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Developmentally Regulated Transcription in a Cloned Segment of the *Bacillus subtilis* Chromosome

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We describe a model system for studying developmentally regulated transcription during spore formation in *Bacillus subtilis*. This model system is a cloned cluster of genes known as *0.4 kb*, *ctc*, and *veg* from the *purA-cysA* region of the *B. subtilis* chromosome. Each gene exhibited a distinct pattern of transcription in cells growing in glucose medium and in cells deprived of nutrients in sporulation medium. The *0.4 kb* gene was transcribed at a low level in growing cells but was actively transcribed during nutrient deprivation in sporulation medium. This ribonucleic acid (RNA) synthesis was dependent upon the products of five *B. subtilis* genes that are involved in the initiation of spore formation: *spo0A*, *spo0B*, *spo0E*, *spo0F*, and *spo0H*. A mutation in any one of these regulatory genes severely restricted transcription of the *0.4 kb* sequence. Transcription of the *ctc* gene was also turned on by nutrient deprivation, but this RNA synthesis was not impaired in Spo0 mutants. Although not under *spo0* control, the *ctc* gene probably corresponds to a locus, *spoVC*, whose product is required at a late stage of sporulation. Finally, the *veg* gene was actively transcribed both in growing cells and in nutrient-deprived cells. Like *ctc* RNA synthesis, transcription of the *veg* gene was not dependent upon the *spo0* gene products. We propose that the *spo0A*, *spo0B*, *spo0E*, *spo0F*, and *spo0H* gene products are components of a pathway(s) that senses nutrient deprivation in *B. subtilis* and translates this environmental signal into the transcriptional activation of a subset of developmental genes.

The process of sporulation in *Bacillus subtilis* is triggered by depriving growing cells of a source of nutrients. Nutrient-limited bacteria cease normal cell division and form instead polar septa that partition the sporulating cells into mother cell and forespore compartments. After engulfment by the mother cell, the forespore matures into a dormant endospore which is ultimately liberated by lysis of the mother cell. The initiation of this developmental progression is dependent upon the products of at least seven genetic loci known as the *spo0* genes (12, 13, 21). A mutation in any one of these genes blocks sporulation before any of the morphological changes characteristic of early spore development. Despite their crucial role in development, however, little is known about the *spo0* genes and how they control the initial events of spore formation.

We describe here a model system for studying the role of the *spo0* genes in gene transcription. This model system is a cloned cluster of three genes from the *purA-cysA* region of the *B. subtilis* chromosome (6, 24). Each of these genes—the *veg* gene, the *ctc* gene, and the *0.4 kb* gene—exhibited a distinct pattern of transcription. The *veg* gene was actively transcribed in growing cells as well as in sporulating bacteria. Transcription of the *ctc* gene was strongly stimulated during sporulation, but this RNA synthesis did not require the products of the *spo0* loci. Finally, the *0.4 kb* gene exhibited a third pattern of transcription: its transcription was markedly enhanced during sporulation, but this RNA synthesis was noticeably restricted by mutations in five of the seven *spo0* genes. Based on this model system, we propose that at least five of the *spo0* gene products are components of a regulatory pathway that influences the pattern of developmental gene transcription in *B. subtilis*.

Haldenwang and Losick (8, 9) have described a modified form of *B. subtilis* RNA polymerase that differs in its transcriptional specificity from the usual form of RNA polymerase holoenzyme. This modified enzyme lacks sigma factor (herein termed σ^{55}), but contains a novel sigma-like subunit of 37,000 daltons termed σ^{37} (8, 9). Here we report that both the *0.4 kb* gene and the *ctc* gene were transcribed selectively in cloned DNA by σ^{37}-containing RNA polymerase; σ^{55}-containing RNA polymerase, in contrast, directed specific transcription of the *veg* gene. The role of σ^{37} and

the *spo0* gene products in sporulation-controlled transcription is discussed.

MATERIALS AND METHODS

Bacterial strains. *B. subtilis* SMY, a Marburg strain, was obtained from P. Schaeffer. The stage 0 mutants JH646 (*spo0A12*), JH648 (*spo0B136*), JH695 (*spo0C9V*), JH647 (*spo0E11*), JH649 (*spo0F221*), JH651 (*spo0H81*) and JH693 (*spo0J93*) were a gift of J. Hoch, who constructed them from a sporulating 168 strain, JH642 (*trpC2, phe-1*).

Hybrid plasmid DNAs. The construction of p213 was described previously (24). p105 and p63 contain 1.8- and 5-kb segments of *B. subtilis* DNA, respectively, inserted by "DNA tailing" into the *Hpa*I site of the *E. coli* plasmid pMB9. These hybrid plasmids were identified in a clone bank of *B. subtilis* DNA shear fragments (kindly provided by Hutchison and Halvorson [15]) by means of colony hybridization, using radioactively labeled p213 DNA as a probe. p213-1, which contains the *veg* gene, was constructed by inserting an *Eco*RI* 2-kilobase (kb) fragment of p213 into pMB9. p63-1, which contains the *ctc* gene, was constructed by inserting the right-hand *Hin*dIII-*Eco*RI segment of p63 (containing both *B. subtilis* and vector DNA) into the *E. coli* vector pBR322. pLS5, which contains the *0.4 kb* gene, was constructed by A. L. Sonenshein, who inserted *Hin*dIII 700-bp DNA of p63 into the *Hin*dIII cleavage site of the *E. coli* plasmid pBR322. pLS5-11ΔR1, derived from pLS5 by M. Fine and A. L. Sonenshein, contains the *Hin*dIII-*Eco*RI 420-base pair (bp) segment from the promoter end of the *0.4 kb* gene.

Mapping endonuclease restriction sites. p105, p63, and p213 were cleaved with *Eco*RI, *Hin*dIII, *Hin*cII, *Hpa*I, and *Hpa*II, and the size of the fragments was determined by electrophoresis in agarose and polyacrylamide. Overlapping fragments (Fig. 1) were identified by sizing the products of double and triple digestions of total plasmid DNA, of purified restriction fragments and of subcloned *Eco*RI* fragments. Overlapping sequences were also identified by the two-dimensional DNA-DNA hybridization procedure of Hutchison as described in reference 20. Finally, *Hpa*II fragments were ordered by gel electrophoresis of a partial *Hpa*II digest of end-labeled *Eco*RI and *Hin*dIII fragments (14, 26). These mapping data are recorded elsewhere (J. F. Ollington, Ph.D. thesis, Harvard University, 1981, and T. Huynh, undergraduate Honors thesis, Harvard University, 1979).

