# *RECOGNITION OF RIBOSOMAL RNA SITES IN DNA, I. ANALYSIS OF THE E. COLI SYSTEM**

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The evidence recently presented of specific hybridization between bacterial ribosomal RNA and homologous DNA[1-3] has indicated the possibility of a biochemical approach to the problem of the identification of ribosomal RNA sites in DNA. A difficulty in this approach is that while the specific role of the RNA in the hybridization is open to experimental test, the critical evidence of the unique involvement of presumptive DNA sites is not easily attainable.

Convincing arguments for hybridization with specific sites would be provided by the demonstration that the RNA molecules are regularly and completely base-paired with DNA. A possible criterion for systematic hydrogen bonding may be given by the resistance to RNase of the RNA complexed with DNA,[1, 4-6] and therefore by the recovery from the hybrid, after extensive RNase treatment, of the intact RNA as judged from its size and base composition. The development of an appropriate experimental procedure has allowed us to perform this type of analysis on the *E. coli* system and on the HeLa cell system. Here the experimental approach and the results obtained with *E. coli* will be reported.

*Materials and Methods.*—The bacterial strains used were *E. coli* ML308, *S. marcescens* $F_D$1-3, and *B. subtilis* SB19B.

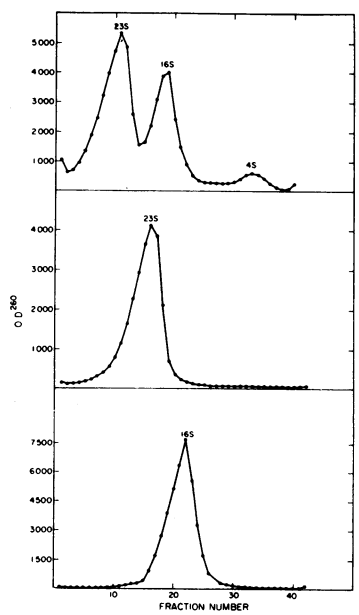


FIG. 1.—Fractionation of *E. coli* ribosomal RNA into 16S and 23S components by repeated cycles of sucrose density gradient centrifugation.

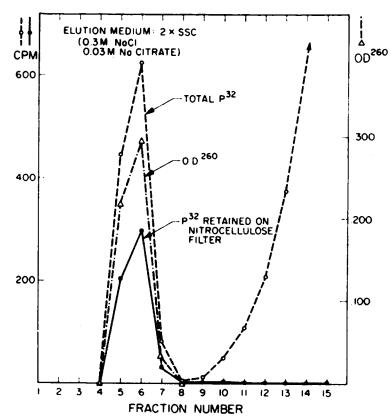


FIG. 2.—Separation of RNA-DNA hybrids by Sephadex chromatography. A mixture containing 25 μg/ml DNA and 1.25 μg/ml 16S $P^{32}$-RNA was annealed as described in the text. After RNase treatment, the mixture was run through Sephadex G-100 in 2 × SSC, and 2-ml fractions were collected. After UV-absorption reading, one aliquot of each fraction was directly plated; the rest was filtered through a nitrocellulose membrane.

Uniform labeling of *E. coli* ML308 was carried out by growing the cells for four generations in a casamino acid-glycerol medium containing $10^{-4}$ *M* phosphate, in the presence of 80 $\mu$c/ml of carrier-free $P^{32}$-orthophosphate. The cells were harvested, washed, and allowed to chase for one generation in medium containing $10^{-2}$ *M* phosphate in order to reduce the specific activity of the messenger RNA fraction. The cells were alumina-ground in the presence of $10^{-2}$ *M* $Mg^{++}$; the extract was centrifuged to remove cell debris and treated with DNase (10 $\mu$g/ml) for 30 min in the cold. Ribosomes were pelleted by centrifugation at 105,000 $\times$ *g* for 90 min. The RNA was extracted with phenol and fractionated into the 23S and 16S components by repeated cycles of centrifugation in a linear (5–20%) sucrose gradient containing $10^{-2}$ *M* acetate buffer, pH 5.0, and 0.1 *M* NaCl. Figure 1 illustrates the separation of the two RNA components thus obtained.

