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*DISTINCT CISTRONS FOR THE TWO RIBOSOMAL RNA COMPONENTS**

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Previous investigations[1, 2] have established that *E. coli* DNA contains sequences complementary to homologous ribosomal RNA. The proof depended on demonstrating the formation of specific RNAase resistant complexes between labeled ribosomal RNA and heat-denatured DNA. It was further shown, by the use of two identifying isotopic labels, that nonribosomal RNA from the same organism does not compete for the DNA sites complementary to ribosomal RNA.

All of these experiments were carried out with the 23S ribosomal RNA and they left unanswered the relation of these findings to the 16S RNA component. The similarity in base composition[3, 4] and the fact that the molecular weights[5] of the 23S and 16S are almost in the relation of 2:1 suggest the possibility of a common origin, the 23S being perhaps a dimer of the 16S RNA. Definitive evidence on whether they do, in fact, derive from the same sequence can be readily obtained with the hybridizing technique of Hall and Spiegelman[6] as modified in the ribosomal RNA investigations[1, 2] cited.

The following sorts of information are pertinent to a resolution. (a) *Saturation plateaus:* If the 16S and 23S are derived from the same sequence, the RNA/DNA ratio found in the hybrid at saturation should be the same for each RNA. (b) *Additivity:* At the saturation RNA/DNA ratio of either, the addition of the other should lead to no further complex formation if they are derived from the same sequences. If the sequences of origin are different, additional hybrids should be observed. (c) *Competitive interaction:* By the use of two identifying isotopic labels the presence or absence of competition during hybridization can be established. Absence of competitive interaction would indicate distinct sequences and its existence would argue for identity.

The present paper describes experiments which provide the data necessary for a decision. To alleviate somewhat the current monotony of molecular biology and to extend our understanding of these matters beyond *E. coli,* the experiments to be described were performed with *B. megaterium.* The results indicate that the sequences of the 23S and 16S RNA components are dissimilar. They must therefore possess different genetic origins.

Materials and Methods.—(a) *Bacterial strain:* Strain 219, a pyrimidine-requiring derivative of KM isolated by the technique of Mangalo and Wachsman[7, 8] was kindly provided by Dr. J. T. Wachsman.

(b) *Media:* A basal medium[7] supplemented with 10 to 30 μg/ml uridine was generally used. For P^{32} incorporation experiments, the phosphate concentration was reduced from 0.024 *M* to 0.0012 *M* and 0.05 *M* Tris (pH 7.3) added for buffering.

(c) *Preparation of cells:* Cells suspended in basal medium supplemented with 20–30 μg/ml in uridine were shaken overnight at 37°C, harvested, and resuspended at an $O.D._{660}$ of 0.200 in fresh medium containing 14 μg/ml uridine. When they attained an $O.D._{660}$ of 0.400, the cultures were harvested, washed, and resuspended in basal medium to an $O.D._{660}$ of about 1.000 for use as inocula in incorporation experiments.

(d) *Steady state isotope incorporation:* (1) *H^3-uridine:* Log phase cells, prepared as described, were suspended in basal medium to an $O.D._{660}$ of 0.035 and shaken at 37°C for 15 min. Then 10.3 μg/ml of H^3-uridine (New England Nuclear Corp., 3.0 mc/mM) was added and the culture shaken at 37°C until growth stopped at an $O.D._{660}$ of 0.240. The culture was harvested, washed, and resuspended in twice the original volume of basal medium containing 90 μg/ml of unlabeled uridine. The cells were incubated with aeration at 37°C for 0.8 generations to eliminate H^3 counts from the unstable RNA fraction, then harvested. (2) *P^{32}-orthophosphate:* Pyrophosphate-free, neutralized P^{32}-orthophosphate was added (630 μc/ml) to log phase cells at an $O.D._{660}$ of 0.07 in basal medium, containing 0.0012 *M* phosphate, and incorporation continued until an $O.D._{660}$ of 0.400 was reached. The culture was then "chased" for one generation in a medium adjusted to 0.024 *M* in nonradioactive phosphate.