Pulse-labeling cells during growth and sporulation. Vegetative cells were grown in Sterlini-Mandelstam (29) resuspension medium (3 ml) modified to contain 0.8% glucose and 0.1 mM K_2HPO_4 and labeled for 3 min during midexponential growth by the addition of 7 mCi of "carrier-free" $^{32}PO_4$ (New England Nuclear Corp.). Sporulation was induced by transferring exponentially growing cells from DS medium (23) or hydrolyzed casein medium (29) to Sterlini-Mandelstam (29) resuspension medium (3 ml) lacking phosphate. Sporulating cells (or stationary-phase cells of the stage 0 mutants) were labeled for 3 min (or for 5 min, in the experiments of Fig. 6 and Fig. 7B) with 7 mCi of $^{32}PO_4$ at h 4 after resuspension in the sporulation medium.

Isolation of pulse-labeled RNA. Pulse-labeled cells were poured over frozen 121 solution (27) to chill rapidly, transferred to protoplasting buffer (0.015 M Tris-hydrochloride [pH 8], 8 mM EDTA, 0.45 M sucrose, 400 μg of lysozyme per ml) and incubated for 40 min at 0°C. The protoplasts were collected by low-

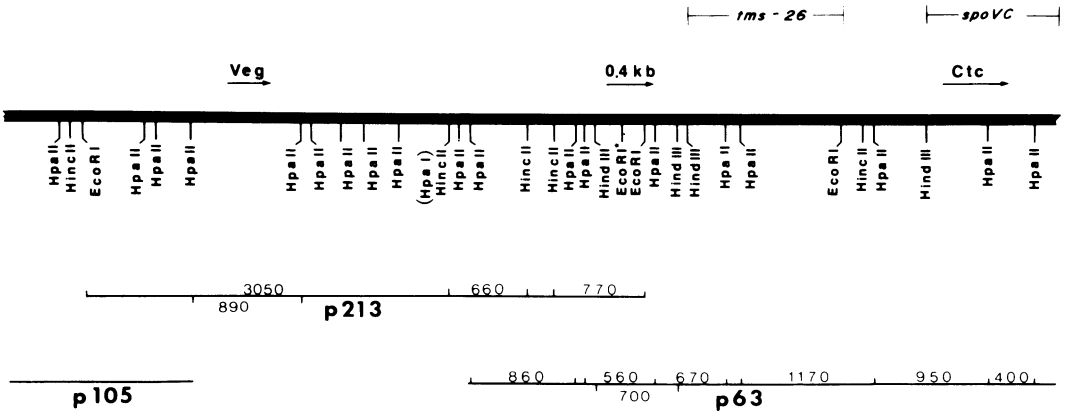

FIG. 1. *Physical and genetic map of the cloned gene cluster. Sites of cleavage for the restriction endonucleases EcoRI, HindIII, HincII, HpaI and HpaII are shown. The position of an EcoRI* site within the 0.4 kb gene is also identified, but a complete map of EcoRI* sites has not been determined. The positions on the physical map of the B. subtilis DNA inserts in p105, p213, and p63 are shown at the bottom of the figure. EcoRI-HincII and HpaII fragments of p213 that are referred to in the text are identified (in base pairs) above and below the DNA insert line, respectively. Similarly, specific HpaII and HindIII fragments of p63 are identified above and below the p63 DNA insert line, respectively. The location and orientation of the vegetative (veg), 0.4 kb, and ctc genes are identified above the physical map. The upper portion of the figure shows the boundaries within which the genetic loci tms-26 and spoVC lie. The genetic map is from the data of Haldenwang et al. (10), Moran et al. (17), and Moran and Stephens as described by R. Losick, in press.*

speed centrifugation (5,000 rpm for 5 min) and then rapidly frozen over dry ice-ethanol. The protoplasts were then suspended in lysing buffer (0.01 M Tris-hydrochloride [pH 8], 0.01 M NaCl, 0.001 M sodium citrate, 1.5% [wt/vol] sodium dodecyl sulfate, 3% [vol/vol] diethylpyrocarbonate) and incubated at 0°C for 30 min. Next, after the addition of 0.5 volume of 40% (wt/vol) NaCl solution, the mixture was incubated at 0°C for 10 min and clarified by centrifugation at 10,000 rpm for 10 min. RNA was then precipitated from the clarified supernatant fluid by addition of ethanol to 65% (vol/vol) and further purified by one of two methods.

In the experiments of Fig. 5 and 7A, the ethanol-precipitated RNA was suspended in 0.1 ml of TSE buffer (0.05 M Tris-hydrochloride [pH 6.9], 0.1 M NaCl, 0.001 M EDTA), brought to 35% (vol/vol) ethanol, and loaded onto a 2-ml CF-11 cellulose (Whatman) column prepared as described by Franklin (4). The column was then washed with TSE buffer brought to 35% (vol/vol) ethanol. (This step removes tRNA and DNA.) mRNA and rRNA were subsequently eluted with TSE buffer brought to 15% (vol/vol) ethanol. This RNA was precipitated with ethanol and suspended in 1 ml of 0.01 Tris-hydrochloride (pH 7.5)–0.5 M KCl, passed through a nitrocellulose filter, precipitated with ethanol, suspended in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and filtered through G-15 Sephadex beads (1 ml). Finally, formamide (MCB) was added to 50% (vol/vol), and the mixture was incubated with a blank strip of nitrocellulose for 60 min at 37°C to remove radioactive material that adhered nonspecifically to nitrocellulose.

