

High-level transcription of the *cryIII*A toxin gene of *Bacillus thuringiensis* depends on a second promoter located 600 bp upstream of the translational start site

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ABSTRACT

The *cryIII*A gene expression was analyzed in a low-copy number plasmid background by using deletion derivatives of a larger DNA fragment carrying the toxin gene and additional adjacent regions. The results indicated that a 1-kb DNA segment (the H₂-H₃ fragment) located 400 bp upstream from the presumed promoter strongly increases *CryIII*A toxin production in *Bacillus thuringiensis* sporulating cells at the transcriptional level (De-Souza *et al.*, *J. Bacteriol.* 175: 2952-2960, 1993). The delineation and functional analysis of this region showed that a functional promoter is located within the H₂-H₃ fragment, situated 400 bp from the promoter previously described and about 600 bp upstream of the *cryIII*A gene coding region. The association of the region containing this promoter with the DNA sequence directly upstream from the *cryIII*A gene is required to obtain full expression of the transcriptional fusions. The putative -35 e -10 regions of this upstream promoter do not match the sporulation-specific *cry* genes promoter sequences, but are similar to those recognized by the RNA polymerase bound to σ^A .

INTRODUCTION

Bacillus thuringiensis (Bt) is characterized by its capacity to synthesize insecticidal proteins during sporulation. These proteins, also called δ -endotoxins, are classified according to their amino acid sequence and toxic activity towards the larvae of insects within the orders Lepidoptera, Diptera, and Coleoptera (Höfte and Whiteley, 1989). The *Cry* proteins are synthesized in large amounts (in some subspecies it can account for 20-30% of the dry weight of sporulated cells), ultimately

accumulating as a parasporal crystalline inclusion. The crystal protein (*cry*) genes are developmentally and spatially controlled (Ribier and Lecadet, 1973). The temporal gene expression of the *cryI* genes is, at least in part, assured by the successive activation of the sporulation-specific sigma (σ) factors (σ^{35} and σ^{28}), which transcribe lepidopteran specific genes from dual overlapping promoters (Adams *et al.*, 1991). Similar promoter regions are found upstream of *cryII*, *cryIV*, and *cyt* genes (Lereclus *et al.*, 1989). However, the coleopteran-specific *cryIII*A genes seem to bear a distinct regulatory mechanism.

Toxin production was found to be low in an acrySTALLIFEROUS (*Cry*⁻) *B. thuringiensis* strain containing the *cryIII*A gene cloned with its promoter region, and its putative terminator located in a low-copy number plasmid (Arantes and Lereclus, 1991). We previously (De-Souza *et al.*, 1993) identified a ~1-kb DNA fragment (hereafter designated H₂-H₃ fragment) required for full

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expression of the *cryIIIA* gene in *B. thuringiensis*. The 3'-end of this fragment is located ca. 400 bp upstream of the *cryIIIA* gene promoter (P1) (Sekar et al., 1987). Analysis of the start sites, the sizes, and the amounts of the *cryIIIA*-specific mRNA showed that enhancement occurs at the transcriptional level by increasing the number of these transcripts from the onset of sporulation (t_0) to about six hours after (t_6) with maximum production between t_3 and t_6 . In this paper, we report an outline and functional analysis of the H₂-H₃ fragment.

MATERIAL AND METHODS

Bacterial strains and growth conditions

Escherichia coli K-12 TG1 [$\Delta(lac-proAB)$ *supE thi hdsD* (*F'* *traD36 proA⁺ proB⁺ lacI^q lacZ* M15)] (Gibson, 1984) was used as the host for M13 bacteriophage. *E. coli* MC1061 [*hsdR mcrB araD139* $\Delta(araABC-leu)$ 7679 $\Delta lacX74 galU galK rpsL thi$] (Meissner et al., 1987) was the host for construction of the plasmids described in Figure 2. The *cryIIIA* gene was isolated from *B. thuringiensis* strain LM79 (Chaufaux et al., 1993). The synthesis of the corresponding gene product in this strain matches that of other *B. thuringiensis* strains carrying the *cryIIIA* gene (Herrnstadt et al., 1987; Sekar et al., 1987; Donovan et al., 1988). *B. thuringiensis* subsp. *kurstaki* strain HD1 Cry⁻ B (kindly provided by A.I. Aronson) was used as the host for the *lacZ* fusion analysis. The bacteria were cultured, transformed, and screened as previously described (De-Souza et al., 1993).

