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Hybridization Analysis of Restriction Endonuclease DNA Fragments of *Bacillus cereus* Transcribed During Spore Outgrowth

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Transcribing *Bacillus cereus* DNA was visualized by means of autoradiography of electrophoretically separated *Eco*RI restriction endonuclease DNA fragments hybridizing ^{32}P-labeled RNA. Hybridization of RNA of dormant spores, vegetative cells, and outgrowing spores indicates the following. (i) A large fraction of the nonribosomal RNA in dormant spores is transcribed at a limited number of regions on the bacterial chromosome. (ii) After induction of spore germination, transcription activity is not limited to a single short region on the chromosome, but rather is distributed along the chromosome. The DNA/RNA hybridization technique has been used to identify restriction endonuclease DNA fragments homologous to RNA species that are present in dormant spores but absent from vegetative cells, RNA species that are synthesized immediately after germination induction and are present at a relatively low concentration in vegetative cells, and RNA species that are transcribed at a late stage of outgrowth but are absent or present at low concentration at an early stage of outgrowth.

A temporal order of protein synthesis has been demonstrated in outgrowing bacterial spores. The synthesis of a small number of protein species is initiated after induction of germination. As outgrowth proceeds, the number of synthesized proteins increases, and temporal changes in the population of peptides being synthesized at any period are observed. These changes have been documented by analysis of pulse-labeled peptides of outgrowing spores (8, 20) as well as by following the kinetics of synthesis of individual enzymes during outgrowth (7, 8, 16).

The dependency of protein synthesis on labile RNA synthesis and the inhibition of de novo enzyme synthesis by chloramphenicol or actinomycin have led to the conclusion that the periodicity of protein synthesis during outgrowth is determined at the transcription level (5, 8) rather than by post-transcriptional control. The following data are consistent with this hypothesis. (i) Competitive hybridization of pulse-labeled RNA of outgrowing spores with total RNA extracted at different periods during outgrowth indicates that the distribution of the predominant mRNA species fluctuates during outgrowth (5). (ii) A positive correlation has been observed between the time of synthesis of individual inducible enzymes and the time of their inducibility (16).

The mechanism which determines the temporal order of gene expression during outgrowth is not yet understood. The time of expression of individual genes may be determined by the presence of regulatory factors in the outgrowing spores at specific stages of development (3). Alternatively, timing of gene expression may be controlled by sequential transcription of the genome (7). For the latter case, if transcription is initiated after induction of germination from a defined site on the chromosome, as proposed by Kennett and Sueoka (7), then at an early stage of outgrowth, transcription would be limited to genes clustered on a small fraction of the chromosome. This hypothesis can be tested by hybridizing pulse-labeled RNA from different periods during outgrowth and vegetative growth to electrophoretically separated restriction endonuclease DNA fragments by Southern's technique (14).

Since genes expressed at a defined stage of outgrowth are not necessarily clustered on individual restriction fragments, differences in the population of RNA species being synthesized at different stages of outgrowth would not necessarily be reflected in the differences between the hybridization patterns of RNA pulse-labeled at the corresponding periods. Nevertheless, comparison of the hybridization patterns should allow the detection of DNA fragments enriched

for sequences homologous to RNA being synthesized at defined stages of development. Subsequently, such detection should facilitate the cloning of such fragments for the purpose of further studies of control of gene expression during spore outgrowth.

MATERIALS AND METHODS

Organism. *Bacillus cereus* T was used in all experiments. Spores were prepared by the method of Vary and Halvorson (21) and stored at −20°C. Before germination, spores were activated at 65°C for 1 h.

Media. Growth of cells and spores was carried out in the low-phosphate medium PGAT (G. B. Spiegelman, Ph.D. thesis, University of Wisconsin, Madison, 1972) containing 0.1% peptone (Difco), 0.4% glucose, 0.24% NH_4Cl, and 0.01 M tris(hydroxymethyl)-aminomethane (Tris)-hydrochloride, pH 7.2. To induce germination and outgrowth, PGAT was supplemented with 100 μg of L-alanine per ml, 100 μg of adenosine per ml, and 0.05% Tween 80.

