Positive regulation of pertussis toxin expression

(Bordetella species/promoter/deletion analysis/chloramphenicol acetyltransferase assay)

ROY GROSS AND RINO RAFFUOLI*

Sclavo Research Center, Via Fiorentina 1, 53100 Siena, Italy

Communicated by A. M. Pappenheimer, Jr., January 11, 1988 (received for review November 1, 1987)

ABSTRACT Although the genus Bordetella contains several closely related species, pertussis toxin (PT) is produced only by phase I Bordetella pertussis. In this work we have studied the regulation of expression of the PT operon and investigated why PT is produced by phase I and not by phase III B. pertussis despite the presence of the PT genes. We have constructed a vector for Bordetella species that contains the PT promoter fused to the coding region of the chloramphenicol acetyltransferase (CAT) gene, and we have used it to identify the regulatory elements involved in the transcription of the PT operon. Efficient transcription of these genes requires at least two features: (i) the 170-base-pair DNA sequence upstream from the start site of transcription and (ii) a trans-activating factor encoded by the vir locus. Bordetella parapertussis and Bordetella bronchiseptica, although endowed with a functional trans-activating system, do not produce PT because of mutations within their PT promoter regions. In contrast, phase III Bordetella species do not show any trans activity.

The human pathogens Bordetella pertussis and Bordetella parapertussis and the animal pathogen Bordetella bronchiseptica are closely related species that produce many closely related virulence factors (1). Each of the three species can switch from the virulent phase, phase I, to an avirulent phase, phase III, in which none of these factors are produced. In the case of B. pertussis it has been shown that a single gene locus, vir, controls the coordinate expression of many factors associated with virulence (2).

Pertussis toxin (PT), an oligomeric protein composed of five different subunits, believed to play a major role in the pathogenesis of whooping cough, is produced only by phase I B. pertussis (3–5). B. parapertussis and B. bronchiseptica contain transcriptionally silent PT genes (6). Comparison of the nucleotide sequences of the PT genes in B. pertussis (7, 8) and B. parapertussis and B. bronchiseptica (6) has shown that the last two species, although containing several base-pair substitutions scattered all over the genes, could encode functional toxins (6). We previously proposed that the presence of an unusual accumulation of mutations common to the two species within the promoter of the PT operon and its upstream region could explain their inability to transcribe their PT genes (9).

To study the expression of the PT operon in the three Bordetella species, we have constructed a promoter-probe vector for Bordetella containing a B. pertussis PT promoter fused to the chloramphenicol acetyltransferase (CAT) gene; we used this fusion to show that at least 170 base pairs (bp) upstream from the start site of transcription, together with a functional vir locus, are required for PT expression. Furthermore, we demonstrate that B. parapertussis and B. bronchiseptica contain a functional vir locus and that the lack of transcription of their PT genes is due to mutations in their regulatory region.

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MATERIALS AND METHODS

Strains. The vir+ B. pertussis strain BP356 (ptx::Tn5) and the vir− strain BP347 (vir::Tn5) were obtained from S. Falkow (10). B. parapertussis P14 was isolated in 1986 in the United States (6). B. bronchiseptica CCUG 7865 was obtained from the Culture Collection of the University of Göteborg, Sweden (6). Spontaneous phase III derivatives of these Bordetella strains were isolated in our laboratory. For the conjugation experiments, spontaneous nalidixic acid-resistant and streptomycin-resistant mutants of these strains were used. The Escherichia coli strains JM101 and SM10 have been described elsewhere (ref. 11, p. 506; ref. 12).

Cloning and Sequencing. The CAT gene–promoter fusions were made in the low copy number vector pLAFLR2, a derivative of the broad host range plasmid RK2 (13). A HindIII/Xba I DNA fragment containing the promoterless CAT gene of plasmid pA10-CAT2 (14) and the BamHI/HindIII fragments containing the PT promoters were ligated with pLAFLR2 that had been digested with BamHI/Xba I, resulting in the pPB plasmid series (Fig. 1). In the case of the negative control plasmid pLAFLR-CAT, the promoterless CAT gene was cloned in this vector by using the BamHI/HindIII linker of pEMBL18 (15). As a second control, we constructed the plasmid pBP255, in which the region upstream of the Kpn I site (Fig. 3) was replaced with the 600-bp BamHI/HindIII fragment of pTE255 (16), which encodes the S1 subunit of PT but does not contain a promoter. The deletions in the upstream region of the PT promoter were generated with the exonuclease BAL-31 (17). For this purpose the EcoRI/HindIII fragment (Fig. 3) containing the PT promoter with its 5′ region was cloned in the vector Bluescript SK (Stratagene, San Diego, CA) that had been digested with EcoRI/HindIII. The resulting plasmid was linearized at its EcoRI site, partially digested with BAL-31, treated with the Klenow fragment of DNA polymerase I to make blunt ends (ref. 11, p. 113), and digested with HindIII. The restriction fragments were then separated on a 4% polyacrylamide gel. Fragments 50–250 bp long were electroeluted and cloned in the vector Bluescript SK digested with SmaI/HindIII for the sequence analysis. The BamHI/HindIII fragments of the deletion mutants shown in Fig. 3 were isolated and cloned in pLAFLR2 as described above. The deletion plasmid pBP1 has been constructed by deleting the region upstream from the Kpn I site at sequence position −62 (Fig. 3). For the transfer of these constructions into Bordetella species, the mobilizing strain SM10 (12) was used. The conjugations were carried out with fresh cultures of the recipient and donor strains on Bordet–Gengou plates (18) for at least 6 hr. Exconjugants were selected on Bordet–Gengou plates containing tetracycline at 10 µg/ml, streptomycin at 20 µg/ml, and nalidixic acid at 20 µg/ml.

