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Transcriptional Control of *dacB*, Which Encodes a Major Sporulation-Specific Penicillin-Binding Protein

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Sporulation-specific sigma factor E (σ^E) of *Bacillus subtilis* is both necessary and sufficient for transcription of the *dacB* gene, which encodes penicillin-binding protein 5*. Evidence in support of this conclusion was obtained by primer extension analysis of *dacB* transcripts and the induction of active σ^E with subsequent synthesis of PBP 5* in vegetative cells.

The *dacB* gene of *Bacillus subtilis* encodes penicillin-binding protein (PBP) 5*, a membrane-bound enzyme that is required for synthesis of the cortical peptidoglycan and for the development of heat-resistant spores (2, 3). This PBP is never produced by vegetatively growing cells or stationary-phase cells under nonsporulating conditions (19). PBP 5* first appears late in stage II of sporulation; it is produced exclusively in the mother cell, and it can be found in both the mother cell (sporangial) membrane and the outer forespore membrane (2, 4). The protein, which has a mass of 40 kDa after cleavage of its amino-terminal signal sequence, is believed to be anchored in the membrane at its carboxy terminus with the bulk of the PBP on the outer surface, where peptidoglycan is assembled and cross-linked (3). PBP 5* has a weak D,D-carboxypeptidase activity in vitro, but its actual function in vivo is not yet known (21).

The aim of the present study was to determine the mechanism by which *dacB* gene expression is regulated. We started with the hypothesis that *dacB* has a promoter that is only recognized by a sporulation-specific form of RNA polymerase. Many such genes are already known to exist (8). In the case of *dacB*, there is circumstantial evidence pointing to its dependence on RNA polymerase associated with sigma factor E (σ^E), e.g., both the timing and cellular location of *dacB* expression are consistent with the activation of pro-σ^E, which occurs during stage II in the mother cell only; *dacB* has what appears to be a σ^E recognition sequence in its putative promoter region; and *dacB* is not expressed in mutants in which σ^E is absent or unprocessed (3, 13, 20, 22). In contrast, PBP 5* is still produced when either the forespore-specific σ^G or the late-stage, mother-cell-specific σ^K is absent (3). The results described herein provide additional and more direct evidence of the σ^E-dependent expression of *dacB*.

Primer extension analysis of *dacB* transcripts. *B. subtilis* 168 *trpC2* was induced to sporulate by the nutrient exhaustion method (18, 19), and both RNA and membranes were prepared from samples collected at various times thereafter (6, 14, 19). The PBPs in the membranes were assayed as described previously (1, 5, 19). The primer extension assay was performed on each RNA sample with an oligonucleotide primer that was complementary to a region of the *dacB* mRNA that extended from nucleotide 73 through nucleotide 93 of the coding sequence (3). Its 5′ end was labeled with ^{32}P by using T4 polynucleotide kinase from Promega Corp. (Madison, Wis.) and [γ-^{32}P]ATP from Dupont NEN (Boston, Mass.). The primer extension reaction was done with an AMV reverse transcriptase primer extension system purchased from Promega Corp. on 80 μg of each RNA preparation according to the directions provided by the manufacturer. The primer extension products were separated from the bulk of the RNA by purification with a MERmaid kit purchased from BIO 101 (La Jolla, Calif.).

The same predominant primer extension product was generated from RNA samples collected after 2.5, 3, 3.5, and 4 h of sporulation (Fig. 1A). By comparison with the results of a DNA sequencing assay of *dacB* using the same primer, the apparent +1 nucleotide was determined to be a C in the region downstream from a sequence that strongly resembled the σ^E promoter consensus sequence (12). This nucleotide was 26 bp upstream from the AUG start codon for PBP 5* (3).

The time at which *dacB* transcripts could first be detected by primer extension analysis coincided with the appearance of PBP 5* in the membranes (compare Fig. 1A with 1B). Moreover, the amount of PBP 5* per milligram of membrane protein approached its maximum at roughly the same time as the amount of *dacB*-specific RNA began to decline. These observations indicate that the accumulation of membrane-bound PBP 5* is transcriptionally regulated, and it is very likely that the same promoter is used throughout the time of *dacB* expression.

Production of sporulation-specific PBP 5* during vegetative growth. A direct test to determine if *dacB* can be transcribed in vivo by σ^E-associated RNA polymerase was performed with vegetative cells in which neither σ^E nor PBP 5* is normally found. The plasmid pDG180 carries a truncated *spoIIGB* sequence, which encodes the mature form of σ^E and is under the control of the isopropyl-β-D-thiogalactoside (IPTG)-inducible *spac* promoter (10, 16). This plasmid was introduced by transformation into *B. subtilis* MO367, which has a *spoIID-lacZ* transcriptional fusion integrated at the *amyE* locus (20). This fusion is efficiently expressed during vegetative growth when 1 mM IPTG is used to induce σ^E production from the *spac* promoter (11, 20). Thus, the production of β-galactosidase served as a positive control for σ^E activity.

