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In vivo and in vitro processing of the *Bacillus subtilis* transcript coding for glutamyl-tRNA synthetase, serine acetyltransferase, and cysteinyl-tRNA synthetase

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ABSTRACT

In *Bacillus subtilis*, the adjacent genes *gltX, cysE*, and *cysS* encoding respectively glutamyl-tRNA synthetase, serine acetyl-transferase, and cysteinyl-tRNA synthetase, are transcribed as an operon but a *gltX* probe reveals only the presence of a monocistronic *gltX* mRNA (Gagnon et al., 1994, *J Biol Chem 269*:7473–7482). The transcript of the *gltX-cysE* intergenic region contains putative alternative secondary structures forming a ρ-independent terminator or an antiterminator, and a conserved sequence (T-box) found in the leader of most aminoacyl-tRNA synthetase and many amino acid biosynthesis genes in *B. subtilis* and in other Gram-positive eubacteria. The transcription of these genes is initiated 45 nt upstream from the first codon of *gltX* and is under the control of a σ^A-type promoter. Analysis of the in vivo transcript of this operon revealed a cleavage site immediately downstream from the ρ-independent terminator structure. In vitro transcription analysis, using RNA polymerases from *Escherichia coli*, *B. subtilis*, and that encoded by the T7 phage, in the presence of various RNase inhibitors, shows the same cleavage. This processing generates mRNAs whose 5′-end half-lives differ by a factor of 2 in rich medium, and leaves putative secondary structures at the 3′ end of the *gltX* transcript and at the 5′ end of the *cysE/S* mRNA, which may be involved in the stabilization of these mRNAs. By its mechanism and its position, this cleavage differs from that of the other known transcripts encoding aminoacyl-tRNA synthetases in *B. subtilis*.

Keywords: *cysE*; *cysS*; cysteine biosynthesis; *gltX*; RNA cleavage

INTRODUCTION

The aminoacyl-tRNA synthetases (aaRS) catalyze the accurate charging of tRNAs with their cognate amino acids. The aaRS are present in high levels, as are most macromolecules involved in protein biosynthesis, and these levels increase with growth rate, a phenomenon known as “metabolic regulation” (Neidhardt et al., 1977). In *Bacillus subtilis* and in other Gram-positive bacteria, many of these genes and many amino acid biosynthetic operons are controlled by tRNA-mediated antitermination in response to starvation for their cognate amino acid (Henkin et al., 1992; Putzer et al., 1992, 1995b; Grundy & Henkin, 1993). Their transcripts have an untranslated leader mRNA of about 300 nt, including a ρ-independent terminator just upstream from the translation initiation site (Putzer et al., 1992, 1995a; Grundy & Henkin, 1994; Henkin, 1994). A highly conserved sequence, called T-box, is situated just upstream from the terminator structure and can form, with the 5′-strand of the terminator stem, an alternative and mutually exclusive antiterminator structure (Grundy & Henkin, 1993). The much weaker antiterminator conformation is thought to be stabilized by base pairing of the cognate uncharged tRNA with its NCCA-3′ end to a UGGN′ sequence in the T-box (Grundy & Henkin, 1994; Putzer et al., 1995b), and with its anticodon to a specifier codon present near the 5′ end of the leader (Grundy & Henkin, 1993).

Following this tRNA-mediated antitermination, the leader region of many aaRS mRNAs is cleaved between the T-box consensus sequence and the terminator structure leaving a stable secondary structure at the 5′ end of the processed aaRS mRNA. For the *B. subtilis thrS* mRNA this processing increases its half-life and was reported to be catalyzed in vivo and in vitro by *Escherichia coli* RNase E (Condon et al., 1996, 1997); the ratio of the amounts of processed and full-length

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thrS transcripts is influenced by threonine starvation, indicating that this processing participates in *thrS* regulation (Condon et al., 1996).

The *B. subtilis gltX* gene encoding glutamyl-tRNA synthetase is followed on the chromosome by the *cysE* and *cysS* genes, coding respectively for serine acetyl-transferase (SAT) and cysteinyl-tRNA synthetase (CysRS; Gagnon et al., 1994). The transcript of the region between *gltX* and *cysE/S* contains alternative and mutually exclusive secondary structures forming either a ρ-independent terminator or an antiterminator, the conserved T-box sequence and a specifier domain for $tRNA^{Cys}$. This intergenic region regulates the expression of *cysE/S*, as demonstrated by the repression of a *cysE-lacZ* transcriptional fusion in response to the overproduction of *B. subtilis* CysRS *in trans* (Gagnon et al., 1994). Moreover, studies with a transcriptional fusion and the lethality of a Campbell-type integration interrupting this intergenic region indicate that no promoter downstream of *gltX* allows the expression of *cysE/S* in vivo. All these genes are thus expressed from the σ^A-type promoter of *gltX* as a tricistronic transcript starting 45 nt upstream from the first codon of *glt*X. However, Northern analysis with a *gltX* probe revealed only the presence of the monocistronic *gltX* mRNA (Gagnon et al., 1994).

To understand the coregulation of the biosynthesis of these two aminoacyl-tRNA synthetases and of the first enzyme for cysteine biosynthesis (SAT), we have investigated the possibility of a processing in the intergenic region between *gltX* and *cysE*. Here we show that this intergenic region is cleaved in vivo and in vitro immediately downstream from the ρ-independent terminator structure. This processing leaves stable putative secondary structures at the 3′ end of the *gltX* transcript and at the 5′ end of the *cysE/S* mRNA.

