Outgrowth of *Bacillus cereus* Spores Harboring Bacteriophage CP-51 DNA

I. Initiation of Bacteriophage Development

AMIKAM COHEN, HANNAH BEN-ZE'EV, AND JUDITH YASHOUV

The Hebrew University-Hadassah Medical School, Department of Microbiological Chemistry, Jerusalem, Israel

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Time of initiation of bacteriophage CP-51 RNA synthesis, inhibition of host DNA synthesis, initiation of CP-51 DNA synthesis, and release of phage was determined in infected *Bacillus cereus* cells and outgrowing spores harboring phage DNA. The development of phage in outgrowing spores is initiated 45 min after induction of spore germination and then proceeds at a rate similar to that observed in infected cells. Although spore RNA synthesis is inhibited by the presence of chloramphenicol or puromycin during germination, these antibiotics do not inhibit initiation of phage DNA transcription in outgrowing spores.

When bacteriophage CP-51 infects sporulating *Bacillus cereus* cells, the lytic cycle does not take place; instead, phage DNA is trapped until spore outgrowth. Then phage development is initiated, and the spore becomes an infective center (16). Recent studies indicate that bacterial failure to initiate phage DNA transcription during sporulation (18) may be due to changes in template specificity of bacterial RNA polymerase; these changes result in the reduction of the enzyme's affinity toward promoters of genes which are transcribed only during vegetative growth (7), as well as toward phage DNA (9). If phage transcription products are not present in spores harboring phage DNA (13), initiation of trapped phage DNA transcription during spore germination must be controlled by the transcription mechanism of its host.

The development of *B. cereus* spore to a vegetative cell is controlled by sequential transcription of its genome. Initiation of transcription during the first minutes of germination is followed by the synthesis of a small number of protein species (17), and later, during outgrowth, there is a sequential synthesis of different species of mRNA, followed by the synthesis of the corresponding proteins (14). Although this sequence of events has been described and its dependence on de novo mRNA synthesis has been demonstrated, the mechanism which controls the sequence of transcription of different segments of the bacterial genome at different stages of spore outgrowth is not yet understood (6). If the control of initiation of trapped phage

DNA transcription in outgrowing spores is related to the control of sequential transcription of the spore genome during outgrowth, elucidation of the mechanism which controls initiation of phage development may contribute to the understanding of the control of the sequence of events observed in outgrowing spores. It is, therefore, the purpose of this work to determine (i) whether initiation of phage development in a population of outgrowing spores harboring phage DNA is a synchronous event; (ii) at what stage of spore germination or outgrowth this event takes place; and (iii) what relation, if any, this event has to the control of transcription of outgrowing spore DNA.

MATERIALS AND METHODS

Bacteria and phage. *B. cereus* T and CP-51 phage were used in all experiments.

Media and growth conditions. Bacteria were grown in PA (16) medium, or GC medium which contained G salts (12), 0.2% glucose, 0.2% vitamin-free Casamino Acids, and 0.5% NaCl. Sporulation medium contained 0.16% nutrient broth, 0.5% NaCl, 0.02% $\text{MgSO}_4 \times 7\text{H}_2\text{O}$, 0.005% MnSO_4 , and 0.035% CaCl_2 . Unless indicated otherwise, all incubations were at 30°C with aeration by shaking. Heat activation of dormant spores was in water suspension at 65°C for 60 min.

Preparation of *B. cereus* spores containing trapped CP-51 DNA. Spores containing phage DNA were prepared by the method of Issachary, Aventchick, and Hartman (*unpublished data*). Heat-activated spores of *B. cereus* were inoculated onto PA medium supplemented with 100 μg of L-alanine and 100 μg of adenosine per ml to give a suspension of

3×10^7 spores per ml. After growth for 4 h at 37 C, the cells were harvested, suspended in sporulation medium at one-third the original culture volume, incubated for an additional 30 min, and then infected by CP-51 phage at a multiplicity of 5. The infected culture was allowed to sporulate for 20 h, and the spores were collected by centrifugation, washed in water, and subjected to Renografin density banding (15). This procedure yielded 80 to 95% infected spores. Spores were stored in water suspension at -20 C. Efficiency of trapping is defined as the percentage of spores in a preparation which harbor phage DNA.

