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The Pertussis Toxin Liberation Genes of *Bordetella pertussis* Are Transcriptionally Linked to the Pertussis Toxin Operon

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The DNA sequence of the pertussis toxin operon (*ptx*) of *Bordetella pertussis* predicts that transcription of the operon ends downstream from the *ptxS3* gene at a possible stem-loop structure. Secretion of the assembled pertussis toxin into the culture medium requires the expression of 8 genes arranged in an operon (*ptl*) and lying 55 bp downstream from the *ptx* operon. We have investigated the role of this 55-bp intergenic region in transcriptional regulation of the *ptl* operon. Our results show that *ptx* transcripts transverse this DNA region and that the *ptx* and *ptl* operons are cotranscribed and coregulated by the P_{TOX} promoter. Deletion of the 55-bp DNA region caused an increase in the amount of the *ptl* transcripts. It is likely that this DNA region is involved in regulation of the *ptx-ptl* expression.

The bacterium *Bordetella pertussis* is the causative agent of whooping cough, an acute respiratory disease in humans. During infection, the bacterium releases a number of toxins that cause the systemic symptoms of the disease. In response to environmental stimuli, the expression of these proteins, and of many others, is coordinately regulated at the transcriptional level by the *bvgAS* locus (1, 18). Virulence factors are synthesized at 37°C, while their expression is repressed at 25°C or upon addition of $MgSO_4$ or nicotinic acid to the culture medium (4, 9, 14, 15).

Pertussis toxin is a major factor released into the extracellular environment by the bacterium and is composed of five subunits, S1, S2, S3, S4, and S5 (10). In order to be exported into the external environment, pertussis toxin requires the expression of an operon encoding 8 genes, spanning approximately 9.5 kb and named *ptlA* through *ptlH* (5, 6, 19). These genes are located 55 bp downstream from the pertussis toxin operon, *ptx* (10), and each of the deduced amino-acid sequences of the PTL proteins shares a high degree of similarity with the VirB proteins of *Agrobacterium tumefaciens* (5, 19). The VirB proteins are thought to be involved in the transport of single-stranded DNA across bacterial membranes (8). Recently, Kotob and coworkers (7) and Baker and coworkers (2) have provided evidence that the *ptl* operon is controlled by the *ptx* promoter (P_{TOX}).

A schematic representation of the *ptx-ptl* locus structure is shown in Fig. 1A. Downstream from the S3 gene of *ptx* there is a potential stem-loop structure (Fig. 1B) that recalls a rho-independent termination signal of transcription (10). To analyze the transcriptional regulation in this DNA region, we carried out S1 nuclease mapping experiments (3) with total RNA extracted from cultured *B. pertussis* cells.

The results of urea-acrylamide gel electrophoresis shown in Fig. 2A are from an S1 nuclease protection experiment carried out by hybridizing the 286-bp DNA probe (Fig. 1A), labeled at the *Mlu*I site, to RNA of wild-type *B. pertussis* BPW28 grown at 35 or 25°C. RNA extracted from strain BPC (13), grown at 35°C and defective in BpH1 protein (wild type with respect to virulence gene expression at 35°C), was also included. Figure 2A shows a major S1 protected band of 232 nucleotides (nt), a minor band of 170 nt, and several bands migrating to a position between 130 and 148 nt (lanes 4 and 5). Interestingly, the same RNA sample shows a full-length protection (286 nt) of the DNA probe from S1 nuclease digestion, suggesting that longer RNA molecules cover the entire length of the DNA probe. RNA extracted from *B. pertussis* cells grown at 25°C (Fig. 2A, lane 6) does not show DNA bands protected from S1 digestion. Consequently, transcription of this DNA region is repressed at this temperature. The 232- and 170-nt RNA bands define 3′-end RNAs mapping downstream from the ATG start codon of *ptlA*. These RNA bands are likely to arise from intermediate products of in vivo RNA degradation. The 130- to 148-nt RNA bands map 10 to 28 nt downstream from the stem-loop structure (Fig. 1B), suggesting that this structure functions in vivo as a transcriptional termination signal. However, since the DNA probe was fully protected by longer RNA molecules and the 3′-end RNAs were found downstream from this signal, this termination signal should have a poor transcription termination efficiency. In order to estimate the efficiency of transcription termination at this stem-loop structure, we evaluated the amount of radioactivity retained by these bands by laser scanning the autoradiogram of Fig. 2A. The total radioactivity of lane 4 was evaluated, subtracted from the background of the experiment (lane 3), and set to 100. The relative intensity of the fast-migrating bands (130 to 148 nt) was evaluated as 26% of the total RNA-dependent signals. This value is likely to represent the in vivo efficiency of the transcriptional termination signal.

