Promoter of the Pertussis Toxin Operon and Production of Pertussis Toxin

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Pertussis toxin (PT), the major virulence factor of Bordetella pertussis, is composed of five different subunits whose genes are organized as an operon. We report the mapping of the promoter region of the PT operon and show that this promoter is only weakly active in *Escherichia coli. Bordetella parapertussis* and *Bordetella* bronchiseptica, which do not produce any PT, are shown to have a weaker promoter sequence for this operon and not to produce any detectable PT mRNA. We show that transcription of the PT operon in B. pertussis was constant throughout until the late stationary phase, when transcription signifieantly decreased. Analysis of the transposon TnS mutant BP347 showed that the product of the vir locus was required for transcription of the PT operon. Characterization of the TnS mutant BP356 showed that subunit S3 was required for the release of PT into the extracellular medium.

Pertussis toxin (PT) is produced by phase ^I (virulent) Bordetella pertussis strains, but not by Bordetella parapertussis, Bordetella bronchiseptica, phase III B. pertussis, or phase ^I organisms grown in high-magnesium or low-sodium medium (19). PT, an oligomeric protein composed of five different subunits, can be divided into two functionally different moieties: A, which comprises subunit S1, and B, which comprises subunits S2, S3, S4, and S5 arranged in two dimers, Dl (S2 plus S4) and D2 (S3 plus S4), which are held together by S5. The B moiety binds the receptors on the surface of eucaryotic cells and allows the entry of the enzymatically active subunit S1 into the cells (18, 21). The toxicity is due to Si, which is an ADP-ribosyltransferase which ADP-ribosylates a family of GTP-binding proteins involved in the transduction of signals across the membrane of eucaryotic cells (12, 14, 17, 22). The genes coding for the five subunits of PT, which have recently been cloned and sequenced (15, 18), have been shown to be clustered in an operon in the following order: Si, S2, S4, S5, and S3. Subunits S2 and S3 have been shown to have 67% homologous amino acids. A sequence resembling the Escherichia coli promoter consensus sequence found upstream from the first gene has been tentatively proposed as the promoter for the PT operon (15, 18). The genes coding for each of the five subunits have been shown to be preceded by a sequence coding for a typical bacterial leader peptide, suggesting that the five subunits are secreted separately into the periplasm of B. pertussis cells (15, 18).

Analysis of the transcription of the PT genes and identification of some crucial steps in PT production should provide useful information on the regulation of expression of such a complex bacterial protein.

In this paper we identify the promoter of the PT operon and show that in E. coli it is very weak. We confirm that, as previously proposed by Weiss et al. (23), a trans-acting factor encoded by the vir locus is required for expression of the PT operon in B. pertussis and show that regulation is at the transcriptional level. B. parapertussis and B. bronchiseptica are shown to produce no detectable PT mRNA, most probably because of severe down-mutations in the promoter region which make their promoters very inefficient. We also show that transcription of the PT operon in B. pertussis is constant throughout the different phases of growth until the late stationary phase, when PT mRNA is almost absent. Characterization of the transposon TnS mutant of B. pertussis BP356, which does not produce any active toxin, allowed us to conclude that PT release into the extracellular medium requires assembly of the five subunits, which are secreted separately into the periplasm.

MATERIALS AND METHODS

Strains and media. B. pertussis BP165 was obtained from the Bureau of Biologics, Bethesda, Md. (20). B. pertussis Tohama has been described (13). The nonvirulent B. pertussis TnS mutants BP347 vir and the BP356 (which does not produce any active toxin) were obtained from Stanley Falkow (24). The strains of B. parapertussis and B. bronchiseptica were obtained from the American Type Culture Collection (ATCC ⁹³⁰⁵ and ATCC 4617, respectively). E. coli JM101 has been described (25). B. pertussis strains were grown in Bordet-Gengou plates (19) or Vervey liquid medium containing cyclodextrin (1 mg/ml) (13). Vervey medium contains (per liter) 14 g of Casamino Acids, 0.2 g of KCl, 0.5 g of KH_2PO_4 , 0.1 g of $MgCl_2 \cdot 6H_2O$, 20 mg of nicotinic acid, 10 mg of glutathione, and ¹ g of starch. E. coli strains were grown in LB plates or LB liquid medium (16).

