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Preferential Transcription of *Bacillus subtilis* Light Deoxyribonucleic Acid Strands During Sporulation

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Messenger ribonucleic acid (RNA) from log and sporulation stages of growth were transcribed mainly from the heavy strand of the complementary strands of *Bacillus subtilis* deoxyribonucleic acid (DNA). During sporulation a slight transcription shift from heavy to light DNA strands was observed. RNA-DNA hybrid competition experiments revealed that this shift was due to sporulation-specific transcription from light-DNA strands.

During bacterial sporulation, a series of events occur resulting in many morphological and biochemical changes (13, 16, 21). Therefore bacterial sporulation is a good model system for the study of cytodifferentiation at the unicellular level. Investigators have demonstrated that differential expression of the genome occurs during sporulation (1, 4, 5) and that a messenger ribonucleic acid (RNA) fraction is formed which is not present in vegetative cells. We investigated messenger RNA synthesis during bacterial sporulation to characterize the nature of gene transcription and to obtain more precise information about the specificity of transcription during bacterial morphogenesis.

The main approach we have taken is to test for hybrid formation between the separated complementary strands of *Bacillus subtilis* deoxyribonucleic acid (DNA) and messenger RNA from log-phase and sporulating cells. Methods to separate complementary DNA strands by CsCl density gradient centrifugation with synthetic polynucleotides (25) and methylated albumin kieselguhr (MAK) column chromatography (28, 29) have been reported. By use of separated complementary DNA strands, the asymmetric transcription of DNA strands has been demonstrated in bacteriophage λ (33), T7 (31), T4 (10), φ80 (20), SPP1 (27), mitochondria (2) and bacteria (11, 15, 17, 18, 24, 25, 32). These results have revealed an orientation and specificity of the transcription process.

This paper describes an analysis of sporulation messenger RNA of *B. subtilis* by use of these techniques. The results reveal that transcription occurs preferentially from one (the L strand) of the complementary strands of *B. subtilis* DNA during sporulation.

MATERIALS AND METHODS

Bacterial strain and media. *B. subtilis* 168 wild type was used in all experiments. The cells were grown at 37 C in Penassay (Difco) medium for preparation of DNA and in Y medium (34) for the sporulation studies.

Preparation of complementary DNA strands. The cells at the late log stage were converted to protoplasts by treatment with lysozyme in maleate (0.05 M, *p*H 6.0)-ethylenediaminetetraacetic acid (2 mM)-sucrose (15%) buffer containing NaCN (10^{-3} M), β-mercaptoethanol (60 mM) and Na_2SO_4 (10 mM). After the preparation of protoplasts, DNA was prepared essentially by the method of Marmur (23). Molecular weight of the native DNA preparations was determined by the sedimentation velocity method (7) and found to be 1.8×10^7. Denaturation of DNA with alkali was performed by addition of one-tenth volume of 1 M NaOH to the DNA solution which was diluted to 40 μg/ml with 0.1 SSC (0.15 M NaCl containing 0.015 M sodium citrate). After 10 min at room temperature, the solution was neutralized to *p*H 6.8 by the addition of 1 M NaH_2PO_4 and then dialyzed against saline buffer (0.6 M NaCl in 0.05 M phosphate buffer, *p*H 6.8). Separation of complementary strands of DNA was accomplished by MAK column chromatography at 4 C using a linear gradient of NaCl (0.6 to 0.9 M; reference 28). The elution rate was 35 ml/hr, and 3-ml fractions were collected. Two components were obtained from the MAK column, and each component was purified further by two passes through a MAK column under the conditions described above to yield a light (L)-DNA and a heavy (H)-DNA preparation. Each preparation was dialyzed against 2 × SSC. Before immobilization of each DNA preparation on membrane filters for the hybridization studies, trace contamination by the other strand was eliminated by incubating each preparation

for 24 hr at 68 C. This caused annealing of the contaminating strand to its complementary strand and effectively prevented it from hybridizing with RNA in the annealing mixture.

Properties of the separated DNA strands. Renaturation of the separated DNA strands was performed by incubating a mixture of the two strands at 68 C for 24 hr in 2 × SSC. Heat denaturation and hyperchromicity of the renatured DNA strands were followed spectrophotometrically at 260 nm.

