JOURNAL OF BACTERIOLOGY, Aug. 1981, p. 443-451
0021-9193/81/080443-09$02.00/0

Vol. 147, No. 2

A Cloned Gene That Is Turned on at an Intermediate Stage of Spore Formation in *Bacillus subtilis*

JAMES F. OLLINGTON AND RICHARD LOSICK*

The Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138

Received 6 March 1981/Accepted 8 May 1981

Cells of *Bacillus subtilis* synthesize a relatively long-lived ribonucleic acid (RNA) of about 300 bases during the course of spore formation. This transcript does not appear until an intermediate stage (III or IV) of development but is the predominant sporulation-specific transcript among RNAs of discrete size in late (stages IV to VI) developing cells. Appearance of the 300-base RNA is under sporulation control as this transcript could not be detected in cells of an early-blocked sporulation mutant (Spo0A). We have located the coding sequence for the 300-base RNA within a cloned chromosomal segment from the *purA-cysA* region that was previously shown to contain a cluster of genes that are actively transcribed during sporulation. The coding sequence for the 300-base RNA (designated as the *0.3 kb* gene) mapped between a gene (*veg*) that was actively transcribed during growth and development and a gene (*0.4 kb*) that was turned on at the onset of sporulation. Although clustered within a small segment of the chromosome, the *veg*, *0.3 kb*, and *0.4 kb* transcription units exhibited, therefore, distinct patterns of temporally programmed gene expression. Models for the activation of the *0.3 kb* gene at an intermediate stage of development are discussed.

The process of endospore formation in *Bacillus subtilis* proceeds according to a temporally defined program of gene expression (2, 11, 16, 17, 23). This is visualized most clearly by changes in the electrophoretic pattern of pulse-labeled polypeptides during the course of sporulation; sporulating cells synthesize at least five temporally defined classes of polypeptides during the first 5 or 6 h of development (12). The acid-soluble, spore core peptides (3, 20, 24) and at least one species of coat polypeptide (12), for example, appear at an intermediate stage of development. Is this temporally ordered program of protein synthesis regulated at the level of gene transcription, and if so, what are the molecular mechanisms that govern the timing of gene expression?

To investigate programmed gene expression in *B. subtilis*, we (7, 19) previously cloned in *Escherichia coli* a DNA segment from the *purA-cysA* chromosomal region that contains a cluster of genes that are actively transcribed during sporogenesis. Two genes (*0.4 kb* and *ctc*) in this cluster are induced at the onset of sporulation, whereas a third gene (*veg*) is actively transcribed during both growth and development (15). The *0.4 kb* gene is the site of a mutation that impedes spore formation at stage V to VI (A. Rosenbluh, C. Banner, and R. Losick, unpublished data), whereas *ctc* is close to or identical with the site of a mutation (*spoVC285*; 17, 25) that blocks development at stage IV to V. (The transcription of these genes is turned on earlier than the stage at which their products are apparently required in morphogenesis.) The *0.4 kb* and *ctc* (*spoVC*) genes are controlled from novel promoters since their transcription in vitro is catalyzed by two newly discovered species of *B. subtilis* sigma factor of 37,000 (σ^{37}) and 29,000 (σ^{29}) daltons but not by the usual transcriptional determinant of 55,000 (σ^{55}) daltons (8-10).

Here, we report on the discovery of a third sporulation-controlled gene (the *0.3 kb* gene) in this cloned gene cluster whose transcription is turned on at h 3 to 4 of development (stage III to IV). Although located among genes that are actively transcribed early in sporulation, the *0.3 kb* gene exhibits, therefore, a new pattern of temporally regulated gene expression.

MATERIALS AND METHODS

Bacterial strains and hybrid plasmids. *B. subtilis* SMY, a Marburg strain, and its asporogenous mutant Spo0A-5NA were obtained from P. Schaeffer. The construction of plasmids p213 and p213-1 was described previously (7, 19).

Pulse-labeling cells during sporulation. Sporulation was induced by transferring exponentially growing *B. subtilis* cells from DS medium (18) to Sterlini-Mandelstam (22) resuspension medium lacking phosphate. Cells in resuspension medium were

transferred to a prewarmed 50-ml Erlenmeyer flask containing "carrier-free" $^{32}PO_4$ (New England Nuclear Corp.) (2 mCi/ml). Cells were pulse-labeled for 5 min at 37°C, followed in all cases, except that of the hybridization experiment of Fig. 7, by a 5-min incubation with rifampin (12.5 μg/ml).

Purification of pulse-labeled RNA. Pulse-labeled RNA was isolated as described in the accompanying paper (15). If the RNA was to be fractionated by polyacrylamide gel electrophoresis, the radioactive RNA was eluted with the mRNA fraction from a CF-11 cellulose (Whatman) column (5), precipitated and washed with ethanol, and suspended in RNA sample buffer (4 mM Tris-hydrochloride [pH 7.2], 2 mM sodium acetate, 0.1 mM EDTA, 0.02% [wt/vol] sodium dodecyl sulfate, 0.02% [wt/vol] bromophenol blue, and 15% [vol/vol] glycerol). If unfractionated RNA was to be employed in a hybridization reaction (see Fig. 7), then after elution from cellulose and precipitation with ethanol, the pulse-labeled RNA was filtered through a nitrocellulose filter, precipitated and washed with ethanol, and suspended in hybridization buffer (50% [vol/vol] formamide and 5× SSC, pH 7.3 [1× SSC is 0.15 M NaCl and 0.015 M sodium citrate]).

In vitro RNA synthesis. ^{32}P-labeled RNA was synthesized in vitro by RNA polymerase holoenzyme (provided by either W. Haldenwang or N. Lang) as described previously (15).

Gel electrophoresis; elution of RNA and preparation of Southern strips. Methods and buffers for polyacrylamide and agarose gel electrophoresis and for the elution of ^{32}P-labeled RNA from polyacrylamide gel slices were described previously (19). Transfer of electrophoretically separated restriction fragments from agarose gels to nitrocellulose was modified from the procedure of Southern (21) as described previously (19). Nitrocellulose strips were 0.5 cm wide and contained 0.5 μg of plasmid DNA or 5 μg of total *B. subtilis* DNA.