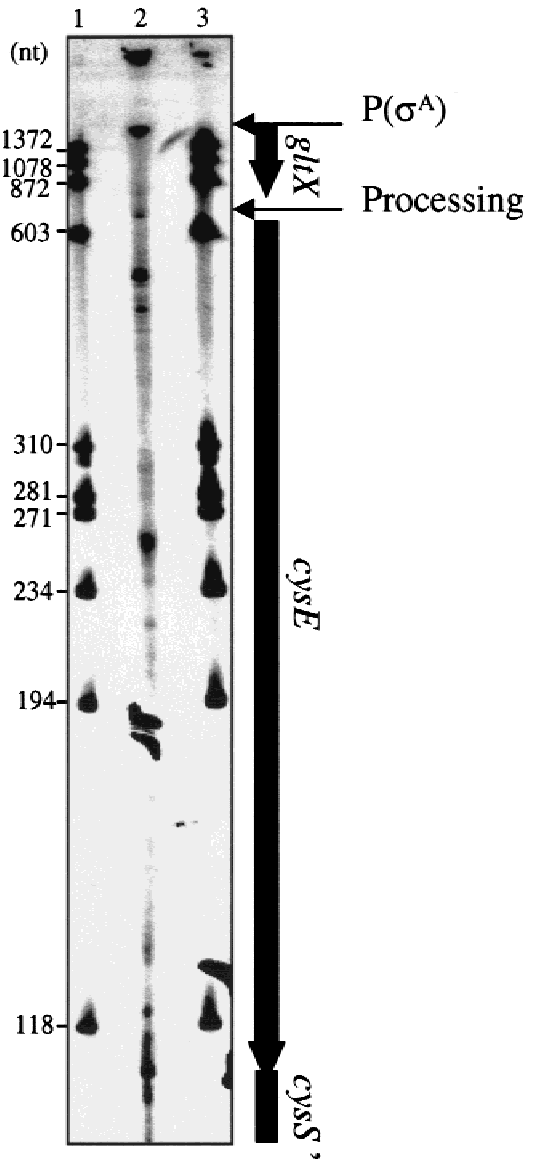

FIGURE 1. Primer extension analysis of the *gltX-cysE-cysS* transcripts. An oligonucleotide (CYSS1) complementary to nucleotides +96 to +80 from the beginning of *cysS* was extended on *B. subtilis* RNA (10 μg) from cells grown exponentially in LB medium using 4 U of avian myeloblastosis virus reverse transcriptase (lane 2). ^{32}P-labeled ϕX174/*Hae*III fragments were used as DNA molecular weight markers (lanes 1 and 3).

RESULTS

Detection of the full-length in vivo transcript and of a shorter in vivo product of the *gltX-cysE-cysS* operon

In spite of the absence of a promoter in the intergenic region between *gltX* and *cysE*, indicating a probable cotranscription of *gltX*, *cysE*, and *cysS* in *B. subtilis*, Northern analysis with a *gltX* probe did not show a full-length *gltX-cysE-cysS* mRNA but revealed only the presence of a 1,700-nt-long monocitronic *gltX* mRNA (Gagnon et al., 1994). To determine the origin of the transcription of *cysE* and *cysS*, we extended an oligonucleotide complementary to nucleotides +96 to +80 from the beginning of *cysS* (Fig. 1). This analysis revealed the existence of in vivo RNAs including *cysE-cysS*, as well as *gltX-cysE-cysS*, thereby proving the presence of a large operon including the three genes, and providing evidence for the processing of its transcript between *gltX* and *cysE*. Some signals upstream from *cysS* (Fig. 1) may be due to pauses in the reverse transcription reaction, as they appear in GC-rich regions and are not observed by S1 mapping analysis (data not shown). To identify the 5′ end of the processed *cysE-cysS* mRNA, we extended the oligonucleotide MP168 (Fig. 2), whose 3′ end is 37 nt downstream from the beginning of *cys*E, on *B. subtilis* total RNA. The signal obtained shows that the 5′ end of this mRNA is located 49 nt upstream from the beginning of the *cys*E initiation codon (Fig. 3), just downstream from the ρ-independent terminator secondary

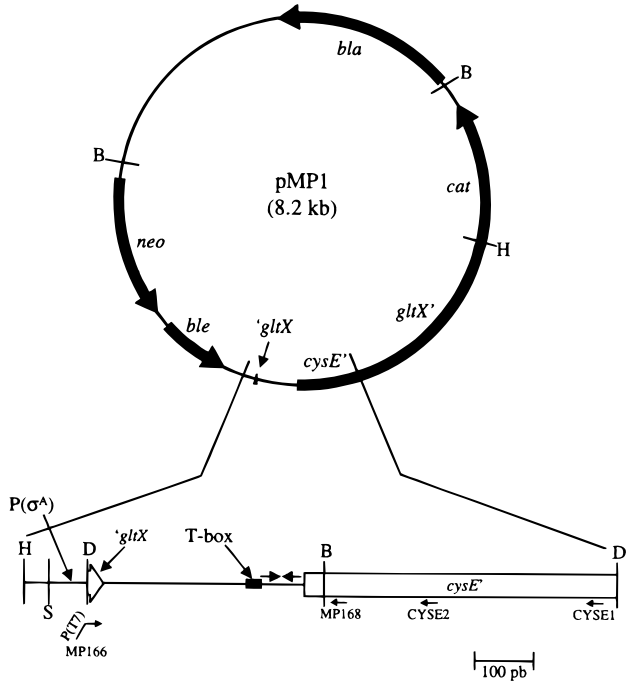

FIGURE 2. Physical map of plasmid pMP1 containing the *gltX-cysE* intergenic region. The small arrows indicate the position and orientation of the oligonucleotides used. The amplicon made with MP166 and CYSE2 was used for S1 mapping on in vitro and in vivo RNA (Fig. 6), and the one made with MP166 and MP168 was used for in vitro transcription using T7 RNA polymerase. P(σ^A) is followed by the first 12 nt of the transcribed region and the last 13 nt of *gltX*. Restriction endonucleases: H: *Hin*dIII; D: *Bsu*36I/*Dra*I; B: *Bbs*I; S: *Sca*I.

