# *THE IDENTIFICATION OF THE RIBOSOMAL RNA CISTRON BY SEQUENCE COMPLEMENTARITY, I.* SPECIFICITY OF COMPLEX FORMATION*

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*Communicated by H. S. Gutowsky, May 7, 1962*

Despite the fact that ribosomal ribonucleic acid constitutes the bulk (85%) of cellular RNA, its mode of origin is little understood. While clearly not exhaustive, two alternatives can, at present, be entertained. One, would assume a DNA-dependent reaction[1–3] and the other would invoke a synthetic mechanism independent of DNA. The fact that the base composition of ribosomal RNA shows no tendency to correlate[4, 5] with homologous DNA is irrelevant to a choice between the two hypotheses. The presumed DNA segment involved might be so small as to constitute a statistically inadequate sample of the over-all base composition.

Posing the problem in the form of these two alternatives suggests the following question, pertinent to a decision and amenable to experimental resolution: *Does DNA contain a sequence complementary to homologous ribosomal RNA?* An approach to questions of complementarity is in principle provided by Hall and Spiegelman's[6] demonstration that specific hybrid formation can be exhibited between T2-DNA and the RNA synthesized in *E. coli* infected with T2. These experiments used labeled nucleic acids for ease of identification and took advantage of equilibrium centrifugation in density gradients[7] to separate free RNA from that which hybridized to DNA.

The technical difficulties inherent in using hybridization to establish the existence of complementarity between ribosomal RNA and some sequence in the DNA have already been discussed.[5] The major complications stem from the numerology of the situation. For example, the 23S RNA component of the ribosomes is $1.1 \times 10^6$ in molecular weight, so that even if a specific complex were formed, it might

involve only 0.02 per cent of the DNA available in the genome of *E. coli*. One is, therefore, faced with the problem of designing experiments which will detect hybridization at this level. This can theoretically be accomplished by raising the specific radioactivity of the RNA used in the hybridization to suitable levels. Indeed, in principle, the test could be made definitive in both a positive and negative sense. However, magnification of the sensitivity of hybrid detection by these means carries with it the attendant danger that complexes will be observed which are irrelevant to the question being examined. Apparent "hybrids" might represent any one of the following: (a) complexes between DNA and small amounts of informational RNA contaminating the ribosomal preparations; (b) mechanical trapping of small amounts of ribosomal RNA in the strands of DNA; (c) partial hybridization resulting from accidental coincidences of complementarity over short sequences.

In view of these possible complications, observations of "labeled" RNA accompanying the DNA in a density gradient must be supplemented with independent information which establishes that the RNA, so complexed, is ribosomal and that it is specifically hybridized to the DNA. Experimental procedures which provide the requisite information have been devised. A central device depends on the resistance of hybrid to nucleolytic enzymes[8, 9] for differentiating between chance pairing in restricted regions and specific hybridization. It is the purpose of the present paper to describe the data thus obtained. The results indicate that a sequence does exist in homologous DNA which is complementary to ribosomal RNA. In view of the base composition of ribosomal RNA, this further implies that only one of the two DNA strands is used for transcription.

*Materials and Methods.—Bacterial strains:* In addition to *Pseudomonas aeruginosa* (ATCC-10197) two strains of *E. coli* were used, BB and A-155, a uracilless derivative of B.

*Media: E. coli* BB was grown on the modified SC medium described by Hayashi and Spiegelman;[9] *E. coli* A-155 was grown on the same medium supplemented with 20 $\mu$g/ml uridine. *Ps. aeruginosa* was grown on the SC-glutamate medium used by the above investigators. When growth on minimal media was not required, Bacto Nutrient Broth (Difco Laboratories) was used.

*Buffers:* The buffers and their designations are: (1) Tris-magnesium (TM); 0.01 *M* tris, pH 7.3–0.005 *M* $MgCl_2$; (2) Tris-magnesium (TMS); 0.03 *M* tris, pH 7.3–0.001 *M* $MgCl_2$–0.3 *M* NaCl; (3) Hundredth molar phosphate (HMP); 0.0025 *M* $Na_2HPO_4$–0.0050 *M* $NaH_2PO_4$–0.001 *M* EDTA; (4) Low Salt (LS); 0.01 *M* tris, pH 7.3–0.005 *M* $MgCl_2$–0.007 *M* NaCl; (5) Low Salt versene (LSV); 0.01 *M* tris, pH 7.3–0.005 *M* $MgCl_2$–0.007 *M* NaCl-0.0005 *M* EDTA.

