RIBOSOMAL RNA GENES IN BACTERIA: EVIDENCE FOR THE NATURE OF THE PHYSICAL LINKAGE BETWEEN 16S AND 23S RNA GENES IN BACILLUS SUBTILIS*

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Abstract.—The nature of the arrangement of 16S and 23S ribosomal RNA genes on the Bacillus subtilis chromosome was studied by means of a Cs2SO4 density gradient centrifugation technique after complexing DNA-(ribosomal)RNA hybrids with mercuric ions. It was observed that the same fragments of single-stranded DNA with an average molecular weight of 1.9 \times 10^6 which hybridize with 23S RNA also hybridize with 16S RNA. This indicates that genes for both these species of ribosomal RNA are physically linked on such DNA fragments. Some evidence suggests that the two types of genes are interspersed with each other, perhaps in sets, each of which contains a 16S and a 23S ribosomal RNA gene.

Previous work using a technique of synchronous gene replication during spore germination\(^1\) has located the structural genes for ribosomal RNA and transfer RNA on the B. subtilis chromosome.\(^3\) These studies demonstrated that the majority of both 16S and 23S ribosomal RNA genes as well as transfer RNA genes are confined to the proximal region of the chromosome. More recently, Smith et al.\(^5\) have located 5S RNA genes at about the same place and they suggest the presence of a second minor group of RNA genes on a more distal part of the chromosome. None of these experiments, however, provide information about the fine structural organization of these genes. It would be difficult to map the fine structure of the ribosomal RNA genes by conventional techniques. Therefore, we have approached the problem biochemically, taking advantage of the fact that Cs2SO4 density gradient centrifugation is able to produce an unambiguous separation of single-stranded DNA from double-stranded DNA in the presence of the mercuric ion.\(^6\) The hybridizing of ribosomal RNA with single-stranded DNA and the separation of these hybrids from the nonhybridized single-stranded DNA, provided evidence which indicates that the genes coding for 16S and 23S ribosomal RNA are physically linked on fragments of DNA sheared to a molecular weight of 1.5 to 2.3 \times 10^6 daltons. Preliminary results of this work were previously presented.\(^7\)

Materials and Methods.—Bacterial strains: A uracil-requiring B. subtilis mutant strain (168 ura\(^-\) — his\(^-\)) was used for the preparation of \(^3\)H-labeled ribosomal RNA. A thymine-requiring strain (168 thy\(^-\), ind\(^-\), Smr, Er\(^-\)) was used for preparation of DNA.

Preparation of labeled RNA: \(^3\)H-labeled 16S (spec. act. = 1.27 \times 10^6 cpm/\mu g) and 23S (spec. act. = 1.30 \times 10^6 cpm/\mu g) ribosomal RNA were prepared as previously described.\(^2\) Each preparation was essentially free of contamination by other species of ribosomal RNA (cf. ref. 8). Pulse-labeled RNA (\(^4\)H) was prepared according to the technique of Dubnau et al.\(^9\)
Isolation, shearing, and denaturation of DNA: DNA was isolated by the method of Saito and Miura and further purified by isopropanol precipitation. The DNA was dissolved in 1/10 SSC (50 μg/ml) and sheared by a Sorvall mechanical mixer to a molecular weight of 11 to 12 × 10⁶. NaOH (0.1 N) was then added and after 20 min at room temperature the solution was neutralized with NaH₂PO₄ and dialyzed overnight against 0.5 M NaCl-0.05 M sodium phosphate (pH 6.7) and 0.001 M EDTA.

Separation of complementary DNA strands by methylated albumin kieselguhr column chromatography: The preparation of the column was essentially as described by Mandell and Hershey. The elution was performed with a linear gradient of NaCl ranging from 0.6 to 1.2 M containing 0.05 M sodium phosphate buffer, pH 6.7. The rate of elution was approximately 1 ml/min and the total vol. was 500 ml.

Preparation of hydroxyapatite: Hydroxyapatite was prepared by the method of Miyazawa and Thomas.