Radioactive labeling of cells and spores. Vegetative cells containing uniformly ^{32}P-labeled RNA were prepared as follows. Heat-activated spores were germinated in PGAT supplemented with L-alanine and adenosine, diluted in PGAT medium at a concentration of 0.05 mg/ml, and were incubated on a rotary shaker at 30°C. When the culture reached an optical density at 600 nm of 0.3, carrier-free $H_3{}^{32}PO_4$, which had been neutralized, was added at a final concentration of 100 μCi/ml. The cells were harvested after two more generations and washed once with 0.01 M Tris-hydrochloride–0.01 M NaCl–0.0001 M sodium acetate, pH 8.0 (Tris-NaCl). Preparation of uniformly labeled spores was as follows. Cells were labeled at an optical density at 600 nm of 0.3 with 100 μCi of $H_3{}^{32}PO_4$ per ml, grown for two generations, and then another portion of 100 μCi of $H_3{}^{32}PO_4$ per ml was added, and the cells were allowed to sporulate for 48 h, after which they were centrifuged, washed twice in water, and subjected to a Renographin step-wise density gradient centrifugation (17). The fraction containing the refractile spores was thoroughly washed in distilled water and kept at −20°C. For preparation of pulse-labeled cells, cells were grown as above. At an optical density at 600 nm of 0.600, $H_3{}^{32}PO_4$ was added to a final concentration of 100 μCi/ml, and after 3 min the suspension was poured over frozen Tris-NaCl buffer at one-third of the volume of the cell suspension. The cells were centrifuged and washed once in the same buffer. For preparation of pulse-labeled outgrowing spores, spores were heat activated at 65°C for 1 h and diluted in PGAT medium supplemented with L-alanine and adenosine at a final concentration of 0.5 mg of spores per ml. At the times indicated, $H_3{}^{32}PO_4$ was added to a final concentration of 100 μCi/ml for 5 min. The termination of the labeling and the washing of the spores were carried out as described for cells.

Purification of bulk RNA from cells and spores. Cells or spores were suspended in 10 ml of Tris-NaCl buffer containing 150 μl of diethylpyrocarbonate. The cells were disrupted in the presence of 15 g of 0.1-mm glass beads in a Braun homogenizer (B. Braun Melsungen Apparatebau, Melsunger, Germany) for 3 to 4 min. Dry ice was used to keep the temperature below 10°C. The beads were washed once in 2.5 ml of Tris-NaCl buffer containing 1% sodium dodecyl sulfate, the suspension was centrifuged at 12,000 × *g* for 10 min, and 2 volumes of absolute ethanol were added to the supernatant. The precipitate was centrifuged, and the pellet was dissolved in 0.15 M sodium acetate–0.01 M magnesium acetate (pH 5) buffer and subjected to deoxyribonuclease at 10 μg/ml for 2 h in ice. Three phenol extractions were performed, followed by three chloroform extractions and Sephadex G-50 gel filtration. The RNA was precipitated by 2 volumes of ethanol.

Preparation of rRNA. Cells were suspended in 0.01 M Tris-hydrochloride–5 mM $MgCl_2$, pH 7.0 (TM) and disrupted in a Braun homogenizer as above. After the disruption, the supernatant was centrifuged at 27,000 × *g* for 15 min, to remove cell debris and glass beads, and then centrifuged at 105,000 × *g* for 2 h. The ribosomal pellet was suspended in TM, incubated at 37°C for 3 h, and dialyzed against 500 volumes of 10 mM Tris-hydrochloride, pH 7.4. Ribosomal subunits were centrifuged at 105,000 × *g* for 2 h, suspended in Tris-NaCl buffer containing 1% sodium dodecyl sulfate, and subjected to phenol and chloroform extractions as above.

Preparation of DNA. DNA was prepared by a modification of the Marmur procedure (9). Vegetative cells were suspended in 0.15 M NaCl, 0.1 M ethylenediaminetetracetate, 20% sucrose (pH 8.0), and 1 mg of lysozyme per ml and incubated at 20°C for 90 min. Sodium dodecyl sulfate was added to the suspension to a final concentration of 1%, and sodium perchlorate was added to a final concentration of 1.0 M. After chloroform-isoamyl alcohol (24:1) extractions, the DNA was precipitated by adding 2 volumes of ethanol. The precipitated DNA was dissolved in 0.1 × SSC (SSC = 0.15 M NaCl + 0.015 M sodium citrate) and subjected to CsCl density gradient centrifugation, followed by extensive dialysis against 10 mM Tris-hydrochloride (pH 7.4)–1 mM ethylenediaminetetraacetate.

Enzyme digestion. Endonuclease *Eco*RI was prepared as described by Tanaka and Weisblum (18). The digestion mixture contained 200 μg of DNA per ml, 90 mM Tris-hydrochloride (pH 7.4), 10 mM $MgCl_2$, and enzyme. Reactions were terminated by adding 0.2 volume of 60% sucrose–0.05% bromophenol blue–0.1 M ethylenediaminetetraacetate.