Plasmid constructions

Plasmids pHT304'*lacZ* and pHT7901'*lacZ* (De-Souza et al., 1993) were used to construct the transcriptional fusions. Except for the H₂-H₃ fragment present in the recombinant pHT7920'*lacZ*, all the fragments used in these constructions are in the 5' → 3' orientation with regard to the *lacZ* gene coding sequence. Plasmid pHT7903'*lacZ* was obtained by cloning the D₃-D₄ fragment (Figure 1) in the unique *Sma*I site of pHT304'*lacZ* vector. Plasmids pHT7904'*lacZ* and pHT7905'*lacZ* were constructed as follows: i) the plasmid pHT304'*lacZ* was digested with *Hind*III restriction enzyme and treated with the Klenow fragment of DNA polymerase I and deoxyribonucleoside triphosphates, ii) the linearized and blunt-ended pHT304'*lacZ* was digested with *Pst*I restriction enzyme, and iii) the B-P₁ and A₂-P₁ fragments (Figure

1) were cloned in ii, respectively. Plasmid pHT7920'*lacZ* was obtained by cloning the H₂-H₃ fragment in the opposite orientation (3' → 5'; see Figure 1) into the unique *Hind*III site of pHT7901'*lacZ* (De-Souza et al., 1993). Plasmid pHT7922'*lacZ* was obtained by cloning two copies of the H₂-H₃ fragment in tandem in the same vector. The orientation of the H₂-H₃ fragment and derivatives was determined using convenient restriction endonuclease sites present in the recombinant plasmids. The general structure of the recombinant plasmids carrying the *lacZ* fusions is shown in Figure 2.

DNA manipulation

Standard procedures were used to extract plasmids from *E. coli*, for the transfection of M13 recombinant phage DNA and to purify single-stranded DNA (Sambrook et al., 1989). Restriction enzymes, T4 DNA ligase, T4 polynucleotide kinase were used as recommended by the manufacturers. DNA restriction fragments were purified from agarose gels using a Prep A gene kit (Bio-Rad). Nucleotide sequences were determined by the dideoxy-chain termination method (Sanger et al., 1977) using M13mp18 phage templates, Sequenase version 2.0 kit (US Biochemical Corp., Cleveland, Ohio, USA), and [α -³⁵S] dATP (15 TBq, Amersham, UK).

RNA extraction and primer extension analysis

B. thuringiensis subsp. *kurstaki* strain HD1 Cry⁻ B (pHT305P) was cultured and the RNA manipulated as previously described (De-Souza et al., 1993). A sample was taken at t_3 . For the primer extension experiment a 32 mer oligonucleotide primer (3'-CAATCTATTCGTAACCTCCATCTCAGGCAGGC-5') complementary to nucleotides (nt) 1190 to nt 1222 was synthesized and 5'-end labelled with [γ -³²P] dATP (110 TBq/mmol) by T4 polynucleotide kinase. The oligo was purified on a G25 Sephadex (Pharmacia) column (incorporation was about 70%). A primer extension experiment was performed as previously described (De-Souza et al., 1993). The same 32 mer primer was used to extend the H₃P₁ fragment cloned in M13mp19 and the resulting reaction was run on gels in parallel with the products of transcription.

β -galactosidase assay

E. coli and *B. thuringiensis* strains containing *lacZ* transcriptional fusions were manipulated, stored, and assayed for β -galactosidase activity, as described by

De-Souza *et al.* (1993). Sampling was at t_{-2} , t_{-1} (two and one hour before the onset of sporulation), t_0 , $t_{1.5}$, t_3 , $t_{4.5}$, t_6 and $t_{7.5}$. The specific activities presented (expressed in Miller units per milligram of protein) are the mean of at least two independent experiments.