Hybridization of DNA with RNA: Usually the hybridization reaction was performed at 68°C for 2 hr in 0.3 M NaCl (pH 7.0). The ³H-16S RNA or ³H-23S RNA was added in a concentration of 0.1 μg/μg of denatured DNA unless otherwise specified. When the hybridization reaction was to be immediately followed by Cs₂SO₄(Hg²⁺) density gradient centrifugation, NaCl was replaced by 0.15 M Na₂SO₄ and 0.01 M borate buffer (pH 8.0) was used. After the 2-hr incubation period, 4 μg/ml of RNase A and 5 units/ml of RNase T₁ were added and the mixture was further incubated for 15 min at 37°C. The hybridized material was assayed as described by Nygaard and Hall.

Self-annealing and hydroxyapatite chromatography: Fractions from methylated albumin kieselguhr column chromatography containing single-stranded DNA complementary to the ribosomal RNA were pooled (620 μg) and incubated at 68°C for 3 hr in a solution containing 0.3 M NaCl and 0.01 M sodium phosphate (pH 7.4)-0.001 M EDTA. After the incubation phosphate buffer (pH 7.0) was added to a final concentration of 0.1 M NaCl and 0.05 M sodium phosphate and the mixture was applied to a hydroxyapatite column (1.9 × 5.0 cm).

Cs₂SO₄ (Hg⁺⁺) density gradient centrifugation of the DNA-RNA hybrid: The method used for Cs₂SO₄(Hg⁺⁺) density gradient centrifugation was essentially the same as described by Nandi et al. After extensive dialysis of all components of the reaction mixture against 0.05 M Na₂SO₄-0.01 M borate buffer (pH 8.0), hybridization was performed as described above. The solution was then mixed with 30 μg of native DNA (double-stranded marker), borate buffer (final concentration 0.02 M, pH 9.0), solid Cs₂SO₄ and HgCl₂ in this order to give a final density of 1.580 gm/cm³ and a R₁ (Hg⁺⁺/phosphorus) = 0.25-0.30. The mixture (usually 3.8-4.0 ml) was centrifuged at 4°C for 40 hr at 36,000 rpm in the Spinco SW50 rotor. After centrifugation, 5-drop fractions were collected and mixed with 0.3 ml of 0.015 M NaCl and 0.001 M EDTA. The absorbance and radioactivity of each fraction were then monitored.

Determination of molecular weights: The molecular weight of each DNA sample was determined by alkaline sucrose gradient centrifugation (5-20% sucrose, 0.9 M NaCl, 0.1 M NaOH, 0.001 M EDTA) using T₇ phage DNA as standard. For the determination of small molecular weight DNA, sheared T₄ phage DNA, whose molecular weight (1.5 × 10⁶) had been determined in the Spinco model E analytical centrifuge, was used as standard.

Results.—Separation of the complementary strands of DNA by methylated albumin kieselguhr column: Methylated albumin kieselguhr column chromatography had been found to separate the two complementary strands of D. pneumoniae and B. subtilis DNA. Hybridization studies with B. subtilis DNA have shown that ribosomal RNA and transfer RNA, hybridize exclusively with the strand eluted at higher salt concentration (H strand). It was desirable to separate the complementary strands of the sheared B. subtilis DNA, before hybridization with RNA and density gradient centrifugation in Cs₂SO₄(Hg⁺⁺),
Fig. 1.—Methylated albumin kieselguhr column chromatography of denatured DNA. 4 mg of denatured DNA were applied to the column (3.2 × 10.0 cm) and eluted by a linear gradient of NaCl (0.6–1.2 M, 500 ml total) in the presence of 0.05 M sodium phosphate buffer, pH 6.7, and 5.5 ml fractions were collected. After measuring absorbance at 260 nm (●), 0.1 ml of each fraction was taken for hybridization with $^3$H-16S RNA (○) and $^3$H-23S RNA (▲). Saturating amounts of labeled ribosomal RNA (0.2 µg/ml) were used during hybridization. The relative concentration of the DNA which is competitive with ribosomal RNA during hybridization was assayed by incubating (68°C for 2 hr) 0.2 ml of each fraction in tubes containing 2.3 µg of pooled DNA (tubes 37–43), 0.2 µg of $^3$H-23S RNA and 0.3 M NaCl in a final volume of 1 ml. DNA from the first peak was omitted in the control tube. The assay of hybridized radioactivity was performed as described in Materials and Methods. The values ($\alpha$, ○) were obtained, after normalizing the relative inhibitory activity ($\beta$), by using the following equation $\alpha = \beta/(1 - \beta)$ ($\beta = 1$ when inhibition is 100%).