In the experiments of Fig. 6 and 7B and Table 1, the ethanol-precipitated RNA was dialyzed against DNase buffer (0.01 M sodium acetate, 0.05 M NaCl, 0.001 $MnCl_2$ [pH 5.2]) and incubated with 100 μg of DNase (RNase-free; Worthington Diagnostics) for 90 min. After extraction three times with hot phenol (60°C), the final aqueous phase was brought to 0.5 M KCl–0.01 M Tris-hydrochloride (pH 7.5), passed through a nitrocellulose filter, and precipitated with ethanol. The DNase-treated radioactive RNA was then suspended in 200 μl of 10× SSC and filtered through Sephadex G-15 beads (1 ml). Finally, formamide was added to 50% (vol/vol).

In vitro RNA synthesis. RNA polymerase holoenzyme and σ^{37}-containing RNA polymerase were purified from *B. subtilis* as described by Shorenstein and Losick (25) and by Haldenwang and Losick (8). RNA polymerase (1.5 μg) was prebound to plasmid DNA template (2.5 μg) in 50-μl reaction mixtures lacking ribonucleotides but containing 20% (vol/vol) glycerol (30, 31). RNA synthesis was initiated by the addition of 0.15 mM CTP, GTP, and ATP and 0.4 μM (5 μCi) [α-^{32}P]UTP. (In the experiment of Fig. 8B, heparin [30 μg] was added 30 s after the addition of ribonucleotides to block reinitiation.) After 4 min of incubation at 37°C, unlabeled UTP (0.15 mM) was added. The reactions were then stopped after 2 min and RNA was isolated as described by Talkington and Pero (30, 31).

Gel electrophoresis and transfer of DNA fragments to nitrocellulose. Methods and buffers for polyacrylamide and agarose gel electrophoresis were described previously (24). Transfer of electrophoretically separated restriction fragments to nitrocellulose was modified from the procedure of Southern (28) as described previously (24). Nitrocellulose strips were 0.5 cm wide and contained 0.5 μg of DNA.

Hybridization reactions. Nitrocellulose strips, wet with hybridization buffer (5× SSC [pH 7.4] and 50% formamide), were wound into coils and placed in 1-cm-diameter vials. Portions (0.4 ml each) of radioactive RNA in hybridization buffer were added to each vial, completely covering the coil, and the vials were then sealed with Saran Wrap. The reaction mixtures were incubated with gentle shaking at 37°C for 18 h. The strips were then washed with hybridization buffer for 90 min at 37°C and washed twice with 2× SSC for 15 min at room temperature. Next, the strips were incubated with heat-treated pancreatic RNase (20 μg/ml) for 1 h at room temperature and then washed with 2× SSC for 1 h at room temperature. Finally, the strips were flattened, dried in vacuo at 80°C and exposed to Kodak XR-5 X-Ray film with an intensifying screen at −80°C.

RESULTS

Physical map of the cloned gene cluster. Figure 1 shows the location and direction of transcription of the three differentially regulated genes within a cloned segment of the *B. subtilis* chromosome. DNA-RNA hybridization experiments establishing the position and orientation of the *0.4 kb* gene and the *veg* gene are described below and in the accompanying report (19), respectively; the identification and positioning of the *ctc* gene are presented separately below.

Figure 1 also shows sites of cleavage for the restriction endonucleases *Eco*RI, *Hin*dIII, *Hin*cII, *Hpa*II, and *Hpa*I. This map was determined from the overlapping DNA inserts in hybrid plasmids p213, p105, and p63 (see above). The *B. subtilis* DNA insert in p213 is a 4.6-kb *Eco*RI fragment that contains the *veg* and *0.4 kb* genes (24). Plasmids 105 and 63 contain, respectively, 1.8- and 5-kb inserts that overlap the left- and right-hand ends of cloned DNA in p213. These plasmids were identified in a clone bank (15) of sheared *B. subtilis* DNA by reaction with radioactive p213 DNA (see above). Together, the overlapping DNA sequences in p213, p105, and p63 span an 8.5-kb segment of the *B. subtilis* chromosome (Fig. 1).

Mapping and orienting the *0.4 kb* gene. To position the *0.4 kb* gene, radioactively labeled 0.4-kb RNA was hybridized by the Southern (28) procedure to electrophoretically separated restriction fragments of p213 DNA. The hybridization experiment of Fig. 2 shows that radioactive 0.4-kb transcript reacted with *Hin*cII and *Hpa*II fragments from the extreme right-hand end of cloned DNA in p213. These and other Southern hybridizations to *Eco*RI, *Eco*RI*, *Hin*dIII, *Hin*cII, and *Hpa*II fragments of p63

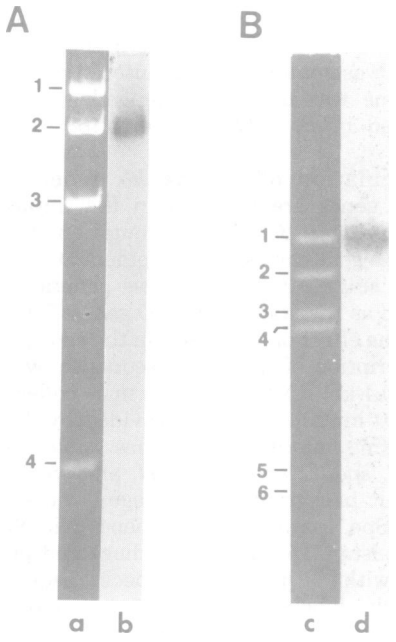

FIG. 2. *Mapping the 0.4 kb gene. Lanes a and c show HincII and HpaII fragments, respectively, of p213 that were separated electrophoretically in agarose (see text) and stained with ethidium bromide. The sizes in bp of the HincII fragments were: (1) 4,000; (2) 3,220; (3) 2,210; and (4) 660. The sizes in bp of the HpaII fragments were: (1) 1,140; (2) 1,000; (3) 890; (4) 860; (5) 520; and (6) 500. HincII fragment 1 and HpaII fragment 1 overlapped the left-hand EcoRI junction of B. subtilis DNA and vector pMB9 DNA in p213; HincII fragment 2 and HpaII fragment 1 overlapped the right-hand junction of B. subtilis and vector DNA. Electrophoretically separated HincII (lane b) and HpaII (lane d) were transferred to nitrocellulose by the Southern (28) procedure and hybridized with ^{32}P-labeled 0.4-kb RNA (30,000 cpm) purified as described by Segall and Losick (24). Hybrids were visualized by autoradiography.*

and p213 DNAs (data not shown) place the 0.4-kb sequence within a 500-bp segment bounded by *Hin*dIII and *Hpa*II sites as indicated in Fig. 1.