RNA preparation. Pulse-labeled tritium RNA was prepared by incorporation of [5- 3 H]uracil (15 Ci/mmol; 5 μ Ci/ml) for 1 min in GC medium. Incorporation was terminated by the addition of one volume of frozen (-20 C) 0.01 M Tris-1.0 mM MgCl₂-0.1 M NaCl buffer (pH 7.9), centrifugation, and suspension in the same buffer. RNA was prepared from infected cells or from outgrowing spores by disintegration in the Braun cell disintegrator. Phenol extractions were followed by precipitation in ethanol, DNase (10 μ g/ml), and Pronase (25 μ g/ml) treatments, additional phenol extractions, and ethanol precipitation. RNA preparations were stored as ethanol precipitates at -20 C.

Hybridization procedure. Competitive hybridization was carried out by the procedure of Bolle et al. (1) except that the total reaction volume was 0.1 ml, and hybrids were collected on 13-mm (0.45- μ m pore size) Sartorius membrane filters. Hybridization of pulse-labeled RNA to membrane-bound denatured DNA was carried out by the method of Gillispie and Spiegelman (5) with 40 μ g of heat-denatured DNA bound to 25-mm (0.45- μ m pore size) Sartorius membrane filters. Filters were suspended in Denhardt solution (4) before hybridization to reduce background.

RNA was eluted from DNA-RNA hybrids by incubating the filters for 5 min in $0.1 \times$ SSC (0.015 M NaCl-0.0015 M sodium citrate, pH 7.0) at 85 C. After elution, the RNA was subjected to phenol extraction, DNase treatment, and additional phenol extraction.

Incorporation experiments. Incorporation of labeled nucleotide precursors to phage or bacterial DNA was determined by assaying for trichloroacetic acid-insoluble, alkali-resistant label. Incorporation of labeled [5- 3 H]uracil to RNA was determined by assaying for trichloroacetic acid-insoluble label.

RESULTS

Development of CP-51 phage in outgrowing *B. cereus* spores. *B. cereus* spores harboring phage DNA (efficiency of trapping, 90%) were heat activated and incubated at 30 C in GC medium supplemented with L-alanine (100 μ g/ml) and adenosine (100 μ g/ml) to induce germination. Samples were taken at time intervals and assayed for PFU. Release of phage under these conditions is synchronous and takes place 135 to 140 min after induction of germination (Fig. 1B). Release of phage in the same medium at 37 C takes place 100 min after

FIG. 1. Lytic cycle of phage CP-51 in infected cells and outgrowing spores of *B. cereus* T. A, Cells at the logarithmic phase of growth in GC medium were infected at a multiplicity of 0.2, and the number of PFU was determined on a lawn of *B. cereus* T heat-activated spores (15) at the indicated time intervals after infection. B, Spores of *B. cereus* T harboring CP-51 DNA (efficiency of trapping, 90%) were heat activated and transferred to GC medium supplemented with L-alanine (100 μ g/ml) and adenosine (100 μ g/ml); samples were assayed for PFU at the indicated time intervals after induction of germination.

induction of germination (not shown). The length of the lytic cycle of phage CP-51 in *B. cereus* vegetative cells growing at 30 C in GC medium is 90 to 100 min (60 min at 37 C). Burst size of phage developing in vegetative cells or in outgrowing spores is about 90.

The longer period required for phage release from outgrowing spores may be due to the slower rate of phage development in outgrowing spores or to delayed initiation of phage DNA transcription. To discriminate between these two possibilities, the time of initiation of phage RNA synthesis was determined in infected vegetative cells and outgrowing spores harboring phage DNA. In addition, the pace of phage development in infected cells and outgrowing spores after initiation of transcription was studied by determining the time of host DNA synthesis cessation and initiation of phage DNA synthesis.

Initiation of phage RNA synthesis in infected cells and outgrowing spores. *B. cereus* cells at the logarithmic phase of growth were infected with CP-51 phage at a multiplicity of 10. RNA labeled by 1-min pulse of [^3H]uracil at time intervals after infection was prepared, and the percent of label hybridizable to heat-denatured CP-51 DNA was determined. All hybridizations were carried out under conditions of DNA excess, and in all cases label retained on the filters was demonstrated to be proportional to RNA added. Efficiency of hybridization of in vitro-prepared CP-51 RNA (3) to denatured CP-51 DNA under the conditions used in the experiment was 65 to 70%. RNA hybridizable to CP-51 DNA was detected in infected vegetative cells 1 min after infection, and its proportion of the total label increases up to 40 min after infection (Fig. 2A).