Agarose gel electrophoresis. Electrophoresis of the DNA sample was conducted according to the method of Sharp et al. (13) on 1% agarose gel (Sigma, electrophoresis grade). Electrophoresis buffer contained 0.04 M Tris-acetate (pH 8.05), 0.02 mM sodium acetate, 2 mM sodium ethylenediaminetetraacetate, and 0.018 M NaCl. Gels were prepared in glass tubes (0.6 by 14 cm). A 5-μg amount of DNA in a total volume of 50 μl was loaded on one gel; electrophoresis was at 2 mA/gel (constant current) for 14 h. DNA bands were stained by immersing the gels in 0.5 μg of ethidium bromide per ml of solution. Photographic records were made with a Polaroid Land camera, using two 4-W UV lamps (UVS-11, Ultraviolet Products, Inc., San Gabriel, Calif.) and a Wratten 12 yellow gelatin filter (Kodak). Polaroid 107c film was used.

Hybridization conditions. Electrophoretically separated *Eco*RI restriction endonuclease DNA fragments were transferred from agarose gels onto nitrocellulose membranes by the method of Southern (14). The efficiency of transfer of ^{3}H-labeled DNA was determined to be above 80%. All experiments described in this paper were conducted with a single preparation of digested *B. cereus* DNA. Hybridization conditions were: 2×10^{4} cpm of ^{32}P and 5 to 10 μg of RNA in 15 μl of 2× SSC per cm^{2} of nitrocellulose membrane. Incubation, filter washing procedure, and autoradiography were as described by Southern (14).

RESULTS

Hybridization of rRNA and pulse-labeled RNA of vegetative cells. DNA of *B. cereus* was digested to completion by *Eco*RI restriction endonuclease and subjected to gel electrophoresis. The separated fragments were transferred onto nitrocellulose sheets and hybridized to ^{32}P-labeled rRNA of *B. cereus* under the conditions described in Materials and Methods. The autoradiogram of the hybridization filter (Fig. 1a) contains one dense band, which corresponds to a DNA fragment of 1.5 kilobases (kb), and about 18 bands of lower intensity, which correspond to *Eco*RI fragments of higher molecular weights.

Most of the *Eco*RI DNA fragments which are resolved by agarose gel electrophoresis hybridize to pulse-labeled RNA of vegetative cells to form a reproducible pattern (Fig. 1b). Whereas nonlabeled rRNA (600 μg/ml) fully competes with ^{32}P-labeled rRNA upon hybridization with *Eco*RI DNA fragments of *B. cereus,* the same amount of nonlabeled rRNA does not reduce the intensity of any of the resolved hybridization bands of pulse-labeled RNA (Fig. 1d). Most of the RNA of the pulse-labeled preparations consists of rRNA, which is present in the cell before labeling. Therefore, hybridization of pulse-labeled RNA appears in this system to be under conditions of RNA excess and relatively low specific activity, even without the addition of competing rRNA to the hybridization mixture. Consequently, hybridizing rRNA is not represented in the pattern of hybridizing pulse-labeled RNA. Nonlabeled bulk RNA of *B. cereus* cells was added to the hybridization mixture containing pulse-labeled RNA in the same amount as rRNA (Fig. 1c). Total cell RNA competes with the hybridizing pulse-labeled RNA to reduce the intensity of all the bands, which are resolved by autoradiography.

Spore nonribosomal RNA. In an attempt to characterize the population of RNA species in dormant spores before germination, uniformly labeled RNA of dormant spores was hybridized to *B. cereus* DNA *Eco*RI fragments. The pattern of DNA fragments hybridizing spore RNA is similar to that of DNA fragments, which hybridize rRNA. To eliminate the background of DNA fragments transcribing rRNA, hybridization was conducted in the presence of 750 μg of nonlabeled rRNA per ml. About eight *Eco*RI DNA