Sizing the 0.3-kb RNA. ^{32}P-labeled RNA extracted from the 0.3-kilobase (kb) band of an aqueous polyacrylamide gel was rerun under denaturing conditions on (i) a 5% polyacrylamide gel in 98% formamide, as described by Segall and Losick (19), with insulin mRNA, 5.8S rRNA (gifts from A. Efstratiadis), and 5S rRNA used as standards, and (ii) a 5% polyacrylamide gel containing 7 M urea, with denatured pMB9-*Hae*III restriction fragments run as standards.

Hybridization reactions. Hybridization reactions were carried out as described in the accompanying paper (15). Unlabeled vegetative RNA was prepared by C. Herring from *B. subtilis* SMY cells growing exponentially in Sterlini-Mandelstam (22) resuspension medium supplemented with glucose (0.8% [wt/vol]) by the procedure of Pero, Nelson, and Losick (16). In the "blocking" hybridization experiments, Southern strips were incubated with 500 μg of unlabeled vegetative RNA in 0.4 ml of hybridization buffer (50% [vol/vol] formamide, 5× SSC [pH 7.3]) at 37°C for 18 to 24 h, at which time the vegetative RNA (in the hybridization fluid) was removed and the strips were washed briefly two to three times with fresh, prewarmed hybridization buffer. Radioactively labeled RNA was then added to the vials containing these strips. Control strips were incubated with hybridization buffer only before hybridization with ^{32}P-labeled RNA.

RESULTS

Sporulation-specific RNAs. To identify a transcript that appeared at an intermediate stage of sporulation, we examined changes in the pattern of pulse-labeled RNAs during the course of development. At 1.5-h intervals after their resuspension in Sterlini-Mandelstam medium, *B. subtilis* cells were radioactively labeled for 5 min with [^{32}P]phosphate and were then incubated for 5 additional min with rifampin, a specific inhibitor of RNA polymerase. As shown previously by Segall and Losick (19), this procedure enriches for relatively long-lived RNAs of discrete size by allowing less stable transcripts to decay. Pulse-labeled RNAs from drug-treated cells were then subjected to electrophoresis though a slab of polyacrylamide and exposed to X-ray film to visualize a wide range of discrete-sized transcripts (Fig. 1).

A comparison of the pattern of pulse-labeled RNAs from an early-blocked sporulation mutant Spo0A-5NA (Fig. 1, lanes e through h) with that of its wild-type parent SMY (Fig. 1, lanes a through d) revealed two RNAs of about 400 and 300 bases whose appearance was apparently under sporulation control; neither of these transcripts could be detected in stationary-phase cells of the asporogenous mutant. Although more readily visualized in RNA from rifampin-treated cells (Fig. 1), the 400- and 300-base RNAs could also be detected in pulse-labeled RNA from cells that had not been drug treated (J. F. Ollington, Ph.D. Thesis, Harvard University, Cambridge, Mass., 1981; and below); thus, their appearance during sporulation was not simply a consequence of rifampin treatment.

The 400-base RNA corresponds to a previously described sporulation-specific transcript that is encoded by a gene (*0.4 kb*) from the *purA-cysA* region of the chromosome (7, 19). This was confirmed directly by hybridizing 400-base RNA from the polyacrylamide gel of Fig. 1 to cloned *0.4 kb* DNA. The 400-base or "0.4-kb" RNA was synthesized through the first 6 h of development; an overexposure of the autoradiograph of Fig. 1 readily revealed this transcript in lanes 1 ($T_{1.5}$) and 2 (T_3) as well as in lanes 3 ($T_{4.5}$) and 4 (T_6) (data not shown, but see Fig. 1 and 6 of reference 19).

The 300-base or "0.3-kb" RNA, in contrast, could not be detected at $T_{1.5}$ (even after prolonged exposure of the X-ray film [data not shown]) and could be detected in only low amounts at T_3. By h 6 (lane d) of development, however, the 0.3-kb RNA was the dominant sporulation-specific transcript. These data sug-