To determine whether CP-51 RNA is present in spores harboring phage DNA before induction of germination, competitive hybridization

FIG. 2. CP-51 RNA synthesis by infected cells and outgrowing spores harboring phage DNA. RNA was pulse labeled at the indicated periods after cell infection (A) or induction of spore germination (B), and percent of label hybridized to CP-51 DNA bound to membrane filters was determined. Hybrid retained on filters is expressed as percent of labeled RNA added to the hybridization mixture.

experiments with RNA of spores harboring phage DNA against pulse-labeled RNA of infected cells to CP-51 DNA were conducted (Fig. 3). Although RNA extracted at different periods after infection and RNA extracted from outgrowing spores harboring phage DNA compete against RNA labeled at 15 and 50 min after infection of vegetative cells, RNA extracted from spores which harbor phage DNA does not compete with labeled RNA for sites on denatured CP-51 DNA, thus indicating that dormant spores of *B. cereus* which harbor CP-51 DNA have little or no CP-51 RNA.

Time of initiation of CP-51 RNA synthesis in outgrowing spores was determined by the following procedures. Spores harboring phage DNA (efficiency of trapping, 90%) were heat

FIG. 3. Competitive hybridization with CP-51 DNA between ^3H -pulse-labeled RNA prepared at 15 min (A) and 50 min (B) after infection of vegetative *B. cereus* cells, and nonlabeled RNA of: vegetative cells 15 min after CP-51 infection (O); vegetative cells 50 min after infection (●); spores harboring CP-51 DNA (efficiency of trapping, 90%) (□); outgrowing spores harboring CP-51 DNA 80 min after induction of germination (■). A 0.1-ml amount of $6 \times \text{SSC}$ (0.0 M NaCl - 0.09 M sodium citrate, pH 7.0) 0.6 μg of heat-denatured CP-51 DNA, 0.1 μg of ^3H -labeled RNA, and indicated amounts of nonlabeled RNA were incubated at 66 C for 4 h and then subjected to RNase treatment (25 $\mu\text{g}/\text{ml}$ at 30 C for 60 min). This was then diluted into 6 ml of 0.01 M Tris, 0.5 M KCl (pH 7.3), filtered through Sartorius membrane filters which were washed with an additional 25 ml of the same buffer and 2 ml of ethanol, and dried. Activity retained on filters was determined in a scintillation spectrometer and is expressed as percent of label retained on filter in the absence of nonlabeled RNA. Ten percent of RNA labeled 50 min after infection and 6% of RNA labeled 6 min after infection was retained on filter when hybridization was performed in the absence of nonlabeled RNA.

activated and incubated in GC medium supplemented with 100 μ g of L-alanine per ml and 100 μ g of adenosine per ml. Pulse-labeled RNA was prepared, and the percent of label hybridizable to denatured CP-51 phage DNA was determined. Labeled RNA hybridizable to CP-51 DNA does not increase above 0.3% of the total label at early stages of germination and outgrowth, whereas bacterial RNA synthesis starts immediately after induction of spore germination (17). Increase in the percentage of RNA hybridizable to CP-51 DNA is observed 45 min after induction of germination, and its proportion of total RNA increases up to 100 min after induction of germination.

The low percentage of label hybridizable to CP-51 DNA, observed when RNA of spores harboring phage DNA is pulse labeled at early stages of outgrowth (0.2 - 0.3% of total label in different experiments), is consistently higher than that observed when pulse-labeled RNA of outgrowing spores which do not harbor phage DNA is assayed for label hybridizable to phage DNA (0.05 - 0.15%). To determine whether this difference is due to the low rate of phage RNA synthesis or to nonspecific annealing of *B. cereus* RNA to CP-51 DNA, the experiment presented in Table 1 was conducted. RNA, pulse labeled 20 min after germination induction of spores harboring CP-51 DNA, was hybridized to CP-51 DNA; hybridized RNA was then eluted from the membrane filters, and the eluent was assayed for percent of label hybridizable to *B. cereus* or CP-51 DNA. The results show that more than 50% of the label is homologous to CP-51 DNA but not to *B. cereus* DNA, thus indicating that a low level of phage RNA synthesis takes place at early stages of spore outgrowth.

The percentage of label hybridizable to phage DNA is lower in outgrowing spores than in infected cells throughout the period of phage development. This may be due to a rate of RNA synthesis in the fraction of the outgrowing spore population which do not harbor phage DNA higher than in that fraction which harbor phage DNA, or to a difference in RNA synthesis profile between infected cells and outgrowing spores harboring phage DNA. The observation that the rate of RNA synthesis in outgrowing spores harboring phage DNA is similar to that of outgrowing spores which do not harbor phage DNA (H. Ben-Ze'ev, M.Sc. thesis, The Hebrew University, 1972) is consistent with the second alternative.