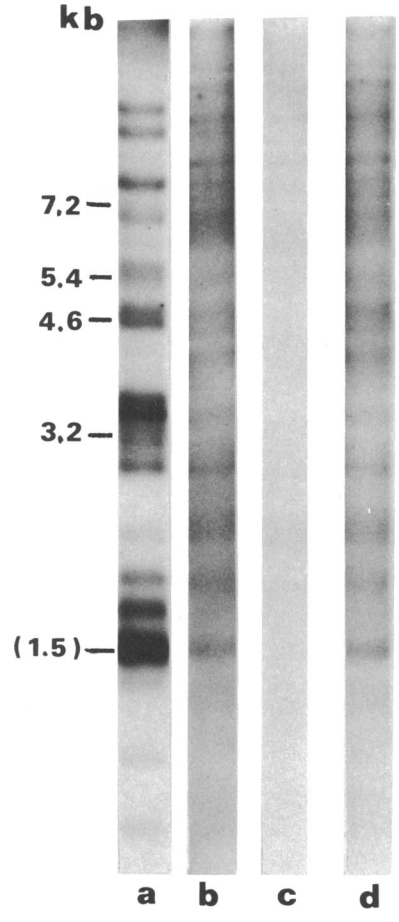

FIG. 1. *Hybridization of vegetative-cell RNA to EcoRI DNA fragments. B. cereus DNA was digested with EcoRI restriction endonuclease and subjected to agarose gel electrophoresis as described in the text. The electrophoretically separated EcoRI DNA fragments were hybridized to ^{32}P-labeled RNA preparations, and the hybridization pattern was visualized by autoradiography. (a) B. cereus rRNA hybridizing EcoRI DNA fragments; (b) pulse-labeled vegetative-cell RNA hybridizing EcoRI DNA fragments; (c) EcoRI DNA fragments hybridizing pulse-labeled, vegetative-cell RNA in the presence of 750 μg of nonlabeled bulk vegetative-cell RNA per ml; (d) EcoRI DNA fragments hybridizing pulse-labeled, vegetative-cell RNA in the presence of 750 μg of nonlabeled rRNA per ml. Kilobase values designate the position of molecular-size markers of phage λ EcoRI restriction fragments (19). The kilobase value in parentheses designates the position of the EcoRI fragment referred to in the text.*

fragments hybridize to spore RNA in the presence of nonlabeled rRNA (Fig. 2c) to reveal autoradiographic bands of low intensity. The intensity of these bands does not decrease when the ratio of nonlabeled rRNA to ^{32}P-labeled spore RNA is increased. Hybridization of *Eco*RI fragments to ^{32}P-labeled spore RNA in the presence of 750 μg of bulk, nonlabeled, vegetative-cell RNA eliminated all but one of the hybridization bands (Fig. 2d). This hybridization band corresponds to an *Eco*RI DNA fragment of about 8.5 kb.

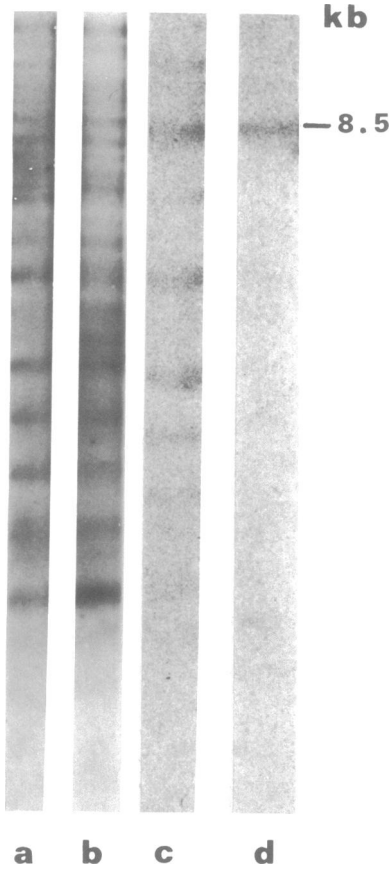

FIG. 2. *Spore and vegetative-cell RNA hybridizing EcoRI DNA fragments. Uniformly labeled vegetative-cell or spore RNA was hybridized to electrophoretically separated EcoRI DNA fragments in the presence or absence of competing nonlabeled RNA. Autoradiograms are of EcoRI DNA fragments hybridizing the following preparations: (a) uniformly labeled, vegetative-cell RNA in the presence of 750 μg of nonlabeled RNA per ml; (b) uniformly labeled vegetative-cell RNA in the presence of 750 μg of nonlabeled dormant spore RNA per ml; (c) uniformly labeled spore RNA in the presence of 750 μg of nonlabeled rRNA per ml; (d) uniformly labeled spore RNA in the presence of 750 μg of nonlabeled, vegetative-cell RNA.*

Hybridization of continuously labeled vegetative-cell RNA with *Eco*RI DNA fragments also forms a pattern similar to that of rRNA. Addition of 750 μg of competing nonlabeled rRNA per ml to the hybridization mixture reveals the hybridization pattern presented in Fig. 2a. A similar pattern is obtained upon addition of 750 μg of competing nonlabeled bulk RNA of dormant spores to the hybridization mixture (Fig. 2b).