structure. The position of this processing differs from that observed for several other *B. subtilis* aaRS gene transcripts, which are cleaved upstream from the ρ-independent terminator (Condon et al., 1996, 1997). Confirmation of this result was also obtained by S1 mapping analysis using a polymerase chain reaction (PCR) probe covering this region (data not shown).

The *gltX-cysE-cysS* transcript is cleaved in vitro

We further characterized the *gltX-cysE-cysS* mRNA by analysis of in vitro transcription products labeled with [α-^{32}P]UTP. Using purified *B. subtilis* RNA polymerase, in conjunction with an RNase inhibitor (Promega's RNAsin), in vitro transcription of the 420 bp isolated *Sca*I–*Bbs*I fragment of pMP1 containing the intergenic region placed downstream from the σ^A *gltX* promoter (Fig. 2) yielded three RNAs (Fig. 4A, lane B): one ending immediately after the stem of the putative terminator (281 nt); a 323-nt transcript corresponding to the read-through of the DNA template; and, finally, a small fragment whose length (81 nt) corresponds to the distance between the processing site and the end of the DNA template. Other signals between the 281 nt and the 81 nt are also observed, and correspond to pauses in the in vitro transcription, as they are not observed by in vivo analysis. Similar results were obtained with purified RNA polymerase from *E. coli* (using the same isolated *Sca*I–*Bbs*I fragment of pMP1) (Fig. 4A, lane E) and with that encoded by the T7 phage using Pharmacia's RNAguard as RNase inhibitor. In the latter case, we used the isolated *Eco*RI fragment of pMP8, which is nearly identical to the former fragment except for the replacement of P(σ^A) by a T7 RNA polymerase promoter (which allows transcription initiation at the same position) and by a 20-bp extension at its 3′ end, when compared to the fragment used for in vitro transcription using *B. subtilis* or *E.coli* RNA polymerases (Fig. 4A, lane T7). This extension is reflected on the length of the small RNA (99 nt) present in lane T7 of Figure 4B versus RNA of 81 nt in lanes E and B, indicating that this signal comes from the downstream fragment of the cleaved transcript, and is not due to an early pause nor to transcription termination. To look at this cleavage reaction directly, uncoupled from transcription, we tried to isolate the read-through transcript by doing transcription time courses and transcription at various temperatures and salt concentrations, but no significant variation in the efficacy of the cleavage reaction was detected (data not shown).

To provide more evidence that the 99-nt RNA fragment coming from in vitro transcription using T7 RNA polymerase is the downstream RNA fragment of the cleaved transcript, S1 mapping analysis was performed (Fig. 5). We used an amplicon covering the intergenic region (obtained with the oligonucleotides MP166 and CYSE2; see Fig. 2) on RNA obtained by in vitro transcription of the same amplicon with T7 RNA polymerase, and on *B. subtilis* total RNA. This analysis shows that the cleaved RNA obtained from the in vitro transcript has the same 5′ end as that observed in vivo, demonstrating that the observed in vitro processing position is the same as that observed in vivo (see above).

Stability of the 5′ ends of *cysE* and *gltX* mRNAs

As Northern analysis of this region of the *B. subtilis* chromosome did not detect polycistronic mRNAs for *gltX-cysE*-cysS nor for *cysE-cysS*, it was suggested that the *cysE-cysS* mRNA may be unstable (Gagnon et al., 1994). To determine the role of processing on this mRNA degradation and to gain some insight on its biological significance, we studied the in vivo stability of the 5′ ends of the two resulting mRNAs by measuring their half-lives. Primer extension of oligonucleotides complementary to the beginning of their coding sequences showed that in exponentially growing cells in rich medium, the half-life of the 5′ end of *cysE* mRNA (1.4 min) is about twice as long as that of *gltX* mRNA