since any contaminating complementary DNA strand would obscure the results by undergoing renaturation during DNA-RNA hybridization. Figure 1 shows that denatured, sheared B. subtilis DNA can be partially separated into its complementary strands by methylated albumin kieselguhr column chromatography and that $^3$H-labeled 16S and 23S ribosomal RNA hybridize with the DNA fractions eluted in the later portion of the second peak (H strand). These results agree with a previous observation but show even more clearly the location of the ribosomal RNA genes in the eluted fractions. The method of Rudner et al. was modified by use of a shallower linear gradient, producing broader peaks which increased the resolution of the separation. The position of the DNA fraction complementary to the H strand of the ribosomal RNA genes was determined by competing each DNA fraction from the first peak against $^3$H-23S RNA in annealing experiments with the pooled H strands of the ribosomal RNA genes (tubes 37–43) eluted in the second peak. As shown in Figure 1, the DNA strands complementary to the H strands of the ribosomal genes are eluted at an early position in the first peak. This result demonstrates that the complementary strands of the DNA which code for ribosomal RNA can be completely separated into two essentially nonoverlapping fractions.
Hydroxyapatite chromatography of the DNA: The fractions which hybridize efficiently with ribosomal RNA after methylated albumin kieselguhr column chromatography (tubes 37–43, Fig. 1) were pooled and annealed to renature any complementary DNA present. The mixture was then subjected to hydroxyapatite column chromatography. Less than 10 per cent of the DNA was eluted as renatured DNA.

Demonstration of the gene linkage: Mercuric ions (Hg++) bind more effectively to single-stranded DNA than to double-stranded DNA. Therefore, formation of a complex with mercuric ions allows good separation of single from double-stranded DNA by Cs2SO4 density gradient centrifugation. Consequently, we would expect a DNA-(ribosomal) RNA hybrid to band at a lower density position (toward the double-stranded DNA position) than that of single-stranded DNA. The purified single-stranded DNA was therefore hybridized (initial hybridization) with saturating amounts of purified tritium-labeled 16S RNA, 23S RNA or both, and the mixtures were subjected to Cs2SO4(Hg++) density gradient centrifugation in the presence of a reference double-stranded DNA. As predicted, the hybrids obtained with 16S RNA (Fig. 2A, open squares) or 23S RNA (Fig. 2B, black triangles) banded at a lower density position than that of nonhybridized single-stranded DNA. Moreover, the 23S RNA-DNA hybrid was shifted more than the 16S RNA-DNA hybrid, reflecting the difference in size of the hybrid region on the DNA fragments. When both RNA's were added simultaneously to the hybridization reaction (initial hybridization) the shift of the double hybrid (Fig. 2C, open squares) was greater than the shift observed for the hybrids containing either 16S RNA or 23S RNA alone (Fig. 2A and 2B). The quantitative results of these experiments are summarized in Table 1 (Expts. 1 and 2). The formation of the hybrid introduces a given amount of double-stranded structure with a size proportional to the molecular weight of the RNA. Since the molecular weight of 23S RNA (mol wt = 1.1 × 10^6) is twice that of the 16S RNA (mol wt = 0.56 × 10^6) the density shift of the 23S RNA-DNA hybrid should be twice as great as the one observed with the 16S RNA-DNA hybrid just as we found (Table 1, Expts. 1 and 2). The differences in the extent to which the hybrid molecules are shifted in the three experiments of Table 1 are probably due to the different molecular weights of the DNA used. Density differences reported in Table 1 were not changed by use of increased amounts of the same RNA species during hybridization. Furthermore, the displacement of the hybrid obtained by simultaneous initial hybridization with 16S and 23S RNA is the sum of the displacements obtained with the individual hybrids (Table 1, Expts. 1 and 2). The molecular weight of the DNA in these two experiments at this final step was found to be 1.5–2.3 × 10^6 daltons. The additivity in hybrid displacements was not found for the small molecular weight DNA of Experiment 3 of Table 1 (0.3–0.5 × 10^6 daltons). This fact will be discussed later. Since there are different loci for the two species of ribosomal RNA, the experiments described above suggest a close linkage between the two types of genes within DNA fragments with a molecular weight averaging 1.9 × 10^6. The starting single-stranded DNA had a molecular weight of approximately 5.5 × 10^6 daltons. Degradation of the DNA molecules occurred during hybridization or reannealing conditions probably due to high temperatures.
Fig. 2.—Cs2SO4(Hg^2+) density gradient centrifugation of the RNA-DNA hybrids. In each experiment 15 μg of DNA were used and the incubation conditions were as described in Materials and Methods. After addition of a double-stranded DNA marker, Cs2SO4 and HgCl2, the mixtures were centrifuged. (●), absorbance at 260 μm of the collected fractions. The peak to the left is single-stranded DNA and the peak to the right is the double-stranded DNA marker. Each fraction was divided into aliquots for the different assays described below.