Transcribing DNA fragments of outgrowing spores. RNA was pulse-labeled at 5 min after germination induction and during vegetative growth and hybridized to electrophoretically separated restriction endonuclease DNA fragments of *B. cereus*. The hybridization pattern of early-outgrowth RNA was similar to that of vegetative-cell RNA. The number of resolved hybridization bands was the same for both preparations, and most bands were at parallel positions. This indicates that transcription activity at this stage of development is not restricted to genes clustered on a single short region, but rather is distributed along the chromosome, the same as is transcription activity during the vegetative phase of growth.

An apparent difference between the hybridization pattern of germinating-spore RNA and vegetative-cell RNA is in the distribution of radiointensity among the hybridization bands. Hybridization bands which correspond to *Eco*RI fragments of 1.5, 1.8, 2.9, and 3.5 kb have a relatively higher intensity when hybridizing outgrowing-spore RNA pulse-labeled at 5 min after germination induction than vegetative-cell RNA (Fig. 3). To detect restriction fragments homologous to RNA species, which may be present in outgrowing spores at a relatively higher concentration than in vegetative cells, hybridization of pulse-labeled, outgrowing-spore RNA was conducted in the presence of competing nonlabeled RNA of outgrowing spores and of vegetative cells (Fig. 4). Addition of 120 μg of nonlabeled RNA of outgrowing spores per ml to the hybridization mixtures reduced the intensity of all the hybridization bands (Fig. 4c), and at 500 μg of competing early-outgrowth RNA per ml the bands were barely visible under the conditions of the experiment (Fig. 4d). On the other hand, addition of 1,000 μg of competing vegetative-cell RNA per ml to the hybridization mixture (Fig. 4b) led to the reduction of intensity of the major hybridization bands, which correspond to *Eco*RI fragments of 1.5, 1.8, and 3.5 kb, but no marked reduction in the radiointensity of the band which corresponds to an *Eco*RI fragment of 2.9 kb was

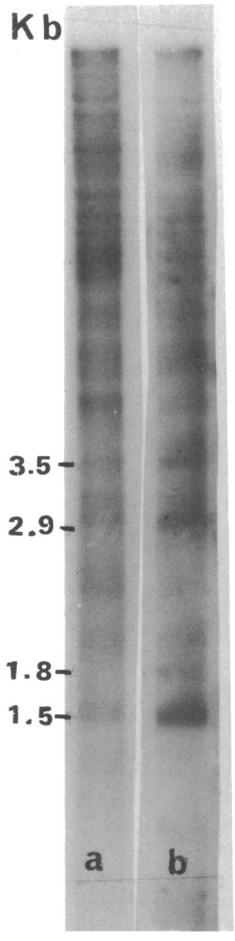

FIG. 3. *EcoRI DNA fragments hybridizing pulse-labeled vegetative-cell and outgrowing-spore RNA. Electrophoretically separated EcoRI DNA fragments were hybridized to RNA pulse-labeled (a) during vegetative growth and (b) starting at 5 min after germination induction. Estimated kilobase values designate EcoRI fragments referred to in the text. Hybridization conditions are as described in Materials and Methods.*

observed. This observation indicates that at least part of the RNA homologous to the 2.9-kb fragment is present in an exceedingly lower concentration in vegetative cells than in spores at an early stage of outgrowth.

To detect possible changes in the pattern of *Eco*RI DNA fragments that are transcribed at different stages of outgrowth, pulse-labeled RNA of spores at an early stage (0 to 5 min after germination induction) and a late stage (60 to 65 min after germination induction) was prepared and hybridized to electrophoretically separated *Eco*RI DNA fragments. Autoradiograms of the respective hybridization patterns are presented in Fig. 5a and d. Most of the major hybridization bands of the two preparations are at parallel positions. However, a few differences are observed. At least one band, corresponding to an *Eco*RI fragment of 5.1 kb, appears as a major band in the hybridization pattern of RNA pulse-labeled at 60 min (Fig. 5d) but not in the hybridization pattern of RNA pulse-labeled at 0 to 5 min (Fig. 5a). To determine whether hybridiza-

