*THE TRANSCRIBING STRANDS OF BACILLUS SUBTILIS DNA FOR RIBOSOMAL AND TRANSFER RNA**

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Abstract and Summary.—H^3-labeled purified 16*S* and 23*S* ribosomal RNA's and transfer RNA's were found to hybridize exclusively with the H strand of the separated complementary strands of *B. subtilis* DNA. These results indicate that these RNA's are transcribed *in vivo* from a strand with a composition similar to that from which messenger RNA's are copied and presumably by the same mechanism.

Previously we reported upon applications of the phenomenon of synchronous DNA replication during germination of *Bacillus subtilis* spores.[1] In those reports,[2, 3] we presented information about the structural genes for ribosomal (16*S* and 23*S*) RNA's and transfer (soluble) RNA's. The conclusions from these experiments were as follows: (1) The information for the structure of the 16*S* and 23*S* RNA species is encoded in different genes. (2) There are equal numbers of 16*S* and 23*S* RNA structural genes (3–10 of each per chromosome according to variations in the calculated molecular weight of the *B. subtilis* chromosome). (3) Most of the ribosomal RNA genes are located close together between two adenine markers and proximal to the point at which the chromosome begins replication. (4) Most of the transfer RNA genes are also located together, close to the ribosomal RNA genes. Dubnau *et al.*[4–6] also mapped the ribosomal RNA and transfer RNA genes and located them at about the same place on the chromosome. They showed that these RNA genes are located close to several antibiotic resistance markers and that the nucleotide sequence structure of both the RNA's and antibiotic resistance genes tends to be conserved among many species of bacilli. More recently, Smith *et al.*, in an extensive study of all the RNA genes, have located 5*S* RNA genes at about the same place on the chromosome.[7] Furthermore, they suggest the presence of a second group of RNA genes on a more distal part of the chromosome.

Opara-Kubinska *et al.* have separated complementary strands of *B. subtilis* DNA by density gradient centrifugation in the presence of guanine-rich polyribopolymers,[8] and Kubinski *et al.* found that the heavier strand hybridizes with pulse-labeled RNA.[9] Essentially the same results were obtained in *B. megatherium*.[10] More recently, Rudner *et al.* succeeded in separating complementary strands of *B. subtilis* DNA by a modified technique of methylated albumin kieselguhr (MAK) column chromatography,[11] and they showed that the base composition of RNA which was transcribed from *B. subtilis* DNA by RNA polymerase *in vitro* is complementary to the denser (H strand) of the two DNA strands, which suggests that RNA was transcribed from that strand.[12]

In this paper, I want to report evidence, obtained from hybridization experiments with separated complementary DNA strands of *B. subtilis*, that indicates that essentially all of highly purified ribosomal RNA's (16*S* and 23*S* RNA) and

transfer RNA's are transcribed from the denser (H strand) of the DNA. Similar results have been observed in other laboratories.[13, 14]

Materials and Methods.—Bacterial strains: A wild-type strain (W1C8) of *B. subtilis* was used for preparation of DNA. A uracil-requiring derivative (168 ura-his) of W168 was used for preparation of labeled RNA.

Isolation, denaturation, and strand separation of DNA: DNA was isolated from *B. subtilis* (W168) by the method of Saito and Miura,[15] followed by isopropanol precipitation.[16] Alkaline denaturation of DNA was performed by adding NaOH (final concentration 0.1 *M*) to the DNA solution (50 μg/ml in $^1/_{10}$ SSC). After 5 min at room temperature, the solution was neutralized by NaH_2PO_4. Strand separation was carried out by MAK column chromatography[17, 18] as recently modified by Rudner *et al.*[11] (Fig. 2).

Preparation of H^3-labeled transfer RNA and ribosomal RNA: H^3-labeled ribosomal RNA's (16*S* and 23*S* RNA) and transfer RNA's were prepared as described previously.[2, 3] The specific radioactivities of these preparations were transfer RNA, 320,000 cpm/μg; 16*S* RNA, 135,000 cpm/μg; and 23*S* RNA, 128,000 cpm/μg.