RESULTS

Partial characterization of the minimal *cryIII A* gene full expression region

The *cryIII A* gene was poorly expressed in a recombinant reproducing wild-type strain conditions (low-copy number plasmids; Arantes and Lereclus, 1991), where the gene was flanked only by the promoter (P1) described by Sekar *et al.* (1987) and a putative terminator (Donovan *et al.*, 1988). This observation led us to map a DNA fragment of about 1 kb, located ca. 400 bp upstream of P1 (De-Souza *et al.*, 1993). This segment of DNA (H₂-H₃ fragment) contained an element which strongly enhanced the CryIII A protein production in *B. thuringiensis*. Analysis of the start sites, the sizes, and the amounts of the *cryIII A*-specific mRNA revealed that the improvement in toxin production occurred at the transcriptional level by increasing the *cryIII A* transcription from the onset of sporulation (t_0) to about six hours (t_6). A schematic representation of the H₂-H₃ fragment as well as the adjacent 713 bp of the H₃-P₁ fragment is presented in Figure 1. To go further in the analysis of the *cryIII A* expression region, a new set of *lacZ* transcriptional fusions was constructed. The H₂-H₃ fragment was cloned in the opposite orientation into the unique *Hind*III site of pHT7901'*lacZ* (Figure 2A) to give

the plasmid pHT7920'*lacZ* (Figure 2B). Two copies of this fragment were cloned in tandem (both in the wild-type orientation) to provide the plasmid pHT7922'*lacZ* (Figure 2B). The minimal fragment involved in the transcription of the *cryIII A* gene was assessed by cloning different restriction fragments upstream of the *cryIII A* gene. The plasmid pHT7903'*lacZ* (Figure 2B) was generated by cloning the D₃D₄ fragment (Figure 1) in the unique *Sma*I site of the plasmid pHT304'*lacZ* (Figure 2A). Finally, the plasmids pHT7904'*lacZ* and pHT7905'*lacZ* (Figure 2B) were constructed by cloning the B-P₁ and A₂-P₁ fragments (see Figure 1) in the plasmid pHT304'*lacZ* (Figure 2A), respectively. Plasmids pHT304'*lacZ*, pHT7901'*lacZ*, pHT7920'*lacZ*, pHT7922'*lacZ*, pHT7903'*lacZ*, pHT7904'*lacZ*, and pHT7905'*lacZ* were introduced into *B. thuringiensis* subsp. *kurstaki* strain HD1 Cry⁻ B by electroporation. Each strain was induced to sporule and samples were harvested at t_{-2} and t_{-1} and from t_0 to $t_{7.5}$, at 1.5-h intervals and assayed for β -galactosidase activity (Figure 3). The β -galactosidase specific activities of the strains carrying the parental plasmid pHT304'*lacZ* or the derivative pHT7920'*lacZ* were constant, at about 800 and 350 Miller units from t_{-2} to $t_{7.5}$, respectively (Figure 3). In the strain bearing the other parental plasmid, pHT7901'*lacZ*, the level of enzyme synthesis slightly increased from about 250 Miller units at t_{-2} , t_0 to about 1200 Miller units at $t_{7.5}$. Due to the scale used, this enhancement is not apparent in Figure 3. The profiles of enzyme production for the recombinants carrying the plasmids pHT7903'*lacZ*, pHT7904'*lacZ*, pHT7905'*lacZ*, and pHT7922'*lacZ* were similar both in shape and level. The β -galactosidase specific activity was at about 30,000 Miller units between t_6 and $t_{7.5}$ (Figure 3) and enhancement was