Next, we determined the orientation of the *0.4 kb* gene. By DNA-RNA hybridization to electrophoretically separated strands of denatured DNA, we (8) had previously shown that the "slowly migrating" strand of a 770-bp *Hin*cII-*Eco*RI fragment (Fig. 1) that terminates within the *0.4 kb* gene is the DNA coding strand. To establish the direction of gene transcription, it was only necessary, then, to determine the 5′—3′ orientation of the slowly migrating DNA strand. This was accomplished by radiolabeling selectively the 5′ strand terminus at the *Eco*RI end of the 770-bp fragment as outlined in Fig. 3. Electrophoresis of denatured 770-bp fragment that contained radioactive phosphate at one (Fig. 3, left side) or both (Fig. 3, right side) 5′ termini identified the slowly migrating DNA strand as the strand whose 5′ terminus was at the *Eco*RI end of the 770-bp fragment (Fig. 4). Therefore, as the slowly migrating strand was the coding DNA and as RNA is synthesized in a 5′ to 3′ direction, the direction of transcription of the *0.4 kb* gene was from left to right (Fig. 1).

Induction of the *0.4 kb* gene. Because the *veg* and *0.4 kb* genes are separated by cleavage sites for *Hin*cII, transcription of each gene could be monitored separately by hybridization to electrophoretically separated *Eco*RI-*Hin*cII fragments of p213 DNA. The *veg* gene was contained within a *Hin*cII-*Eco*RI fragment of 3,050 bp, whereas the *0.4 kb* sequence was largely contained in a fragment of 770 bp (Fig. 1). Pulse-labeled RNA from vegetative cells (growing in Sterlini-Mandelstam [29] resuspension medium plus glucose) hybridized specifically to the *Eco*RI-*Hin*cII 3,050-bp fragment; little hybridization could be detected to the 770-bp fragment that contains the *0.4 kb* gene (Fig. 5A, lane a, and 5B, graph a). In contrast, pulse-labeled RNA from cells induced to sporulate by resuspension in Sterlini-Mandelstam medium (without added glucose) hybridized to both the 3,050- and 770-

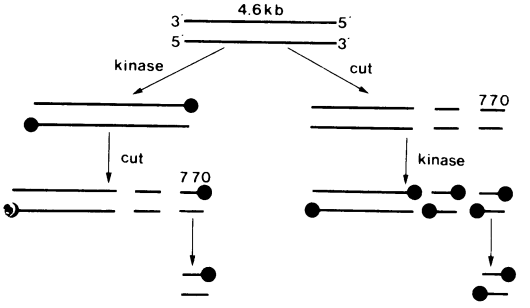

FIG. 3. *Strategy for radioactively labeling the DNA strands of EcoRI-HincII 770-bp fragment. The figure outlines the strategy for labeling the EcoRI-HincII 770-bp fragment either at its EcoRI 5′ terminus or at both its EcoRI and HincII 5′ termini. For labeling only the EcoRI terminus (left side of figure), EcoRI-cut p213 was radioactively labeled by reaction with polynucleotide kinase as described by Talkington and Pero (31). Next, the end-labeled DNA was cut with HincII, and finally, radioactive 770-bp DNA, labeled on only one DNA strand, was purified by agarose gel electrophoresis. For labeling both 5′ termini (right side of figure), EcoRI-cut p213 DNA was first cut with HincII and then labeled at both EcoRI and HincII 5′ termini by reaction of polynucleotide kinase. Finally, radioactive 770-bp DNA, labeled on both DNA strands, was purified by agarose gel electrophoresis.*

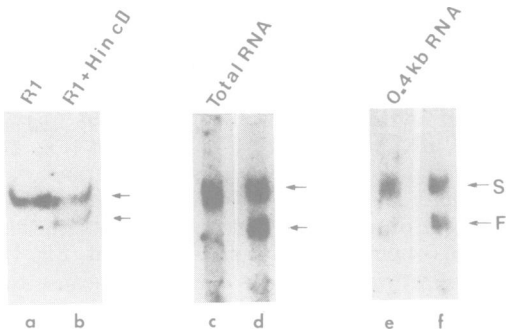

FIG. 4. *Direction of transcription of the 0.4 kb gene. A 770-bp fragment that contains 0.4 kb sequence was purified from p213 DNA that had been cleaved with EcoRI and HincII. (The EcoRI-HincII 770-bp DNA is identified in Fig. 1.) Next, the strands of the 770-bp fragment were separated by electrophoresis of denatured DNA through a 5% polyacrylamide gel as described by Hayward (11). The separated DNA strands were then transferred by the Southern (28) procedure to nitrocellulose. Lanes a and b are Southern imprints of the separated strands of 770-bp DNA that had been radioactively labeled either at its 5' terminus (lane a) or at both its EcoRI and HincII 5' termini as outlined in Fig. 4. Lanes c through f are Southern imprints of unlabeled DNA strands of EcoRI-HincII 770-bp DNA (0.05 μg per strip). In lanes d and f, both DNA strands were visualized by annealing Southern imprints (that had been treated by the Denhardt [3] procedure to prevent nonspecific DNA binding) with p213 DNA that had been radioactively labeled in vitro by the nick-translation activity of DNA polymerase I (22). In lanes c and e, the Southern imprints were hybridized either with total pulse-labeled sporulation RNA (lane c; 5×10^5 cpm) prepared as described in the text or with purified 0.4-kb RNA (lane e; 2×10^4 cpm) prepared as described by Segall and Losick (24). S is the slowly migrating DNA strand and F is the fast-migrating DNA strand.*

bp fragments (Fig. 5A, lane b, and 5B, graph b). Thus, hybridization to the 770-bp fragment served as a measure of *0.4 kb* gene transcription.

To measure quantitatively transcription from these genes, we hybridized pulse-labeled RNAs to DNA subclones of p213 that contained the *veg* gene (p213-1) or the *0.4 kb* gene (pLS5-11ΔR1). The *veg* gene was transcribed about as actively in sporulating cells as in growing bacteria (Table 1). The *0.4 kb* gene, in contrast, was transcribed about 10 times more actively in the sporulating bacteria than in the growing cells.