Hybridization of DNA with H^3-labeled ribosomal RNA's and transfer RNA's: (1) *Competitive hybridization:* The procedure was essentially the same as reported previously.[2] The reaction mixture (1.0 ml) was composed of alkaline-denatured DNA (4 μg), NaCl (300 μmoles), EDTA (0.1 μmole), and H^3-labeled 16*S* RNA (0.17 μg) or 23*S* RNA (0.17 μg) plus various amounts of cold 16*S* or 23*S* RNA. After incubation at 72°C for 3 hr, RNase A (2 μg, Worthington Biochemical Corp.) and RNase T_1 (5 units, Sankyo Co., Tokyo) were added and incubation was continued further at 37°C for 20 min, followed by the addition of 0.5 *M* KCl solution. Hybridized material was collected on a membrane filter (B-6, Schleicher and Schuell Co., Keene, N.H.) according to the method of Nygaard and Hall.[19]

(2) *Hybridization of separated DNA strands with H^3-labeled ribosomal and transfer RNA's:* Hybridization was carried out basically according to the method of Gillespie and Spiegelman.[20] From each 5-ml DNA fraction from a MAK column a 0.3-ml sample was immobilized on a membrane filter (B-6, Schleicher and Schuell Co.). Such loaded filters were incubated in vials that contained 0.2 μg of H^3-labeled 16*S* RNA or 0.3 μg of H^3-labeled 23*S* RNA or 0.06 μg H^3-labeled transfer RNA's in 1.5 ml of 2 × SSC. Hybridization was carried out at 68°C for 18–20 hr, and after incubation each filter was rinsed with 2 × SSC and then incubated further with 20 μg/ml of RNase A and 20 units/ml of RNase T_1 for 60 min at room temperature. After both sides of the filters had been rinsed with 100 ml of 2 × SSC, radioactivity was assayed with a scintillation counter.

Results.—Tests for purity of the H^3-labeled RNA: Before being used, the H^3-labeled 16*S* and 23*S* ribosomal RNAs were tested for their purity by competitive hybridization with denatured DNA, cold homologous or heterologous RNA being used to compete. As is shown in Figure 1, the hybridizability of H^3-labeled 16*S* and 23*S* RNA's was affected only by competition from cold homologous RNA, an indication that these RNA preparations are each free from cross-contamination by the other species of RNA. The H^3-labeled transfer RNA preparation was also tested for purity by competitive hybridization with the other two RNA's and by an analysis of the saturation curve of hybridizability.

DNA strand separation: Strand separation of *B. subtilis* DNA was accomplished by intermittent gradient techniques[11] on a MAK column. As is shown in Figure 2, the two complementary strands (L and H) were separated almost perfectly into two peaks. The molecular weight of single-stranded DNA applied to the column ranged from 13 million to 18 million (determined by sucrose gradient centrifugation[21]).