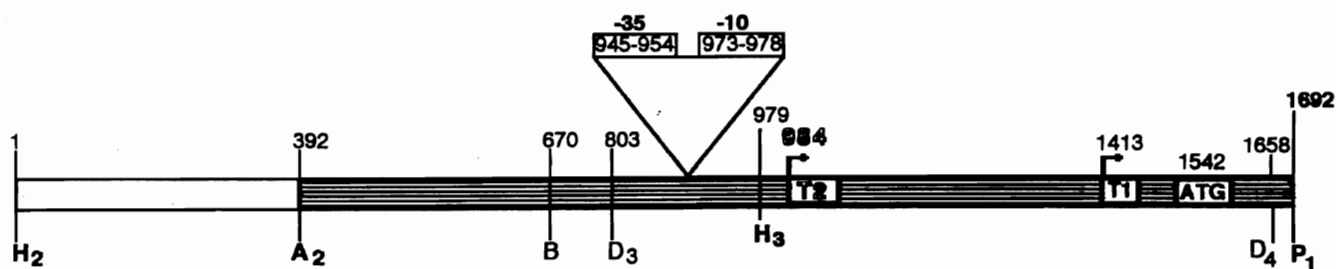


Figure 1 - Upstream region of *cryIII A*. The physical map of the H₂-P₁ fragment (H₂-H₃ plus H₃-P₁) is described in the 5' → 3' orientation with regard to the *cryIII A* gene coding sequence (De-Souza *et al.*, 1993). Note that the numbers of the nucleotides are not related to the initiation of transcription, but to the fragments sequenced for this study. The nucleotide positions of the two *Hind*III sites (H₂ and H₃) and the *Pst*I site (P₁) defining the H₂-P₁ fragment are indicated. A₂, B, D₃, and D₄ are the *Hpa*I, *Bal*I and the two *Dra*I sites, respectively. The nt positions of the ATG initiation codon (Herrnstadt *et al.*, 1987; Höfte *et al.*, 1987; Sekar *et al.*, 1987; Donovan *et al.*, 1988), of the transcription start sites from P₁ and P₂ promoters, of the P₂-35 and -10 regions, and of the restriction sites, are also shown. The arrows indicate the start site positions and direction of transcription of *cryIII A* gene from P₁ and P₂. Transcripts are designated T1 and T2, respectively. The cross-lined part contains the H₂-H₃ derivative fragments utilized in this study.

