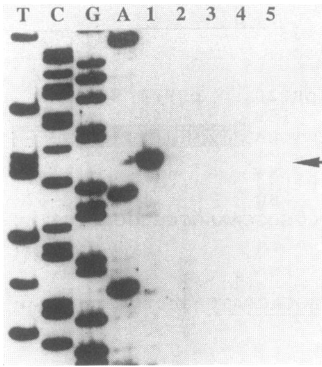

FIG. 2. Primer extension analysis of CAT mRNA from phase I *B. bronchiseptica* BB7865 containing plasmid pBP2 grown in normal SS medium at 35°C (lane 1), in SS medium containing 50 mM $MgSO_4$ at 35°C (lane 2), in SS medium containing 5 mM nicotinic acid at 35°C (lane 3), or in SS medium at 25°C (lane 4) and from phase III *B. bronchiseptica* BB7866 containing pBP2 grown in normal SS medium at 35°C (lane 5). The sequencing gel used to map the start point of transcription (TCGA) is shown on the left. The sequence used is part of the coding region of the S1 subunit (15, 18). A primer, 3'-ATCTGGACCGGGTCGGG-5', complementary to sequence positions 1314 to 1331 was used (18). The arrow indicates the extended primer in lane 1 which comigrates with the sequence gctTtcg, which is complementary to the sequence cgaAagc of the S1 coding region. This sequence is 85 base pairs upstream from the beginning of the sequencing primer used. From the sequence of the primer used to extend the CAT mRNA shown in Fig. 1, it can be deduced that the transcription of the CAT mRNA starts on the adenine residue indicated with +1 in Fig. 1.

min. Therefore, the proteins encoded by the *vir trans*-activated genes are no longer synthesized. The proteins which are already present are diluted during cell divisions or degraded until they are no longer detectable. In the case of the CAT enzyme, at least four cell generations were required for the disappearance of the CAT activity. This observation agrees with the work of Lacey, who showed that several cell divisions were required for the complete change of X-mode (virulent) cells to C-mode (avirulent) cells after the addition of modulating agents (13). This explains the detection of different phenotypes (I mode) which are somehow intermediate between the X and C modes.

In the experiment in which cells were grown under modulative conditions (50 mM $MgSO_4$), washed, and suspended in normal medium, both the resumption of transcription of the CAT mRNA and resumption of the CAT activity were unexpectedly slow (Fig. 3B). In fact, a faint band of CAT mRNA was detected only after 60 min and reached approximately 25% of the maximum value shown in Fig. 3A after four cell generations. At this stage the cell culture was entering the stationary phase, and the rise in CAT activity could therefore not be detected. These observations agree with the studies of Lacey, who showed that the change from C mode to X mode was slow and required 7 to 15 cell generations. The reason for such a slow resumption of the transcription of the CAT mRNA is not clear. It could be calculated that after removal of the medium containing 50 mM $MgSO_4$ and washing of the cells, not more than 0.5 mM $MgSO_4$ is left in the medium. Because this concentration is far below the concentration required to produce a modulative effect (10 mM $MgSO_4$), the resumption of transcription may be influenced by other factors due to excess residual $MgSO_4$ within the cells.

Modulation requires *vir* and a promoter region of 170 base pairs. The pBP plasmids (Fig. 1) containing a series of 5' deletions of the PT promoter were previously used for the identification of the PT promoter sequences required for positive regulation (6) and were used in this study to establish whether modulation also requires the same *trans*-activating system (*vir*) and the same promoter sequences in *cis*. The deletion analysis shown in Fig. 4 demonstrates that, as previously described (6), *vir*-dependent activation of the PT promoter is mediated by the direct repeated sequence at positions −157 to −117 (pBP20, pBP24, and pBP46) and that at least 170 base pairs of the 5' region are necessary for strong promoter activity in phase I bacteria (pBP2, pBP14,