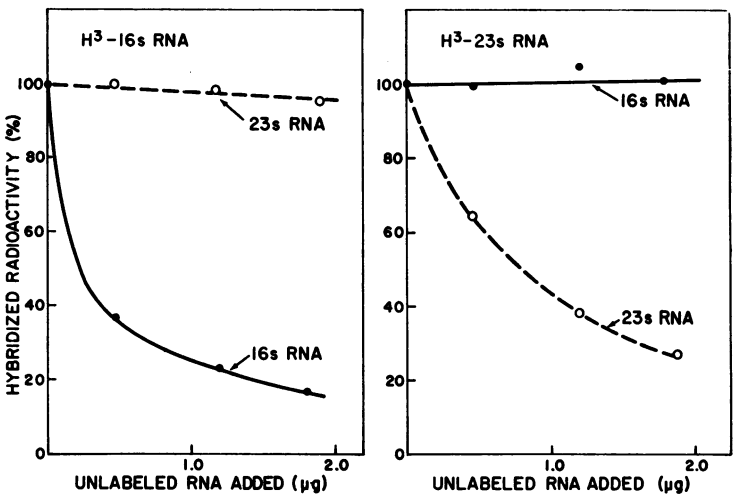

FIG. 1.—Competitive hybrid formation between 16*S* and 23*S* ribosomal RNA's. Each reaction mixture (1 ml) contained 4.0 μg of alkaline-denatured *B. subtilis* (W168) DNA, NaCl (300 μmoles), EDTA (0.1 μmole), and 0.17 μg of H^3-labeled 16*S* RNA or 23*S* RNA plus increasing amounts of unlabeled 23*S* RNA or 16*S* RNA as indicated on the abscissa. Incubation was carried out at 72°C for 3 hr and hybridized radioactivity was measured under conditions described in *Materials and Methods*.

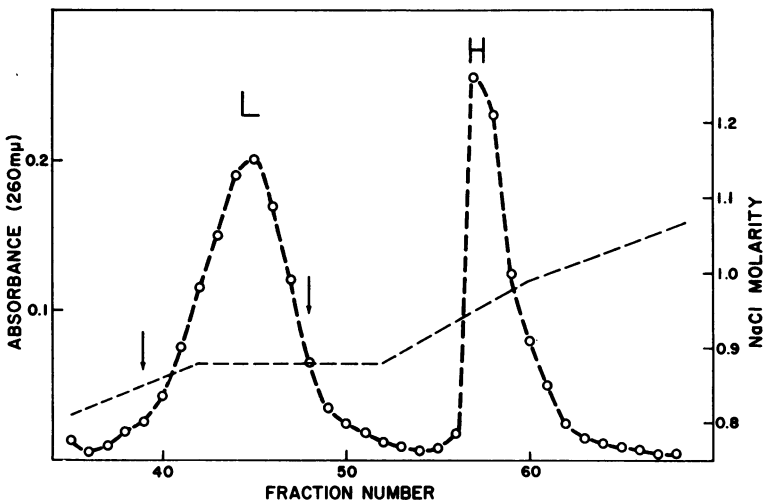

FIG. 2.—Separation of the complementary strands of *B. subtilis* DNA by an intermittent gradient technique of methylated albumin column chromatography.[11] Alkaline-denatured *B. subtilis* (W168) DNA (1.2 mg) was applied to a MAK column (1.9 cm × 18 cm) and was eluted with an intermittent salt gradient ranging between 0.6 and 1.2 *M* (total volume 400 ml collected as 5-ml fractions, flow rate 25 ml per hour) in a conventional two-cell gradient-former starting with the same water level in each cell. As indicated by the arrows in the figure, the gradient was disconnected at tube 39 and reconnected at tube 48. Just before reconnection, to avoid a sharp increase in gradient, buffer with an NaCl concentration equal to that of the mixing chamber was added to the mixing chamber up to a level equal to half the difference between the water levels of the two chambers. The recovery of DNA after chromatography was 42% (L fraction 25%, H fraction 17%). The salt concentration of each fraction was determined by an Abbe-3L refractometer.

Hybridization of separated strands with H³-labeled 16S, 23S, and transfer RNA's: DNA fractions (from MAK columns) of the separated complementary strands (Fig. 2) were hybridized with H³-labeled RNA of the three different species. As is shown in Figure 3, H³-labeled 16S ribosomal RNA hybridizes exclusively with the DNA from the second peak (H strand). The same is true in the case of 23S ribosomal RNA, as shown in Figure 4. These results indicate that in the cells