By use of a Beckman Spinco model E ultracentrifuge, the buoyant density of each of the separated DNA strands was compared with native and unfractionated denatured DNA in a CsCl gradient. The CsCl solution containing 0.02 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, *p*H 8.5, had an initial density of 1.710. About 2 to 5 μg of DNA was used, and the centrifugation was performed at 20 C for 18 hr at 44,770 or 42,040 rev/min.

To determine the binding of synthetic polynucleotides to the separated DNA strands, polycytidylate or polyguanylate (Miles Laboratories, Inc.) was added to solutions of each of the strands at room temperature, and the density of the DNA was determined as mentioned above.

Preparation of messenger ^{3}H-RNA and unlabeled bulk RNA. Maximum growth of this strain in Y medium was reached at 6 hr and endospore formation occurred at 12 hr. Labeling of RNA with ^{3}H-uridine was carried out with 100-ml cultures at two stages of growth. At log stage (1.5 hr), 2 mCi of ^{3}H-uridine (uridine-5-^3H, 28.3 Ci/mmole) was added for 3 min and, at sporulation stage III (10 hr), 10 mCi was added for 10 min. The frozen labeled cells, washed previously with a glutamate (150 μg/ml)-$MgCl_2$ (50 mM) solution containing NaCN (10^{-3} M), were ground with 100 μg of deoxyribonuclease I in the presence of 6 mg of macaloid and sodium dodecyl sulfate at a final concentration of 1%. The RNA fraction was extracted three times with phenol (8) and standard buffer (10 mM $MgCl_2$, 60 mM KCl, 6 mM β-mercaptoethanol, 10 mM Tris-hydrochloride, *p*H 7.6). The RNA was precipitated from the extraction buffer by addition of two volumes of ethanol containing 2% potassium acetate. The precipitation procedure was repeated twice. The ^{3}H-labeled RNA was suspended in and dialyzed against 0.1 × SSC containing potassium polyvinylsulfate (20 μg/ml).

The preparation of bulk RNA from unlabeled cells at log and sporulation stages followed the same procedure.

Hybridization experiments. The method of Gillespie and Spiegelman (9) was used for the hybridization of ^{3}H-RNA with DNA. DNA immobilized on nitrocellulose membranes (B-6, coarse, 13 mm, Schleicher and Schuell Co.) was incubated for 21 hr at 68 C in vials containing 2 × SSC, ^{3}H-RNA, and 1 mg of yeast RNA (soluble A grade, Calbiochem) in a total volume of 1.0 ml. The yeast RNA reduced nonspecific adsorption of ^{3}H-RNA to the membrane. After incubation, each filter was washed with 100 ml of 2 × SSC and then incubated with 20 μg of heat-treated pancreatic ribonuclease A (Worthington Biochemical Corp.) per ml for 60 min at room temperature. After treatment with ribonuclease, the filter was rinsed with 100 ml of 2 × SSC. The radioactivity was counted with a Packard scintillation counter.

The hybrid competition experiments were performed as above except for the addition of increasing amounts of unlabeled homologous or heterologous bulk RNA.

RESULTS

Separation and characterization of the complementary strands of DNA. Strand separation of alkali-denatured *B. subtilis* DNA was performed with a MAK column by using a linear salt gradient at 4 C (28). The two complementary strands (L and H strands) were purified by repeating the column fractionation twice on each of the components (Fig. 1). The DNA fractions were tested for complementarity by their ability to renature under the proper conditions and for their buoyant densities in CsCl gradients.

The L-DNA, H-DNA, and a mixture of L- and H-DNA preparations were incubated at 68 C for 24 hr in 2 × SSC buffer to allow renaturation, and then the preparations were heated to obtain DNA melting profiles. The melting curve of the L- plus H-DNA mixture was typical of double-stranded DNA. The results with either the L or H preparation indicated very little, if any, renaturation during heating. The results with the L- plus H-DNA mixture showed that the L-DNA and the H-DNA were complementary and were able to renature.