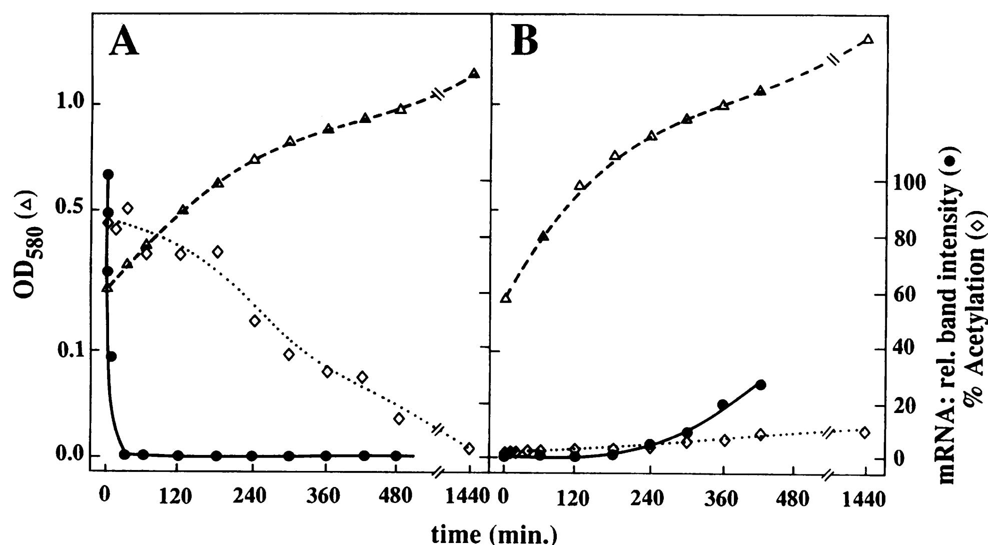

FIG. 3. CAT mRNA detected by densitometric scanning of Northern blots (●) and CAT activity in cultures (◇) of *B. bronchiseptica* BB7865 containing plasmid pBP2. (A) The strain was grown to an OD_{580} (△) of 0.3 in SS medium, and then $MgSO_4$ was added to a final concentration of 50 mM. (B) The strain was grown to an OD_{580} of 0.3 in SS medium containing 50 mM $MgSO_4$ and then centrifuged, washed, and suspended in normal SS medium. The time was measured from the change in the culture conditions.

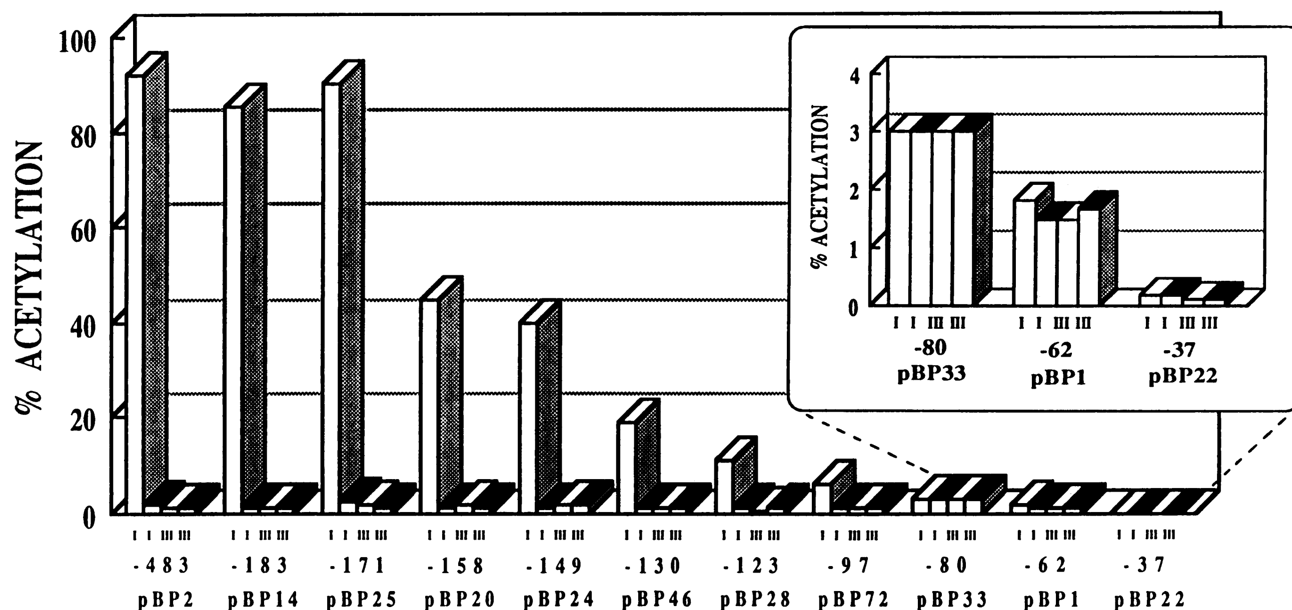

FIG. 4. CAT activities of the various deletion mutants (expressed as percent acetylation) in relation to the length of the 5′ region of the PT promoter. Results with strains grown in normal SS medium at 35°C (white-tipped columns) and strains grown in SS medium containing 50 mM $MgSO_4$ at 35°C (black-tipped columns) are shown. The phases of the strains (I, BB7865; III, BB7866) are indicated. The names of the plasmids and the amount of the 5′ region of the PT promoter fused to the CAT gene are shown. The enlargement shows results obtained with pBP33, pBP1, and pBP22 to demonstrate that the PT promoter containing the −35 and −10 boxes has approximately 10 times more *vir*-independent promoter activity than the promoter of pBP22, for which the −35 box has been destroyed. The standard deviations are 6 to 10% for columns representing 85 to 95% relative acetylation, 6% for columns representing about 40% acetylation, 4% for columns representing 10 to 20% acetylation, 1.0% for columns representing 2 to 5% acetylation, and about 0.2% for the results obtained with plasmid pBP22.