*Preparation of cells:* Overnight cultures from a given medium were harvested and resuspended in the same medium at an $O.D._{660}$ of 0.100. If the cells were to be subsequently exposed to $P^{32}$, the phosphate level was lowered to $2 \times 10^{-3}$ *M* at this point. The cultures were allowed to attain an $O.D._{660}$ of 0.200 while shaking at 37°C, then harvested and resuspended in the same medium to an $O.D._{660}$ of 1.000. Such freshly prepared, log-phase cells were used as initial inocula for radioactive isotope incorporation experiments.

*Uniform labeling of cells for ribosomal RNA preparations free of informational RNA:* High-specific-activity ribosomal RNA, free of significant contamination with labeled informational RNA, was prepared by a procedure suggested by the studies of Hayashi and Spiegelman.[9] Because of the metabolic instability of informational RNA, labeled components of this variety can be eliminated from the 23S and 16S regions by subjecting a uniformly labeled culture to a "chase" in nonradioactive medium. Due to their very diverse size distribution, the presence of labeled informational RNA components is readily detected[9] by discordancies between the distribution of radioactive and optically dense materials in a linear sucrose gradient. Success of the chase is easily assured by the absence of such discrepancies. A sample protocol is described here.

Log-phase cells of *Ps. aeruginosa* were suspended at an $O.D._{660}$ of 0.02 in SC-glutamate medium made $2 \times 10^{-3}$ *M* in phosphate. After 15 min incubation with aeration at 37°C, 400 $\mu$c/ml of

neutralized $P^{32}$ orthophosphate, freed of pyrophosphate by acid hydrolysis, were added and the incubation continued until an $O.D._{660}$ of 0.320 was attained. The cells were harvested, washed, and resuspended at an $O.D._{660}$ of 0.150 in the same medium containing phosphate at $5 \times 10^{-3}$ *M*. It was then allowed to chase for one generation.

The same procedure was followed in preparing $H^3$-labeled RNA. In this case, $H^3$-uridine (2.56 curies per mM) was used with the uracilless strain, *E. coli* A-155. Chasing was permitted for 0.9 generation in the presence of 100 $\mu$g/ml of nonradioactive uridine.

*Preparation of purified ribosomal RNA:* Cells were lysed by the freeze-thaw lysozyme method[9] and the bulk RNA purified by the phenol procedure.[10] Following repeated (3 times) precipitation with 2 volumes of alcohol, the final product was dissolved in and dialyzed against TM buffer. Whenever pertinent, all labeled RNA preparations were examined for alkali stable counts. None were found in the preparations used. Isolation of the 23S components was achieved by centrifugation in linear sucrose gradients (2.5 per cent–15 per cent).[9]

*Extraction and purification of DNA:* Bacterial and viral DNA was purified according to the procedure of Marmur.[11] Calf-thymus DNA was obtained from the Worthington Biochemical Corporation. To remove contaminating RNAase, all DNA preparations were treated three times with 1 volume of water-saturated phenol at room temperature. Phenol was removed and the DNA precipitated with two volumes of ethanol.

*DNA-RNA hybridization and analysis by CsCl equilibrium density gradient centrifugation:* Heat-denatured DNA, prepared according to the procedures of Eigner,[12] was mixed with radioactive RNA in TMS buffer. The DNA concentration never exceeded 115 $\mu$g/ml. In some experiments, EDTA was added to the reaction mixture to a final concentration of $5 \times 10^{-4}$ *M*. The mixtures were placed in an insulated water bath at 55°C and allowed to slowly cool to 35°C, or below, according to a cooling schedule previously reported.[6] In some instances, noted, the reaction mixtures were held at 40°C for 36 hr.

The procedures for CsCl density gradient centrifugation are those described by Hall and Spiegelman.[6] Final densities were adjusted to between 1.70 and 1.75.