When the densities of the L and H preparations were examined by use of a CsCl gradient, it was found that the average densities of the L and H strands were 1.716 and 1.719, respectively. A mixture of the two preparations gave an average density of 1.718 which was identical with the density of unfractionated heat- or alkali-denatured DNA. When the affinity of each of the preparations with synthetic polynucleotides was tested, it was found that only the H strand had an affinity with poly G; however, the L strand did not bind to poly C. These results indicate that the separation of unfractionated heat-denatured DNA into two density components in the presence of poly G was due to the binding of poly G by the H strand.

Transcription from the complementary DNA strands during the log and sporulation stages. Pulse-labeled RNA was obtained from log-phase vegetative cells and from stage 3 sporulating cells. To determine the capacity of both DNA strands to hybridize with messenger ^{3}H-RNA from the log and sporulation stages, hybridization experiments were performed initially between unfractionated DNA and increasing levels of messenger ^{3}H-RNA (Fig. 2). Saturation of DNA by ^{3}H-RNA was approached when the ratio of RNA to DNA was about 150:1 to 200:1.

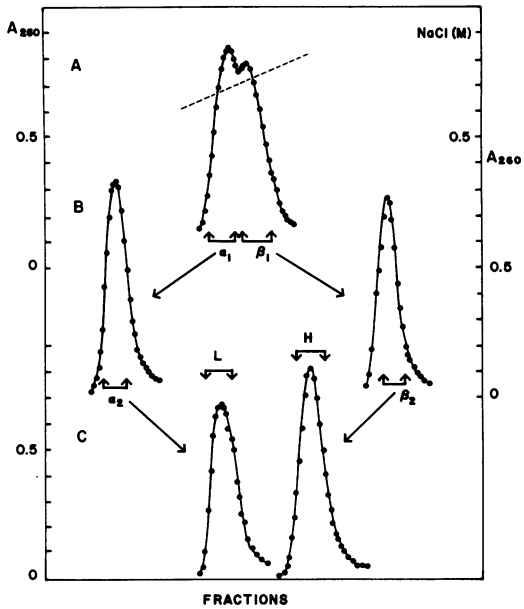

FIG. 1. *Separation of complementary DNA strands of B. subtilis by MAK column chromatography. (A) Alkali-denatured DNA (3.0 mg) was applied to a MAK column (1.9 × 17 cm) and was eluted with a linear NaCl gradient from 0.6 to 0.9 M at 4 C. Three-milliliter fractions were collected. Fractions indicated by the arrows were pooled and labeled α_1 and β_1. Each of the pooled fractions was passed through another MAK column under the conditions described above and in Materials and Methods. (B) After passage of fractions α_1 and β_1 through MAK columns, fractions indicated by the arrows were pooled and labeled α_2 and β_2, respectively. (C) After passage of fractions α_2 and β_2 through separate MAK columns, the fractions indicated by the arrows were collected and labeled L and H, respectively.*

From these results it was decided to use saturation levels of labeled RNA for the experiments. Ratios of labeled RNA to DNA of 170:1 and 190:1 were used for the log-phase RNA and sporulation-phase RNA, respectively.

The results in Table 1 illustrate that the transcription of DNA occurred mainly from the H strand at both growth phases. However, a slight shift of transcription from the H to L strand occurred during sporulation since the percentage of count hybridizing with the H strand decreased from 87.8 to 85.5%, and the count hybridizing with the L strand increased from 12.2 to 14.5%.

Transcription shift from H to L DNA strand during sporulation. To analyze the apparent transcription shift from H to L strands during sporulation, a series of hybrid competition experiments was carried out between pulse-labeled and unlabeled log-phase and sporulation RNA with the L and H DNA strands (Fig. 3). In Fig. 3A is shown the competition between log-phase ^{3}H-RNA and unlabeled log-phase and sporulation RNA with H-DNA. The results in these experiments indicate that the log-phase RNA species hybridizing with H-DNA are also present in the sporulation RNA preparations. The results in Fig. 3B show that all RNA transcribed from the L-DNA strands during the log phase are also transcribed during sporulation. In Fig. 3C the results demonstrate that all the RNA species made from the H strand during sporulation are also made during the log phase. However, in Fig. 3D the results indicate that approximately 22% of the RNA made during sporulation from the L strand is not made during the log phase of growth. The average of four experiments gave a value of 18 ± 3%. These results suggests strongly that differential transcription during stage 3 of sporulation occurs primarily from the L-DNA strands.