To map the 5′-end RNA in this region, we used the 286-bp DNA fragment labeled at the *Hin*cII site as a probe (Fig. 1A) in S1 nuclease mapping experiments (Fig. 2B). The RNA extracted from cells grown at 35°C shows a major band migrating at a position corresponding to 234 nt (Fig. 2B, lane 4). This band defines a major 5′ end of RNA located 114 bp upstream from the *ptlA* ATG start codon and mapping within the coding region of the S3 gene. The same RNA sample shows a full-length protection (286 nt) of the DNA probe from S1 digestion, confirming that longer RNA molecules cover the entire length of the probe used. RNA extracted from *B. pertussis* cells grown at 25°C does not show DNA bands, nor does it show

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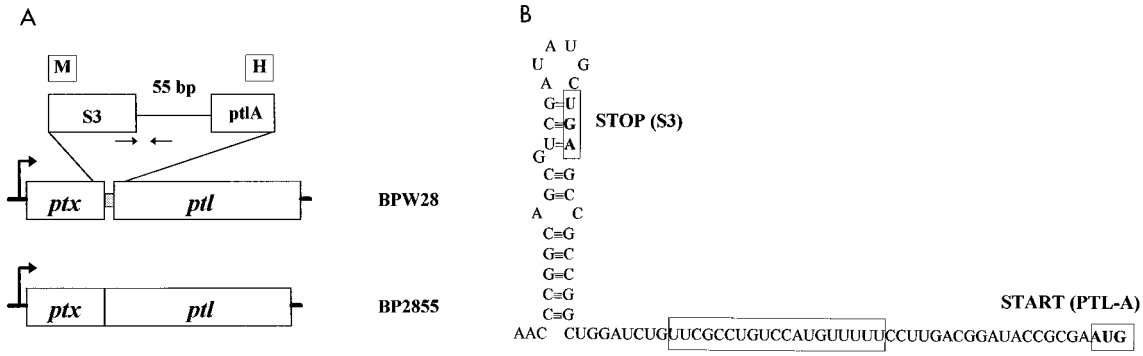

FIG. 1. (A) Schematic representation of the strains used (not drawn to scale). BPW28 is a wild-type strain; the region between *ptxS3* and *ptlA* has been enlarged. *B. pertussis* BPW28 (Bvg^+ Sm^r) (13) and its derivative BP2855 were grown on Bordet-Gengou agar plates containing 15% defibrinated blood (11) or in Stainer-Scholte-modified liquid medium (17). *E. coli* SM10 (16) was used for conjugation with *B. pertussis*. The chromosomal deletion of the 55-bp intergenic region was obtained by conjugation with *E. coli* transformed with plasmid pSS1129 (18) containing 485 bp of the 3′-end region of the *ptxS3* gene ligated to the 956-bp 5′ end of the *ptlA* gene. These fragments, obtained by PCR with *Taq* DNA polymerase (Perkin-Elmer) and synthetic oligonucleotides as primers, joined the stop codon of the *ptxS3* gene to the start codon of the *ptlA* gene, thus creating the deletion of the 55-bp region between *ptS3* and *ptlA*. DNA manipulations were carried out by standard techniques (12). Antibiotics (Sigma) were used at the following concentrations: ampicillin, 100 μg/ml; streptomycin, 400 μg/ml; kanamycin, 25 μg/ml; gentamicin, 10 μg/ml; nalidixic acid, 20 μg/ml. Symbols: open boxes, the indicated operons; thick arrow, the direction of transcription of the P_{TOX} promoter; M, *Mlu*I; H, *Hin*cII. (B) The nucleotide sequence including the 55-bp region between the *ptx* and *ptl* operons. The stem-loop structure at the end of the *ptxS3* gene is shown along with its translational stop codon and the PTLA start codon (in boldface type). The framed nucleotides indicate sites of transcriptional termination (see the text).