CAT Assay. Bordetella strains were grown in Stainer–Scholte medium (19) containing tetracycline at 10 µg/ml to an
optical density at 580 nm of 0.8. Then 1.5 ml of the culture was centrifuged briefly and the cells were resuspended in 300 μl of 0.25 M Tris-HCl, pH 7.8. The cells were then disrupted by sonication and centrifuged at 12,000 × g for 10 min. The supernatant was incubated at 65°C for 10 min, centrifuged, and used in the CAT assay (20, 21). Fifteen microliters of the lysate was diluted to 150 μl in 0.25 M Tris-HCl, pH 7.8, and 1 μCi (1 Ci = 37 GBq) of [14C]chloramphenicol was added. After incubation at 37°C for 5 min, 20 μl of 4 mM acetyl-coenzyme A was added and the reaction mixture was incubated at 37°C for 1 hr. The reaction was then stopped by extracting the chloramphenicol and its derivatives with 2 ml of cold ethyl acetate. The organic phase was evaporated and the different chloramphenicol forms were separated by thin-layer chromatography using silica gel plates and a 95:5 (vol/vol) chloroform/ethanol mixture. Qualitative results were then visualized by autoradiography of the thin-layer plates. For the quantitative assays, dilutions of each sample were analyzed to identify the linear range of the reaction and then assayed. After autoradiography the nonacetylated and monoacetylated chloramphenicol forms were cut out and their radioactivities were measured.

RESULTS

A Region Upstream from the Promoter Is Required for PT Expression in B. pertussis. We have previously defined the promoter region of the PT operon as a region homologous to the −35 and −10 consensus sequence of E. coli promoters, spanning approximately 40 bases from the start site of transcription (7, 20). This promoter is inefficient in E. coli (20). To study the properties of this promoter in Bordetella species, we used the CAT indicator gene cloned in the broad host range vector pLAFR2 (Fig. 1). Two plasmids containing 62 and 483 bp upstream from the start site of transcription cloned in front of the promoterless CAT gene (pBP1 and pBP2, respectively) (Fig. 3), were introduced by conjugation into the vir+ B. pertussis strain BP356 and into the vir− strain BP347. As shown in Fig. 2, strong promoter activity was found only in BP356 containing the construction pBP2. The absence of the region upstream from the promoter, as in pBP1, or the absence of the active vir locus, as in BP347, caused a strong decrease of the CAT activity. Primer extension experiments showed that the natural start site of transcription of the PT promoter is also used in pBP2 (data not shown).

To verify that the results obtained are due only to the specific sequence of the region upstream from the PT promoter, we used two controls: pLAFR-CAT, in which no promoter is present upstream from the CAT gene, and pBP255, in which a fragment of Bordetella DNA without a promoter was cloned upstream from the PT promoter. In both a vir+ and a vir− background, no CAT activity could be detected in pLAFR-CAT, whereas pBP255 had the same activity as pBP1 (data not shown), indicating that the specific sequences upstream from the PT promoter are required for trans-activation.

Mapping of the Promoter Upstream Region. To further define the region required for the PT expression, we used the exonuclease BAL-31 to generate a series of progressive deletions of the promoter upstream region (Fig. 3). Several of the deletion mutants were then introduced into the B. pertussis strains BP356 and BP347 and their CAT activities were determined. As shown in Fig. 4, in the vir+ background there is a high expression of the CAT activity when at least 170 bp of the upstream region are present. A first reduction to about 45% of the CAT activity is observed when the nucleotides −170 to −158 are deleted. A second decrease to about 10% of the original activity is found when the −149 to −130 region is deleted. The CAT activity goes down to below 1% when a deletion destroys the −35 box of the promoter. All the deletion mutants had a low level (about 10%) of CAT activity in the vir− strain BP347. In this strain also a decrease in CAT activity was observed when the −35 box of the promoter was removed.