(A) Initial hybridization (Hyb) with 3H-16S RNA followed by centrifugation (□). Each fraction was subjected to alkaline hydrolysis and rehybridized (Re-Hyb) with 3H-23S RNA (▲).

(B) Initial hybridization (Hyb) with 3H-23S RNA (▲) and rehybridization (Re-Hyb) with 3H-16S RNA (□).

(C) Initial hybridization (Hyb) with 3H-23S RNA + 3H-16S RNA (□) and rehybridization (Re-Hyb) with 3H pulse labeled RNA (sp act = 16000 cpm/μg; 18 μg/ml) (○). As a control for the alkaline hydrolysis, aliquots of the fractions were hydrolyzed and subjected to the annealing reaction without addition of RNA (●). The density shifts in this experiment are reported in Table 1 under Expt. 2.
Further evidence for the existence of a close linkage between the two types of genes was obtained by the following experiments. After the Cs$_2$SO$_4$(Hg$^{++}$) density gradient centrifugations reported in Figs. 2A, B, and C, each fraction was subjected to alkaline hydrolysis (0.1 M NaOH, 30°C, overnight) to destroy any hybridized RNA. After neutralization, each of these fractions was rehybridized with the RNA species which had not been used for the initial hybridization (e.g., if the initial hybridization was performed with $^3$H-23S RNA the rehybridization was done with $^3$H-16S RNA and vice versa). Therefore each DNA fraction of Figure 2A was rehybridized with $^3$H-23S RNA (●), those of Figure 2B with $^3$H-16S RNA (□) and, as control, those of Figure 2C with $^3$H-pulse labeled RNA (○). As can be seen the patterns of rehybridization coincide with the patterns of the initial hybridization. In other words, the DNA fragments which were shifted to a lower density position by the initial hybridization are the same fragments which hybridize with the alternate species of RNA in the rehybridization (Figs. 2A, B). This indicates that the DNA structures complementary to both RNA's (16S and 23S) are located in the same DNA fragment. In contrast, when the DNA frac-