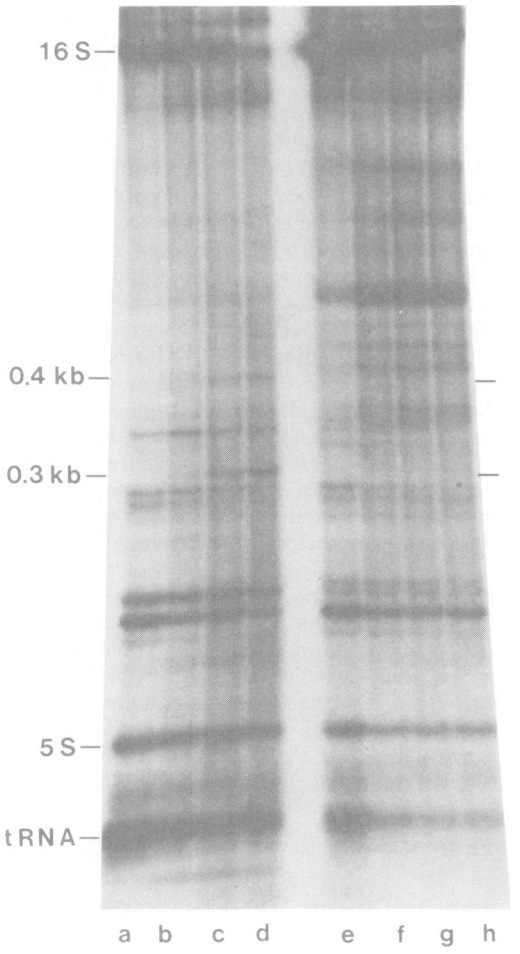

FIG. 1. *Polyacrylamide gel electrophoresis of RNA from pulse-labeled, rifampin-treated cells of B. subtilis. Cells of the wild-type sporulating strain SMY (lanes a through d) and an asporogenous mutant Spo0A-5NA (lanes e through h) were pulse-labeled with $^{32}PO_4$ and treated with rifampin at various times after transfer to Sterlini-Mandelstam resuspension medium as described in the text. RNA was extracted from these cells and resolved by slab gel electrophoresis through a 2.5 to 10.5% gradient of polyacrylamide and visualized by autoradiography. Cells were pulse-labeled at the following times after resuspension: SMY at h 1.5 ($T_{1.5}$) (lane a); SMY at T_3 (lane b); SMY at $T_{4.5}$ (lane c); SMY at T_6 (lane d); Spo0A-5NA at $T_{1.5}$ (lane e); Spo0A-5NA at T_3 (lane f); Spo0A-5NA at $T_{4.5}$ (lane g); Spo0A-5NA at T_6 (lane h).*

gested that syntheses of the 0.4- and 0.3-kb RNAs were subject to distinct temporal controls, an interpretation that is confirmed below by the hybridization of pulse-labeled RNAs to the cloned genes that encode these sporulation-specific transcripts.

In addition to their failure to synthesize the 0.4- and 0.3-kb RNAs, Spo0A cells synthesized several RNAs which were either absent or present in low levels in cells of the sporulating parent (Fig. 1), a finding which is consistent with previous observations on the pleiotropy of *spo0A* mutations (1, 12, 19).

Identification of the 0.3-kb coding sequence in cloned DNA. To identify the coding sequence for the 0.3-kb RNA in chromosomal DNA, gel-purified transcript was hybridized by the procedure of Southern (21) to electrophoretically separated fragments of EcoRI-digested *B. subtilis* DNA (Fig. 2, lane a). To our surprise, ^{32}P-labeled 0.3-kb RNA hybridized to DNA corresponding in size (4.6 kb) to a previously cloned *Eco*RI fragment that encodes the 0.4-kb RNA. That the 0.3- and 0.4-kb RNAs were encoded by the same *Eco*RI fragment was demonstrated directly by hybridizing radioactive 0.3-kb RNA

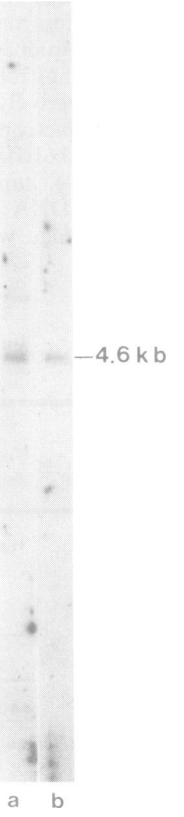

FIG. 2. *Southern hybridization of 0.3-kb RNA to B. subtilis DNA and to p213 DNA. Southern strips of EcoRI-cleaved total B. subtilis DNA (lane a) or EcoRI-cleaved p213 DNA (lane b) were hybridized with ^{32}P-labeled 0.3-kb RNA (40,000 cpm); the hybridization reaction in lane a also contained 4 μg of unlabeled rRNA. Hybrids were visualized by autoradiography.*

to a Southern strip containing cloned 4.6-kb DNA from the previously constructed hybrid plasmid p213 (Fig. 2, lane b). Finally, to confirm that the 0.3-kb RNA itself (rather than, for example, contaminating 0.4-kb sequences) was responsible for this hybridization, radioactive RNA was eluted from polyacrylamide gel slices cut from above, below, and over the band of electrophoretically purified 0.3-kb RNA and then hybridized to Southern strips of p213 DNA; only [^{32}P]RNA from the gel slice containing the 0.3-kb transcript hybridized to the cloned *B. subtilis* DNA (Ollington, Ph.D. thesis).

Mapping the 0.3-kb coding sequence in cloned DNA. The 4.6-kb *Eco*RI fragment in p213 DNA contains two previously identified transcription units: the *0.4 kb* gene, which is induced at the onset of sporulation (15), and the *veg* gene, which is transcribed both during growth and sporulation. The position and direction of these genes in cloned DNA is shown in the endonuclease restriction map of Fig. 3. To map the 0.3-kb coding sequence (hereafter referred to as the *0.3 kb* gene) relative to the *0.4 kb* and *veg* genes, radioactively labeled 0.3-kb RNA was hybridized to electrophoretically separated fragments of *Eco*RI-*Hin*cII double-cleaved p213 DNA (Fig. 4, lanes a through c) and *Hpa*II-cleaved p213 DNA (Fig. 4, lanes d through f). Double cleavage with *Eco*RI and *Hin*cII generated a 3,050-base pair (bp) fragment that contains the *veg* gene and a 770-bp fragment that contains the promoter-proximal portion of the *0.4 kb* gene (upper line in Fig. 3). Similarly, *Hpa*II cleavage generated a 1,140-bp fragment (overlapping the right-hand joint of *B. subtilis* and pMB9 vector DNA) that contains *0.4 kb* sequence and a 890-bp fragment containing the *veg* gene (not shown in Fig. 3, but see the *Hpa*II map in reference 15). In both cases, radioactive 0.3-kb RNA hybridized to the *veg* gene-containing DNA (*Eco*RI-*Hin*cII 3,050-bp fragment, Fig. 4, lane a; and *Hpa*II 890-bp fragment; Fig. 4, lane d), whereas radioactive 0.4-kb RNA hybridized as expected to the *0.4 kb* gene-containing DNAs (*Eco*RI-*Hin*cII 770-bp fragment, Fig. 4, lane b; and *Hpa*II 1,140-bp fragment, Fig. 4, lane e). Thus, the *0.3 kb* gene was separated by at least 2,000 bp from the *0.4 kb* gene but was located in close proximity to the *veg* gene.