Using DNA probes to measure specific transcription, we examined the induction of the *0.4 kb* gene under a variety of conditions in several different strains of *B. subtilis*. In summary, the *0.4 kb* gene was efficiently induced in Sterlini-Mandelstam (29) resuspension medium, 121B medium (27), and Difco sporulation medium (23). However, the previously reported (24) repression of the *0.4 kb* gene by phosphate addition was not observed. Transcription of the *0.4 kb* gene was not strain dependent as it was induced in *B. subtilis* strains 168, 3610, and SMY.

Regulation of the *0.4 kb* gene. At least seven genes are involved in the initiation of sporulation in *B. subtilis*; a mutation in any one of these genes arrests development at its earliest stage, stage 0 (13, 22). These genetic loci are known as the stage 0 or *spo0* genes. To investigate the effect of mutations in the *spo0* genes on transcription of the *0.4 kb* sequence, we examined 0.4-kb RNA synthesis in a collection of stage 0 mutants (kindly provided by J. Hoch) harboring mutations in genes *spo0A*, *spo0B*, *spo0C*, *spo0E*, *spo0F*, *spo0H*, and *spo0J* in an isogenic background. The stage 0 mutants and their Spo^+ parent were suspended in Sterlini-Mandelstam sporulation medium and pulse-labeled with [^{32}P]phosphate. Specific sequences in the pulse-labeled RNAs were then detected by Southern hybridizations to *Eco*RI-*Hin*cII fragments of p213 DNA.

We distinguish two categories of Spo0 mutants (Fig. 6 and 7, which is considered below). Mutations in genes *spo0A*, *spo0B*, *spo0E*, *spo0F*, and *spo0H* severely impaired 0.4-kb RNA synthesis, whereas *spo0C* and *spo0J* mutations apparently had little or no effect on *0.4 kb* gene transcription. (It is conceivable, however, that the *spo0C* and *spo0J* mutations prevent 0.4-kb transcription but that a sequence other than the *0.4 kb* gene is transcribed on the *Hin*cII-*Eco*RI 770-bp segment of Fig. 6 and the *Hpa*II 560-bp segment of Fig. 7.) Neither category of mutation had a measurable effect on transcription from the 3,050-bp segment containing the *veg* gene. We conclude that efficient transcription of the *0.4 kb* gene was apparently dependent upon the products of at least five of the seven *spo0* genes.

Identification of a new sporulation-induced gene. By means of the colony hybridization procedure of Grunstein and Hogness (5), we searched in the clone bank of Hutchison and Halvorson (15) for *B. subtilis* sequences that are actively transcribed during sporulation. This search identified the *B. subtilis* DNA inset in p63 (Fig. 1) as a chromosomal region of exceptionally active, sporulation-induced transcription (data not shown). Since p63 contained the *0.4 kb* gene, we investigated whether the *0.4 kb* gene was sufficient to account for this active transcription or whether p63 contained additional sporulation-induced transcription units.

To map transcribed sequences in p63 DNA, pulse-labeled RNA was hybridized to electrophoretically separated *Hpa*II fragments of the

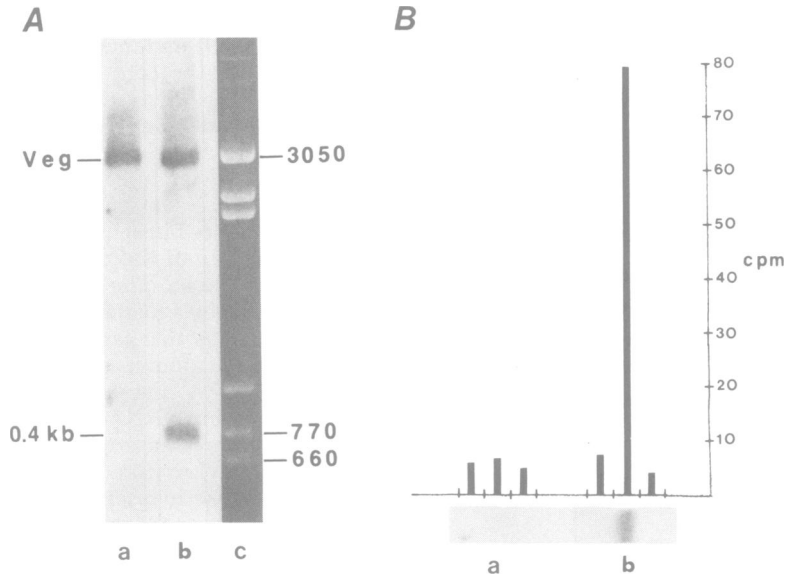

FIG. 5. *Transcription of the veg and 0.4 kb genes. (A) Pulse-labeled RNA (8 × 10⁵ cpm) from vegetative (lane a) and from sporulating (lane b) cells of B. subtilis strain SMY was hybridized to Southern strips containing EcoRI-HincII fragments of p213 DNA. Hybrids were visualized by autoradiography. Lane c shows EcoRI-HincII fragments of p213 that were separated electrophoretically in agarose and stained with ethidium bromide. The labeled fragments are B. subtilis DNAs and are identified in Fig. 1; the unlabeled fragments are from the vector pMB9. (B) Radioactivity in 4-mm slices cut from across the 770-bp region of the Southern strips of (A) was measured in a scintillation counter. Radioactivity from the hybridization of pulse-labeled vegetative RNA (a) and of pulse-labeled sporulation RNA (b) is plotted above the region of the Southern strip from which the slice was cut.*

TABLE 1. *Quantitative hybridizations*[a]

DNA probe	% Radioactivity retained in hybrid	
	Growth	Sporulation
p213-1 (*veg*)	0.12	0.10
pLS5-11ΔR1 (*0.4 kb*)	0.011	0.10
p63-1 (*ctc*)	0.010	0.14