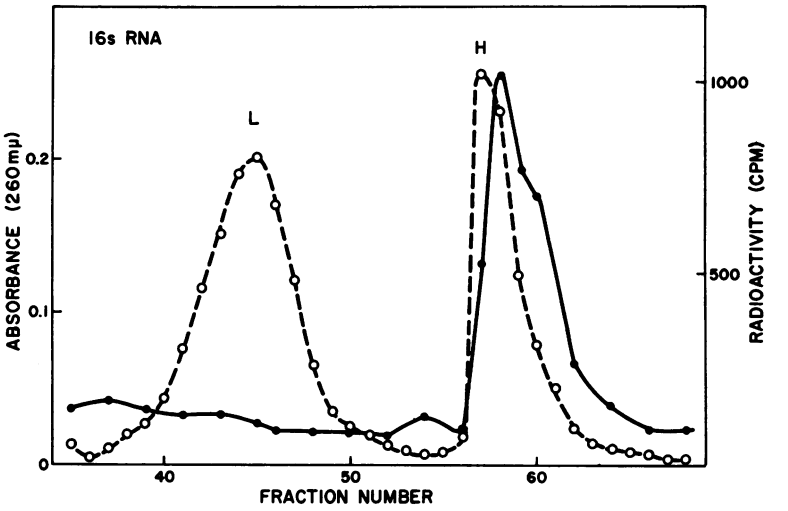

FIG. 3.—Hybridization of separated complementary DNA fractions with H³-labeled 16S ribosomal RNA. From each 5-ml fraction, the DNA of a 0.3-ml sample was immobilized on a membrane filter and incubated with H³-labeled 16S ribosomal RNA. After incubation, ribonuclease-resistant radioactivities were determined as an assay of hybridized radioactivity.

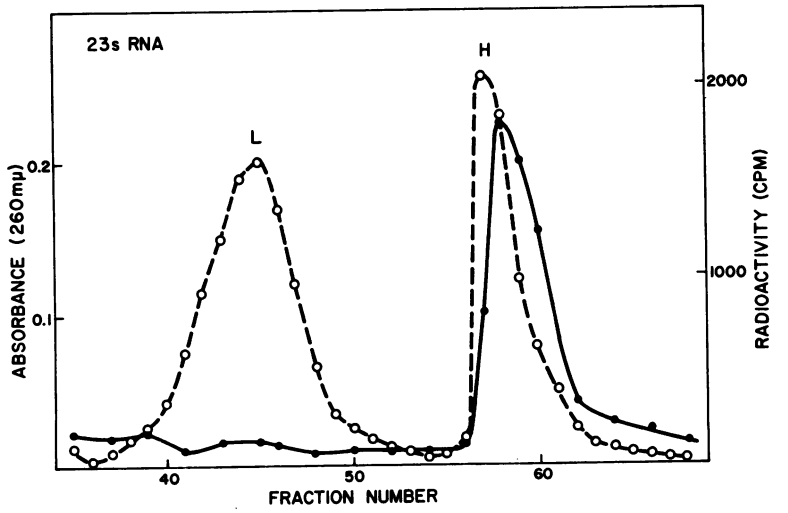

FIG. 4.—Hybridization of separated complementary DNA fractions with H³-labeled 23S ribosomal RNA. From each 5-ml fraction, the DNA of a 0.3-ml sample was immobilized on a membrane filter and incubated with H³-labeled 23S ribosomal RNA. After incubation, ribonuclease-resistant radioactivities were determined as an assay of hybridized radioactivity.

all the RNA making up the two major ribosomal RNA components is transcribed from the H strand of the DNA, which also is known to be responsible for transcription of messenger RNA.