In vitro transcription of the *veg* gene. To map more precisely the location of the *0.3 kb* sequence, the position of the *veg* gene as determined from the products of its transcription in vitro by σ^{55}-containing RNA polymerase must first be considered. Hybridization of in vitro-synthesized RNA to separated endonuclease restriction fragments and to separated DNA strands (Ollington, Ph.D. thesis) has shown that RNA polymerase selectively copies RNA from

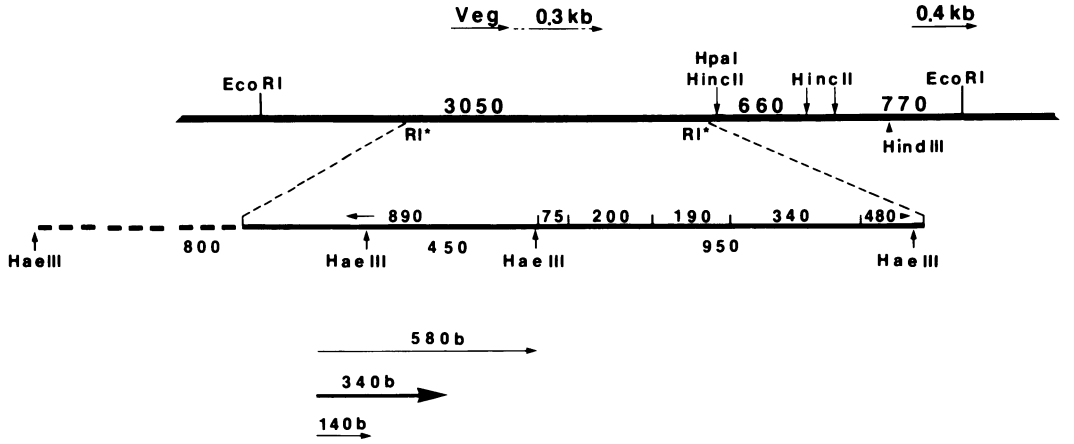

FIG. 3. *Endonuclease restriction map of cloned B. subtilis DNA. The thick line in the upper portion of the figure is a physical map of the veg and 0.4 kb gene region of the B. subtilis chromosome (15). The outermost EcoRI sites define the ends of the 4.6-kb DNA insert within p213; sites of cleavage for the endonucleases HpaI, HincII, and HindIII within this EcoRI fragment are indicated with arrows. The sizes of the fragments produced by EcoRI-HincII cleavage are shown (in bp) above this insert line. The two EcoRI* (RI*) sites beneath the upper restriction map define the 2-kb subcloned DNA insert in the hybrid plasmid p213-1 (7). An expanded physical map of the 2.0-kb EcoRI* fragment is shown in the middle of the figure. HaeIII segments (in bp) are shown below the line; the 800-bp HaeIII fragment contains vector DNA (represented by the horizontal dotted line) as well as B. subtilis DNA. HpaII segments (in bp) are shown above the p213-1 restriction map; since the EcoRI* sites occur within the 890- and 480-bp HpaII segments of p213, only a portion of these terminal fragments are presented in p213-1, and this is indicated by the horizontal arrows extending leftward from the 890-bp and rightward from the 480-bp HpaII fragments.*

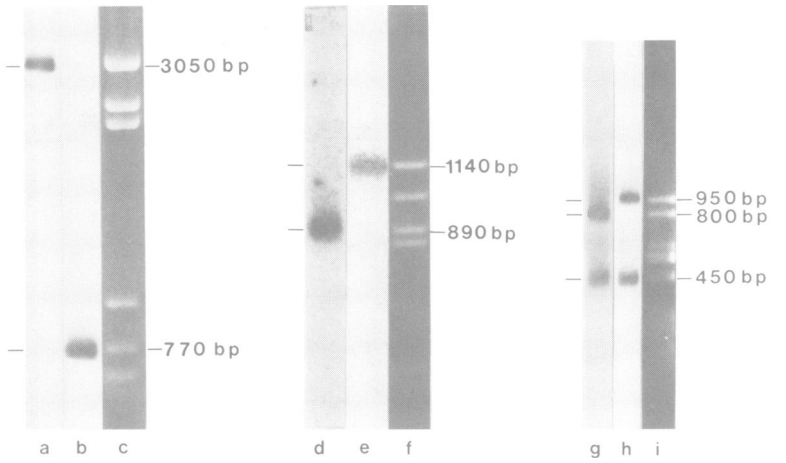

FIG. 4. *Mapping the 0.3 kb gene.* ^{32}*P-labeled 0.3-kb RNA and 0.4-kb RNA, eluted from polyacrylamide gel slices, and in vitro-synthesized veg gene RNA (transcribed from HpaII-cut p213) were incubated under hybridization conditions with Southern strips of the indicated endonuclease restriction fragments. Each reaction contained approximately* 5×10^4 *cpm of* ^{32}*P-labeled probe. The hybrids were visualized by autoradiography. Hybridizations of:* ^{32}*P-labeled 0.3-kb RNA to p213 EcoRI-HincII DNAs (lane a);* ^{32}*P-labeled 0.4-kb RNA to p213 EcoRI-HincII DNAs (lane b); ethidium bromide-stained gel of p213 EcoRI-HincII DNAs (lane c);* ^{32}*P-labeled 0.3-kb RNA to p213 HpaII DNAs (lane d);* ^{32}*P-labeled 0.4-kb RNA to p213 HpaII DNAs (lane e); ethidium bromide-stained gel of p213 HpaII DNAs (lane f);* ^{32}*P-labeled veg gene RNA to p213-1 HaeIII DNAs (lane g);* ^{32}*P-labeled 0.3 kb to p213-1 HaeIII DNAs (lane h); and ethidium bromide-stained gel of p213-1 HaeIII DNAs (lane i).*