CAT assay. Cell extracts for the chloramphenicol acetyltransferase (CAT) assay were prepared by sonicating cells from 1 ml of culture in 150 μ l of 0.25 M Tris, pH 8. The lysate was then centrifuged for 10 min in an Eppendorf centrifuge, and the supernatant was heated for 7 min at 60°C. After 10 min of centrifugation, the supematant was used for the CAT assays, which were perforrned as described previously (8).

DNA procedures. Manipulations of DNA were performed by standard procedures (16). Oligonucleotide synthesis was performed with the Applied Biosystems model 380A synthesizer. Oligonucleotide-directed mutagenesis was performed as described previously (27).

RNA procedures. To prepare RNA, samples were taken at intervals from a culture of B. pertussis growing in liquid medium and centrifuged, and the pellet was frozen in a dry ice-ethanol bath. The pellets were then suspended in ¹ ml of cold 10 mM Tris-1 mM EDTA, pH 7.5, containing 500 μ g of proteinase K per ml, vortexed, and mixed with ¹ ml of 0.2%

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FIG. 1. Primer extension analysis of PT mRNA from B. pertussis Tohama phase I Tohama (TOX⁺), the BP347 mutant which does not produce $PT (TOX⁻)$, and a control without RNA. To the right is the sequencing gel used to map the beginning of the mRNA. Next to this is the nucleotide sequence containing the most relevant features: -35 and -10 promoter boxes, beginning of the mRNA, Shine-Dalgarno (S.D.) sequence, and start codon of the S1 subunit.

sodium dodecyl sulfate (SDS) to lyse the cells; 0.8 g of CsCl was then added to the lysate, which was layered over 2 ml of a 5.7 M CsCl cushion in an SW65 ultracentrifuge tube and centrifuged at 35,000 rpm for 12 h. The pellet containing the RNA was then suspended in 200 μ l of 10 mM Tris-1 mM EDTA-0.2% SDS, pH 7.5, phenol-chloroform extracted, precipitated with ethanol, and suspended in water. Primer extension was performed as described previously (3). For the dot-blot hybridization, the RNA was suspended in 10 μ I of a solution containing 130 μ l of 40% formaldehyde in 330 μ l of water. Then, $10 \mu l$ of 4 M NaCl was added, and the RNA was spotted onto the nitrocellulose filter. Three fourfold dilutions (2 μ g, 0.5 μ g, and 0.125 μ g) were used for each RNA sample. Filters were hybridized with ^a single-stranded RNA probe complementary to the S1 mRNA which was obtained from a clone of the S1 gene in the SP6 vector. The 5P6 vector was obtained from Promega Biotech (Madison, Wis.) and used as specified by the manufacturer. Hybridization was performed under standard conditions (16), followed by extensive washings at high stringency in $0.2 \times$ SSC $(1 \times$ SSC is 0.15 M NaCl and 0.015 M sodium citrate) at 68°C.

Assays for PT. The Chinese hamster ovary cell assay (10) was used to test the biological activity of PT. Quantitative determination of PT in culture supernatants of cell extracts was performed by enzyme-linked immunosorbent assay (ELISA) (20). Purified goat anti-PT immunoglobulins or

fetuin was used to coat microtiter plates. Following incubation with antigen-containing preparations, a conjugate made of affinity-purified anti-PT rabbit gammaglobulins covalently coupled to alkaline phosphatase was added. PT was estimated by the resulting alkaline phosphatase activity.

RESULTS AND DISCUSSION

Identification of the PT promoter. The nucleotide sequence of the PT operon showed that a sequence closely resembling that of E . *coli* promoters is present upstream from the $S1$ subunit gene (15, 18). The -35 and -10 regions of this putative promoter are in good agreement with the consensus sequence of $E.$ coli, but their spacing is 21 base pairs (bp) instead of the optimal ¹⁷ bp. We used primer extension analysis to confirm that the sequence previously identified by Nicosia et al. (18) is in fact the promnoter of the PT operon. The oligonucleotide 5'-AGTCATAGCGGTATAC-GGTG-3', which is complementary to the S1 mRNA (from hucleotides 642 to 623 [18]), was chemically synthesized and used as a primer for extension of the PT mRNA. The results (Fig. 1) show that transcription of the PT operon starts at the adenine residue located 7 bp downstream from the -10 region. This finding definitively identifies the promoter of the PT genes and is in agreement with the general concensus sequence of E. coli, in which transcription starts at a purine residue located 7 to 10 bp downstream from the -10 region, possibly within a 5'-CAT-3' sequence (1, 9).