*Base ratio analysis of hybridized RNA:* Peak tubes containing radioactivity in the DNA region of the density gradient were pooled, dialyzed into TM buffer in the cold, and digested with RNAase free DNAase (50 $\mu$g/ml) for 30 min at 37°C. To the resulting digest was added 4 mg/ml of nonradioactive *E. coli* ribosomal RNA (prepared by the phenol method) followed by ethanol precipitation in the cold. The precipitate was hydrolyzed with alkali and the 2′-3′ nucleotides separated and analyzed by anion exchange chromatography.[9]

*Assay for RNAase:* The method depends on the use of isotopically labeled RNA and checking for loss of acid precipitable counts. All assays were adjusted to correspond to the conditions under which the material was to be employed experimentally. No DNA or DNAase samples were used which showed detectable RNAase activity in a 15-hr incubation at 37°C. DNAase was freed of RNAase activity by chromatography.[13]

*Resistance to nucleolytic enzymes as a test for hybrid specificitiy:* The peak tubes containing RNA radioactivity in the DNA density region were pooled and dialyzed in the cold for about 15 hr (3 buffer changes) against 100 volumes of LS or LSV buffer. If the apparent hybrid was labeled with $P^{32}$, free $H^3$-RNA was added as an internal control and vice versa. The volume of the mixture was adjusted to 4 ml and preincubated at 37°C for 5–10 minutes. RNAase (5 $\gamma$/ml final) or RNAase plus DNAase (10 $\gamma$/ml final) were added at zero time and incubation continued at 37°C. 1 ml aliquots were removed for acid insoluble raidoactivity assay at the indicated time intervals. When both enzymes were present, the buffer used was always LS.

*Experimental Results.*—1. *Base composition of the RNA complexed to DNA:* The procedure described under *Methods* made available purified ribosomal RNA of sufficiently high-specific radioactivity to permit a decision on whether or not "hybrids" with DNA were detectably formed. Preliminary experiments quickly revealed that, following incubation with heat-denatured DNA, apparent hybrids containing RNA could be observed in the DNA region of cesium chloride density gradients. To interpret the significance of these observations, it was necessary to test for some of the complicating possibilities noted in the introduction.

The first step was to prove by direct analysis that the RNA, so complexed, was indeed ribosomal and not contaminating informational RNA. *Pseudomonas aeruginosa* was chosen since the base composition of its ribosomal and informational varieties are readily distinguished. Figure 1 shows the distribution of cpm and

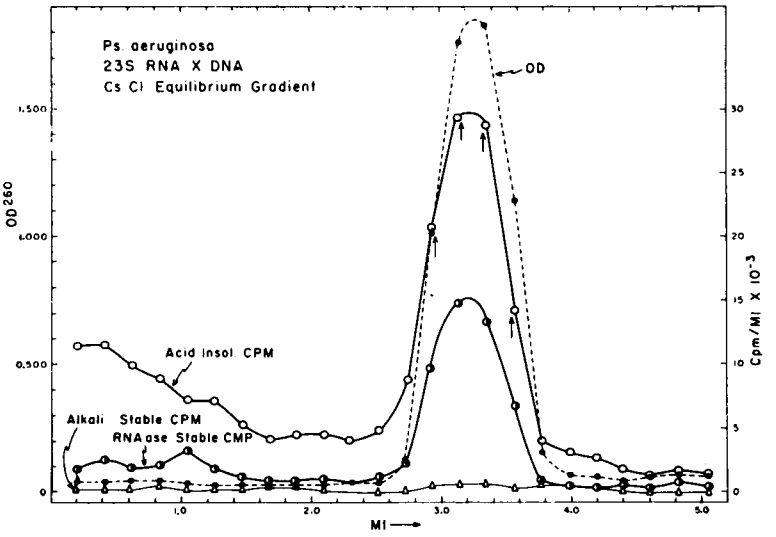


FIG. 1.—Cesium chloride equilibrium density gradient. 800 $\mu$g/ml *Ps. aeruginosa* heat-denatured DNA + 2.95 $\mu$g/ml 23S-$P^{32}$-RNA ($6 \times 10^5$ cpm/$\mu$g) in TMS slow-cooled from 55°C to 34°C. Duration of the centrifuge run was at 33,000 rpm and 25°C for 72 hr. 0.21 ml fractions collected and diluted 1.1 ml in TM buffer. Aliquots were directly taken for digestion with 5 $\mu$g/ml RNAase at 37°C for 30 min or 0.3 *M* NaOH at 30°C for 20 hr.