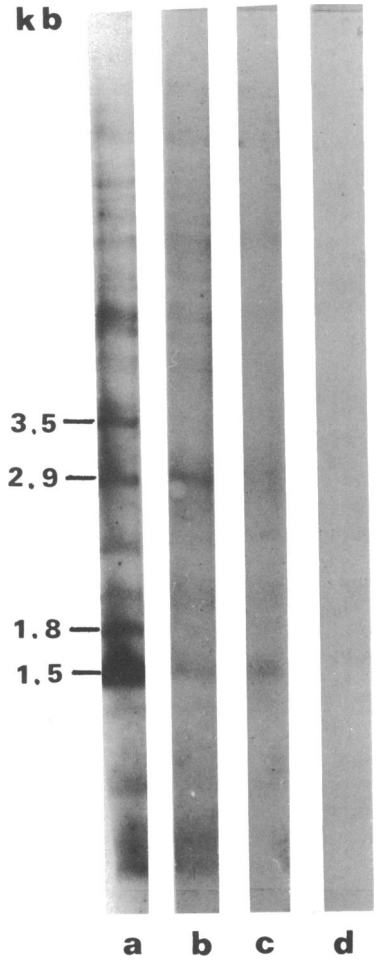

FIG. 4. *Hybridization of pulse-labeled, early-outgrowth RNA in the presence of competing nonlabeled RNA of vegetative cells and outgrowing spores to EcoRI DNA fragments. RNA, pulse-labeled at 5 to 10 min after germination induction, was hybridized to EcoRI fragments of B. cereus DNA (a) in the absence of competing RNA; (b) in the presence of 1,000 μg of vegetative-cell RNA per ml; or in the presence of competing homologous RNA of outgrowing spores at a concentration of (c) 120 or (d) 500 μg/ml.*

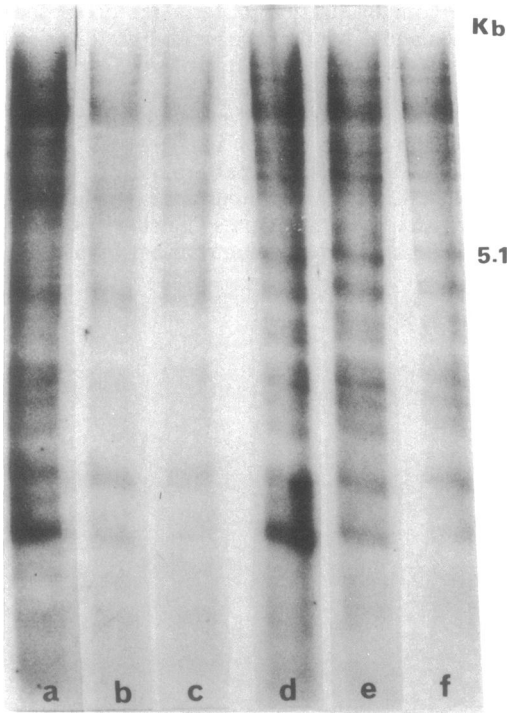

FIG. 5. *Hybridization of pulse-labeled RNA from early and late stages of outgrowth to EcoRI DNA fragments. RNA, pulse-labeled at (a, b, c) 0 to 5 min after germination induction and (d, e, f) 60 to 65 min after germination induction, were hybridized to EcoRI B. cereus DNA fragments (a, d) in the absence of competing RNA or in the presence of a sixfold excess of competing RNA of outgrowing spores at (b, e) 5 min after germination induction or (c, f) 65 min after germination.*

tion at the 5.1-kb *Eco*RI DNA fragment was conducted under conditions of DNA or RNA excess, a sixfold excess of competing nonlabeled RNA of spores at either early or late stages of outgrowth was added to the hybridization mixture. The intensity of the 5.1-kb band in the hybridization pattern of late-outgrowth RNA was not reduced markedly by either of the two competing RNAs. This indicates that in the absence of competing RNA, hybridization of late-outgrowth RNA at this site was under conditions of DNA excess. Excess of competing species in the nonlabeled early RNA preparation would have markedly reduced the intensity of the 5.1-kb band in Fig. 5e. Since the concentration of nonlabeled early-outgrowth RNA in the hybridization presented in Fig. 5e is sixfold the concentration of the homologous labeled RNA in the hybridization presented in Fig. 5a, it could be assumed that hybridization of early-outgrowth, pulse-labeled RNA at this site was under conditions of DNA excess. Thus, the absence of a major hybridization band at the 5.1-kb site from the hybridization pattern of pulse-labeled RNA of spores at an early stage of outgrowth is probably due to a relatively lower rate of synthesis of the corresponding RNA species, rather than to hybridization under conditions of low specific activity and RNA excess.

DISCUSSION

The technique of hybridization of ^{32}P-labeled RNA to restriction endonuclease DNA fragments has been used for the analysis of rRNA, nonribosomal RNA of dormant spores, and RNA of outgrowing spores at different stages of development of *B. cereus*.