DNA synthesis. The results presented in the preceding section indicate that phage development in spores harboring phage DNA is sup-

TABLE 1. Hybridization of 20-min 3 H-RNA of outgrowing spores harboring CP-51 DNA^a

3 H-RNA	Hybridizable to <i>B. cereus</i> DNA (%)	Hybridizable to CP-51 DNA (%)
<i>B. cereus</i> (CP-51) RNA ^a	52	0.35
<i>B. cereus</i> RNA ^b	54	0.10
<i>B. cereus</i> (CP-51) RNA ^c	2.0	50.2

^a RNA of spores harboring CP-51 DNA (*B. cereus* [CP-51]) or spores not harboring phage DNA was purified after 1 min of pulse labeling (10 μ Ci/ml of [3 H]uracil) at 20 min after induction of germination, and then tested for label hybridizable to CP-51 or *B. cereus* DNA. Labeled *B. cereus* (CP-51) RNA was then eluted from filters of CP-51 DNA and retested for percentage of label hybridized to CP-51 or *B. cereus* DNA.

^b Labeled 20 min after induction of spore germination.

^c Eluted from CP-51 DNA filters.

pressed at early stages of spore outgrowth. Once this suppression is lifted, does phage development proceed at a similar pace to that observed in infected cells? To answer this question, we compared the time of cessation of host DNA synthesis and initiation of phage DNA synthesis in infected cells and outgrowing spores harboring phage DNA.

[14 C]uracil added to CP-51-infected bacteria is incorporated into phage DNA as 5-hydroxymethyluracil and cytosine (19) (J. Yashouv, M.Sc. thesis, The Hebrew University, 1971); very little of this isotope is converted to cell DNA precursors and incorporated into cellular DNA. On the other hand, [3 H]thymidine supplied to infected *B. cereus* cells incorporates into cellular DNA and not into phage DNA. The rate of incorporation of label into phage and into host DNA could, therefore, be followed by determination of [14 C]uracil or [3 H]thymidine to alkali-stable trichloroacetic acid-insoluble label (Fig. 4).

Cellular DNA synthesis stops 20 min after infection of vegetative cells with CP-51 phage, and phage DNA synthesis commences 35 min after infection of vegetative cells. On the other hand, incorporation of [3 H]thymidine into *B. cereus* DNA in outgrowing spores harboring CP-51 DNA proceeds at a rate similar to that observed in noninfected outgrowing spores for about 60 min and stops only 65 min after induction of germination. Phage DNA synthesis is initiated 80 min after induction of germination and proceeds for an additional 55 min; spores are then lysed, and phage is released (Fig. 1).

Effect of chloramphenicol and puromycin

FIG. 4. Synthesis of host and phage DNA in infected cells and outgrowing spores harboring phage DNA. [methyl- ^3H]thymidine (5.0 $\mu\text{Ci/ml}$ or 0.75 mCi/mmol) and [^{14}C]uracil (0.62 $\mu\text{Ci/ml}$ or 10.3 $\mu\text{Ci/mmol}$) were added to infected *B. cereus* cells (multiplicity of infection 10) or to outgrowing spores harboring phage DNA (efficiency of trapping, 90%). Samples (0.025 ml) were taken at intervals, added to 1 ml of 0.5 M NaOH, and incubated at 37 C for 20 h. After incubation, the mixtures were chilled, titrated with 0.5 ml of 1 M HCl, and assayed for 5% trichloroacetic acid-insoluble label. Infected cells or spores harboring phage DNA ($\bullet$); noninfected cells or spores ($\circ$). A, Incorporation of [methyl- ^3H]thymidine to DNA in infected and noninfected cells. B, Incorporation of [^{14}C]uracil to DNA in infected and noninfected cells. C, Incorporation of [methyl- ^3H]thymidine in outgrowing spores and outgrowing spores harboring CP-51 DNA. D, Incorporation of [^{14}C]uracil in outgrowing spores and outgrowing spores harboring CP-51 DNA.

on phage RNA synthesis in outgrowing spores. Inhibition of protein synthesis at early stages of *B. cereus* spore germination reduces the rate of net RNA synthesis by 5- to 10-fold (2). This and other observations led to the suggestion that proteins which are synthesized immediately after induction of germination play a role in the control of transcription during spore outgrowth. Since initiation of trapped phage DNA transcription in outgrowing *B. cereus* spores is controlled by its host transcription system, it would be of interest to determine if the dependence of transcription during outgrowth on de novo protein synthesis is general to

all transcription activities in the outgrowing spore, or whether it is specific to transcription of spore DNA.