$O.D._{260}$ in a CsCl gradient of the incubation mixture containing the labeled 23S RNA and heat-denatured DNA of *Ps. aeruginosa*. Clearly, a considerable proportion of the added RNA has moved into the DNA density region. Further, there is no detectable alkali stable radioactive material present in any part of the density gradient. This fact eliminates the possibility of ascribing the apparent hybrid to DNA contaminating the RNA preparation. Finally, the labeled material external to the "hybrid" region is sensitive to RNAase whereas the RNA in the region of the DNA is resistant. This is a characteristic feature of hybridized RNA reported previously[8, 9] which will be examined in further detail below.

We now turn our attention to the question of whether the hybridized material is indeed ribosomal. The fact that such a large proportion (greater than 50%) of the labeled RNA was complexed, virtually precludes that the hybrid involves only "contaminating" informational material. To make completely certain, however, an analysis of base composition of the pooled fractions indicated by the arrows in Figure 1 was completed. The results of this analysis and a companion duplicate experiment are summarized in Table 1. Comparison leads to the conclusion that the RNA found in the DNA region in such hybridization experiments is similar to ribosomal rather than informational RNA in its base composition.

2. *The specificity of the complexes formed as determined by equilibrium density centrifugation:* It became necessary next to examine the specificity of the apparent combination between the ribosomal RNA and the homologous DNA. Figure

TABLE 1

BASE COMPOSITION OF PSEUDOMONAS RNA HYBRIDIZED TO HOMOLOGOUS DNA

The material corresponding to experiment 1 came from the pooled fractions indicated by arrows in Figure 1. Experiment 2 was an independently repeated hybridization. The base composition was determined chromatographically as described previously.[9] The values for ribosomal RNA and informational RNA are those found earlier[9] for *Ps. aeruginosa*.

| | | Moles per cent | | | | | |
|---|---|---|---|---|---|---|---|
| | | C | A | U(T) | G | %GC | Pu/Pyr |
| Hybrid RNA | Exp 1 | 19.3 | 25.4 | 21.1 | 34.2 | 53.5 | 1.48 |
| | Exp 2 | 21.0 | 25.8 | 20.4 | 32.8 | 53.8 | 1.42 |
| Ribosomal RNA | | 22.4 | 26.8 | 20.7 | 30.3 | 53.3 | 1.30 |
| Informational RNA | | 30.3 | 20.9 | 19.0 | 29.8 | 60.1 | 1.02 |
| DNA | | 32.0 | 18 | 18 | 32.0 | 64 | 1.00 |

2 shows the profiles observed when tritium labeled 23S RNA of *E. coli* was incubated with heat-denatured T2, T5 and *E. coli* DNA and then centrifuged in CsCl gradients. No complex was formed with either of the two viral DNA molecules (Figs. 2*A* and *B*). However, excellent apparent hybrid is observed in the DNA region of the homologous mixture (Fig. 2*C*).

The absence of a detectable reaction in the heterologous tests shows that mechanical trapping during incubation of RNA in the DNA strands is not a complication which need be considered. The results would appear to support the conclusion that the complex observed with the homologous RNA and DNA is indeed a reflection of a specificity requirement. However, cogent arguments can be leveled against accepting these data as definitive evidence for the existence of a specific region in coli DNA complementary to its ribosomal RNA. In particular, one might argue that DNA of viral origin is less complicated in terms of the variety of sequences to be found in their genomes as compared with an organism like *E. coli*. The observation, therefore, of an apparent hybrid structure in the coli by coli test might be a consequence of the greater heterogeneity of coli DNA. This feature could provide complementary sequences of restricted length but sufficient to permit enough hydrogen bonding to carry the ribosomal RNA into the DNA density region.