and pBP25). Low but significant *vir*-dependent promoter activity was found in plasmids pBP28 and pBP72, in which the two repeated sequences have been almost (pBP28) or completely (pBP72) deleted. In our previous study the promoter activities of plasmids pBP46, pBP28, and pBP72 were considered negligible, and we concluded that the two direct repeats shown in Fig. 1 were responsible for the full promoter activity (6). In our present work we have been able to lower the background of the system so that we could detect the low activities of these plasmids, showing that the sequences downstream from the second repeat also contribute to the promoter activity.

Promoter activities, as observed in plasmids pBP2 to pBP72, were abolished by the addition of 50 mM $MgSO_4$ to the medium (Fig. 4). This implies that all the *vir*-dependent promoter activities can be inhibited by the addition of modulative agents. On the other hand, *vir*-independent promoter activities were not affected by modulation. This can also be seen in the enlarged area of Fig. 4, where it is evident that the PT promoter containing the −10 and −35 boxes showed a *vir*-independent activity approximately 10 times higher than that in a promoter mutant consisting only of the −10 region (pBP22). Since this promoter activity was not influenced by $MgSO_4$ in any of the pBP plasmids, we can conclude that modulation does not occur in phase III bacteria. We can therefore conclude that, as in the case of positive regulation of the PT promoter, modulation requires a functional *vir* locus and the same promoter sequences. Consequently, *trans*-activation and modulation use the same system.

Furthermore, these results indicate that in addition to the previously identified repeated sequences spanning the region from positions −156 to −117, other *cis*-acting sequences downstream from position −117 are involved in the *trans*-activation of the PT promoter. In order to confirm that the entire PT promoter region is necessary for *trans*-activation and modulation, we used a new plasmid (pBP4), which contained the entire 5′ region but had a deletion of four nucleotides (four cytosine residues) in position −31 (Fig. 1). This deletion was chosen because other putative *B. pertussis* promoters (such as those of the fimbriae [14]) have a series of cytosine residues located in the same position; furthermore, in a previous study it was demonstrated that this deletion increases the strength of the PT promoter in *Escherichia coli*, probably by optimizing the distance between the −35 and −10 regions (19). Interestingly, the promoter of pBP4 is neither *trans*-activated nor modulated, despite the presence of the sequence between positions −170 and −80, which is also essential for *trans*-activation (data not shown). This implies that the integrity of the PT promoter region is important for positive regulation and suggests that the stretches of cytosines observed in other *vir*-dependent promoters play an important role.

The findings that more than one region of a promoter is important for its regulation and that the promoter does not work if one of these is impaired are not new; many parallels exist in both procaryotic and eucaryotic promoters which are positively regulated (17). Usually, multiple factors interact with different regions of the promoter, and all of them are important for its activation. For example, the *E. coli* maltose operons *malE* and *malK* are regulated by the MalT regulatory protein and the cyclic AMP receptor protein (21), and the arabinose operon of *E. coli* is regulated by the AraC regulatory protein and the cyclic AMP receptor protein (7). Interestingly, even in positions −22 to −7 of the PT promoter, we find the sequence 5′-TGTGAtccgtaaaAtA-3′,

which is a putative binding site of cyclic AMP receptor protein (3). It is also remarkable that the entire promoter region contains a series of inverted repeats (Fig. 1) which could be involved in the regulation of gene expression (9).

It is interesting to note that a third copy of the 21-base-pair directly repeated sequence is also present in the S1 gene, starting with base pair 60 of the coding region. Related sequences are also present in the 5′ untranslated region of the structural gene for the filamentous hemagglutinin (D. Relman and M. Domenighini, unpublished data) and in two copies at the beginning of the coding region of the first open reading frame of the *vir* locus (B. Aricò, unpublished data). Although the significance of these sequences remains to be elucidated, examples in which the same regulative sequences are present in the promoter regions and within the coding sequences have been reported elsewhere (10).

ACKNOWLEDGMENTS

We thank Lorena dell'Arciprete and Maria Perugini for technical help, Stefano Ricci for the oligonucleotide, Catherine Mallia for typing the manuscript, and Giorgio Corsi for graphic work.