In Figure 5, it is shown that H^3-labeled transfer RNA's also hybridize almost

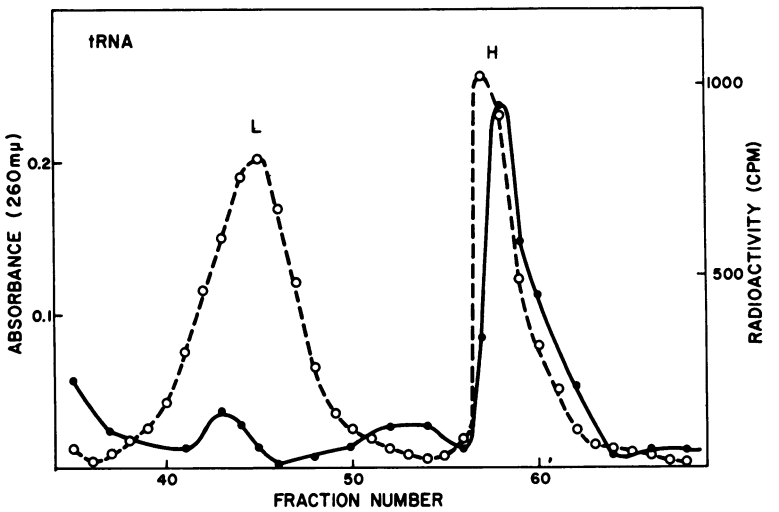

FIG. 5.—Hybridization of separated complementary DNA fractions with H^3-labeled total transfer RNA. From 5-ml fraction, the DNA of a 0.3-ml sample was immobilized on a membrane fraction and incubated with H^3-labeled total transfer RNA. After incubation, ribonuclease-resistant radioactivities were determined as an assay of hybridized radioactivity.

exclusively with the second peak (H strand) DNA, another indication that in cells almost all the transfer RNA's are copied from the H strand of the DNA. However, since the transfer RNA preparation used here was a mixture of all different species of transfer RNA, it is still possible that some small number of them are copied from the L strand of the DNA. The small peak found in the L-strand fraction in Figure 5 is not always reproducible. In all three cases, the shifts of the hybridization peaks away from the peak of the absorbance profile of the DNA H strand is probably due to the difference in base composition of the RNA genes (GC content of ribosomal RNA of *B. subtilis* is 55%[22]) from that of bulk DNA (GC content 43%). The MAK column is also known to be able to fractionate DNA according to its GC content.[18]

Discussion.—The results presented here clearly demonstrate that, in *B. subtilis*, essentially all of 16*S* and 23*S* ribosomal RNA and transfer RNA's are transcribed from only H strands of the DNA that is eluted as a second peak in fractionation by MAK column chromatography. By combining these results with the previous findings about transcription of messenger RNA (which were obtained by either hybridization technique[9, 13] or base analysis of the product of RNA polymerase[12]), we now find it reasonable to conclude that all RNA species—messenger, ribosomal, and transfer—are complementary to the H strands of the *B. subtilis* DNA. It should be noted that the isolated H-strand material from

the second peak does not necessarily represent high-density DNA from only one of the two *in vivo* chromosomal strands, since the chromosome is fragmented into relatively small pieces upon the initial isolation of DNA. Therefore some dense segments may originate from one chromosomal strand and some from the other. Switching of transcription from one strand to the other was demonstrated during messenger RNA synthesis after bacteriophage infection in *E. coli*.[12, 23-25] Evidence for this mode of transcription has been found in bacteria.[26, 27]

From the fact that guanine-rich ribopolymers bind preferentially with the strand that is responsible for transcription, Szybalski *et al.* proposed a model for transcription which stressed the importance of pyrimidine-rich clusters on DNA strands which may be related to initiation of transcription.[13] If one assumes that the H-strand fraction from MAK column chromatography contains the same strands which bind guanine-rich ribopolymers, one might predict that the mechanism of transcription of ribosomal RNA and even such small molecules as transfer RNA is essentially the same as that of messenger RNA. To obtain more information about this matter, an analysis using DNA-RNA hybridization with fractionated highly fragmented DNA preparations is now in progress in our laboratory. It is noteworthy that Lozeron and others[28] recently found that, in *E. coli*, the *Su3* gene—coding for a tyrosine transfer RNA—is transcribed from the same strand as the messenger RNA's for the tryptophan, lactose, and galactose operons.

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The following abbreviations are used: MAK, methylated albumin kieselguhr; EDTA, ethylenediaminetetraacetate; SSC, 0.15 *M* NaCl, 0.015 *M* Na_3 citrate.

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