Methods of growth of HeLa cells and isolation of ribosomes from this source have been described previously.[7] Ribosomal RNA was phenol-extracted and resolved into the 28S and 18S components by two cycles of sucrose gradient centrifugation.

The DNA from different bacterial sources and from animal and plant cells was prepared by the Marmur procedure.[8] T4 DNA was prepared from the purified phage by phenol extraction. Calf thymus DNA was obtained from Worthington Biochemical Corp. The DNA preparations used had a sedimentation coefficient varying between 20S and 35S and were free of any detectable RNase activity after heat denaturation. The DNA from the various sources was denatured in $^1/_{10}$ SSC (SSC, standard saline citrate: 0.15 *M* NaCl, 0.015 *M* Na citrate) at a concentration of about 50 $\mu$g/ml by heating at 98°C for 15 min and quick cooling.

The hybridization experiments were performed by incubating denatured DNA at a concentration of 20–50 $\mu$g/ml with varying amounts of purified 23S or 16S in 0.3 *M* NaCl, 0.015 *M* Na citrate, at the temperature and for the time specified below. After slow cooling to room temperature, the annealing mixture was treated with 5 $\mu$g/ml RNase (freed of DNase activity by heating at 80°C for 10 min) at 21°C for 15 min, then passed through a Sephadex G-100 column equilibrated with 2 $\times$ SSC at room temperature. In some experiments, specified below, the Sephadex was equilibrated with 0.01 *M* Tris buffer, pH 7.4, 0.005 *M* NaCl, 0.0075 *M* Na citrate (TNC), and $MgCl_2$ was added to the eluted fractions to a concentration of $10^{-2}$ *M*. The DNA and RNA associated with it were collected in the first fractions, well separated from the digested free RNA (Fig. 2). These were pooled, and after addition of KCl to 0.5 *M*, were filtered through nitrocellulose membranes,[3] which were then washed with 120 ml of 0.5 *M* KCl, 0.01 *M* Tris buffer, pH 7.4, at 53–54°C. DNA retention by the filter in general varied between 90 and 100% for different DNA preparations. In some experiments the hybridized RNA was eluted from the filter with 0.5 *M* NaOH, digested for 16 hr at 30°C, and chromatographed on Dowex 1-X8 for base composition analysis.[10] In other experiments the hybridized RNA was extracted at 85–90°C for 2–3 min with 0.01 *M* potassium phosphate buffer, pH 8.0, containing 2% $CH_2O$ and 0.1% sodium dodecylsulfate, then completely dissociated from DNA by heating at 98°C for 4 min and quick cooling, and run on a 5–20% linear sucrose gradient in 0.02 *M* potassium phosphate buffer, pH 7.4, 0.1 *M* NaCl, containing 1% $CH_2O$, for 24–40 hr. From 90 to 95% of the RNA could be eluted when the amount of DNA deposited on the filter was less than 30 $\mu$g. In most of these experiments HeLa 28S $H^3$-RNA was included in the elution buffer to serve as a position marker for comparison of different gradients, and to test the possible effect on the eluted RNA of the RNase which had been added to the annealing mixture and which we have shown to become associated with the DNA. Fractions were collected from the bottom of the tube and analyzed for total and acid-insoluble $P^{32}$- and $H^3$-radioactivity by using a Packard scintillation counter.

In some experiments, the RNA-DNA hybrids were separated from uncombined RNA by direct filtration through nitrocellulose membranes after treatment of the annealing mixtures with 30 $\mu$g/ml RNase at 26°C for 30 min.