the *Hpa*II 890-bp segment known to contain the *veg* gene and that the direction of this transcription is from left to right. To position this transcription unit, the products of in vitro RNA synthesis were displayed by polyacrylamide gel electrophoresis. RNA polymerase generated from uncut p213 DNA as template transcripts of 140, 340, and 650 bases as well as heterodisperse products of higher molecular weight (Fig. 5, lane d). With *Hpa*II-cleaved p213 as a template, however, the 650-base and higher-molecular-weight RNAs were replaced by a 580-base transcript (Fig. 5, lane e). We interpret the 580-base RNA to represent "runoff" transcription from the end of the *Hpa*II 890-bp segment of the truncated DNA template. Similarly, when *Hae*III-cleaved p213 was employed as a template, the 140-, 340-, and 650-base transcripts were replaced by a single RNA species of about 100 bases (N. Lang, unpublished data). This indicates that polymerase initiates at a single promoter site (located 100 bp to the left of the *Hae*III site and 580 bp to the left of the right-hand *Hpa*II 890-bp site [Fig. 3]) and that the 140-, 340-, and 650-base transcripts arise from "natural" terminators within the cloned DNA template. Thus, the 340-base transcript must arise from "read-through" at the terminator at the end of the 140-base sequence, and similarly, the higher-molecular-weight RNAs must arise from read-through at the 340-base terminator.

Which of these in vitro transcription units are utilized in vivo? To answer this question we "blocked" the *veg* gene sequences in p213 DNA by incubating under hybridization conditions Southern strips of *Eco*RI-*Hin*cII-cut plasmid DNA with a vast excess of unlabeled RNA that had been purified from growing cells. These blocked Southern strips were then incubated in a second hybridization reaction with ^{32}P-labeled, in vitro-synthesized RNAs. The autoradiograph of Fig. 6 shows that the ^{32}P-labeled 140 (lanes a and b)- and the ^{32}P-labeled 340 (lanes c and d)-base RNAs hybridized to the *veg* gene containing the *Eco*RI-*Hin*cII, 3,050-bp fragment of p213 but not to *veg* DNA that had been blocked by prior hybridization to in vivo-synthesized RNA. Hybridization of the 580 (lanes e and f)-base runoff transcript, in contrast, was only partially blocked by unlabeled RNA from growing cells. These experiments show, therefore, that the 340-bp sequence of the *veg* gene is transcribed in vivo and suggest that this RNA synthesis terminates either at or near the 340-bp terminator.

Ordering the *veg* and *0.3 kb* genes. Are the *veg* and *0.3 kb* genes distinct sequences? The autoradiograph of Fig. 5 (lanes a through e) shows that the 0.3-kb RNA was distinct in size from the products of in vitro transcription of the *veg* gene. It was possible, however, that the sporulation transcript was a stable, processed form of the *veg* gene RNA rather than a distinct

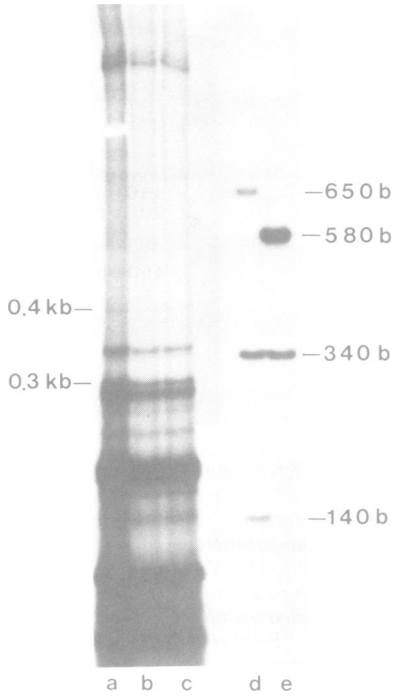

FIG. 5. *Polyacrylamide gel electrophoresis of in vivo-synthesized 0.3-kb RNA and in vitro-synthesized veg gene RNA. Radioactive 0.3-kb RNA (lanes a through c) was extracted from cells of strain SMY that had been pulse-labeled with $^{32}PO_4$ and treated with rifampin at T_5 in Sterlini-Mandelstam resuspension medium. ^{32}P-labeled veg gene RNA was synthesized in vitro by RNA polymerase holoenzyme from p213 (lane d) or HpaII-cut p213 (lane e). The in vivo-synthesized RNAs (2×10^5 cpm per lane) and the in vitro-synthesized RNAs (6×10^4 per lane) were subjected to electrophoresis through a 2.5 to 10.5% gradient gel of polyacrylamide and visualized by autoradiography.*

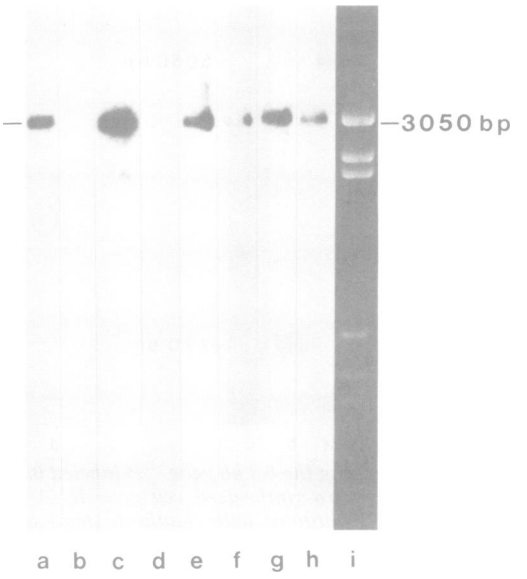

FIG. 6. *Blocking hybridization reactions. Southern strips imprinted with EcoRI-HincII fragments of p213 were incubated under hybridization conditions either with 500 μg of unlabeled vegetative RNA or, as a control, with hybridization buffer as described in the text. Subsequently, these strips were hybridized with ^{32}P-labeled veg gene RNAs or 0.3-kb RNA eluted from slices of polyacrylamide taken from the gel shown in Fig. 5. All hybridization reactions contained approximately 3×10^4 cpm, and the hybrids were visualized by autoradiography (the strips in lanes g and h were exposed to film seven to eight times longer than the other strips). Lanes: (a) ^{32}P-labeled 140-base RNA hybridized to a control strip; (b) ^{32}P-labeled 140-base RNA hybridized to a blocked strip; (c) ^{32}P-labeled 340-base RNA hybridized to a control strip; (d) ^{32}P-labeled 340-base RNA hybridized to a blocked strip; (e) ^{32}P-labeled 580-base RNA hybridized to a control strip; (f) ^{32}P-labeled 580-base RNA hybridized to a blocked strip; (g) ^{32}P-labeled 0.3-kb RNA hybridized to a control strip; (h) ^{32}P-labeled 0.3-kb RNA hybridized to a blocked strip; and (i) ethidium bromide-stained gel of electrophoretically separated EcoRI-HincII restriction fragments of p213.*