LITERATURE CITED

1. **Aricò, B., and R. Rappuoli.** 1987. *Bordetella parapertussis* and *Bordetella bronchiseptica* contain transcriptionally silent pertussis toxin genes. J. Bacteriol. **169:**2847–2853.
2. **Bordet, J., and O. Gengou.** 1909. L'endotoxine coquelucheuse. Ann. Inst. Pasteur (Paris) **23:**415–419.
3. **de Crombrugghe, B., S. Busby, and H. Buc.** 1984. Cyclic AMP receptor protein: role in transcription activation. Science **224:**831–837.
4. **D'Onofrio, C., V. Colontuoni, and R. Cortese.** 1985. Structure and cell specific expression of cloned human retinol binding protein gene: the 5′ flanking region contains hepatoma specific transcriptional signals. EMBO J. **4:**1981–1989.
5. **Friedman, A. M., S. R. Long, S. E. Brown, W. J. Buikema, and F. M. Ausubel.** 1982. Construction of a broad host range cosmid cloning vector and its use in the genetic analysis of *Rhizobium* mutants. Gene **18:**289–296.
6. **Gross, R., and R. Rappuoli.** 1988. Positive regulation of pertussis toxin expression. Proc. Natl. Acad. Sci. USA **85:**3913–3917.
7. **Hahn, S., W. Hendrickson, and R. Schleif.** 1986. Transcription of *Escherichia coli ara in vitro*. J. Mol. Biol. **188:**355–367.
8. **Hodgson, C. P., and R. Z. Fisk.** 1987. Hybridization probe size control: optimized 'oligolabelling.' Nucleic Acids Res. **15:**6295.
9. **Horwitz, M. S. Z., and L. A. Loeb.** 1988. An *E. coli* promoter that regulates transcription by DNA superhelix-induced cruciform extrusions. Science **241:**703–705.
10. **Husslein, V., B. Huhle, T. Jarchau, R. Lurz, W. Goebel, and T. Chakraborty.** 1988. Nucleotide sequence and transcriptional analysis of the *aerCaerA* region of *Aeromonas sobria* encoding aerolysin and its regulatory region. Mol. Microbiol. **2:**507–517.
11. **Idigbe, E. O., R. Parton, and A. C. Wardlaw.** 1981. Rapidity of antigenic modulation of *Bordetella pertussis* in modified Hornibrook medium. J. Med. Microbiol. **14:**409–418.
12. **Kornblum, J. S., S. J. Projan, S. L. Moghazeh, and R. P. Novick.** 1988. A rapid method to quantitate non-labeled RNA species in bacterial cells. Gene **63:**75–85.
13. **Lacey, B. W.** 1960. Antigenic modulation of *Bordetella pertussis*. J. Hyg. **58:**57–93.
14. **Livey, I., C. J. Duggleby, and A. Robinson.** 1987. Cloning and nucleotide sequence analysis of the serotype 2 fimbrial subunit gene of *Bordetella pertussis*. Mol. Microbiol. **1:**203–209.
15. **Locht, C., and J. M. Keith.** 1986. Pertussis toxin gene: nucleotide sequence and genetic organization. Science **232:**560–563.
16. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
17. **Monaci, P., A. Nicosia, and R. Cortese.** 1988. Two different liver specific factors stimulate *in vitro* transcription from the human α 1-antitrypsin promoter. EMBO J. **7:**2075–2087.
18. **Nicosia, A., M. Perugini, C. Franzini, M. C. Casagli, M. G. Borri, G. Antoni, M. Almoni, P. Neri, G. Ratti, and R. Rappuoli.** 1986. Cloning and sequencing of the pertussis toxin genes: operon structure and gene duplication. Proc. Natl. Acad. Sci. USA **83:**4631–4635.
19. **Nicosia, A., and R. Rappuoli.** 1987. Promoter of the pertussis toxin operon and production of pertussis toxin. J. Bacteriol. **169:**2843–2846.
20. **Pittman, M.** 1984. Genus *Bordetella*, p. 388–393. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore.
21. **Schwartz, M.** 1987. The maltose regulon, p. 1482–1502. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 2. American Society for Microbiology, Washington, D.C.
22. **Stainer, D. W., and M. J. Scholte.** 1970. A simple chemically defined medium for the production of phase I *Bordetella pertussis*. J. Gen. Microbiol. **63:**211–220.
23. **Weiss, A. A., and S. Falkow.** 1984. Genetic analysis of phase change in *Bordetella pertussis*. Infect. Immun. **43:**263–269.