The pattern of *B. cereus Eco*RI DNA fragments hybridizing rRNA is consistent with the similar pattern with *B. subtilis* rRNA (10). Potter et al. (10) have proposed that such a pattern reflects the presence of several copies of rRNA genes separated by DNA spacers that vary in size and sequence. Accordingly, the 1.5-kb hybridization band of high radiointensity would represent part of the rRNA gene defined by two *Eco*RI sites, and the other hybridization bands would correspond to fragments defined by one site in the rRNA gene and one site in the external environment of the gene or in an adjacent rRNA gene.

mRNA in dormant spores has been previously demonstrated by the presence of polyribosomes in spores extracts (1, 4) and by comparing the saturation level of DNA-hybridizing bulk RNA of spores to the saturation level of DNA-hybridizing spore rRNA and tRNA (6). Hybridization of uniformly labeled RNA from spores and vegetative cells to *Eco*RI DNA fragments of *B. cereus* in the presence of saturating levels of nonlabeled rRNA allows the comparison of the population of DNA fragments homologous to nonribosomal RNA in vegetative cells and in dormant spores. Whereas the hybridization profile of nonribosomal RNA of vegetative cells is complex, a large fraction of the nonribosomal RNA of dormant spores hybridizes to a limited number of fragments of the bacterial DNA. Most spore nonribosomal RNA hybridization bands could be eliminated by competing vegetative-cell RNA. Only one of the hybridization bands carries sequences hybridizing spore RNA species which could not be fully competed by vegetative-cell RNA. These observations indicate that the predominant species of nonribosomal RNA in dormant spores are transcribed from a limited number of regions on the bacterial chromosome. It suggests that one of these regions codes for RNA species which are present in dormant spores and absent or present at low concentrations in vegetative cells. Further studies should

determine the number of RNA species coded by this DNA segment and the nature of these RNA species. It would also be of interest to determine the time of synthesis of RNA homologous to this band during sporulation and the stability of this RNA during spore germination and outgrowth.

It has been proposed that timing of enzyme synthesis during spore outgrowth is determined by sequential transcription of the genome, starting from the origin of replication (7). This theory is supported by the data of Kennett and Sueoka (7), who observed that the temporal sequence of enzyme synthesis was directly related to the distance of their structural gene from the origin of replication. One prediction of this proposal is that immediately after germination induction, transcription activity would be limited to a short segment of the chromosome. RNA homologous to a short region of the chromosome should hybridize to a small fraction of the fragments hybridizing pulse-labeled RNA of vegetative cells or pulse-labeled RNA of spores at a late stage of outgrowth. Therefore, the observation that the pattern produced by hybridization of pulse-labeled RNA of spores at an early stage of outgrowth is as complex as the pattern of hybridizing vegetative-cell RNA is inconsistent with the proposal that sequential transcription of the genome, which is initiated at a defined location on the chromosome, takes place during outgrowth. Yeh and Steinberg (22) have studied the effect of translocations of structural genes on the time of their expression during *Bacillus subtilis* spore outgrowth. They have demonstrated that translocation of a structural gene to a position distal to the origin of replication does not change the time of its expression during outgrowth. Thus, initiation of transcription after germination induction appears to take place at sites distributed along the length of the chromosome.

If enzyme timing during outgrowth is controlled on the transcription level, the population of RNA species available for translation should change temporally during this phase of the bacterial life cycle. The observations that some RNA species present in spores immediately after germination induction are absent or present at lower concentrations in vegetative cells and that RNA homologous to some *Eco*RI fragments appears at a late stage of outgrowth are consistent with this prediction.

Genes expressed at a specific stage of spore development are not necessarily clustered; therefore, individual *Eco*RI DNA fragments may be homologous to RNA species synthesized at different stages of outgrowth. Consequently, the differences observed between hybridization patterns represent a minimal estimation of the differences between the population of RNA species present in the cell at different developmental stages. The use of two-dimensional restriction analysis (1) coupled with hybridization by the Southern technique should greatly increase the resolution of the method. The electrophoretic separation and identification of *Eco*RI DNA fragments homologous to chromosomal regions which are being transcribed at a defined period during outgrowth should facilitate the enrichment and cloning of these fragments in bacterial plasmids. *B. subtilis* DNA containing a gene that is activated during sporulation has been recently identified and cloned by a similar procedure (12). Cloned fragments could be used as probes in hybridization experiments and contribute to the understanding of the mechanism which determines the timing of gene expression during bacterial spore outgrowth.