[a] Cells were pulse-labeled during growth or after resuspension in sporulation medium as described in the text. ^{32}P-labeled RNAs were then incubated for 48 h at 40°C in vials containing 0.1 ml of hybridization buffer (40% formamide, 2× SSC, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.2% sodium dodecyl sulfate, and 0.02% bovine serum albumin) and nitrocellulose filters containing 1 μg of the following plasmid DNAs: p213-1, a probe for the *veg* gene; pLS5-11ΔR1, a probe for the *0.4 kb* gene; p63-1, a probe for the *ctc* gene; and as a control, pBR322, an *Escherichia coli* plasmid vector. In all cases, the amount of radioactivity retained on the filters was shown to be linearly dependent upon the amount of input RNA (1×10^5 to 5×10^5 cpm for growth RNA and 4×10^5 to 1×10^6 for sporulation RNA). The percent of the input retained on the filters was calculated after subtracting the background radioactivity retained on the control filters (this background radioactivity was less than 0.01% of the input in all cases).

hybrid plasmid. Radioactive sporulation RNA annealed to *Hpa*II fragments of 950, 560, and 400 bp (Fig. 7A, lane b). Transcription from all three DNA segments was induced in sporulation medium as little hybridization could be detected with pulse-labeled RNA from cells growing in glucose medium (Fig. 7A, lane a). Sporulation-induced transcription from within the 560-bp segment represented 0.4-kb RNA synthesis as the *0.4 kb* gene was contained within the *Hpa*II 560-bp fragment (Fig. 1). Hybridization to 950- and 400-bp fragments, which are adjacent to each other in p63 (Fig. 1), indicated that these segments contain at least one new transcription unit. Southern hybridization to *Hin*dIII fragments of p63 (data not shown) further localized this new unit of transcription (designated hereafter as the *ctc* gene) to the right of the *Hin*dIII site within the *Hpa*II 950-bp segment (Fig. 1). The quantitative hybridizations of Table 1 show that the *ctc* gene was transcribed about 14 times more actively in sporulating cells than in growing bacteria.

In contrast to 0.4-kb RNA synthesis, transcription from the *Hpa*II 950-to-400-bp region was not greatly impaired in any of the Spo0

mutants (Fig. 7B). (Notice, however, that RNA synthesis from the *0.4 kb* gene-containing *Hpa*II 560-bp fragment was markedly restricted in Spo0A, Spo0B, Spo0E, Spo0F, and Spo0H [Fig. 7B, lanes a through e] but not in Spo0J [lane f].) Because *ctc* transcription was induced by nutrient deprivation in wild-type bacteria as well as in the stage 0 mutants, this RNA synthesis was not under the control of the *spo0* genes tested. Nevertheless, as discussed below, the *ctc* transcription unit was close to or identical with (17; Fig. 1; R. Losick, *in* D. A. Dubnau, ed., *Molecular Biology of the Bacilli*, volume 1: *Bacillus subtilis*, in press) a locus *spoVC*, whose product is required in sporulation (32).

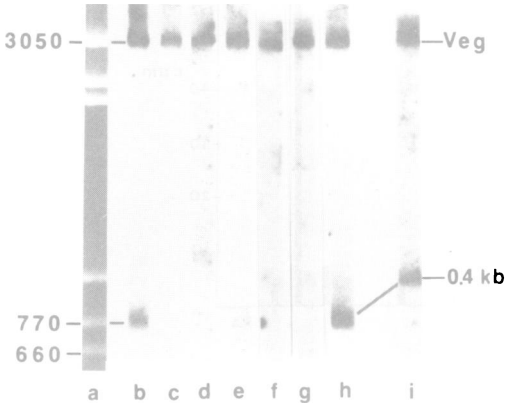

FIG. 6. *Effect of spo0 mutations on transcription of the 0.4 kb gene. Cells of the stage 0 mutants spo0A (lane c), spo0B (lane d), spo0E (lane e), spo0F (lane f), spo0H (lane g), spo0C (lane h), and spo0J (lane i) and of the sporulating parent (lane b) were pulse-labeled with [^{32}P]phosphate in Sterlini-Mandelstam sporulation medium. The pulse-labeled RNAs (10^6 cpm) were hybridized to Southern imprints of EcoRI-HincII fragments of p213. The position of the EcoRI-HincII fragments on the Southern imprints of lanes b through h is shown in the ethidium bromide-stained gel of lane a. The nitrocellulose strip of lane i was from a different batch of Southern strips than that of lanes b through h. Hybrids were visualized by autoradiography.*

Transcription of the *ctc* gene by modified RNA polymerase. The *0.4 kb* gene is transcribed in vitro by a modified form of *B. subtilis* RNA polymerase that lacks σ^{55} but contains a novel sigma-like subunit termed σ^{37} (8, 9). Figure 8A (lane b) shows that with p63 DNA as template, σ^{37}-containing RNA polymerase appeared to direct specific transcription of the *ctc* gene; RNA copied in vitro from p63 DNA by modified RNA polymerase hybridized selectively to the *Hpa*II 950-bp fragment that is known to contain *ctc* sequences. In some experiments, we also observed a low level of hybridization to the *Hpa*II 400-bp segment that is adjacent to the *Hpa*II 950-bp DNA in p63. (To our surprise, however, σ^{37}-containing polymerase failed to transcribe significantly the *0.4 kb* gene-containing *Hpa*II 560-bp region of p63, a finding which we consider further below.) To localize the start point for in vitro transcription by modified RNA polymerase, we employed *Hpa*II-cut DNA as a template for in vitro RNA synthesis. Figure 8B shows that with the truncated DNA as a template, modified RNA polymerase generated a 365-base "runoff" RNA (lane a) that was absent in RNA copied from *Hin*dIII-cut p63 DNA (lane b). Southern hybridizations of the run-off tran-