DISCUSSION

These experiments illustrate that transcription occurs primarily on the H strand of DNA during both log phase and sporulation of *B. subtilis*. Asymmetric transcription of the H strand has been demonstrated previously in bacteria with messenger RNA of *B. megaterium* (11), ribosomal RNA (17, 24, 32), and transfer RNA (24) of *B. subtilis*, and the product of RNA polymerase in vitro (15) of *B. subtilis*. In most of these cases, a small amount of transcription was also noted from the L strand. Several investigators have proposed that pyrimidine-rich clusters

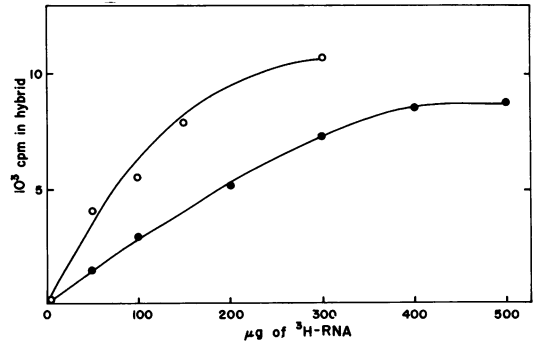

FIG. 2. *Hybridization of unfractionated DNA with pulse-labeled RNA from log-stage and sporulating cells. Each annealing mixture contained 2 μg of alkali-denatured DNA immobilized on membrane filters and log-stage ^{3}H-RNA (11,200 counts per min per μg) or sporulation ^{3}H-RNA (5,000 counts per min per μg). The radioactivity of the controls ranged from 36 to 148 counts/min for log RNA and 27 to 144 counts/min for sporulation RNA, and have been subtracted. Log RNA (○); sporulation RNA (●).*

TABLE 1. *Hybridization of light (L) and heavy (H) strands of B. subtilis DNA with H³-labeled log-stage and sporulation RNA*[a]

Source	Amt of DNA (μg)	³H-labeled RNA	Hybridized RNA[b] (counts/min)	Relative hybridization to L and H strands (%)
L strand DNA	1.5	Log stage	3,499 ± 63	12.2 ± 0.2
H strand DNA	1.5	Log stage	25,291 ± 455	87.8 ± 1.6
L strand DNA	4.0	Sporulation	2,047 ± 59	14.5 ± 0.4
H strand DNA	4.0	Sporulation	12,094 ± 351	85.8 ± 2.5

[a] Annealing mixture contained 1.5 μg of DNA and 255 μg of ³H-labeled log-stage RNA (11,200 counts per min per μg) and 4 μg of DNA and 760 μg of ³H-labeled sporulation RNA (2,900 counts per min per μg) in 1.0 ml, respectively. An RNA-to-DNA ratio was used which approached saturation (see Fig. 2).

[b] Activity of the blank has been subtracted; this is an average of four experiments.