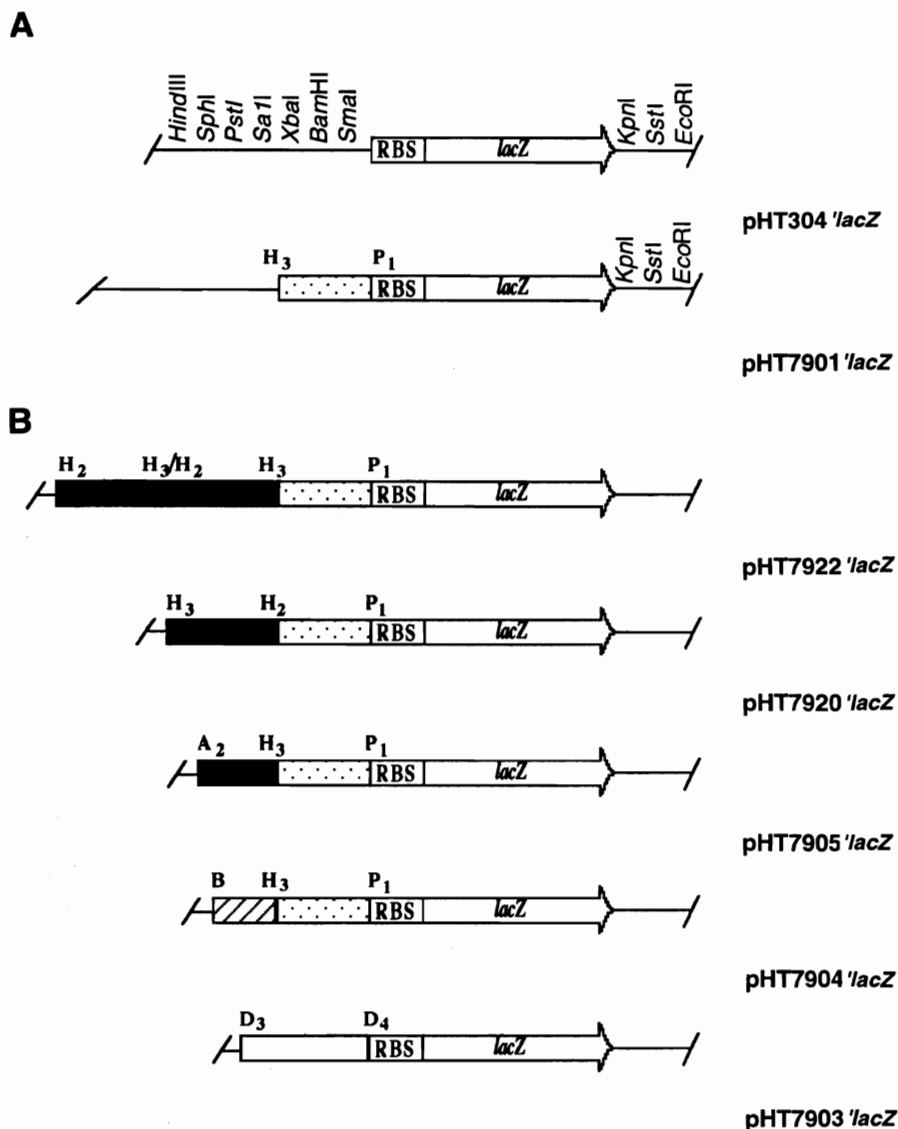


Figure 2 - Diagram of the *lacZ* gene transcriptional fusion constructs. The restriction sites indicated represent the multiple cloning site of pUC19. The elements presented in this diagram are not to scale. A) Parental plasmids (De-Souza *et al.*, 1993). Only the relevant regions of the parental plasmids pHT304'*lacZ* and pHT7901'*lacZ* are detailed (the remaining parts are indicated as solid bars). Arrowheads indicate the direction of transcription of the *lacZ* gene; RBS represents the DNA region of the ribosome binding site of the *spoVG* gene, as previously described for the plasmid pTV32 (De-Souza *et al.*, 1993). B) *lacZ* transcriptional fusions. H₂, H₃, P₁, A₂, B, D₃, and D₄ sites, which represent the restriction fragments used in these constructs, are described in the text and in the legend to Figure 1.

about 20-fold between t_0 and $t_{7.5}$. Thus, it was also similar to the expression profile obtained with the plasmid pHT7902'*lacZ* carrying an entire copy of the H₂-H₃ fragment in the wild-type orientation (De-Souza *et al.*, 1993).

Second start point of the *cryIIIA* gene transcription

The results described above suggest that there is no additive effect on *cryIIIA* gene transcriptional regulation when the H₂-H₃ fragment is present in two

adjacent copies upstream of the H₃P₁ fragment. Furthermore, deletions of this fragment in the 5' → 3' direction, up to nt position 803 (Figure 1), do not interfere with enzyme synthesis. The β -galactosidase specific activity from the strain containing the H₂-H₃ fragment in the opposite orientation (pHT7920'*lacZ*) showed that the effect is orientation-dependent.

We previously mapped a start point for *cryIIIA* gene transcription (De-Souza *et al.*, 1993) located 130 bp upstream of the ATG start codon, one nucleotide upstream of that published by Sekar *et al.* (1987). Northern blot analysis identified a single *cryIIIA* specific transcript of about 2.5 kb (De-Souza *et al.*, 1993). This size is consistent with a transcript delimited by the transcriptional start point referred to above and the putative terminator sequence described by Donovan *et al.* (1988). These experiments were performed on total RNA extracted from the strain carrying the plasmid pHT305P, which bears an entire copy of the H₂-H₃ fragment in its wild-type orientation with regard to the *cryIIIA* gene

adjacent promoter, RBS, and coding sequence. The kinetics of *cryIIIA* mRNA production showed that transcriptional enhancement starts between t_0 and t_3 and is stronger between t_3 and t_6 .