Analysis of PT promoter efficiency in E . coli. To analyze the strength of the promoters in E . coli, we cloned them into the promoterless plasmid vector pKK232-8 (6), which contains the *cat* gene. The four promoters which have been used in this study are shown in Fig. 2. BP is the wild-type promoter of the PT operon; BP17 is a derivative of the wild-type promoter from which we deleted the sequence 5'-CCCC-3' by site-directed mutagenesis in order to have optimal spacing (17 bp) between the -35 and the -10 regions. BPP is the promoter of the PT operon of B. parapertussis, which is identical to the PT promoter of B. bronchiseptica (2). The BPP promoter contains two changes, one T-to-C transition in the -10 region and one in the -35 region, which are

FIG. 2. Analysis of PT promoter efficiency in E. coli. Panels: C, control plasmid without any promoter; BP, promoter from B. pertussis; BP17, promoter from B. pertussis in which the distance between the -35 and -10 regions has been reduced to the optimal 17 bp by site-directed mutangenesis; BPP, promoter of B. parapertussis; SP, strong promoter. The thin-layer chromatography of the CAT assay, the CAT activities (expressed as percent acetylation), and the sequence of the promoters are shown. Lowercase letters indicate bases that are different in the B. parapertussis promoter. The -35 and -10 promoter regions are underlined.

considered severe downmutations for E. coli promoters (26). SP (strong promoter), a Salmonella typhimurium promoter whose efficiency in E . *coli* has been described $(4, 5)$, was used as a positive control.

The wild-type PT promoter gave ¹⁹ times more CAT activity than the control plasmid without any promoter (Fig. 2). As expected, BP17, which has optimal spacing between the -35 and -10 regions, was more efficient than the wild-type BP promoter. The BPP promoter, which contained severe down-mutations in the -10 and the -35 regions, was almost four times less efficient than the wild-type BP promoter. The SP promoter was about 1,000 times more efficient than the BP promoter. By primer extension of the CAT mRNA, we confirmed that transcripts from the above constructions started at the expected base downstream from the -10 region and therefore that the CAT activity we measured was in fact due to the promoters that we were analyzing.

These results showed that the PT promoter, although weak compared with the strong promoter, behaved as predicted in \vec{E} . coli: when the spacing between the -35 and the -10 regions was optimized, it became stronger; when downmutations were introduced, it became weaker. If the requirements for a good promoter are the same in B. pertussis and E. coli, the PT promoter should also be rather inefficient in B. pertussis, and therefore the efficient expression of this gene which is normally observed in phase ^I B. pertussis would imply an additional regulatory mechanism. The positive regulatory element encoded by the vir locus which has been proposed to explain the phenomenon of phase variation in B. pertussis (23) is probably required for efficient expression of the PT operon. On the other hand, the low efficiency of the B. parapertussis promoter is in agreement with the observation that B. parapertussis and B. bronchiseptica do not produce any PT (19).

Transcription of PT operon and production of PT. The production of PT is influenced by several factors, such as phase change of B. pertussis and the presence or absence of ions such as magnesium and sodium (19). We investigated whether transcription of the PT operon and the production of PT vary during different phases of growth.

B. pertussis Tohama was grown in liquid medium, and at intervals, a sample of the culture was taken, centrifuged, and used to prepare RNA. The PT content in the culture supernatant was determined by ELISA. The RNA was used in ^a dot-blot hybridization to determine the amount of PT mRNA. The results (Fig. 3) show that PT mRNA was produced at the same rate throughout growth until the late stationary phase, when there was a sharp decrease. PT was not detected in the supernatant until the late log phase and then rapidly accumulated, reaching a maximum in the early stationary phase, after which there was a sharp decrease.