sequence. To investigate this possibility, we asked whether radioactive 0.3-kb RNA would hybridize to p213 DNA whose *veg* gene sequence had been blocked by prior hybridization with unlabeled RNA from growing cells. Figure 6 (lanes g and h) shows that authentic *veg* gene RNA could not prevent the hybridization of purified 0.3-kb transcript. We conclude, therefore, that the *0.3 kb* gene is distinct from or only partially overlapping with the *veg* gene sequence.

If these two genes are at least in part distinct, what is their order in p213 DNA? To answer this question, we employed a subclone of p213 known as p213-1 that contains a 2-kb *Eco*RI* fragment from the *veg* and *0.3 kb* gene region. *Hae*III cleavage of p213-1 partitions the *veg* gene region into three segments (Fig. 3): a promoter-proximal region (*Hae*III 800-bp segment, which represents the junction of *B. subtilis* and vector DNA in p213-1), a promoter-distal segment (*Hae*III 450-bp segment), and a segment which lacks *veg* sequences (*Hae*III 950-bp segment). Which of these segments contains 0.3-kb sequences? As expected, radioactive *veg* RNA hybridized to the *Hae*III 800-bp and the *Hae*III 450-bp segments of p213-1 but not to the *Hae*III 950-bp fragment (Fig. 4, lane g). Radioactive 0.3-kb RNA, in contrast, hybridized to the *Hae*III 450-bp and *Hae*III 950-bp DNAs but not to the *Hae*III 800-bp segment (Fig. 4, lane h). We conclude, therefore, that the *0.3 kb* gene is to the

right of the *veg* gene and that it overlaps with the *Hae*III site at the junction of the 450- and 950-bp segments (Fig. 3).

Finally, we determined the direction of *0.3 kb* gene transcription by hybridizing the sporulation RNA to electrophoretically separated strands of cloned DNA, a procedure previously employed to orient the *0.4 kb* gene (8, 15). Both *veg* and 0.3-kb RNAs hybridized to the "slowly migrating" strand of the *Eco*RI 4.6-kb insert of p213 (data not shown), the DNA coding strand for 0.4-kb RNA. Thus, as shown in Fig. 3, all three genes are transcribed from left to right.

Regulation of the *0.3 kb* gene. Even though we have not yet been able to separate entirely *0.3 kb* and *veg* gene sequences by endonuclease restriction cleavage, blocking of the *veg* gene by unlabeled RNA from growing cells provided a hybridization assay for transcription of the *0.3 kb* gene that was independent of the size or stability of its RNA product. In the experiment of Fig. 7, total RNA from cells that had been pulse-labeled (in the absence of rifampin) early (T_1; lane a) and late (T_5; lane b) in sporulation was hybridized to Southern strips of *Eco*RI-*Hin*cII-cut p213 DNA that had been blocked with unlabeled competitor RNA. Despite a background of nonspecific binding of radioactive material to the Southern strips, hybridization could be readily detected to the 3,050- and 770-bp fragments that contained the *0.3 kb* and *0.4 kb* genes, respectively. Pulse-labeled RNA from both early and late sporulating cells hybridized actively to the *Eco*RI-*Hin*cII 770-bp DNA, a finding consistent with previous observations on the persistent transcription of the *0.4 kb* gene during spore formation (15, 19). The *0.3 kb* gene, in contrast, exhibited a different pattern of transcription: although late-labeled RNA hybridized significantly to the *0.3 kb* gene-containing DNA, little hybridization of early-labeled RNA could be detected. These observations are consistent with the idea that the *0.3 kb* gene is controlled at the level of transcription and that the *0.3 kb* and *0.4 kb* genes are subject to distinct temporal controls.

(A comparison of the patterns of 0.3- and 0.4-kb RNA synthesis in the gel electrophoresis experiment of Fig. 1 and the hybridization experiment of Fig. 7 warrants special comment. The 0.3-kb RNA was more abundant [as compared with 0.4-kb RNA] when visualized by gel electrophoresis than when measured by hybridization. However, the gel electrophoresis experiment visualized the sporulation transcripts as discrete-sized RNAs among long-lived RNAs from rifampin-treated cells; thus, the relative abundance of the 0.4- and 0.3-kb RNAs in Fig. 1 reflected their processing and stability as well as their rates of synthesis. The hybridization experiment of Fig. 7, in contrast, measures more directly the relative rates of synthesis of 0.4- and 0.3-kb sequences.)