Induction of σ^E synthesis during vegetative growth of *B. subtilis* in 2XYT medium (17) resulted in the production of PBP 5* and its localization in the cell membrane (Fig. 2A, dashed line). At 90 min after induction, which roughly corresponded to the end of logarithmic growth (Fig. 2B), the activity of β-galactosidase reached its maximum value (approximately 80 Miller units), while the amount of PBP 5* continued to increase for at least another hour. We surmise that, upon entry

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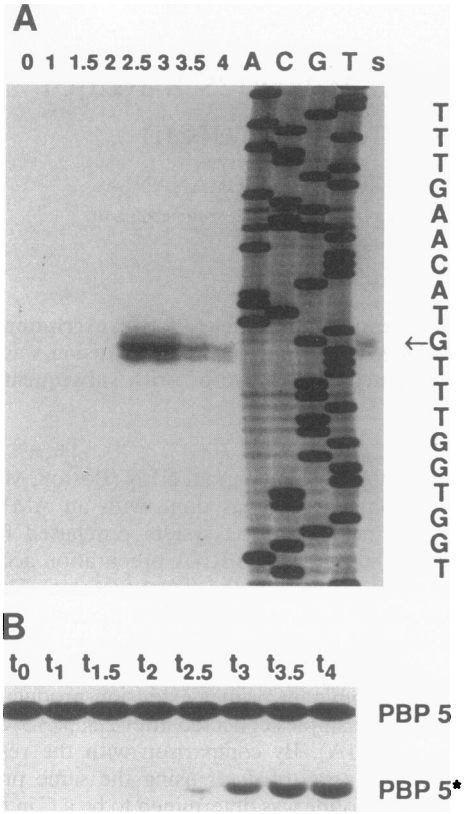

FIG. 1. (A) Primer extension analysis of RNA samples collected at various times (0 through 4 h) after t_0 of sporulation. The DNA sequence at the right (read from sequencing lanes A, C, G, and T) corresponds to the template strand of *dacB*; the apparent +1 nucleotide is indicated by the arrow. Lane s contains surplus sample from the extension products loaded into the lanes on the left side of the figure. (B) Fluorograph of [^{3}H]penicillin-labelled PBPs 5 and 5* in membrane samples prepared from cells collected at various times during sporulation. PBP 5 is the most abundant PBP in vegetative cells. The same culture of *B. subtilis* 168 *trpC2* served as the source of RNA for the primer extension analyses.

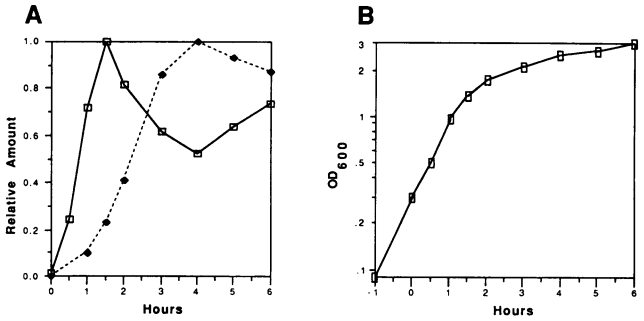

FIG. 2. (A) Activity of β-galactosidase (solid line) and amount of membrane-bound PBP 5* (dashed line) at various times relative to the maximum value achieved for each product in the 6 h following induction of σ^E synthesis. (B) Growth curve for *B. subtilis* MO367 (pDG180) in 2XYT from 1 h before induction to 6 h postinduction. IPTG was added to the culture at zero hour (optical density of 0.3). β-Galactosidase activity was assayed as described previously (15). OD_{600}, optical density at 600 nm.

into stationary phase, the rate of turnover of β-galactosidase began to exceed its synthesis, whereas PBP 5* was a more stable product and thus continued to accumulate in the membrane as long as any synthesis was possible. In a parallel culture that was not induced, there was a very low background level of β-galactosidase activity (<1.5 Miller units), which increased less than twofold over the time period examined. At no time was any PBP 5* detected in the uninduced culture. This result clearly indicates that σ^E is sufficient for *dacB* transcription in vivo, and it is consistent with the results of previous genetic dependency studies (3). The vegetative cells seemed to be tolerant of this inappropriately expressed protein and other σ^E-dependent proteins that must have been made. Their presence had no apparent effect on cell growth or gross cellular morphology, as observed by phase-contrast microscopy.

Effect of premature synthesis of PBP 5* on sporulation. Since production of PBP 5* by vegetative cells had no obvious effect on them, we wondered if premature synthesis of this PBP would also have no effect on sporulation. We discovered that we could not induce expression of either σ^E-dependent gene between the beginning of sporulation (t_0) and stage II. Moreover, when these genes were induced and expressed prior to t_0 in a medium that supported sporulation, their expression ceased when the culture arrived at t_0 and resumed later in stage II (data not shown). Immunoassays for the presence of mature σ^E confirmed that the *spac* promoter was inducible in this time interval (9), and so evidently the sigma factor was not functional. Although induction before t_0 did not result in continuous synthesis of PBP 5*, it did produce cells that initiated sporulation with roughly 20% of their final concentration of PBP 5* already in the membrane. The premature presence of PBP 5* had no effect on the percentage of heat-resistant spores in the culture at t_{24}.

We conclude that σ^E-associated RNA polymerase is both necessary and sufficient for transcription of the *dacB* gene of *B. subtilis*. This explains why PBP 5* has never been detected in nonsporulating cells, because σ^E itself is not activated until stage II of sporulation. Regulation of *dacB* expression by this mechanism guarantees that PBP 5* will be synthesized at the appropriate time and in the appropriate compartment of the sporulating cell for synthesis of the cortical peptidoglycan. The *dacB* gene thus belongs to a subset of σ^E-dependent genes that are essential for normal cortex synthesis (8). Among these genes is at least one other gene, *spoVD*, that encodes a sporulation-specific PBP (7).

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