It was also of interest to study in the same system three other strains of Bordetella which have been reported not to produce PT. Therefore, we analyzed the mutant strain B. pertussis BP347 vir, which contains a TnS insertion in the chromosome that abolishes the expression of all the virulence factors of B. pertussis (23, 24), one strain of B. parapertussis, and one strain of B. bronchiseptica. None of them produced detectable levels of PT mRNA or PT (Fig. 3). These findings show that the Tn5 mutation of BP347 (possibly affecting the production of the positive regulatory element encoded by the vir locus) abolished transcription of the PT operon and therefore that the PT promoter is inactive in B. pertussis unless it is activated by the positive regulatory element. Preliminary data (not shown) indicate that the alternative explanation, mRNA instability, is unlikely.

FIG. 3. Transcription of the PT operon and production of PT. The optical density of the culture (\bullet) , the amount of PT in the supernatant (\blacksquare) , and the amount of PT mRNA for B. pertussis Tohama are shown. For BP347, B. parapertussis, and B. bronchiseptica, we show only the dot hybridization of the RNA extracted at mid-log phase; the other points of the growth curve were also negative by ELISA and dot-blot hybridization.

For B. parapertussis and B. bronchiseptica, we can conclude that transcription of the PT operon is affected by the low efficiency of the promoter (as shown in Fig. 2) and possibly by alteration of the target of a positive regulatory element, because these strains are in phase ^I and therefore are likely to produce a regulatory element analogous to that encoded by the vir locus of B. pertussis.

Subunit S3 required for extracellular release of PT. Weiss et al. (24) described a TnS mutant of B. pertussis (BP356) which does not produce any active toxin. By nucleotide sequencing of the region containing the TnS insertion in the chromosome of BP356, we found that the Tn5 element is located within the gene coding for subunit S3, at nucleotide position ³⁴³⁶ (18), and that the sequence GCGCCAGCCC is repeated at both ends of the TnS insertion. Since S3 is the last of the PT genes, strain BP356 should produce the S1, S2, S4, and S5 peptides and the first 136 amino acids of subunit S3. Tamura et al. (21) have shown that at least in vitro, both dimers Dl and D2 are required to assemble the toxin. If this is also true in vivo, the incomplete sets of subunits produced by BP356 should not be assembled, and this might be the reason for the lack of toxicity of BP356 supernatants seen in the CHO cell assay (24).

To test this assumption, we performed the following experiment. Cultures of the wild-type B. pertussis BP165 and of the BP356 mutant were grown in parallel. Samples were taken at intervals and centrifuged. Each pellet was then suspended and divided into two samples; one was incubated in Tris buffer, and the other was incubated in the same buffer containing ² mg of polymyxin B per ml, which releases the periplasmic content of the cell (7). After centrifugation, the toxin content in the Tris extract, the polymyxin B extract, and the culture supernatant was determined by ELISA. The results (Fig. 4) show that the BP356 mutant produced some of the subunits of PT but did not release them into the supernatant. Once released from the periplasm by polymyxin B treatment, the toxin subunits produced by BP356 were still active in the CHO cell assay. Other laboratories have confirmed by Western blotting that the BP356 mutant is able to produce subunits S1, S2, S4, and S5 but not

FIG. 4. PT (as determined by ELISA) in the supernatants $(①)$, the Tris extract (\triangle) , and the polymyxin B extract (\blacksquare) of strains BP165 and BP356. The amount of toxin is reported in arbitrary optical density units at 405 nm.

S3 (S. Falkow, personal communication). This experiment showed that the BP356 strain of B. pertussis is defective for the synthesis of the S3 subunit, which is essential for the release of PT into the culture medium, and implies that in B. pertussis the five subunits are collected separately in the periplasm of the cell, where they are assembled and released. In the absence of the S3 subunit, PT is not released into the extracellular medium, suggesting that this protein is necessary for complete assembly of the toxin. This would be similar to the situation described for Vibrio cholerae, in which the two subunits of the cloned E . *coli* heat-labile enterotoxin are secreted in the periplasm of the cell and released into the culture medium only after assembly (11).

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