To test whether the transcriptional positive effect is due to another promoter activity present in the H₂-H₃ fragment, a second primer extension was performed using a primer which hybridizes to a sequence located upstream of the promoter P₁. Total RNA extracted from the recombinant strain bearing the plasmid pHT305P (De-Souza *et al.*, 1993) was harvested at t_3 and subject to primer extension analysis. A second

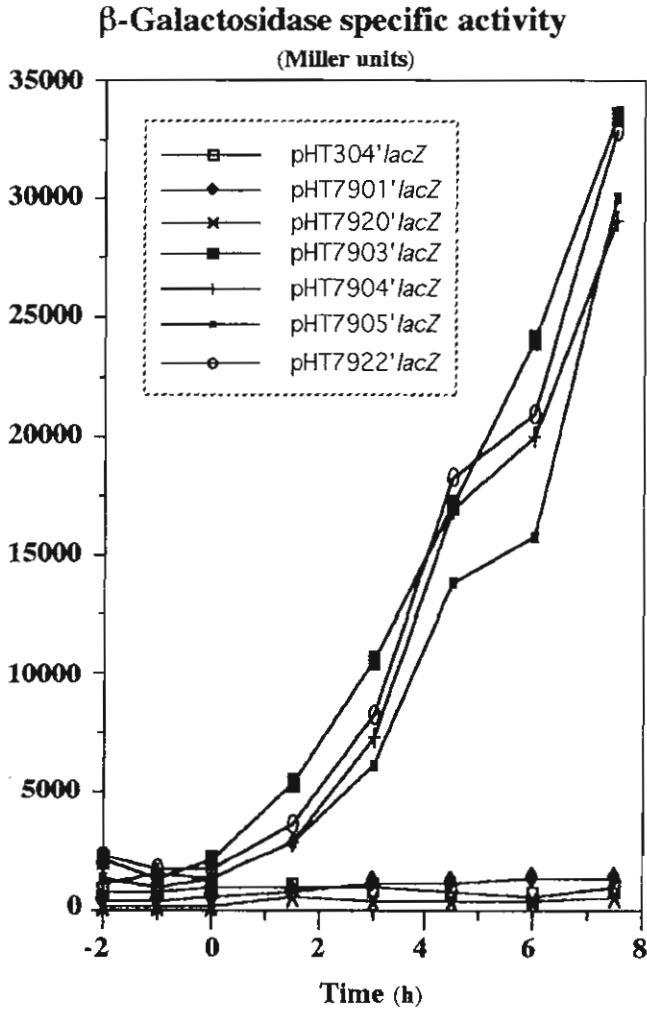


Figure 3 - β -Galactosidase activity profiles. The *Bacillus thuringiensis* cell growth, sample conditions, and the assays used to determine the enzyme specific activity are described in Material and Methods. Time zero (t_0) indicates the onset of sporulation. T_n is the number of hours before (-) or after t_0 .

transcription start point, designated T2, was mapped at nt position 984, about 400 bp upstream of the first putative start site (Figure 4). The putative -35 and -10 regions of this upstream promoter are TTGCAA (nt 949-954, in Figure 1) and TAAGCT (nt 973-978, in Figure 1), respectively; and therefore it resembles a promoter recognized by the $E\sigma^A$ form of the RNA polymerase.

DISCUSSION

Most of the *cry* genes are transcribed by RNA polymerases associated with sporulation-specific sigma factors. This was demonstrated recently for the lepidopteran specific *cryIA* gene (Brown and Whiteley, 1988, 1990; Adams *et al.*, 1991). It seems that the other *cryI* genes, the *cryII*, *cryIV* and *cyt* genes are transcribed