*Results.*—Figure 2 illustrates the separation of RNA-DNA hybrids obtained by Sephadex chromatography. It is apparent that a substantial proportion of radioactivity eluted with the DNA peak is not retained on the nitrocellulose membrane after washing with warm buffer, independently of any effect on DNA retention. This radioactivity probably represents RNA segments which are hydrogen-bonded with regions in the DNA sufficiently short as to form complexes unstable at the temperature and ionic strength used in washing the nitrocellulose membrane.

The bulk (80–90%) of this radioactivity can be removed at temperatures lower than 45°C: only a few per cent decrease in the level of radioactivity retained on the filter is observed between 45 and 55°C.

The stability to RNase of the RNA-DNA complex retained on the nitrocellulose membrane was tested by carrying out a second RNase digestion at 37°C with 5 μg/ml RNase. Under conditions where free added tritiated RNA was digested to acid-soluble products to the extent of 80 per cent, the amount of complex retained on the filter appeared to be essentially unchanged.

The efficiency of hybridization reaches its maximum between 40°C and 55°C, and drops considerably (to 40–50%) at temperatures approaching the optimum (about 72°C) for *E. coli* DNA renaturation at the ionic strength employed here.[11] This is probably due to the fact that at these higher temperatures the renaturation of DNA strands competes effectively with the formation of RNA-DNA hybrids. Unless otherwise specified, the temperature of 53°C was used in the experiments described below.

*Saturation experiments:* Several saturation experiments were carried out by incubating a constant amount of DNA with varying amounts of 23S or 16S RNA. In these experiments and in other single determinations, the levels of DNA saturation for 23S RNA varied between 1.7 and 2.8 $\times 10^{-3}$ (9 determinations) with an average of 2.4 $\times 10^{-3}$; those for 16S RNA varied between 2.5 and 3.7 $\times 10^{-3}$ (7 determinations) with an average of 3.2 $\times 10^{-3}$.

TABLE 1

COMPARATIVE HOMOLOGY OF *E. coli* 23S RNA WITH VARIOUS DNA'S

| DNA source | μg 23S RNA complexed/μg DNA × $10^3$ |
|---|---|
| *E. coli* | 2.10 |
| *S. marcescens* | 0.76 |
| *B. subtilis* | 0.40 |
| T4 | 0.003 |
| *Neurospora crassa* | 0.14 |
| Pea embryo | 0.03 |
| Duck blood | 0.05 |
| Calf thymus | 0.03 |
| HeLa | 0.05 |
| *E. coli* 23S (without annealing) | 0.04 |

Each annealing mixture contained 34 μg/ml DNA and 1.7 μg/ml 23S RNA. Incubation was at 53°C for 15 hr.

*The specificity of the complexes:* Table 1 shows the summary of hybridization experiments carried out between 23S RNA and different DNA's. It is apparent that while there is an appreciable cross hybridization (about 25%) with the DNA from another bacterium of the family Enterobacteriaceae, *S. marcescens*, and to a lesser extent with DNA from *B. subtilis*, there is essentially no complex formation with phage DNA or with DNA from higher organisms.

*Analysis of base composition and size of hybridized RNA after RNase digestion:* It is apparent from Table 2 that, if the hybrid is isolated by Sephadex chromatography in 2 × SSC, the hybridized RNA has a base composition substantially indistinguishable from that of the 23S RNA, and that a second RNase digestion after the Sephadex run (Expt. 2) has no detectable effect on the base ratios. If the hybrid is isolated by Sephadex chromatography in low ionic-strength medium (TNC) and subjected to a second RNase treatment, the amount of radioactivity retained on the filter is somewhat reduced (about 30%), and the base composition of the hybridized RNA differs significantly from that of the 23S RNA in the sense to be expected from a partial attack on the hybrid by RNase, namely, with higher purine to pyrimidine ratio. The base composition of the small amount of RNA complexed with HeLa DNA differs markedly from that of the 23S RNA and is close to the one