An obvious test of this possibility is to carry out similar tests with heterologous DNA which is as complicated as that derived from *E. coli*. It might, perhaps, be natural to turn to the other bacteria. However, in view of the similarity in base composition of all bacterial ribosomal RNA, a positive finding would be open to the interpretation that all bacterial genomes possess in common a sequence complementary to ribosomal RNA. It was decided, therefore, to extend the examination to completely foreign biological material. The results obtained will be illustrated with thymus DNA. Figure 3 describes the results of a cesium chloride gradient density centrifugation of a hybridization test between the 23S coli RNA and heat-denatured calf-thymus DNA. It is evident that a significant amount of apparent "hybridization" has occurred. It should further be noted that very little (less than 1%) of this sort of complex is observed unless the mixture of the two nucleic acids is heated and slow-cooled, suggesting the necessity of hydrogen bond formation.

The existence of this phenomenon makes it clear that the appearance of RNA in the DNA density region with one type of DNA and not another cannot *per se* be accepted as evidence for specificity of the complex observed. One may well be examining rather the comparative complexity of the DNA molecules being used.

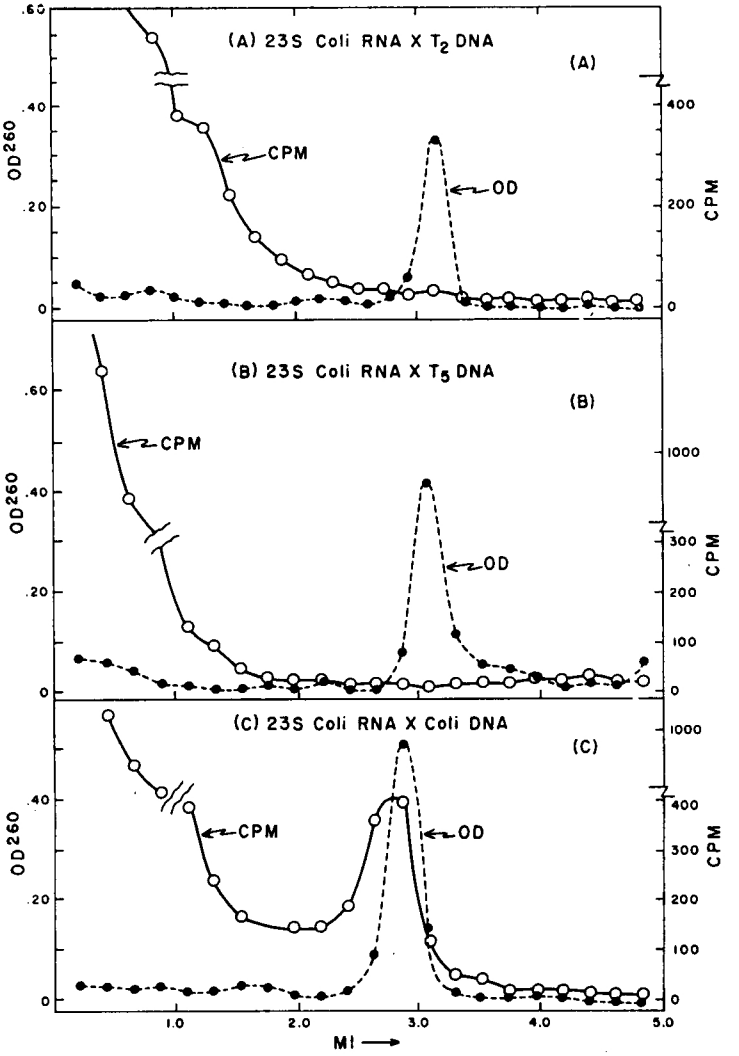


FIG. 2.—CsCl density gradient profiles. (A) 76 $\mu$g/ml T2 heat-denatured DNA + 3.4 $\mu$g/ml *E. coli* 23S-$H^3$-RNA (4.8 $\times$ $10^4$ cpm/$\mu$g) in TMS. (B) 76 $\mu$g/ml T5 heat-denatured DNA + 3.4 $\mu$g/ml *E. coli* 23S-$H^3$-RNA (4.8 $\times$ $10^4$ cpm/$\mu$g) in TMS. (C) 84 $\mu$g/ml *E. coli* heat-denatured DNA + 3.4 $\mu$g/ml 23S-$H^3$-RNA (4.8 $\times$ $10^4$ $\mu$g/ml) in TMS. All three reaction mixtures were held at 40°C for 36 hr. Centrifuge was at 33,000 rpm and 25°C for 72 hr.