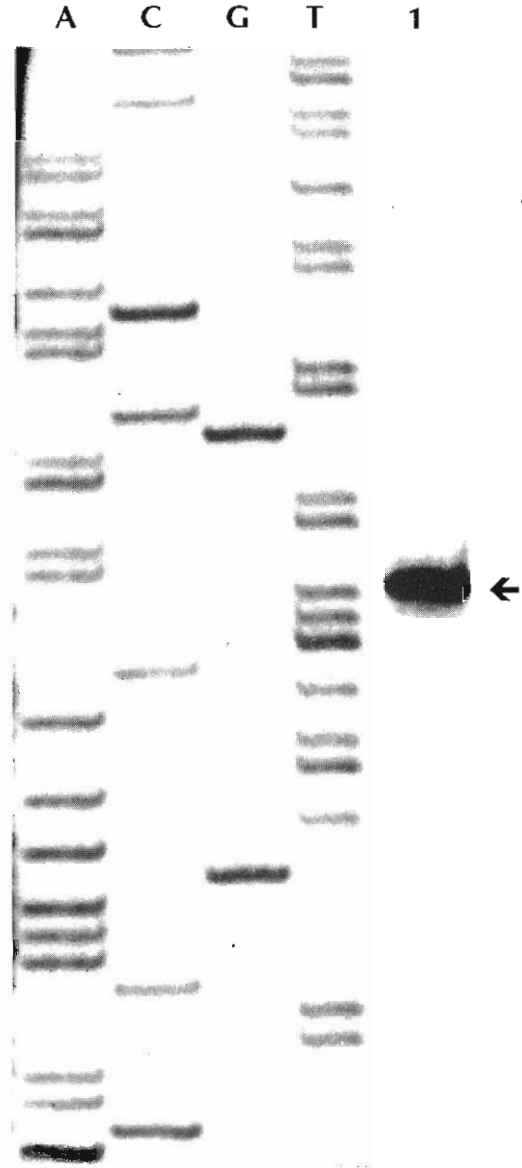


Figure 4 - Determination of the second transcription start site of *cryIII*A. *Bacillus thuringiensis* cells bearing pHT305P growing in HCT medium (De-Souza *et al.*, 1993) were harvested at t_3 , and total RNA extracted. A radiolabelled primer complementary to the nt 1222-1190 was annealed to each RNA sample, extended by avian myeloblastosis virus reverse transcriptase and analyzed by electrophoresis (lane 1). The same synthetic oligonucleotide was used to prime dideoxy sequencing reactions from a single-stranded DNA template which contained the region of interest. The letters above the lanes indicate the dideoxynucleotide used to terminate each reaction. The arrow indicates the 5' end (position 984 in the Figure 1) of the *cryIII*A specific transcript starting from the P2 promoter. The sequence 5'-CTTAATTAAGAT-3' is complementary to the strand determined in the sequencing gel. A in bold represents the second transcription start point.

similarly due to sequence homology between promoter regions (Lereclus *et al.*, 1989). Nevertheless, it appears that the *cryIII* genes, which encode coleopteran-specific toxins of about 70 kDa, exhibit different features for

their transcriptional regulation. Arantes and Lereclus (1991) demonstrated that the *cryIIIA* gene from Bt strain LM79 (Chaufaux *et al.*, 1993) is poorly expressed when cloned with its promoter and putative terminator. This analysis was performed in an environment approaching the natural conditions (low-copy number vector). Regarding these considerations, we analyzed the *cryIIIA* gene adjacent regions (De-Souza *et al.*, 1993) and mapped a 1-kb fragment of DNA localized about 400 bp upstream of the putative promoter P1 described by Sekar *et al.* (1987). This segment of DNA, the H₂-H₃ fragment, contains an element which increases transcription of the *cryIIIA* gene during sporulation. Although it is particularly strong between t₃ and t₆, an appreciable level of *cryIIIA* mRNA is already present at the onset of sporulation. This was unexpected since the transcription of the *cryIA* gene is directed by the σ^E -like and σ^K -like factors of *B. thuringiensis* between t₂ and t₇ of sporulation (Brown and Whiteley, 1988, 1990; Adams *et al.*, 1991). The *cryIIIA* gene promoter P1 is not identical to the *cryIA* gene promoter and could therefore be recognized by a σ factor present during the vegetative phase. Therefore, the key determinant co-ordinating the *cryIIIA* gene expression with sporulation may be the H₂-H₃ fragment.