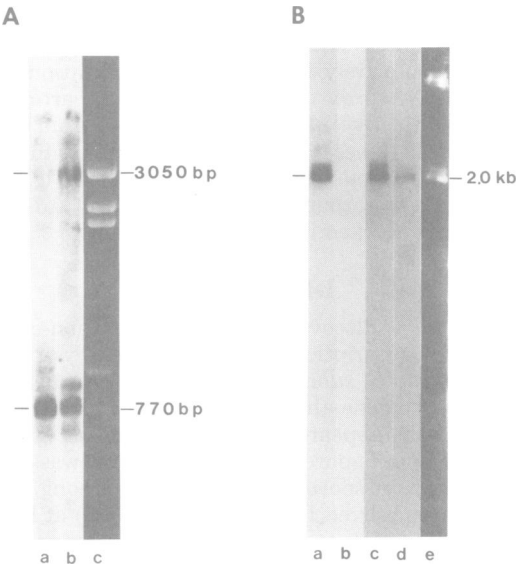

FIG. 7. *Regulation of the 0.3 kb gene. (A) Total RNA was purified from SMY cells that had been pulse-labeled with $^{32}PO_4$ at T_1 or T_5 in Sterlini-Mandelstam medium. The radioactive RNAs were then hybridized to Southern strips of EcoRI-HincII-cut p213 that had been blocked with 500 μg of unlabeled vegetative RNA. Lane a, Total pulse-labeled T_1 RNA from SMY cells. Lane b, Total pulse-labeled T_5 RNA from SMY cells. Lane c, Ethidium bromide-stained gel of the p213 EcoRI-HincII DNAs. (B) Total RNA was purified either from SMY cells or from Spo0A-5NA that had been pulse-labeled with $^{32}PO_4$ at T_5 in Sterlini-Mandelstam medium. The radioactive RNAs were hybridized to Southern strips of EcoRI-cleaved p213-1 DNA that had been previously incubated with 500 μg of unlabeled vegetative RNA or, as a control, with hybridization buffer. Hybridizations were of: total pulse-labeled T_5 RNA from Spo0A-5NA cells to a control strip (lane a); total pulse-labeled T_5 RNA from Spo0A-5NA cells to a blocked strip (lane b); total pulse-labeled T_5 RNA from SMY cells to a control strip (lane c); total pulse-labeled T_5 RNA from SMY cells to a blocked strip (lane d); and ethidium bromide-stained gel of p213-1 EcoRI DNAs (lane e). All of the hybridization reactions contained 1×10^6 to 4×10^6 cpm, and the hybrids were visualized by autoradiography.*

Finally, we employed the blocking hybridization procedure to examine the regulation of the *0.3 kb* gene by the *spo0A* locus. Pulse-labeled RNAs from T_5 cells of Spo0A-5NA and its wild-type parent SMY were hybridized to Southern strips of *Eco*RI-cut p213-1 DNA. (Having been cloned into the *Eco*RI site of pMB9, the 2-kb *Eco*RI* insert in p213-1 can be liberated under

*Eco*RI digestion conditions [7].) Figure 7B (lanes a and b) shows that unlabeled competitor RNA completely blocked the hybridization of [^{32}P]- RNA from the mutant but only partially blocked the hybridization of [^{32}P]RNA from the Spo$^+$ cells (lanes c and d). This finding confirms and extends our previous observations (Fig. 1) and indicates that transcription of the *0.3 kb* gene was blocked in the Spo0A mutant.

DISCUSSION

We have discovered a new sporulation-controlled transcription unit in the *purA-cysA* region of the *B. subtilis* chromosome. This newly discovered gene, the *0.3 kb* gene, encodes a transcript that appeared at an intermediate stage (III to IV) of spore formation and that was the predominant sporulation transcript (among relatively long-lived RNAs of discrete size) at late developmental stages (IV to VI). Appearance of the 300-base RNA was under sporulation control as this transcript could not be detected in an early-blocked sporulation mutant (Spo0A-5NA).

The hybridization experiment of Fig. 7 suggests that the expression of the *0.3 kb* gene is regulated at the level of transcription rather than at the level of RNA processing or stability. In these experiments, *0.3 kb* was visualized indirectly by hybridizing pulse-labeled RNA to cloned probes that had been annealed previously with unlabeled RNA from vegetative cells to block *veg* gene sequences. A direct demonstration of *0.3 kb* gene transcription will require the construction of a cloned probe that contains uniquely *0.3 kb* sequences.

Although located in a cluster of genes that are actively transcribed during sporulation, the *0.3 kb* gene exhibited a strikingly different pattern of temporal regulation than has previously been observed in this cloned chromosomal segment (15). For example, the *veg* gene is actively transcribed during both growth and sporulation, whereas the *0.4 kb* gene is induced at the onset of sporulation. Thus, sequences located on either side of the *0.3 kb* gene are activated well before *0.3 kb* RNA synthesis is initiated.

How might the expression of the *0.3 kb* gene be regulated? In vitro transcription studies have shown that the *veg* gene promoter is controlled by the usual form of *B. subtilis* RNA polymerase containing σ^{55}, whereas the *0.4 kb* gene promoter is recognized by modified forms of RNA polymerase containing σ^{37} or σ^{29}, novel species of sigma factor that are present in early sporulating cells (8–10). Possibly, then, *0.3 kb* transcription is turned on by a sigma-like regulatory protein that is induced during h 3 to 4 of spore formation. Alternatively, however, the juxtaposition of the *0.3 kb* gene just downstream from the *veg* sequence raises the possibility that the *0.3 kb* gene is regulated by an "antitermination" mechanism; relief of transcription termination at the end of the *veg* sequence followed by processing of the resulting run-through transcript could turn on *0.3 kb* RNA synthesis. The availability of the cloned *0.3 kb* gene as a template for in vitro RNA synthesis and as a probe for inserting in vitro-constructed mutations into the chromosome should make it possible to distinguish among these and other possibilities.

Programmed gene expression demands not only specific regulatory proteins for each temporally defined class of genes but also a clock which governs the time of appearance of these proteins during sporogenesis. An understanding of the mechanism by which the *0.3 kb* gene is turned on may still leave unanswered the question of how *B. subtilis* keeps time.

Finally, it is tempting to speculate on the possible product of the *0.3 kb* gene. On the basis of its size, the 0.3-kb RNA could code for a protein of up to about 13,000 daltons. A suitable candidate, then, for the *0.3 kb* gene product might be one of the abundant, low-molecular-weight polypeptides of the mature spore such as the coat polypeptides (6, 14) and the acid-soluble core peptides (3, 20, 24). One of the coat components, a sulfur-containing polypeptide of 11,000 to 13,000 daltons (12, 14), appears during h 3 of sporulation and increases in abundance until a late developmental stage, a time course of synthesis that parallels that of the 0.3-kb RNA. Thus, this coat polypeptide is an attractive candidate for the product of the *0.3 kb* gene. Munoz et al. (14) have, however, detected in extracts of early sporulating cells (T_1) a 25,000-dalton protein that cross-reacts immunologically with the 13,000-dalton coat polypeptide. These authors suggest that the larger polypeptide is a precursor of the mature 13,000-dalton species. If verified (e.g., by peptide mapping or amino acid analysis), this proposed precursor-product relationship would be inconsistent (at least in a simple model) with the idea that the *0.3 kb* gene encodes the 13,000-dalton coat protein. A definitive assignment of the *0.3 kb* gene product must await, therefore, the identification of its translation product in vitro or the determination of its nucleotide coding sequence or both.