(e) *Conversion to spheroplasts:* Log-phase cells were suspended to an $O.D._{660}$ of about 1.2 in a medium consisting of 0.04 *M* Tris, pH 7.3 − 0.002 *M* $MgSO_4$ − 0.3 *M* sucrose, and equilibrated to 37°C. Armour's lysozyme (200 μg/ml) was added and conversion to spheroplasts followed with a phase-contrast microscope. Conversion was virtually complete within 15 min. The spheroplasts were harvested, then washed once in the above medium.

(f) *Lysis and bulk RNA extraction:* Washed spheroplast pellets were lysed by resuspension in 0.01 *M* Tris, pH 7.3 − 0.005 *M* $MgCl_2$ (TM) buffer containing lysozyme (200 μg/ml) and 25 μg/ml of DNAase (Worthington Biochemical). The lysate was then subjected to three freeze-thaw cycles and total cellular RNA was isolated and purified, all as detailed by Hayashi and Spiegelman.[9]

(g) *Purification of ribosomal RNA subclasses:* The two ribosomal RNA components were separated from each other by repeated chromatography on methylated-albumin-kieselguhr (MAK) columns prepared according to Mandell and Hershey.[10] All buffers used during chromatography contained 0.025 *M* NaH_2PO_4 − 0.025 *M* Na_2HPO_4 − pH 6.9. RNA preparations were loaded at 50 μg/ml or less, and elution accomplished with linear NaCl gradients ranging from 0.6 *M* to 1.25 *M* NaCl. The total eluting volume was from 320 to 380 ml, and 5 to 7 ml fractions were collected. The resulting purified RNA fractions were pooled and concentrated to about 50 μg/ml as follows: the ionic strength of the solvent was first changed to 0.01 *M* Tris, pH 7.3 − 0.002 *M* $MgCl_2$ − 0.02 *M* NaCl by dialyzing against at least 100 volumes of this buffer for about 15 hr with two buffer changes. The preparations were next reduced to the appropriate volume in a flash-evaporator at reduced pressure. The sample flask was held at 28°C and the collecting flask at 0°C. Concentrated RNA preparations were finally dialyzed against TMS buffer (0.01 *M* Tris, pH 7.3 − 0.001 *M* $MgCl_2$ − 0.3 *M* NaCl).

(h) *Sucrose gradient analysis:* The size distribution of RNA preparations was routinely determined by centrifugation through linear sucrose density gradients.[9, 11]

(i) *DNA isolation:* DNA was extracted and purified from spheroplasts and heat-denatured as previously described for *E. coli*.[1] Heat-denatured preparations will be designated by 1XDNA.

(j) *DNA sedimentation velocity analysis:* Sedimentation coefficients were determined in the Spinco E analytical ultracentrifuge using UV optics. Runs were performed at 25 μg/ml according to the procedure of Marmur.[12] Observed sedimentation coefficients were corrected to 20°C. No other corrections were applied. Molecular weights were estimated from the measured S_{20} using the empirical relationship of Doty, McGill, and Rice.[13]

(k) *DNA-RNA hybridization:* All experiments described were performed with a heat-denatured DNA derived from a native preparation that had an S_{20} of 21.8 and an estimated molec-

ular weight of 7.3×10^6. DNA from *B. megaterium* undergoes renaturation rather readily; hence, the slow cool from higher temperatures employed in the earlier studies[1, 6] was avoided. Hybridizations were always performed by incubation at 41°–43°C.