Other criteria must be developed if complex formation at this level of detection sensitivity is to be interpreted with certainty.

3. *Sensitivity of ribonuclease as a specificity test of apparent hybrid formation:* It is important to recognize that we are trying to distinguish between the following two possibilities: (1) partial hybridization involving small scattered segments of corresponding complementary sequences; (2) complete hybridization between the ribosomal RNA and an homologous complementary sequence in DNA.

The first possibility would predict that a major portion of each RNA strand in the complex is not actually involved in the hydrogen bonding and should, there-

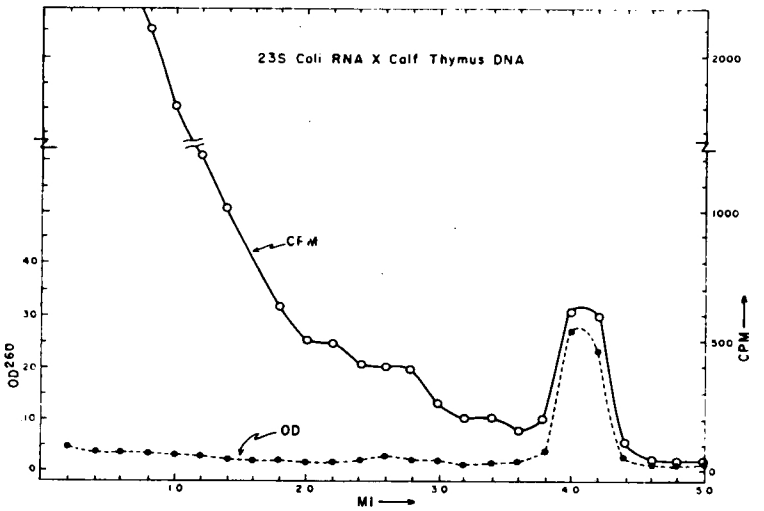


FIG. 3.—CsCl density gradient profile. 84 $\mu$g/ml heat-denatured calf-thymus DNA + 3.4 $\mu$g/ml 23S-$H^3$-RNA ($4.8 \times 10^4$ cpm/$\mu$g) in TMS; slow-cooled from 55°C to 32°C. Centrifugation, at 33,000 rpm and 25°C, for 74 hr.

fore, be accessible to degradation with RNAase. Complete hybrids, involving essentially the entire sequence, would be resistant. Sensitivity to nucleolytic enzymes suggests itself as an obviously convenient differentiating tool. To increase the sharpness of the distinction, preliminary experiments were carried out with T2-specific RNA and DNA to determine the minimal ionic strengths required to maintain the integrity of true RNA-DNA hybrids. This was done to permit functioning of the enzymes at near optimal conditions and to encourage instability of partial hybrids involving only short nucleotide sequences. As a result of these exploratory experiments, the procedures for treating RNA-DNA complexes detailed in *Methods* were finally adopted.

To test whether the nucleolytic enzymes can distinguish between homologous and heterologous complexes, hybridization incubations were carried out between 23S coli RNA mixed with heat-denatured DNA of *E. coli* and thymus. The hybrid regions of the CsCl gradients are shown in Figures 4 (*A* and *D*). Excellent complex formation occurred in each case.

The radioactive fractions in each hybrid region of Figures 4*A* and *D* were pooled and tested for nucleolytic sensitivity as described in *Methods*. In all cases, free ribosomal RNA was included as an internal control to monitor the enzyme activity. The free control RNA was labeled with $P^{32}$ if the hybridized RNA was marked with $H^3$ and vice versa. Examination of the data in Figure 4 reveals an obvious difference between the sensitivities of the two complexes. In the case of the homologous hybridization (Fig. 4*C*), there is a loss of counts in the first 5 min, but the residue is virtually completely resistant to RNAase. On the other hand, in the case of the heterologous complex of thymus DNA by coli ribosomal RNA (Fig. 4*F*), it is impossible to distinguish the sensitivity of the apparently hybridized RNA from that of the internal RNA control. If we examine the sensitivity of the combined action of RNAase and DNAase (Figs. 4*B* and *E*), we see that essentially the same