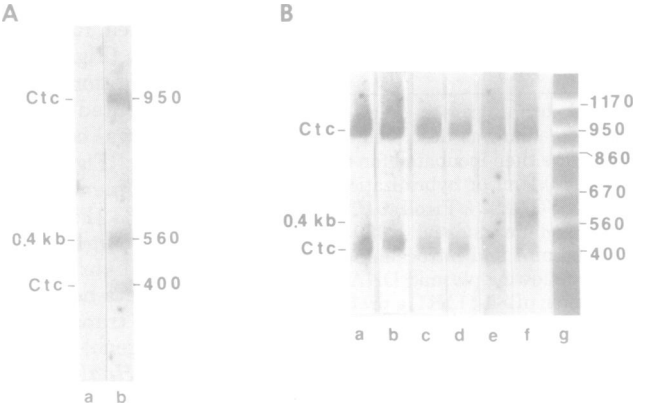

FIG. 7. *Transcription of the ctc gene. (A) Pulse-labeled RNA (10^6 cpm) from vegetative (lane a) and from sporulating (lane b) cells of B. subtilis strain SMY was hybridized to Southern imprints containing HpaII fragments of p63 DNA. (B) Cells of the stage 0 mutants spo0A (lane a), spo0B (lane b), spo0E (lane c), spo0F (lane d), spo0H (lane e), and spo0J (lane f) were pulse-labeled in Sterlini-Mandelstam sporulation medium. The pulse-labeled RNAs (10^6) were hybridized to Southern imprints of HpaII fragments of p63 DNA. The position of the HpaII fragments is shown in the ethidium bromide-stained gel of lane g. The nitrocellulose strips of (A) and (B) were from different batches. Hybrids were visualized by autoradiography.*

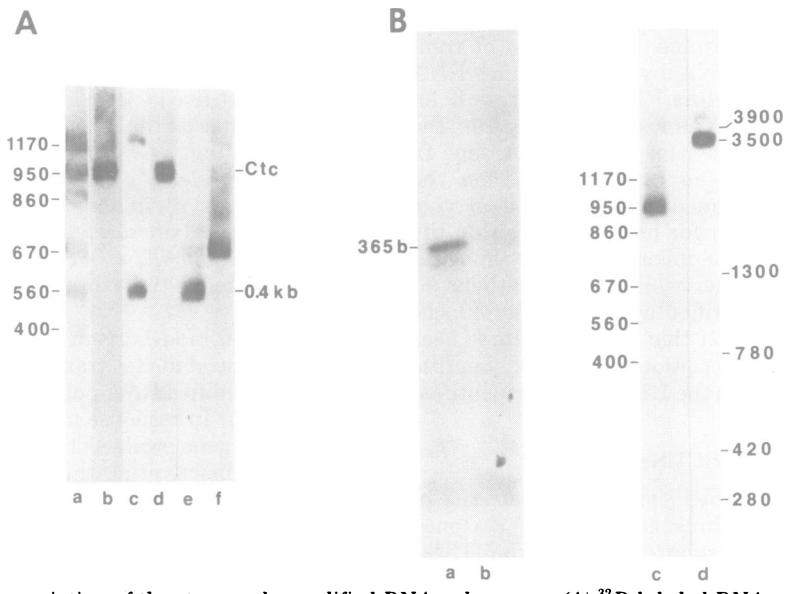

FIG. 8. *Transcription of the ctc gene by modified RNA polymerase. (A)* ^{32}*P-labeled RNA was synthesized in vitro in reaction mixtures (see text) containing: modified RNA polymerase and p63 DNA template (lane b), modified RNA polymerase and p213 DNA template (lane c), the modified RNA polymerase and p63 + p213 DNA template (lane d), modified RNA polymerase and HindIII 700-bp DNA subclone (pLS5) template (lane 3), and RNA polymerase holoenzyme and p63 DNA template (lane f). In vitro-synthesized RNAs were then hybridized to Southern imprints containing HpaII fragments of p63 DNA. The position of the HpaII DNAs was visualized in lane a by hybridizing a Southern strip (that had been treated by the Denhardt [3] procedure to prevent nonspecific DNA binding) with p63 DNA that had been radioactively labeled by the nick-translation activity of DNA polymerase I (22). (B) RNA was copied in vitro by modified RNA polymerase from HpaII-cut p63 DNA template (lane a) or from HindIII-cut DNA template (lane b). The products of in vitro transcription were displayed by electrophoresis through a 5% polyacrylamide gel containing 7 M urea. The 365-base RNA in a slice of polyacrylamide from the gel of lane a was then hybridized with Southern imprints containing HpaII (lane c) or HindIII-EcoRI (lane d) fragments of p63. The HindIII-EcoRI fragment of 3,500 bp was from the right-hand junction of B. subtilis DNA and the vector pMB9 DNA in p63.*

script to *Hin*dIII-*Eco*RI (Fig. 8B, lane d) and *Hpa*II (Fig. 8B, lane c) fragments of p63 localized the 365-base RNA to a 500-bp *Hin*dIII-*Hpa*II segment that contained *ctc* sequences. We deduce, therefore, that σ^{37}-containing RNA polymerase initiated at 135 bp to the right of the right-hand *Hin*dIII site in p63 and that transcription proceeded in a left-to-right direction (Fig. 1). In summary, σ^{37}-containing RNA polymerase transcribed a region of p63 DNA that appeared to correspond closely to the *ctc* gene.

How are we to explain the failure of σ^{37}-containing RNA polymerase to transcribe the *0.4 kb* gene in p63 DNA? As reported previously (8) and confirmed here (Fig. 8A, lane c), with p213 DNA as a template, modified polymerase readily generated RNA sequences complementary to the *Hpa*II 560-bp segment that contains the *0.4 kb* gene. Possibly, then, the *0.4 kb* gene promoter was simply damaged in p63 DNA. To test this, a *Hin*dIII 700-bp fragment containing the *0.4 kb* gene that was subcloned from p63 DNA was employed as a template for modified polymerase. The *Hin*dIII 700-bp DNA served as an effective template for 0.4-kb RNA synthesis (Fig. 8A, lane e). Thus, excision of the *0.4 kb* gene from p63 restored its ability to promote specific RNA synthesis.

A likely explanation, then, for the inability of modified polymerase to transcribe the *0.4 kb* gene in p63 DNA is that the *ctc* gene promoter provides a much stronger binding site for σ^{37}-containing RNA polymerase than does the *0.4 kb* gene promoter. Thus, with p63 DNA as a template, modified polymerase initiated selectively at the *ctc* gene. Consistent with this interpretation, addition of p63 DNA to p213 template strongly inhibited transcription of the *0.4 kb* gene (Fig. 8A, lane d). Moreover, by increasing the concentration of enzyme to a molar excess of polymerase to template (template was in excess in the experiment of Fig. 8), transcription of both the *ctc* gene and the *B. subtilis* gene could be detected (data not shown). A recent DNA binding experiment (9) is also consistent with the notion that the *ctc* promoter is a stronger

initiation site than the *0.4 kb* gene promoter.