Mixtures of 1XDNA at 50 μg/ml and labeled RNA at various concentrations in 0.7 ml of TMS buffer were incubated at 41°–43°C for 12 to 16 hr. Saturated CsCl was added to a final volume of about 3 ml and a density of 1.72. Centrifugation was carried out for 70 hr at 33,000 rpm in an SW 39 rotor of the Model L Spinco ultracentrifuge at a rotor temperature of 25°C. Fractions were collected from the bottom of the tube. Procedures for examining the DNA density region for RNAase resistant radioactivity on millipore membranes in a liquid scintillation spectrometer have been detailed by Yankofsky and Spiegelman.[1]

Results.—Purification of ribosomal RNA subclasses: Separation of labeled 16S and 23S RNA components was achieved by repeated chromatography on MAK columns. The purification was monitored by centrifugation in sucrose linear density gradients with unlabeled bulk RNA of *E. coli* added as size markers. The degree of cross contamination is readily determined by comparison of the radioactivity and $O.D._{260}$ profiles.

An example of bulk *B. megaterium* RNA separation on a MAK column is shown in Figure 1. The profile is similar to those obtained in this laboratory with *E. coli* RNA preparations[14] except that the 16S region appears to be partially resolved into two components.

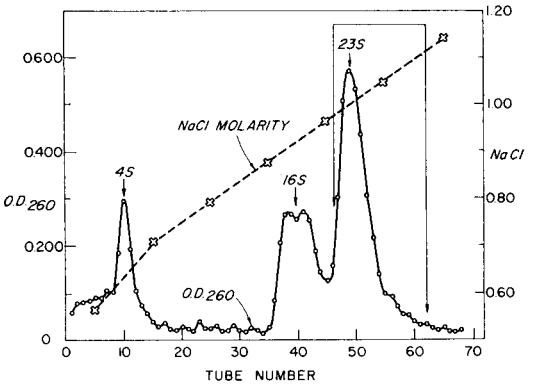

FIG. 1.—*Chromatographic separation of bulk RNA. B. megaterium* RNA was uniformly labeled with H^3-uridine as in *Methods*. The column was equilibrated at 0.66 *M* NaCl; the RNA loaded at 50 μg/ml in 0.66 *M* NaCl and eluted with a 360 ml linear gradient running from 0.66 *M* to 1.25 *M* NaCl. 5 ml fractions collected.

The 23S RNA region, indicated by the arrows in Figure 1, was chromatographed repeatedly, and the profile on the fourth column is shown in Figure 2*A*. Here, the arrows denote the region pooled and concentrated for experimental use, and Figure 2*B* shows its size distribution in a sucrose gradient. As can be seen, the purified labeled component is virtually confined to the 23S region of the carrier bulk RNA added.

The 16S component of Figure 1 was similarly treated, and Figure 3*A* shows a representative profile on MAK. Again, the arrows indicate the region pooled, concentrated, and analyzed for size. Figure 3*B* shows the size distribution of this region compared to that of *E. coli* marker RNA. Although of interest, the abnormality seen in the 16S profile both in Figure 1 and Figure 3*A* is not directly pertinent to the present investigation and its discussion will be deferred for a subsequent publication. Comparison of 3*A* and 3*B* indicates that the asymmetry observed is not due to significant contamination with 23S RNA. All preparations employed in the

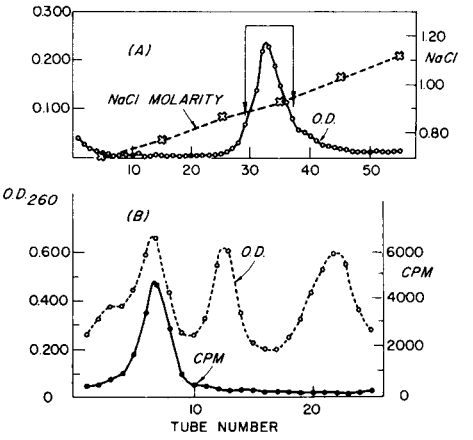

FIG. 2A.—*MAK column: Chromatographic profile after the fourth chromatography of the 23S region shown by the arrows in Figure 1.* The column was equilibrated at 0.68 *M* NaCl; 600 μg RNA loaded at 50 μg/ml in 0.68 *M* NaCl and eluted with a 320 ml linear gradient from 0.72 to 1.22 *M* NaCl.