ACKNOWLEDGMENTS

We thank W. G. Haldenwang, N. Lang, J. Segall, and M. Stephens for helpful discussions.

This work was supported by Public Health Service grant 2 RO1 GM 18568 from the National Institutes of Health.

LITERATURE CITED

1. **Brehm, S. P., F. Le Hegarat, and J. A. Hoch.** 1975. Deoxyribonucleic acid-binding proteins in vegetative

Bacillus subtilis: alterations caused by stage 0 sporulation mutations. J. Bacteriol. **124**:977–984.

2. **DiCioccio, R. A., and N. Strauss.** 1973. Patterns of transcription in *Bacillus subtilis* during sporulation. J. Mol. Biol. **77**:325–336.
3. **Dignam, S. S., and P. Setlow.** 1980. *In vivo* and *in vitro* synthesis of the spore-specific proteins A and C of *Bacillus megaterium*. J. Biol. Chem. **255**:8417–8423.
4. **Doi, R. H.** 1977. Genetic control of sporulation. Annu. Rev. Genet. **11**:29–48.
5. **Franklin, R. M.** 1966. Purification and properties of the replicative intermediate of the RNA bacteriophage R17. Proc. Natl. Acad. Sci. U.S.A. **55**:1504–1511.
6. **Goldman, R. C., and D. J. Tipper.** 1978. *Bacillus subtilis* spore coats: complexity and purification of a unique polypeptide component. J. Bacteriol. **135**:1091–1106.
7. **Haldenwang, W. G., C. D. B. Banner, J. F. Ollington, R. Losick, J. A. Hoch, M. B. O'Connor, and A. L. Sonenshein.** 1980. Mapping a cloned gene under sporulation control by insertion of a drug resistance marker into the *Bacillus subtilis* chromosome. J. Bacteriol. **142**:90–98.
8. **Haldenwang, W. G., and R. Losick.** 1979. A modified RNA polymerase transcribes a cloned gene under sporulation control in *Bacillus subtilis*. Nature (London) **282**:256–260.
9. **Haldenwang, W. G., and R. Losick.** 1980. A novel RNA polymerase sigma factor from *Bacillus subtilis*. Proc. Natl. Acad. Sci. U.S.A. **77**:7000–7004.
10. **Haldenwang, W. G., N. Lang, and R. Losick.** 1981. A sporulation-induced sigma-like regulatory protein from *Bacillus subtilis*. Cell **23**:615–624.
11. **Hoch, J. A.** 1976. Genetics of bacterial sporulation. Adv. Genet. **18**:67–98.
12. **Linn, T., and R. Losick.** 1976. The program of protein synthesis during sporulation in *Bacillus subtilis*. Cell **8**: 103–114.
13. **Moran, C. P., Jr., R. Losick, and A. L. Sonenshein.** 1980. Identification of a sporulation locus in cloned *Bacillus subtilis* deoxyribonucleic acid. J. Bacteriol. **142**:331–334.
14. **Munoz, L., Y. Sadaie, and R. H. Doi.** 1978. Spore coat protein of *Bacillus subtilis*. J. Biol. Chem. **253**:6694–6701.
15. **Ollington, J. F., W. G. Haldenwang, T. V. Huynh, and R. Losick.** 1981. Developmentally regulated transcription in a cloned segment of the *Bacillus subtilis* chromosome. J. Bacteriol. **147**:432–442.
16. **Pero, J., J. Nelson, and R. Losick.** 1975. In vitro and in vivo studies of transcription by vegetative and sporulating *Bacillus subtilis*, p. 202–212. *In* P. Gerhardt, R. N. Costilow, and H. L. Sadoff (ed.), Spores VI. American Society for Microbiology, Washington, D.C.
17. **Piggot, P. J., and J. G. Coote.** 1976. Genetic aspects of bacterial endospore formation. Bacteriol. Rev. **40**:908–962.
18. **Schaeffer, P., J. Millet, and J. Aubert.** 1965. Catabolite repression of bacterial sporulation. Proc. Natl. Acad. Sci. U.S.A. **54**:704–711.
19. **Segall, J., and R. Losick.** 1977. Cloned *Bacillus subtilis* DNA containing a gene that is activated early during sporulation. Cell **11**:751–761.
20. **Setlow, P.** 1975. Identification and localization of the major proteins degraded during germination of *Bacillus megaterium* spores. J. Biol. Chem. **250**:8159–8167.
21. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
22. **Sterlini, J. M., and J. Mandelstam.** 1969. Commitment to sporulation in *Bacillus subtilis* and its relationship to development of actinomycin resistance. Biochem. J. **113**:29–37.
23. **Sumida-Yasumoto, C., and R. H. Doi.** 1974. Transcription from the complementary deoxyribonucleic acid strands of *Bacillus subtilis* during various stages of sporulation. J. Bacteriol. **117**:775–782.
24. **Tipper, D. J., W. C. Johnson, G. H. Chambliss, I. Mahler, M. Arnaud, and H. O. Halvorson.** 1981. Acid-soluble polypeptides of *Bacillus subtilis* spores, p. 178–183. *In* H. S. Levinson, A. L. Sonenshein, and D. J. Tipper (ed.), Sporulation and germination. American Society for Microbiology, Washington, D.C.
25. **Young, M.** 1976. Use of temperature-sensitive mutants to study gene expression during sporulation in *Bacillus subtilis*. J. Bacteriol. **126**:928–936.