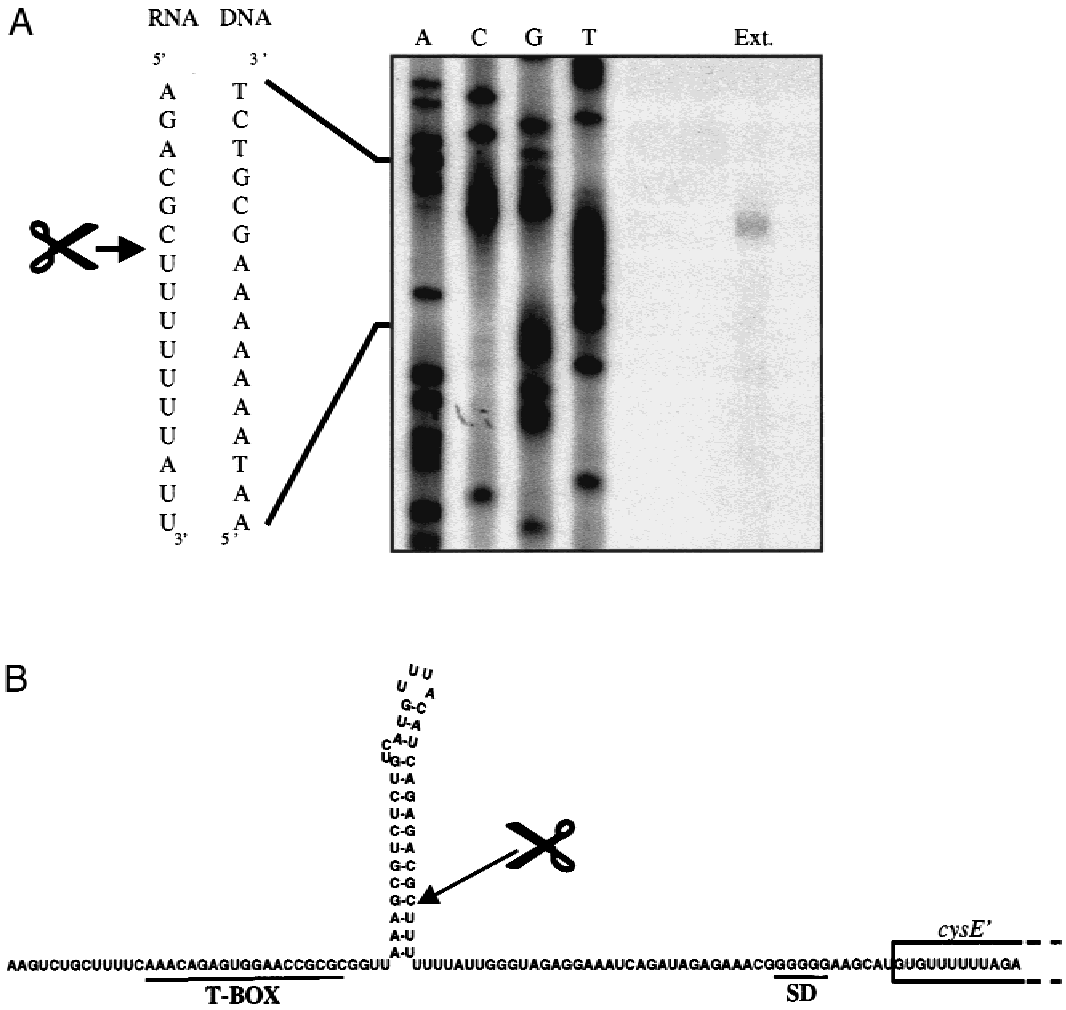

FIGURE 3. Determination of the 5′ end of the *cysE-cysS* mRNA by primer extension. **A**: The oligonucleotide MP168 ending 37 nt from the beginning of *cysE* was extended with 4 U of avian myeloblastosis virus reverse transcriptase, using 10 μg of *B. subtilis* RNA from cells grown exponentially in LB medium. The nucleotide sequence was determined using MP168 on pMP1. **B**: Location of the cleavage site within the ρ-independent terminator, as determined by primer extension (**A**).

(0.8 min) (Fig. 6). This result suggests that this specific cleavage of the *gltX-cysE-cysS* transcript allows a distinct regulation of these two sets of cotranscribed genes.

DISCUSSION

Of the 24 genes encoding aminoacyl-tRNA synthetases in *B. subtilis*, the *gltX-cysE-cysS* operon is the only case where an aminoacyl-tRNA synthetase gene (*cysS* encoding the cysteinyl-tRNA synthetase) is cotranscribed with a gene encoding an enzyme involved in the biosynthesis of the amino acid substrate of this synthetase (*cysE* encoding the serine acetyltransferase; Gagnon et al., 1994). This gene organization was also found in two other Gram-positive bacteria: *Bacillus stearothermophilus* (Gagnon et al., 1994) and *Staphylococcus xylosus* (Fiegler & Brückner, 1997). In *B. subtilis* and *S. xylosus*, *cysE* expression is regulated at the level of transcription attenuation by $tRNA^{Cys}$ (Gagnon et al., 1994; Fiegler & Brückner, 1997) in agreement with the proposed model for the T-box family genes (Grundy & Henkin, 1993). In contrast, in *E. coli* these genes are not adjacent nor are they controlled directly by the level of aminoacylation of a cognate tRNA (Denk & Böck, 1987; Champagne, 1997).

Bacillus subtilis gltX transcription is initiated 45 nt upstream from its initiation codon from a σ^A-type promoter; Northern analysis with a *glt*X probe shows a monocistronic mRNA for *gltX* but no polycistronic *gltX-cysE-cysS* transcript (Gagnon et al., 1994). As the absence of a promoter in the intergenic region was demonstrated previously in vivo (Gagnon et al., 1994), we conclude that a polycistronic *gltX-cysE-cysS* mRNA is transcribed from the σ^A promoter upstream from *gltX* and that this long transcript is rapidly cleaved (Fig. 7A). We showed that this cleavage is located 49 nt upstream from *cysE*, just downstream of the ρ-independent terminator (Fig. 7), and that it can be observed in vitro