FIG. 2B.—*Sucrose density gradient centrifugation.* An aliquot (0.5 μg RNA, 60,000 cpm) of the pooled tubes indicated by the arrows in (*A*) was used. 0.6 mg *E. coli* bulk RNA added as O.D. marker. 1.2 ml fractions collected and 0.3 ml samples from each tube plated for radioactive counts. The O.D. profile identified the known components in the added carrier material. The first major peak on the left is the 23S, the second the 16S, and the last corresponds to the 4S component.

present study were examined before use in sucrose gradients for cross contamination or evidence of breakdown. Samples showing evidence of either were discarded.

We now consider the details of the three types of experiments which can illuminate the origins of the 16S and 23S RNA components.

(1) *Saturation plateaus:* The proportion of RNAase resistant hybrid formed by incubating a fixed amount of 1XDNA with increasing amounts of each ribosomal RNA component are shown in Figure 4. The 23S RNA reaches a plateau when approximately 0.18% of the DNA is occupied, while about 0.14% of the DNA is capable of complexing with 16S RNA. In six repetitions, mean values of 0.179 ± 0.0072 and 0.136 ± 0.014 were obtained for the respective saturation values of 23S and 16S RNA. The fact that the saturation plateaus for the two are different supports the conclusion that the two types of RNA have different origins.

(2) *Additivity:* It will be noted from Figure 4 that for 50 μg DNA, saturation for the 23S RNA is achieved at 3 μg/ml, and 2 μg/ml saturates for the 16S component. We now inquire whether the addition of both at saturating levels to the same reaction mixture increases the amount of complex observed, and, if so, to what extent. The results of such an experiment are presented in Table 1. Addition of the values obtained when saturating amounts of each RNA subclass is complexed alone (mixture 1 + mixture 2) indicates that 0.303 per cent of the DNA would be hybridized. The amount of complex formed when both are incubated together (mixture 3) is within 4 per cent of this value. These results are difficult to reconcile

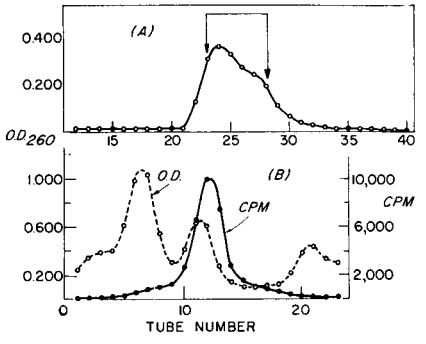

FIG. 3A.—*MAK column: Chromatographic profile of B. megaterium 16S RNA (steady state P^{32} label) after third chromatography.* Column equilibrated at 0.6 *M* NaCl; 760 μg RNA loaded at 50 μg/ml in 0.6 *M* NaCl and eluted with a 340 ml linear gradient from 0.6 *M* to 1.2 *M* NaCl.

FIG. 3B.—*Sucrose density gradient centrifugation: Analysis of an aliquot (0.5 μg of RNA, 80,000 cpm) from the pooled tubes shown under the arrows in* (*A*). 0.6 mg *E. coli* bulk RNA added as O.D. marker. All other details as in Figure 2*B*.

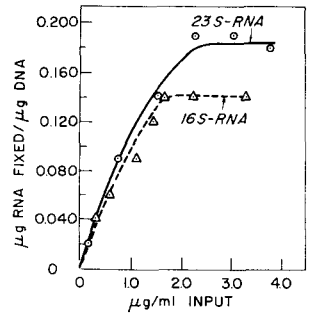

FIG. 4.—*Saturation plateaus: Saturation curves of B. megaterium 16S and 23S RNA hybridized with 50 μg/ml B. megateriumheat-denatured DNA.* Each point represents RNAase resistant counts found in the DNA region after CsCl equilibrium density gradient centrifugation. Annealing and analytical procedures as described under *Methods*.