full-length protection of the probe (Fig. 2B, lane 6). This suggests a *bvg*-dependent regulation of these messages.

To further investigate the role of the 55-bp region between *ptx* and *ptl*, we generated a *B. pertussis* mutant strain carrying the deletion of the 55-bp intergenic region (Fig. 1A). A recombinant fragment was designed to join the stop codon of the *ptxS3* gene to the start codon of the *ptlA* gene, cloned into the conjugative pSS1129 (18) plasmid, and transferred to the *B. pertussis* chromosome by genetic recombination, following conjugation with *Escherichia coli*. Exconjugants were analyzed for chromosomal DNA substitution by Southern blot analysis and for the expected changes of RNA transcription by S1 nuclease mapping or primer extension experiments (data not shown). Selected positive strains were then used to evaluate the relative abundance of the *ptl* transcripts in Northern (RNA) blot analysis.

Northern blots were carried out on total RNA extracted from the wild-type strain grown at 35 or 25°C and on RNA

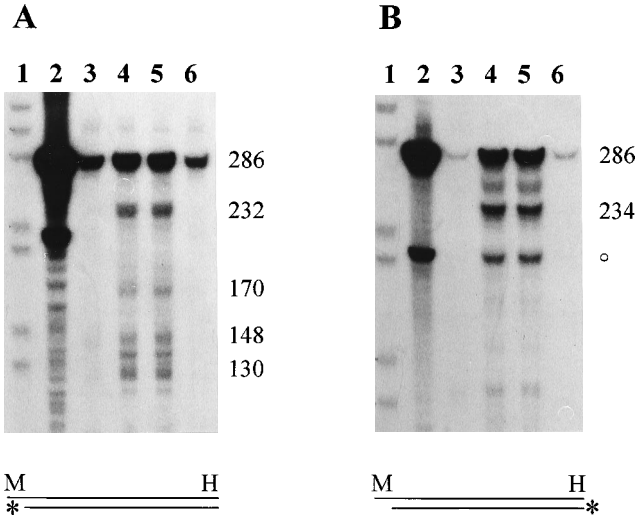

FIG. 2. Mapping RNA ends between *ptx* and *ptl* genes. (A) 3′ ends of RNAs. The 286-bp *Mlu*I-*Hin*cII DNA fragment (schematically represented below the gel) from plasmid pT110 (10) was end labeled with Klenow polymerase (New England Biolabs) and [α-^{32}P]dCTP and used for hybridization to RNA and S1 nuclease digestion analysis (3) as previously detailed (14, 15). RNA was extracted from cells of *B. pertussis* BPW28 (lane 4), strain BPC cells grown at 35°C (lane 5), or strain BPW28 cells grown at 25°C (lane 6). Total RNA was extracted from 25 ml of *B. pertussis* cells grown to an optical density of 0.8 at 590 nm, harvested by centrifugation, and stored at −20°C. Cells were lysed in 3.7 ml of 100 mM Tris-HCl (pH 7.5)–1% sodium dodecyl sulfate–2 mM Na_2EDTA for 5 min at 100°C. After 5 min on ice in the presence of 80 mM KCl, debris was removed by spinning at 8,000 × *g* for 10 min and processed as previously described (14, 15). Control samples were processed in an identical manner but contained no RNA and were (lane 3) or were not (lane 2) digested with S1 nuclease. A denatured 1-kb ladder (Gibco-BRL) served as size markers (lane 1). Bands are indicated by molecular size migration (in base pairs). Hybridization was performed in a water bath for 15 h at 58°C. Asterisks indicate the labeled extremities of the probes. (B) 5′ ends of RNAs. The 286-bp *Mlu*I-*Hin*cII fragment was end labeled with T4 polynucleotide kinase (New England BioLabs) and [γ-^{32}P]ATP (Amersham) and used for S1 nuclease protection experiments. The RNA band marked with a circle was excluded from our analysis because it was also present in the DNA probe (lane 2).