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LITERATURE CITED

1. **Chambon, P., N. P. Deutscher, and A. Kornberg.** 1968. Biochemical studies of bacterial sporulation on germination. X. Ribosomes and nucleic acids of vegetative cells and spores of *Bacillus megaterium*. J. Biol. Chem. **243**:5110–5116.
2. **Chow, L. T., and N. Davidson.** 1973. Electron microscope mapping of the distribution of ribosomal genes of the *Bacillus subtilis* chromosome. J. Mol. Biol. **75**:265–279.
3. **Cohen, A., H. Ben Ze'ev, and Z. Silberstein.** 1975. Control of temporal gene expression during outgrowth of *Bacillus cereus* spores, p. 478–482. *In* P. Gerhardt, R. N. Costilow, and H. L. Sadoff (ed.), Spores VI. American Society for Microbiology, Washington, D.C.
4. **Feinsod, R. M., and H. A. Douthit.** 1970. Ribosomes from spores of *Bacillus cereus* T. Science **168**:991.
5. **Hansen, J. N., G. Spiegelman, and H. O. Halvorson.** 1970. Bacterial spore outgrowth: its regulation. Science **168**:1291–1298.
6. **Jeng, Y. H., and R. H. Doi.** 1974. Messenger ribonucleic acid of dormant spores of *Bacillus subtilis*. J. Bacteriol. **119**:514–521.
7. **Kennett, R. H., and N. Sueoka.** 1971. Gene expression during outgrowth of *Bacillus subtilis* spores. J. Mol. Biol. **60**:31–44.
8. **Kobayashi, Y., W. Steinberg, A. Higa, H. O. Halvorson, and C. Levinthal.** 1965. Sequential synthesis of macromolecules during outgrowth of bacterial spores, p. 200–212. *In* L. L. Campbell and H. O. Halvorson (ed.), Spores III. American Society for Microbiology, Ann Arbor, Mich.
9. **Marmur, J.** 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. J. Mol. Biol. **3**:208–218.
10. **Potter, S. S., K. F. Bott, and J. E. Newbold.** 1977. Two-dimension restriction analysis of the *Bacillus subtilis* genome: gene purification and ribosomal ribonucleic acid gene organization. J. Bacteriol. **129**:492–500.
11. **Potter, S. S., and J. E. Newbold.** 1976. Multidimen-

sional restriction enzyme analysis of complex genomes. Anal. Biochem. **71**:452–458.

12. **Segall, J., and R. Losick.** 1977. Cloned *Bacillus subtilis* DNA containing a gene that is activated early during sporulation. Cell **11**:751–761.
13. **Sharp, P. A., B. Sugden, and J. Sambrook.** 1973. Detection of two restriction endonuclease activities in *Haemophilus parainfluenza* using analytical agarose-ethydium bromide electrophoresis. Biochemistry **12**:3055–3063.
14. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
15. **Spiegelman, G. B., E. Dickinson, J. Idriss, W. Steinberg, S. Rodenberg, and H. O. Halvorson.** 1969. Classes of ribonucleic acids and protein synthesized during outgrowth of spores of *Bacillus cereus,* p. 235–246. *In* L. L. Campbell (ed.), Spores IV. American Society for Microbiology, Bethesda, Md.
16. **Steinberg, W., and H. O. Halvorson.** 1968. Timing of enzyme synthesis during outgrowth of spores of *Bacillus cereus.* I. Ordered enzyme synthesis. J. Bacteriol. **95**:469–478.
17. **Tamir, H., and C. Gilvarg.** 1966. Density gradient centrifugation for the separation of sporulating forms of bacteria. J. Biol. Chem. **241**:1085–1090.
18. **Tanaka, T., and B. Weisblum.** 1975. Construction of a colicin E1-R factor composite plasmid in vitro: means for amplification of deoxyribonucleic acid. J. Bacteriol. **121**:354–362.
19. **Thomas, M., and R. W. Davis.** 1975. Studies on the cleavage of bacteriophage lambda DNA with *Eco*RI restriction endonuclease. J. Mol. Biol. **91**:315–328.
20. **Torriani, A., and C. Levinthal.** 1967. Ordered synthesis of proteins during outgrowth of spores of *Bacillus cereus.* J. Bacteriol. **94**:176–183.
21. **Vary, J. C., and H. O. Halvorson.** 1965. Kinetics of germination of *Bacillus* spores. J. Bacteriol. **89**:1340–1347.
22. **Yeh, E. L., and W. Steinberg.** 1978. The effect of gene position, gene dosage and a regulatory mutation on the temporal sequence of enzyme synthesis accompanying outgrowth of *Bacillus subtilis* spores. Mol. Gen. Genet. **158**:287–296.