Finally, we determined the pattern of transcription of p63 DNA by σ^{55}-containing RNA polymerase. *B. subtilis* RNA polymerase holoenzyme copied RNA almost exclusively from the *Hpa*II 670-bp segment of p63 (Fig. 8A, lane f). Although little or no transcription from the *Hpa*II 670-bp fragment was detected in vivo (Fig. 7), a promoter for holoenzyme within the *Hpa*II 670-bp fragment could be of physiological significance; in other work, we and others (6) have recently identified a known genetic locus (*tms-26*; reference 2) that maps within or near this region of transcription by RNA polymerase holoenzyme (within the 1.3-kb interval identified in Fig. 1).

DISCUSSION

We have described contrasting patterns of transcription for three genes within a cloned segment of the *B. subtilis* chromosome. These are the *veg* gene, the *ctc* gene, and the *0.4 kb* gene. The *veg* gene was actively transcribed both in growing cells and in cells suspended in sporulation medium. This RNA synthesis was apparently not dependent upon the *spo0* gene products. The *ctc* gene was transcribed at a low rate in cells growing in glucose medium, and this RNA synthesis was strongly stimulated during sporulation. Like *veg* gene transcription, *ctc* RNA synthesis was not restricted by mutations at the *spo0* loci. Finally, the *0.4 kb* gene exhibited a third pattern of regulation as its transcription was markedly enhanced during sporulation, but this RNA synthesis appeared to be under *spo0* control; *0.4 kb* transcription was dependent upon the products of five of the seven *spo0* genes.

We propose that at least some *spo0* gene products are components of one or more pathways that sense nutrient deprivation in *B. subtilis* and translate this environmental signal into the transcriptional activation of the *0.4 kb* gene and certain other early sporulation genes. According to this view, one or more of the *spo0* gene products must be present before the onset of sporulation, and hence, we believe that they are "vegetative" proteins. Indeed, mutations in the *spo0A* and *spo0B* genes are known to affect the sensitivity of vegetatively growing cells to certain phages and surface-active antibiotics (reviewed in references 12 and 21). Moreover, mutations in the *spo0A*, *spo0B*, *spo0E*, *spo0F*, and *spo0H* genes alter the pattern of protein synthesis in exponential-phase cells (1, 16).

We have distinguished two categories of *spo0* genes. Mutations in genes *spo0A*, *spo0B*, *spo0E*, *spo0F*, and *spo0H* severely restricted transcription of the *0.4 kb* gene, whereas mutations in genes *spo0C* and *spo0J* apparently had no effect on the pattern of gene expression. This implies that the requirements for *0.4 kb* gene transcription in vivo are complex and involve the products of at least five genetic loci that are located at scattered sites on the *B. subtilis* chromosome. Yet in vitro, the *0.4 kb* gene was transcribed with apparent fidelity by RNA polymerase that simply contained σ^{37} in addition to the subunits of core RNA polymerase. How, then, do the *spo0A*, *spo0B*, *spo0E*, *spo0F*, and *spo0H* gene products influence the expression of the *0.4 kb* gene?

Two general models seem most plausible. In a negative control model, transcription of the *0.4 kb* gene by modified RNA polymerase is blocked by a repressor. In response to nutrient deprivation, the *spo0* gene products turn on 0.4-kb RNA synthesis by inactivating this repressor. Alternatively, one or more of the *spo0* gene products (or a product under *spo0* control) could act as a positive regulator to activate 0.4-kb RNA synthesis. In this positive control model, we would suppose that the *0.4 kb* gene start site is a weak promoter and that active in vivo transcription of the *0.4 kb* gene requires a *spo0* gene product(s) in addition to σ^{37}. Consistent with this model, the experiment of Fig. 8A and a previously published DNA binding experiment (9) indicate that the *0.4 kb* gene initiation site was, in fact, a much weaker promoter for σ^{37}-containing RNA polymerase than was the promoter for the *ctc* gene.

Regulation of *ctc* transcription may be less complicated as this RNA synthesis was not dependent upon the *spo0* gene products. Although at least some σ^{37} can be detected in vegetative cells (10), preliminary experiments indicate that the amount of σ^{37}-containing RNA polymerase is influenced by the growth medium. Conceivably, then, *ctc* transcription is regulated at the level of the synthesis of σ^{37} or its association with RNA polymerase. Alternatively, σ^{37} may only account for the low level (Table 1) of *ctc* RNA synthesis in growing cells, and its replacement by other regulatory proteins could be responsible for the strong stimulation of *ctc* transcription during sporulation. Indeed, Haldenwang, Lang, and Losick (7) have recently found that a form of RNA polymerase containing a sporulation-induced subunit of about 29,000 daltons (σ^{29}; 18 and R. Losick, in press) is capable of transcribing the *ctc* gene in vitro.

Finally, we consider the possible physiological function of the *0.4 kb* and *ctc* genes. By recombinant DNA techniques, we have recently constructed a deletion mutation within the *0.4 kb* gene and inserted this mutation into the *B. subtilis* chromosome (A. Rosenbluh, C. D. B. Banner, and R. Losick, unpublished data). This mutation impedes spore maturation (stage V to

VI) but apparently does not impair vegetative growth, a finding that is consistent with the idea that the *0.4 kb* gene product is specifically involved in the sporulation process. In addition to this newly constructed mutation, at least one known sporulation locus has also been identified within the cloned gene cluster. DNA transformation experiments (17; Losick, in press) have mapped the *spoVC* locus, a gene whose product is required at a late stage of development (32), to within 1 kb of the right-hand edge of the cloned gene cluster (within the terminal *Hin*dIII segment, as indicated in Fig. 1). This places the *spoVC* locus within or very near the *ctc* gene, a gene whose transcription was not dependent upon the *spo0* loci. Nucleotide sequencing should enable us to determine the position of the *spoVC* locus precisely. If *spoVC* is indeed the *ctc* gene, then this could mean that the *spo0* gene products are only required for the transcription of a subset of genes whose products function in sporulation.

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