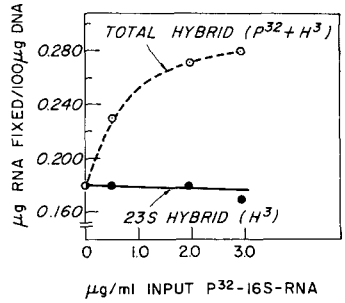

FIG. 5.—*Competitive interaction: Tests for competitive interactions between mixtures of 23S-H³-RNA and 16S-P³²-RNA from B. megaterium complexed with homologous heat-denatured DNA.* All mixtures contain 50 μg/ml DNA, 3 μg/ml purified 23S-H³-RNA (see Fig. 2) and the indicated concentrations of purified 16S-P³²-RNA (see Fig. 3). Annealing and analytical procedures as described under *Methods*.

with a common origin and are clearly consistent with the existence of distinct complementary regions. We come now to the final available experimental test.

(3) *Competitive interaction:* Increasing amounts of P^{32}-labeled 16S RNA were incubated in three tubes, each containing 50 μg of 1XDNA and H^3-labeled 23S RNA at its saturation level (3.0 μg). Because of the identifying isotopic labels, it was possible to determine independently the per cent hybrid formed by each of the two ribosomal RNA size classes in the three mixtures. From the data shown in Figure 5 it is evident that the addition of the P^{32}-16S RNA results in no significant displacement of H^3-23S RNA. Furthermore, as more 16S RNA is added, the total hybrid approaches a level of saturation near that expected for the sum of the two subclasses incubated alone. There is no evidence of competition between the two ribosomal RNA subclasses for common DNA sites.

Discussion.—In previous studies[1, 2] we have shown that specific complexes are formed between homologous ribosomal RNA and DNA in bacterial species having intermediate (52%) and high (64%) contents of guanosine-cytosine (GC) in their DNA. The present study establishes a similar sequence complementarity in *B. megaterium* which has a low (38%) GC content. The fact that ribosomal RNA

TABLE 1

TEST FOR ADDITIVITY DURING HYBRIDIZATION AT SATURATION LEVELS OF 16S AND 23S RNA

Mixture	Contents	RNAase resistant hybrid μg RNA fixed/100 μg DNA
1	50 μg/ml 1XDNA + 3.06 μg/ml 23S RNA	0.186
2	50 μg/ml 1XDNA + 2.21 μg/ml 16S RNA	0.117
		Sum = 0.303
3	50 μg/ml 1XDNA + 3.06 μg/ml 23S RNA + 2.21 μg/ml 16S RNA	0.291

The addition mixture (3) contained the same DNA and RNA preparations as the control mixtures (1 and 2). All three mixtures were annealed under identical conditions, centrifuged together, and the raw hybrids tested for RNAase resistance with the same enzyme preparation. Details of annealing and analytical procedures are given in *Methods*.

complementarity to DNA obtains in organisms of diverse DNA composition, lends credence to its generality.

These findings raise rather forcibly an interesting problem. The base composition of ribosomal RNA shows virtually no correlation with that of homologous DNA.[15, 16] Evidently the ribosomal RNA cistrons have been kept within narrow limits while the rest of the genome has undergone the widest variation in base composition permissible within a triplet coding mechanism. The specification of the selective mechanism which can produce this remarkable outcome poses an interesting problem for experimental resolution.

Sequence complementarity has previously been shown for the unstable messenger RNA,[9] transfer RNA,[17, 18] and 23S RNA.[1, 2] The present study demonstrates that it also holds for 16S RNA. Thus, the synthesis of all known cellular RNA components can be explained in terms of a DNA mediated reaction.