Pulse-labeled RNA was prepared from outgrowing spores harboring CP-51 DNA in the presence or absence of chloramphenicol or puromycin (200 $\mu\text{g/ml}$). This concentration of the antibiotics reduces the rate of total RNA synthesis to 10% of the rate in the absence of antibiotics. Hybridization experiments of pulse-labeled RNA synthesized in outgrowing spores harboring CP-51 DNA in the presence of protein synthesis inhibitors (Fig. 5) indicate that the percent of label hybridizable to CP-51 DNA

increases 10-fold when outgrowth is in the presence of chloramphenicol or puromycin. Since the rate of total RNA synthesis is 10% of the rate in the absence of antibiotics, this result indicates that chloramphenicol or puromycin has little or no effect on trapped CP-51 DNA transcription in outgrowing spores. Competitive hybridization experiments of pulse-labeled phage RNA synthesized in spores outgrowing in the presence of puromycin and RNA synthesized by cells infected in the presence of chloramphenicol to CP-51 DNA (Fig. 6) indicate that at least 90% of the spore-labeled CP-51 RNA is homologous to RNA species present in cells infected in the presence of chloramphenicol.

DISCUSSION

Development of *B. cereus* spores is initiated immediately after induction of germination, whereas transcription of CP-51 DNA trapped in these spores is suppressed at early stages of spore germination and outgrowth. The increase in rate of phage RNA synthesis is detected only 45 min after induction of germination. Once this event takes place, the development of the phage in the outgrowing spores proceeds synchronously and at a rate similar to that observed in infected vegetative *B. cereus* cells. Host DNA synthesis ceases 25 min after initiation of phage development, phage DNA synthesis is initiated

Fig. 5. Effect of chloramphenicol and puromycin on CP-51 RNA synthesis in outgrowing *B. cereus* spores harboring CP-51 DNA. Experimental conditions are as described for Fig. 3 except that 200 µg of chloramphenicol (□) or 200 µg of puromycin (●) per ml was added to the suspension of outgrowing spores before induction of germination. Spores outgrowing in the absence of antibiotics (○).

Fig. 6. Competitive hybridization with CP-51 DNA between ^3H -pulse-labeled RNA of spores harboring CP-51 DNA outgrowing in the presence of puromycin and nonlabeled RNA of cells infected in the presence of chloramphenicol. After hybridization of ^3H -RNA prepared from spores labeled at 30 min after initiation of germination in the presence of puromycin (Fig. 5), RNA was eluted from the filters and subjected to hybridization to CP-51 DNA in the presence of the indicated amounts of nonlabeled cell RNA prepared 30 min after infection by CP-51 in the presence of chloramphenicol (200 µg/ml). ^3H retained in the hybrid is expressed as percent of label retained in the absence of nonlabeled RNA.

at 35 min, and lysis occurs at 100 min after initiation of phage development.

Indications that host failure to support initiation of phage DNA transcription during sporulation is a result of structural changes of host RNA polymerase (9) have suggested the possibility that reinitiation of phage DNA transcription during spore outgrowth may involve restoration of the vegetative enzyme by de novo synthesis of RNA polymerase or part of its subunits. This hypothesis was supported by indications that *B. subtilis* spore RNA polymerase differs from vegetative cell RNA polymerase (11), and by the observation that de novo protein synthesis during germination of *B. cereus* spores is required for RNA synthesis during outgrowth (2). The results presented in this paper demonstrate that initiation of trapped phage DNA transcription in outgrowing spores proceeds in the absence of protein synthesis. Thus, all of the peptides necessary for this function are already present in the dormant spores. This conclusion is consistent with the observations that vegetative cell RNA polymerase peptides are present in dormant spores of *B. cereus* (3a) and *B. subtilis* (8) and that enzyme preparations of *B. cereus* spores are capable of faithful transcription of CP-51 DNA (Z. Mazor and A. Cohen, manuscript in preparation). Our observations do not exclude the possibility that one or more of the peptides necessary for initiation of phage DNA tran-

scription is present in limiting amounts in the spores during early outgrowth, thus affecting the rate of phage RNA synthesis.

Transcription of trapped phage DNA and host DNA in outgrowing spores of *B. cereus* differs in the sensitivity of the DNA to the presence of protein synthesis inhibitors during early stages of outgrowth. This difference may be due to differences in requirements for initiation of transcription of these two species of DNA or to a possible requirement for protein-mediated changes in the state of host DNA to allow its transcription during outgrowth. The results presented in this paper do not offer conclusive evidence to determine which of these two possibilities is correct.

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