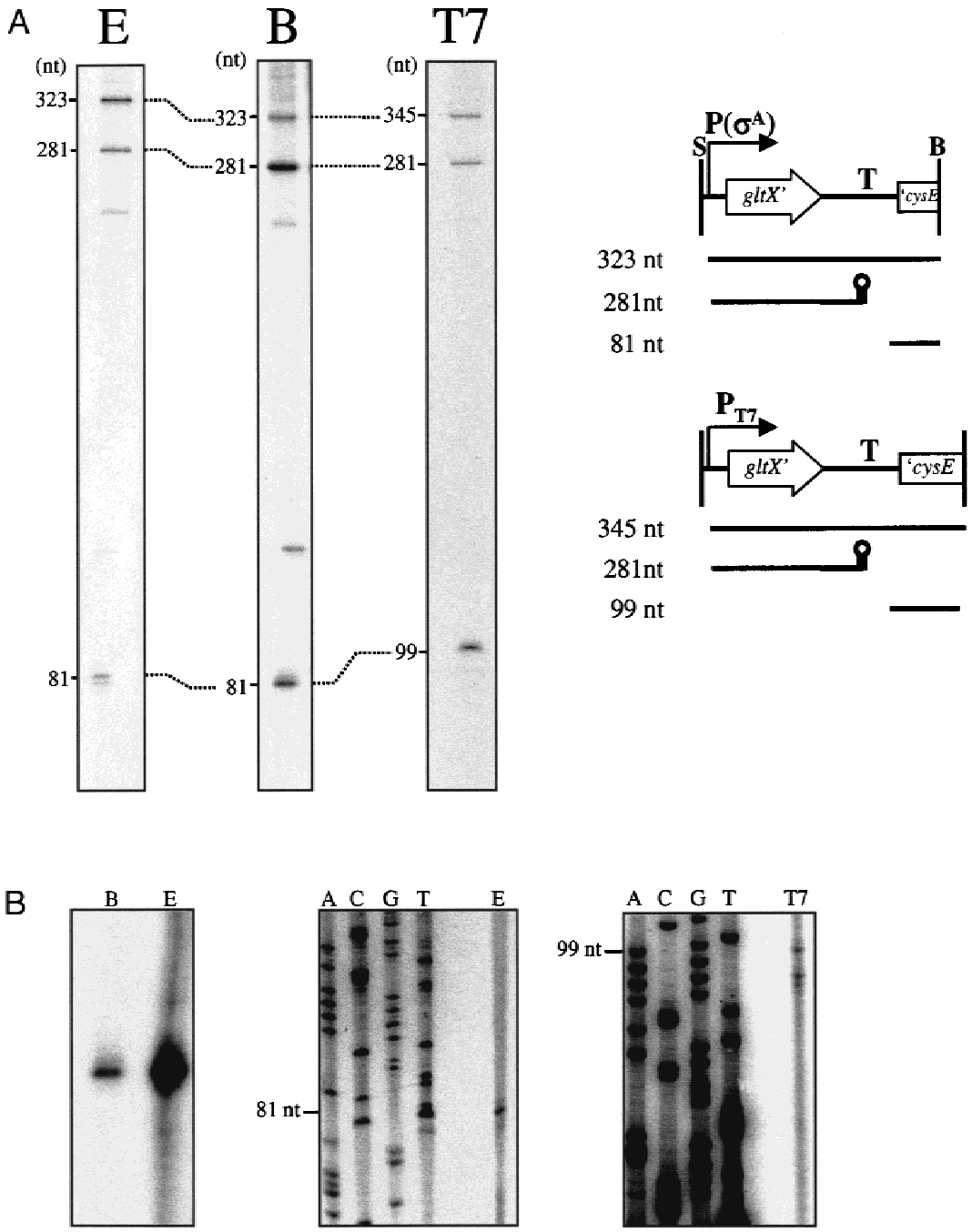

FIGURE 4. In vitro transcription of the *gltX-cysE* intergenic region. The reactions were conducted using RNA polymerases from *Escherichia coli* (lane E), *B. subtilis* (lane B), and that encoded by the T7 phage (lane T7). **A**: Analysis of the in vitro transcription products for the three RNA polymerases used. The products observed are identified by their length with a precision of ±1 nt (by comparison with a DNA sequence (not shown)), below the scheme of the DNA fragment used for template. **B**: Size of the small RNA fragments generated by the cleavage of the transcripts synthesized by the three RNA polymerases used. Restriction endonucleases: B: *Bbs*I; S: *Sca*I.

as well as in vivo. The mechanism of this cleavage is not fully characterized, but the fact that it can be observed in vitro as well as in vivo suggests self-cleavage. However, we cannot rule out the possibility that this highly specific cleavage is due to the RNA polymerase (Surratt et al., 1991) or to the presence of one fragile phosphodiester bond in this long transcript (Watson et al., 1984; Hosaka et al., 1991; Kierzek, 1992).

The mechanism of this cleavage differs from that of the *B. subtilis thrS* transcript, reported to be catalyzed by *E. coli* RNase E in vivo; moreover no processing of this transcript was observed in vitro in the absence of this enzyme (Condon et al., 1997). Furthermore, the processing of the *thrS* mRNA leaves a stable secondary structure (ρ-independent terminator) immediately at its 5′ end (Condon et al., 1996), whereas the cleav-

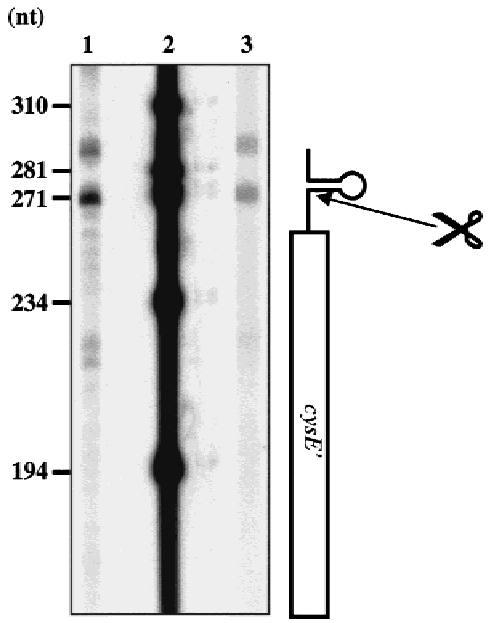

FIGURE 5. Identification of the 5′ end of the *cysE-cysS* mRNA from in vitro and in vivo sources. An amplicon covering the intergenic *gltX-cysE* region (obtained with the oligonucleotides MP166 and CYSE2 on pMP1; see Fig. 2) was used as a probe for S1 mapping analysis using *B. subtilis* total RNA (100 μg; lane 3) or RNA (3 μg) obtained by in vitro transcription with T7 RNA polymerase on the same amplicon (lane 1). ^{32}P-labeled, *Hae*III-digested ϕX174 DNA fragments were used as DNA molecular weight markers (lane 2). The band at about 290 nt corresponds to the double-stranded probe.