Expression of the PT Promoter in B. parapertussis and B. bronchiseptica. The inability of B. parapertussis and B. bronchiseptica to express their PT genes could be due either to the absence of a functional trans-activating system or to an inactive promoter region. To clarify whether the two species are able to trans-activate the B. pertussis PT promoter region, the constructions described in Fig. 3 were introduced into phase I B. parapertussis and B. bronchiseptica and the CAT
activity was determined. Both species were found to be able to activate the PT promoter region; in fact, the results were identical to those found with B. pertussis (Fig. 5). To test the effect of the mutations upstream of the promotor in B. parapertussis and B. bronchiseptica (Fig. 3) the region between the EcoRI site (−483) and the Kpn I site (−62) of the plasmid pBP2 was replaced with the corresponding region of B. bronchiseptica, and the CAT activity of the new plasmid (pBB2) was measured in phase I B. parapertussis and B. bronchiseptica (Fig. 5). The CAT activity of pBB2 was only 40% of the activity of the wild-type pBP2. This demonstrates that the base-pair substitutions reduce the efficiency of the control region upstream of the PT promoter.

Phase III B. parapertussis and B. bronchiseptica showed the same level of CAT activity as the vir− B. pertussis strain BP347 (not shown), confirming that spontaneous phase III mutants and vir− mutants are phenotypically equivalent (2).

**DISCUSSION**

**Properties of the PT Promoter Region.** We have developed a system for the study of gene regulation at the molecular level in Bordetella species and used it to investigate the regulation of pertussis toxic expression. The analysis of the CAT expression in the different deletion mutants shows that the 170-bp DNA sequence upstream from the start site of transcription is necessary for full PT promoter activity. Within this region we have found structures typical of binding sites of regulatory proteins, already described for other genes (22–25): (i) a palindromic sequence in position −182 to −170 (Fig. 3); a 21-bp sequence in position −157 to −137, repeated after two turns of the DNA helix (Figs. 3 and 6).

The CAT activity of the deletion mutants can be grouped into four levels (A, B, C, and D) (Fig. 4), and it correlates well with the structural properties of the DNA sequence. Level A: when at least 170 bp of the 5′ region are present, the CAT activity is maximal. Level B: removal of part of the first repeated sequence (pBP24) or a deletion immediately upstream from it (pBP20) (which might change its conformation) reduces the CAT activity to about 45%. Level C: when the second repeated sequence is partially or totally removed (pBP46, pBP28, pBP72, pBP33, pBP1) the ability of the 5′ region to be trans-activated is lost. The CAT activity is the same as in the vir− background. This indicates that the repeated sequence shown in Fig. 6 is very likely to be the binding site for the trans-activating factor(s). Level D: when the −35 box of the promoter is removed (pBP22), the CAT activity decreases, in both vir+ and vir− backgrounds to below 1%—i.e., to the level observed in pLAFR-CAT, in which no promotor is present.

Note that removal of the sequence between −182 and −171 resulted in a significant increase of the CAT activity (Fig. 4). This correlates with the deletion of the palindromic sequence at position −182 to −170, suggesting that the palindromic sequence might play a role in the regulation of PT transcription.

**B. parapertussis and B. bronchiseptica.** The published data show that the PT promoters of B. parapertussis and B. bronchiseptica are inactive (6, 20). We have previously shown that constructs that include 62 bp upstream from the start site of transcription of B. parapertussis and B. bron-
B. bronchiseptica PT operons show approximately 20% of the transcriptional activity of the corresponding sequence of B. pertussis (20). In this paper we have shown that a hybrid construct between the −62 B. pertussis region and the −483 B. bronchiseptica region also results in an efficiency of transcription lower than that shown by B. pertussis. This suggests that the mutations present in the −62 to −170 region of B. bronchiseptica are altering a sequence that is involved in the regulation of transcription of the PT operon in Bordetella. This is in agreement with the results of the deletion analysis of the 5′ region, which defines the position of the promoter of the PT operon in the sequence between −170 and +1. We conclude that the large number of mutations within this region found in the B. parapertussis and B. bronchiseptica PT operons could explain the absence of pertussis toxin synthesis.

Note that the point mutations common to B. parapertussis and B. bronchiseptica are concentrated within the promoter region (Fig. 7). These data suggest the action of a selective pressure against PT expression in these species and that PT production, strictly required for growth of B. pertussis in vivo, has been selectively eliminated by B. parapertussis and B. bronchiseptica, which have developed the ability of surviving in less stringent growth conditions (1).

We thank Alfredo Nicosia, who commended the work on the functional analysis of the PT promoter, and Beatrice Ariciò, who gave us the cloned PT promoter of B. bronchiseptica and participated in helpful discussions. Furthermore, we thank Maria Luisa Melli, Shona Murphy, and Emanuela Palla for critically reading the manuscript, Giorgio Corsi for preparing the drawings, Maria Perugini for technical help, and Susan Weemys for typing the paper. R.G. was supported by a postdoctoral fellowship of the Commission of the European Communities. This work was supported by a grant of the Ente Nazionale Idrocarburi.

Fig. 6. Comparison of the repeated sequences in the PT promoter upstream region. The bars indicate the identical nucleotides.

-157 GATTCGTC. GTACAAGACCCCTC -137
-136 GATTTCTTCGTAACCCC -117

Fig. 7. Structure of the PT operon, showing the distribution of the mutations that are common to B. parapertussis and B. bronchiseptica (vertical bars above the sequence).