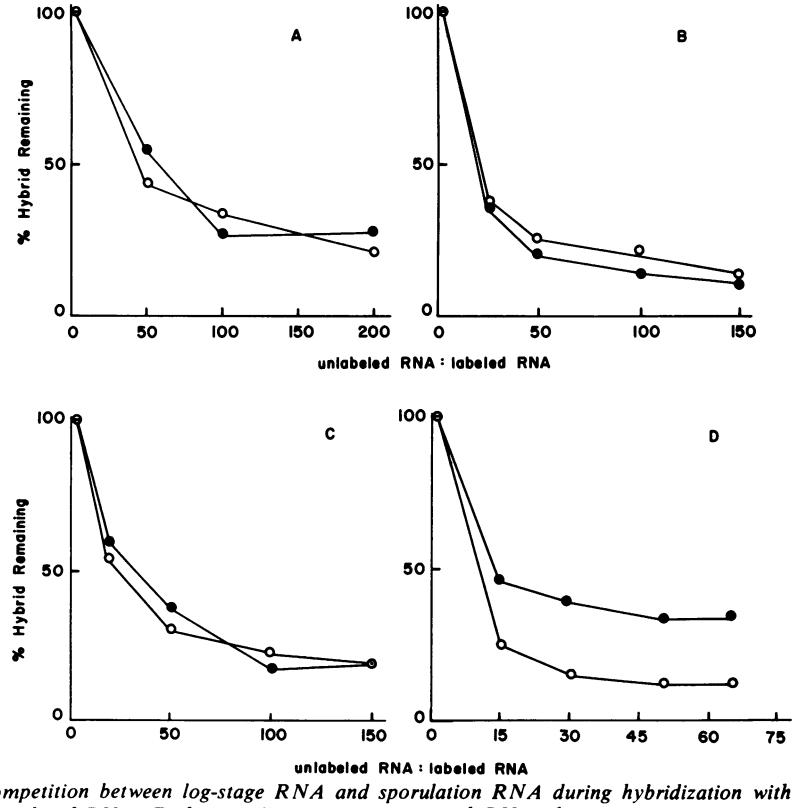

FIG. 3. *Competition between log-stage RNA and sporulation RNA during hybridization with light (L) and heavy (H) strands of DNA. Each annealing mixture contained DNA plus a constant amount of pulse-labeled RNA, and an increasing amount of unlabeled RNA as indicated in the abscissa. The radioactivity of log stage RNA was 11,200 counts per min per μg and of sporulation RNA was 2,900 counts per min per μg. (A) H-DNA, 2 μg, and log stage ³H-RNA, 6 μg. (B) L-DNA, 5 μg, and log stage ³H-RNA, 25 μg. (C) H-DNA, 3.5 μg, and sporulation ³H-RNA, 10 μg. (D) L-DNA, 15 μg, and sporulation ³H-RNA, 150 μg. Competition in the homologous system (○); competition in the heterologous system (●).*

on the DNA strand were involved in directing the initiation of transcription on the H strand (14, 17, 32).

The hybrid competition studies with messenger RNA from log-phase cells (Fig. 3A and 3B) illustrate that sporulation messenger RNA contains essentially all the messenger RNA species found in log-phase cells. These results indicate that sporulating cells continue to transcribe genes which are utilized during vegetative growth and that little or no repression of vegetative genes occurs. One unanswered question is whether all

the vegetative cell messenger RNA made during sporulation is translated into active proteins. The high degree of protein turnover during sporulation (30) suggests that continued synthesis of most cellular proteins may be essential for the basic metabolic functions of the sporulating cell.

The most significant point of the present results is that, although most of the transcription occurs on the H strand during both log phase and sporulation, differential transcription during sporulation occurs primarily on the L strands. The hybrid competition studies (Fig. 3D) with sporulation messenger RNA clearly indicate that approximately 2.5% (14.5% $\times$ 0.18) of the total pulse labeled messenger RNA is being transcribed specifically from the L strand. The messenger RNA made from H strands during sporulation is, however, very similar to or identical with the messenger RNA found in log-phase cells (Fig. 3C). The difficulty of obtaining precise quantitative data from hybridization studies preclude any firm conclusions from data presented in Table 1 alone; however, the general agreement of these data with the results of Fig. 3D lead us to the conclusion that, at stage 3 of sporulation, differential transcription is occurring essentially from L strands. The results in the hybridization experiments are not complicated by the presence of labeled ribosomal and transfer RNA, since they comprise less than 0.5% (6) of the *B. subtilis* genome, the unlabeled RNA preparations used for competition experiments contain a great excess of these stable RNA fractions, and the stable RNA species are transcribed exclusively from H strands of *B. subtilis* (22, 24).

Recent investigations with *Escherichia coli* (3) and *B. subtilis* (19, 26) suggest that regulation of transcription occurs by alteration of RNA polymerase activity. An alteration of promoter recognition specificity of the RNA polymerase may allow a whole set of genes controlled by specific promoters to be turned on or off. The differential transcription of L-DNA strands during sporulation suggests the intriguing possibility that promoter sites for sporulation genes on the L strands are recognized and turned on by such a mechanism. We are currently investigating this possibility with RNA polymerase from sporulating cells.

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