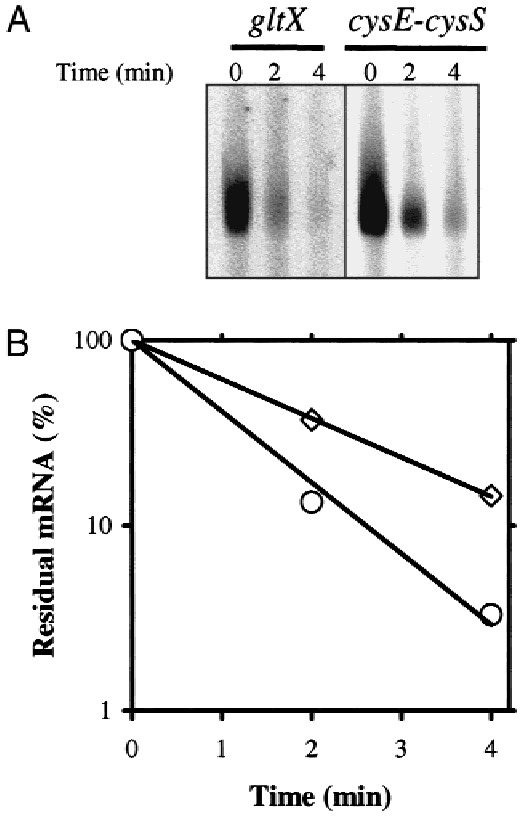

FIGURE 6. Half-lives of the 5′ ends of the *cysE-cysS* and *gltX* mRNAs. RNA samples were isolated from an exponentially growing culture of *B. subtilis* 168T at 0, 2, and 4 min after the addition of rifampicin. **A**: Primer extension analysis of the *cysE-cysS* (◇) and *gltX* (○) mRNA 5′ ends using respectively MP168 and an oligonucleotide complementary to the beginning of *gltX*. **B**: Semi-log plot of the amounts of RNA obtained in **A**, taking as 100% the value obtained at the moment of rifampicin addition.

age of the *B. subtilis gltX-cysE-cysS* mRNA leaves this stable secondary structure ($\Delta G = -20.2$ kcal/mol; Walter et al., 1994) at the 3′ end of the *gltX* transcript (Fig. 7B).

The *gltX* mRNA appears to be more stable than the *cysE-cysS* mRNAs, as only the first was detected by Northern analysis (Gagnon et al., 1994). Surprisingly, the half-life of the 5′ end of the *cysE-cysS* mRNA is twice as long as that of the 5′ end of the *gltX* mRNA. This apparent contradiction may be explained by structures at the ends of these mRNAs after the cleavage of the *gltX-cysE-cysS* transcript. This processing leaves a hairpin structure at the 3′ end of *gltX* mRNA, but downstream of *cysS*, sequence analysis up to the putative promoter (TAGTCA–18 nt–TACAAT) of the next coding sequence (*yacO*; Moszer et al., 1995) did not reveal the presence of a ρ-independent terminator. As the presence of a hairpin structure stabilizes several bacterial mRNAs (Belasco & Higgins, 1998; Emory et al., 1992), these structural differences at the 3′ end are consistent with the facts that *gltX* mRNA was detected by Northern analysis, but that *cysE-cysS* was not. On the other hand, the presence of a putative hairpin ($\Delta G = -6.2$ kcal/mol) at the 5′ end of the *cysE-cysS* mRNA and its proximity with the *cysE* putative ribosome-binding site (SD; Fig. 7B) may protect it against degradation from this end (see Emory et al., 1992), and is consistent with the longer half-life of its proximal part than that of *gltX* mRNA (Fig. 6), which contains no such secondary structure in its short leader.

MATERIALS AND METHODS

Chemicals, enzymes, and oligonucleotides

Restriction endonucleases and modification enzymes were purchased from Bethesda Research Laboratory, Inc., Pharmacia LKB Biotechnology, Boehringer Mannheim, Sigma Chemical Co., New England Biolabs or International Biotechnologies, Inc., and were used according to the manufacturers' instructions. Isopropyl-β-*D*-thiogalactopyranoside (IPTG) was obtained from Bethesda Research Laboratory, Inc. The labeled nucleotides [α-^{32}P]dCTP (800 Ci/mmol), [α-^{32}P]UTP (800 Ci/mmol), and [γ-^{32}P]ATP (3,000 Ci/mmol) were obtained from DuPont Canada, Inc. The following oligonucleotides were synthesized: GLTX (5′-GGTGCATAACGGACGCG-3′), CYSS1 (5′-CCGTGGGTCCGCATAC-3′), MP166 (5′-AGAATTCTAATACGACTCACTATAGTTTTTGCCTGAAAATATC-3′), MP168 (5′-AGAATTCTGCGGGATCTTGATCGAACAC-3′), and CYSE2 (5′-CTCCCGATTGTAGCG-3′) (the