The present study had as its primary purpose to provide evidence which could decide whether the 16S and 23S ribosomal components are derived from the same or different complementary DNA sequences. The experiments reported indicate a difference in saturation plateaus, additivity of hybrid formation at saturation levels of each type, and absence of competitive interaction during hybrid formation. These findings are difficult to reconcile with a common sequence. They provide consistent evidence for distinct cistronic origins.

The further analysis into the nature of ribosomal RNA will require an examination for heterogeneity within each class. We have already pointed out[1, 2] that the level (0.2%) at which DNA is saturated by hybridizing with homologous 23S RNA would suggest that *E. coli* contains about 10 complementary cistrons for this component. The data presented here would suggest that 0.18% of *B. megaterium* DNA is complementary to its 23S ribosomal RNA and 0.14% to its 16S component. On the basis of the DNA content per "nuclear body"[19] one would estimate that the DNA contains approximately 35 stretches complementary to 23S RNA and 45 complementary to the 16S component. The significance of this apparent redundancy may be related to the rather large number of strands required for a full ribosomal complement which constitutes 85 per cent of the total cellular RNA. However, the existence of multiple copies in the genome provides a possibility for variation. It is of no little interest to determine whether this potentiality was exploited. It is evident that the use of column fractionation and competition experiments with identifying labels should provide data pertinent to this problem.

Summary.—The experiments reported were designed to decide whether the 16S and 23S ribosomal RNA components are derived from the same or different complementary sequences in the DNA. Specific hybrid formation, coupled with isotopic labeling was employed as the analytical device. The data establish that (*a*) the maximal amount of RNA which can hybridize per unit of DNA is different for the two; (*b*) at saturation concentrations of each, the amount of hybrid formed is additive when 16S and 23S RNA are both present; (*c*) no evidence of competitive interaction between the two for the same sites can be detected. All these findings are difficult to reconcile with a common origin. We conclude that 16S and 23S ribosomal RNA are derived from DNA sequences unique to each.

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[1] Yankofsky, S. A., and S. Spiegelman, these PROCEEDINGS, **48,** 1069 (1962).
[2] Yankofsky, S. A., and S. Spiegelman, these PROCEEDINGS, **48,** 1466 (1962).
[3] Spahr, P. F., and A. Tissières, *J. Mol. Biol.*, **1,** 237 (1959).
[4] Yankofsky, S. A., and S. Spiegelman, unpublished observations (1962).
[5] Kurland, C. G., *J. Mol. Biol.*, **2,** 83 (1960).
[6] Hall, B. D., and S. Spiegelman, these PROCEEDINGS, **47,** 137 (1961).
[7] Mangalo, R., and J. T. Wachsman, *J. Bacteriol.*, **83,** 27 (1962).
[8] Mangalo, R., and J. T. Wachsman, *J Bacteriol.*, **83,** 35 (1962).
[9] Hayashi, M., and S. Spiegelman, these PROCEEDINGS, **47,** 1564 (1961).
[10] Mandell, J. D., and A. D. Hershey, *Analyt. Biochem.*, **1,** 66 (1960).
[11] Britten, R. J., and R. B. Roberts, *Science*, **131,** 32 (1960).
[12] Marmur, J., *J. Mol. Biol.*, **3,** 208 (1961).
[13] Doty, P., B. McGill, and S. A. Rice, these PROCEEDINGS, **44,** 432 (1958).
[14] Kano-Sueoka, T., and S. Spiegelman, these PROCEEDINGS, **48,** 1942 (1962).
[15] Spiegelman, S., in *Cellular Regulatory Mechanisms*, Cold Spring Harbor Symposia on Quantitative Biology, **26,** 75 (1961).
[16] Woese, C. R., *Nature*, **189,** 918 (1961).
[17] Giacomoni, D., and S. Spiegelman, *Science*, **138,** 1328 (1962).
[18] Goodman, H. M., and A. Rich, these PROCEEDINGS, **48,** 2101 (1962).
[19] Spiegelman, S., A. I. Aronson, and P. C. Fitz-James, *J. Bacteriol.*, **75,** 102 (1958).