<table>
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<th>RNA hybridized prior to centrifugation</th>
<th>Density Difference between Single-Stranded and Hybrid Peaks$^*$</th>
<th>Expt. 1</th>
<th>Expt. 2$^+$</th>
<th>Expt. 3</th>
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<tr>
<td>$^3$H-16S RNA</td>
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<td>0.022</td>
<td>0.012$^+$</td>
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<td>$^3$H-23S RNA</td>
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<td>0.038</td>
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<td><em>E. coli</em> ribosomal RNA</td>
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<td>...</td>
<td>0</td>
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<tr>
<td>Poly-IG</td>
<td></td>
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<tr>
<td>Molecular weight of DNA after Cs$_2$SO$_4$ centrifugation (×10$^{-4}$)</td>
<td>1.5</td>
<td>1.6–2.3</td>
<td>0.3–0.5</td>
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$^*$The conditions of the experiments are as described in the legend of Figure 2 and in *Materials and Methods*.

$^+$Excess cold 16S RNA was added.

$^+$These data were obtained from the experiments described in Figure 2.
to hybridize with $^3$H-23S RNA. It was found (not shown) that the DNA sequences specific for B. subtilis ribosomal RNA were not shifted (see also Table 1, Expt. 2) and remained with the major single-stranded DNA peak. These experiments demonstrate that hybridization of the DNA with homologous ribosomal RNA is a very specific reaction and that the DNA-RNA structures separated from the bulk of nonhybridized single-stranded DNA do contain the genes responsible for the synthesis of both 16S and 23S RNA species.

Discussion.—The results presented demonstrate that the genes coding for 16S RNA and 23S RNA are closely linked on the Bacillus subtilis chromosome. It had been previously calculated that there are three to ten clustered genes for each species of ribosomal RNA per chromosome. However, information about the fine structural relationships among these genes was lacking. The two obvious possibilities are that either a cluster of 16S RNA genes is near a cluster of 23S RNA genes or that the 16S and 23S RNA genes are interspersed among each other in some manner. Our results strongly favor the latter alternative. We have demonstrated linkage between the two gene types on a segment of DNA whose molecular weight averaged 1.9 $\times$ 10$^6$. Since the molecular weight for 23S RNA is 1.1 $\times$ 10$^6$ and for 16S RNA is 0.56 $\times$ 10$^6$, the centrifugation patterns we obtained can only be explained if both gene types are on the same DNA fragment. Experiments similar to those described in Figures 2A, B, and C but using DNA fragments whose molecular weights were approximately 0.3-0.5 $\times$ 10$^6$ showed that the displacement of the 23S RNA genes in the gradient following hybridization with 23S RNA was accompanied by a similar displacement of the 16S RNA genes and vice versa. However, this linkage was not complete and the additivity in density differences reported in experiments 1 and 2 of Table 1 was not observed in this case (Table 1, Expt. 3), indicating that some DNA pieces were only complementary to one of the RNA species and not to the other. These results are expected since the random breakage of the DNA into pieces 3-5 times smaller than the theoretical size of the two genes combined is likely to produce fragments containing sequences complementary to only one of the RNA species. However, the appreciable degree of linkage still observed can only be accounted for if the two gene types are situated very close to each other, possibly as a contiguous unit. The cluster of ribosomal RNA genes would then consist of a series of sets of genes, each set containing one 23S gene and one 16S gene: $\ldots$ 23S-16S $\ldots$ 23S-16S$\ldots$ We do not yet have enough evidence for the existence of a "spacer" between these sets. However, preliminary experiments on the location of 5S RNA genes suggest their linkage to the 23S RNA genes and the existence of such "spaces" between the sets of ribosomal genes. A similar arrangement for the ribosomal RNA genes was discussed in higher organisms and in bacteria.

Our experiments cannot rule out the existence of a small "space" between a 16S and a 23S RNA gene. On the other hand, it is attractive to postulate that in bacteria, ribosomal RNA is synthesized in vivo in a continuous molecule which subsequently is split into the two ribosomal RNA species. Such a mechanism has been suggested for animal cells.

The general features of the techniques described in this paper allow an ex-
tensive purification of the ribosomal RNA genes. The detailed techniques used in the purification of the ribosomal RNA genes will be published subsequently.30

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