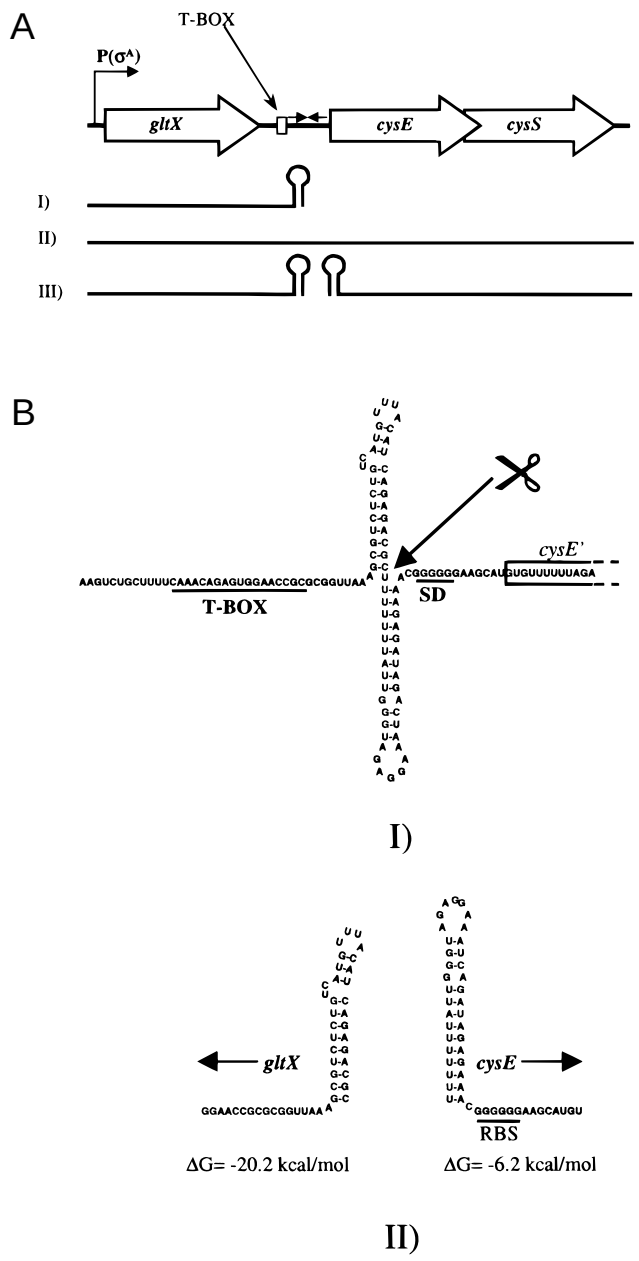

FIGURE 7. Proposed model for the processing of the *gltX-cysE-cysS* mRNA in *B. subtilis*. **A**: As previously described (Gagnon et al., 1994), the intergenic region between *gltX* and *cysE* responds to a terminator-antiterminator mechanism (I and II). In the case of antitermination, the mRNA is cleaved immediately downstream from the terminator secondary structure, which leaves putative stable secondary structures at the 3′ end of the *gltX* mRNA and at the 5′ end of the *cysE/S* RNA (III). **B**: Putative secondary structures located at the 3′ end of *gltX* mRNA and at the 5′ end of the *cysE-cysS* mRNA (II) generated by the cleavage of the transcript (I), and their free energy values calculated according to Walter et al. (1994).

underlined segments are not complementary to *B. subtilis* DNA).

Bacterial strains and culture conditions

The *B. subtilis* 168T (Burkholder & Giles, 1947) strain was used for these studies. The *E. coli* strains used were NovaBlue (λDE3) (*end*A1 *hsd*R17(r$^-$K12 m$^+$K12) *sup*E44 *thi*-1 *rec*A1 *lac*[F′ *pro*AB *lac*I^{q}ZΔM15::Tn10] (λDE3)) from Novagen®, and DH5α (Hanahan, 1983). Strains were grown in LB-medium (Ausubel et al., 1989) at 37 °C. Antibiotic concentrations were 5 μg/mL of chloramphenicol for *B. subtilis*, and 35 μg/mL of chloramphenicol or 100 μg/mL of ampicillin for *E. coli*. IPTG was used at a concentration of 1 mM for the T7 RNA polymerase induction in NovaBlue(λDE3). *E. coli* cells were transformed according to Chung et al. (1989).

Plasmid construction

Plasmid pMP411 (Pelchat et al., 1998) contains the σ^A-type *gltX* promoter *gltX*, the intergenic region *cysE*, and the 5′ end of *cysS* inserted into the *B. subtilis–E. coli* shuttle and promoter probe vector pRB394 (Brückner, 1992). Plasmid pMP205 (Pelchat et al., 1998) contains the *Hin*dIII insert of pLQB205 (Breton et al., 1990) including the σ^A-type *gltX* promoter and the 5′ end of the *gltX* gene, cloned into the *Hin*dIII site of pRB394. On these plasmids, the chloramphenicol resistance gene (*cat*) coming from pRB394 is immediately downstream from the insert, and its transcription is controlled by the *gltX* promoter. Plasmid pMP1 contains the *Dra*I fragment of pMP411 (including the 3′ end of the *gltX* gene, the intergenic region, and the first 537 nt of the *cysE* gene) inserted at the *Bsu*36I site of pMP205 after its blunting with the Klenow fragment of *E. coli* DNA polymerase I (Fig. 2). This plasmid has the *gltX-cysE* intergenic region under the direct control of the *gltX* promoter. Plasmid pMP8 was constructed by inserting at the *Eco*RI site of pRB394 a 412-bp PCR product from pMP1 using the oligonucleotide MP166, including a T7 RNA polymerase promoter initiating the transcription at the +1 site of the transcription of *gltX* and the oligonucleotide MP168 complementary to a sequence situated at the beginning of *cysE* (Fig. 2). Transformants carrying pMP8 were selected by their ability to promote chloramphenicol resistance in *E. coli* NovaBlue(λDE3) in the presence of 1 mM IPTG. Unless otherwise mentioned, the enzymatic reactions were conducted in the buffers provided by the supplier.