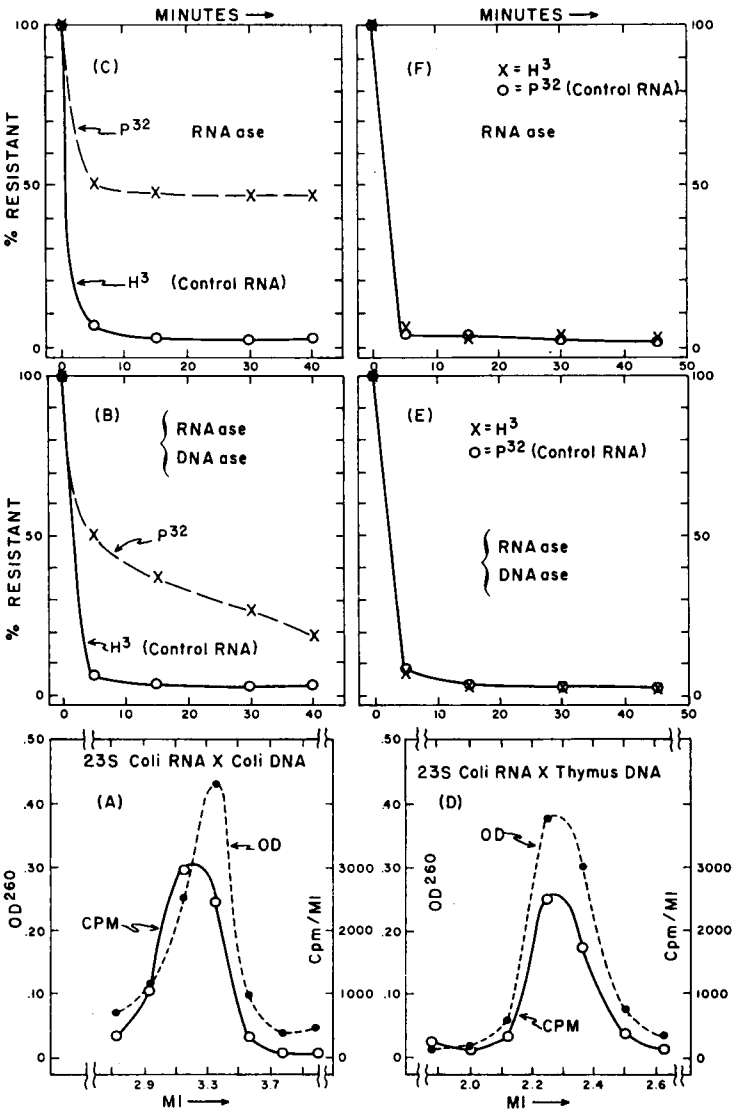


FIG. 4.—(A) Apparent hybrid in DNA region after CsCl density gradient centrifugation. 114 $\mu$g/ml *E. coli* heat-denatured DNA + 3.1 $\mu$g/ml *E. coli* 23S-$P^{32}$-RNA ($1.2 \times 10^5$ cpm/$\mu$g) in TMS slow-cooled from 55°C to 35°C. Centrifugation was at 33,000 rpm and 25°C for 72 hr. (B) Resistance of apparent hybrid (A) to digestion with RNAase + DNAase (A). (C) RNAase resistance of apparent hybrid (A). (D) Apparent hybrid in DNA region after CsCl density gradient centrifugation 100 $\mu$g/ml calf-thymus heat-denatured DNA + 2.72 $\mu$g/ml *E. coli* 23S-$H^3$-RNA ($4.8 \times 10^4$ cpm/$\mu$g) in TMS, slow-cooled from 55°C to 35°C. Duration centrifugation was at 33,000 rpm and 25°C for 97 hr. (E) Resistance of apparent hybrid shown in (D) to digestion with both RNAase and DNAase. (F) Resistance of apparent hybrid (D) to RNAase digestion. See *Methods*.

pattern is obtained. Again, in the case of the homologous hybrid (Fig. 4*B*) there is a rapid loss of counts in the first 5 min bending off again at the 50 per cent resistant residue. This is followed by a slow (3 per cent of free RNA) destruction of the hybridized RNA. Apparently, the presence of both enzymes exposes the hy-

brid material to a double jeopardy which makes complete stability impossible. Turning to the material derived from the heterologous incubation (Fig. 4*E*) we find again no detectable distinction in the sensitivity of the free $P^{32}$-labeled RNA and the apparently hybridized $H^3$-containing material.