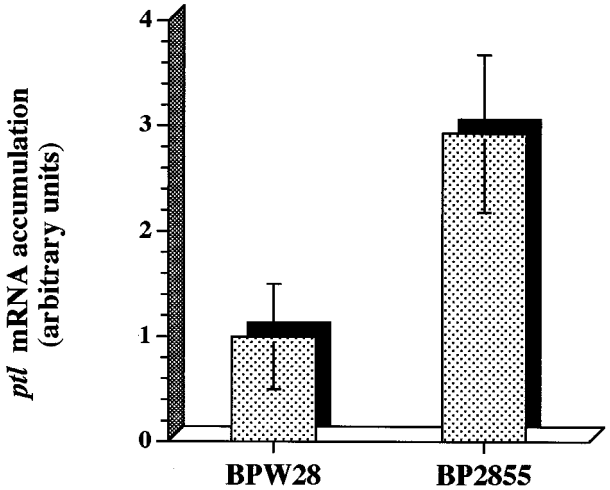

FIG. 3. *ptl* mRNA accumulation. Ten micrograms of total RNA was used for Northern blot experiments. Total RNA was extracted from the same number of wild-type (BPW28) and mutant (BP2855) *B. pertussis* cells (optical density at 590 nm = 0.8) as described in the legend to Fig. 2. Values represent the average of three independent experiments and were obtained by laser scanning the retained radioactivity on Northern blots hybridized to a ca. 600-bp fragment covering *ptlB* and part of the *ptlC* sequence. The amount of radioactivity measured in the RNA sample extracted from the wild-type BPW28 strain was set to 1. Vertical bars represent standard deviations.

from the mutant strain grown at 35°C, and the RNAs were hybridized to a *ptl* probe. As expected, the DNA probe hybridized to RNA from the wild-type strain grown at 35°C but did not hybridize to RNA extracted from the cells grown at 25°C. Therefore, transcription of the *ptl* genes was induced at 35°C and was repressed at 25°C. Thus, the operon is environmentally regulated by the *bvg* locus as the *ptx* operon (14). Interestingly, RNA extracted from the BP2855 mutant strain grown at 35°C hybridized to the *ptl* probe with a different intensity. The relative intensities of the wild-type and mutant RNA bands were evaluated by laser scanning of the autoradiograms, with the results shown in Fig. 3. Strain BP2855, carrying the deletion of the 55-bp fragment, showed that the accumulation of the *ptl* transcripts was at least 30% higher than that for the wild-type BPW28 strain. This is in agreement with the S1 nuclease mapping experiments reported in Fig. 2A, from which we estimated that the efficiency of transcription termination at the stem-loop structure is about 26% of the total message. The same Northern blots were then assayed with a *ptx* probe, and no difference in the amount of *ptx* mRNA was detected among these strains.

We conclude that the 55-bp intergenic region contains information for a weak transcriptional termination of the *ptx* operon, whose read-through is responsible for the transcriptional regulation of the downstream *ptl* operon. We propose that the 55-bp intergenic region is responsible for the correct equilibrium between the available amount of pertussis toxin that has to be delivered outside the cell and the necessary amounts of the PTL factors.

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