RNA isolation

Bacillus subtilis 168T from a 20-mL, exponentially growing culture was harvested by centrifugation at an 0.4 A_{600nm} and frozen with liquid nitrogen. The cells were resuspended in 4 mL ice-cold TE buffer (pH 8.0). RNA was isolated as described by Putzer et al. (1992) by phenol extraction in the presence of glass beads (150–212 μm, from Sigma) and by successive phenol extractions, followed by ethanol precipitations. RNA was resuspended in an appropriate volume of diethyl-pyrocarbonate (DEPC)-treated water.

Reverse transcriptase assays

Reverse transcriptase (RT) assays were carried out as described by Gendron et al. (1994), by heating 10 μg of total RNA for 5 min at 65 °C with 1 pmol of the 5′ end-labeled oligonucleotide in 5 μL of 50 mM Tris-HCl, pH 8.3, 10 mM $MgCl_2$, 80 mM KCl, and then quickly freezing it in a mixture of dry ice and ethanol for 2 min. Four units of avian myeloblas-

tosis virus reverse transcriptase (Pharmacia) in 5 μL of ice-cold 4 mM dNTP, 8 mM DTT, 50 mM Tris-HCl, pH 8.3, 10 mM $MgCl_2$, and 80 mM KCl were added. The mixture was incubated for 30 min at 48 °C, to which 5 μL of formamide-containing dye were added. The DNA products were then analyzed by electrophoresis on a denaturing (8 M urea) 8% polyacrylamide gel in TBE 1× buffer.

S1 nuclease assays

S1 nuclease assays were conducted as described by Ausubel et al. (1989). The DNA probe was produced by PCR in 100 μL of buffer containing 20 ng of pMP1, 4 U of $Vent_R$® (exo^-) DNA polymerase from New England Biolabs, 50 pmol of a 15-mer starting at +229 nt of *cysE* (CYSE2) and 50 pmol of MP166 (Fig. 2). This was purified by electrophoresis on 1.5% agarose gel, extracted with the QIAquick™ Gel Extraction Kit (QIAGEN), and counted for ^{32}P in a scintillation counter. A probe aliquot emitting 50,000 cpm (measured by Cerenkov effect) was coprecipitated with 100 μg of *B. subtilis* total RNA (or 3 μg of RNA produced from in vitro transcription using T7 RNA polymerase on the MP166-CYSE2 amplicon; see above) in ethanol, and resuspended in 20 μL of 80% deionized formamide, 40 mM PIPES, pH 6.4, 400 mM NaCl and 1 mM EDTA. This solution was heated for 10 min at 65 °C and kept overnight at 50 °C to allow hybridization. S1 nuclease (300 U; Pharmacia) in 300 μL of 280 mM NaCl, 50 mM sodium acetate, pH 4.5 and 5 mM $ZnSO_4$ was added and the solution was incubated for 60 min at 30 °C. After ethanol precipitation, the DNA was analyzed by electrophoresis on 12% polyacrylamide gel and autoradiography.

In vitro transcription assays

In vitro transcription analysis was done according to Ausubel et al. (1989) using 20 nmol of a purified plasmid or of a purified DNA fragment, 10 μCi of [α-^{32}P]UTP, 0.5 μg of purified *B. subtilis* RNA polymerase (σ^A), and 8 U of Rnasin (Promega) in 50 μL of 0.4 mM NTP, 40 mM Tris-HCl, pH 8.0, 50 mM KCl, 1 mM DTT and 50 μg/mL of BSA. Samples were incubated for 30 min at 30 °C. The RNA was extracted with water-equilibrated phenol, precipitated, washed, dried, suspended in a formamide loading buffer, and analyzed by electrophoresis on a denaturing (8 M urea) polyacrylamide gel. In vitro transcription was also performed under similar conditions using 10 U of *E. coli* RNA polymerase (Pharmacia) or 10 U of T7 RNA polymerase (Pharmacia); the buffer used with the T7 RNA polymerase was 50 mM Tris-HCl, pH 8.0, 8 mM $MgCl_2$, 2 mM spermidine-$(HCl)_3$ and 25 mM NaCl, along with 10 U of Pharmacia's RNAguard, which was also used as RNase inhibitor with the former two RNA polymerases.

Half-life measurements

Rifampicin (150 μg/mL) was added to 400 mL of an exponentially growing culture of *B. subtilis* 168T. Aliquots of 50 mL of the culture were collected and poured over crushed ice at 0, 2, and 4 min after the addition of rifampicin. The cells were then harvested by centrifugation at 4 °C and frozen with liquid nitrogen. RNA was isolated as described and mRNA half-lives were determined by RT assays using MP168 and GTLX for the 5′ end of the *cysE/S* and *gltX* mRNA, respectively. Residual mRNA was measured by phosphorimager (Fuji-Film) using the MacBas version 2.5 (Fuji Photo Film Co., Ltd.) software.

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