It is apparent that nuclease sensitivity at the ionic strengths employed permits a ready distinction between specific hybridization involving long sequences and the partial pairing of short segments which can occur by chance when the DNA is of considerable complexity. Density centrifugation combined with nuclease sensitivity has yielded completely consistent findings in examinations of the specificity of hybridization between DNA and RNA.

*Discussion.*—The primary purpose of the present paper was to provide evidence pertinent to a decision on the existence of a sequence in DNA complementary to ribosomal RNA. The general experimental approach used has its genesis in the observations of Marmur,[14] Doty *et al.*[15] and the pioneering model experiments with synthetic homopolymers by Rich[16] and Schildkraut *et al.*[8]

The purely optical methods employed by earlier workers provided neither the sensitivity nor the certainty required to detect hybrids between naturally occurring complementary RNA and DNA. Hall and Spiegelman[6] consequently developed the isotopic method, taking advantage of double labeling and equilibrium density centrifugation in preparative swinging bucket rotors. The demands of the present investigation necessitated further increasing the sensitivity of the isotopic method at least 100-fold beyond that found adequate for previous purposes.[9, 17, 18] This, in turn, required the development of experimental devices which would help differentiate between "noise" and relevant observations.

Simple mechanical trapping of ribosomal RNA in the strands of heat-denatured DNA was not observed even at a sensitivity where 0.001 $\gamma$ of RNA per 100 $\gamma$ of DNA would have been readily detected. The more serious difficulty stemming from incidental coincidences over short regions in complex DNA was overcome by testing for nucleolytic sensitivity. Complexes formed between ribosomal RNA and DNA of heterologous origins were completely sensitive to ribonuclease, whereas those which involved homologous nucleic acids were resistant.

The combination of the enzyme technique with the other described permitted the accumulation of data which leads to the conclusion that DNA does contain a localized sequence complementary to its homologous ribosomal RNA. The fact that the ratios of G/C and A/U are not unity would suggest that only one of the DNA strands in this region can serve as the template for the ribosomal RNA. It is impossible to specify the relation, if any, of the sequence identified here with the "RC" locus of Stent and Brenner.[19] As pointed out by these authors, the RC locus could be either a regulator gene, an operator gene, or an operon governing the synthesis of some unspecified transient product necessary for RNA synthesis.

The pairing of ribosomal RNA with a specific region of the DNA leads to the prediction of a maximal ratio of RNA to DNA in the complex. The size of this fraction would yield some insight into the number of complementary sites involved. Experiments along these lines will be reported in a subsequent publication.

It should be noted that the sensitivity and specificity of the methods developed here permit definitive decisions, in both a positive and a negative sense, of the existence of sequences in DNA complementary to any known RNA molecule. The

present study has pushed the detection level to 0.01% of the genome of *E. coli*, and it could be decreased further. It is also evident that the use of nucleolytic enzymes will enable the ultimate isolation of specific DNA sequences corresponding to the RNA molecules used for hybridization.

*Summary.*—Experiments are described that establish the existence of a sequence in *E. coli* DNA complementary to its ribosomal RNA. The proof depends on showing that a hybrid complex, resistant to RNAase, is specifically formed with homologous DNA. The base composition of bacterial ribosomal RNA implies that only one of the two DNA strands is used in the region transcribed.

The procedures developed in the present study provide a method of sufficient specificity and sensitivity to permit the identification, and ultimate isolation, of sequences in DNA complementary to known RNA molecules corresponding to 0.01% of the total genome of *E. coli*.

* This investigation was aided by grants in aid from the U.S. Public Health Service, National Science Foundation, and the Office of Naval Research.

† Predoctoral fellow trainee in Molecular Genetics (USPH 2G-319).

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