The transcriptional positive effect of the H₂-H₃ fragment is not restored when this fragment is cloned downstream of the *cryIIIA* gene coding sequence (De-Souza *et al.*, 1993) or fused with the *lacZ* gene in its original position but in the opposite orientation. Thus, the integrity of the H₂-P₁ region (H₂-H₃ fragment + H₃-P₁ fragment) is crucial for full expression of the *cryIIIA* gene. Another *lacZ* transcriptional fusion was tested to determine whether the positive effect is increased with two copies of the H₂-H₃ fragment. The result showed that there is no additive effect with the H₂-H₃ fragment in tandem. An additional set of transcriptional fusions between the *lacZ* gene and restriction fragments derived from the H₂-P₁ region revealed that at least the 855-bp DNA fragment, mapped between positions 803 and 1658 (see Figure 1), still maintains full expression of the *cryIIIA* gene. Using total RNA sampled at t₃ and a primer located upstream of the promoter P1, a second transcription start site was mapped at nt position 984 (Figure 1), about 400 bp upstream of the first putative transcriptional start point. Thus, in this study, the minimal segment of DNA derived from the H₂-H₃ fragment and required for the full expression of the *cryIIIA* gene was restricted to the D₃-H₃ fragment, which contains the promoter P2 (Figure 1). We could therefore hypothesize that promoter P2 is responsible for the increasing effect of the H₂-H₃ fragment.

A study of the *B. subtilis* succinate dehydrogenase (*sdh*) operon showed that the rate of *sdh* mRNA decay increases during the stationary phase (Melin *et al.*, 1989). The 5' region decays about twice as fast as the 3' region, suggesting the importance of the 5' region for the initiation of decay (Melin *et al.*, 1990). Analysis of *ermC* mRNA stability indicated that mRNA decay in *B. subtilis* generally occurs at or near the 5' terminus (DiMari and Bechhofer, 1993). Interestingly, Northern blot (De-Souza *et al.*, 1993) analysis failed to detect a transcript corresponding to the size delimited by the second transcription start site described here. Thus, the P1 promoter region may be a cleavage site which generates the 2.5 kb stable transcript, whereas the transcript starting at P2 is not detectable.

The putative -35 (TTGCAA) and -10 (TAAGCT) regions of this upstream promoter do not match the respective consensus sequences described for the other *cry* genes, which are transcribed by RNA polymerase associated with sporulation specific sigma factors (σ^{35} and σ^{28}). However, these DNA sequences are highly homologous to the promoter region (-35: TTGACA and -10: TATAAT) recognized by the RNA polymerase bound to σ^A . Thus, the E σ^A form of RNA polymerase or an alternative form of this enzyme containing a stationary phase-stage-specific sigma factor may be involved in the transcription of the *cryIIIA* gene from P2.

ACKNOWLEDGMENTS

We thank G. Rapoport, in whose laboratory this work was conducted. We are grateful to Lindsay Pirrit for revising the English manuscript.

This work was supported by research funds from the Pasteur Institute, the Institute National de la Recherche Agronomique, the Fondation de la Recherche Médicale, and the Centre National de la Recherche Scientifique. M.T. De-Souza was supported by a fellowship from CNPq (Brazil). H. Agaisse was supported by a fellowship from the Ministère de l'Éducation Nationale (France).

RESUMO

A expressão do gene *cryIIIA* foi analisada através de fragmentos de restrição derivados de um fragmento de 6 kb contendo a seqüência do gene e regiões adjacentes. Estes fragmentos foram clonados em plasmídeos contendo baixo número de cópias. Os resultados indicaram que um fragmento de 1 kb (fragmento H₂-H₃), localizado a cerca de 400 pb da extremidade 5' do promotor putativo (previamente descrito), aumenta fortemente a transcrição do gene da toxina CryIIIA, em células de *Bacillus thuringiensis* durante a esporulação (De-Souza *et al.*, *J. Bacteriol.* 175: 2952-2960, 1993).

O delineamento e a análise funcional da região demonstrou que uma seqüência promotora ativa está localizada dentro do fragmento H₂-H₃, estando situada a 400 pb do promotor anteriormente descrito e aproximadamente a 600 pb da região codificadora do gene *cryIII^A*. A associação da região contendo este segundo promotor com a seqüência de DNA localizada diretamente a montante do gene é necessária para obter a expressão integral das fusões transcricionais. As regiões putativas -35 e -10 deste promotor não apresentam homologia com seqüências promotoras de genes específicos da esporulação, mas são similares àquela reconhecida pela forma E σ^A da RNA polimerase.

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(Received May 20, 1994)