TABLE 2

NUCLEOTIDE COMPOSITION OF *E. coli* RIBOSOMAL RNA HYBRIDIZED TO HOMOLOGOUS AND HETEROLOGOUS DNA

| | Moles per cent | | | | | |
|---|---|---|---|---|---|---|
| | A | C | U(T) | G | % GC | Pu/Pyr |
| 23S RNA[12] | 25.4 | 21.5 | 19.6 | 33.5 | 55.0 | 1.43 |
| 23S RNA × *E. coli* DNA (after RNase digestion) | | | | | | |
| Expt. 1* | 25.7 | 21.2 | 18.3 | 34.8 | 56.0 | 1.53 |
| Expt. 2* | 25.6 | 21.4 | 18.9 | 34.1 | 55.5 | 1.48 |
| Expt. 3† | 25.5 | 20.1 | 15.8 | 38.6 | 58.7 | 1.79 |
| 23S RNA × HeLa DNA (after RNase digestion) | 25.2 | 12.8 | 12.1 | 49.9 | 62.7 | 3.02 |
| *E. coli* DNA[13] | 24–25 | 25–26 | 24–25 | 25–26 | 50–52 | 0.96–1.04 |

* Sephadex chromatography in 0.3 *M* NaCl, 0.03 *M* Na citrate (2 × SSC). In expt. 2, the RNA-DNA complex was treated again with RNase (5γ/ml, 37°C, 10 min) after the Sephadex run.
† Sephadex chromatography in 0.01 *M* Tris buffer, pH 7.4, 0.005 *M* NaCl, 0.0075 *M* Na citrate (TNC), followed by second RNase digestion.

to be expected for a "core"-like fraction.

The sedimentation profile of the 23S RNA hybridized with *E. coli* DNA is shown in Figure 3. The dissociation of the secondary structure of the RNA caused by the formaldehyde treatment reduces considerably (about twofold) the sedimentation rate. When the hybrid was isolated by Sephadex chromatography in 2 × SSC, 80–90 per cent of the hybridized RNA eluted from the filter could be accounted for

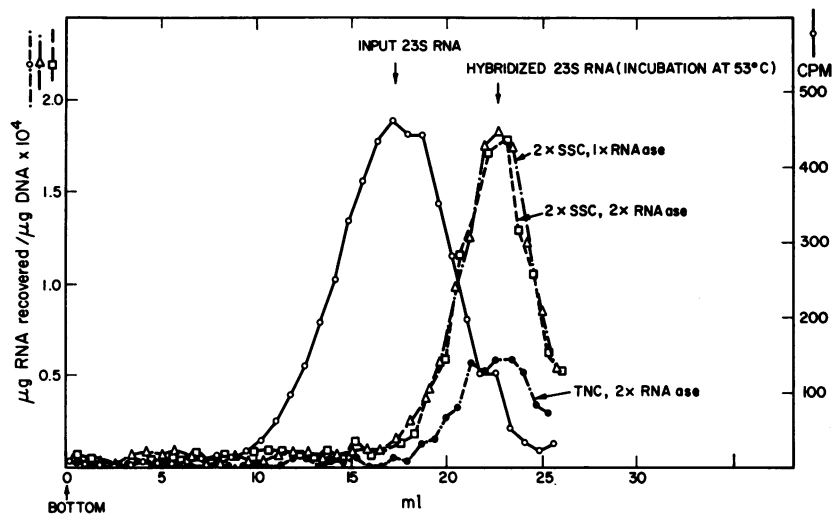


FIG. 3.—Composite diagram showing the sedimentation profile of the 23S $P^{32}$-RNA hybridized with *E. coli* DNA as compared to that of the original RNA. Two mixtures containing 25 μg/ml DNA and 1.25 μg/ml 23S $P^{32}$-RNA were incubated at 53°C for 15 hr. After slow cooling and RNase digestion, one mixture was run through Sephadex G-100 in TNC; the fractions containing DNA, after addition of $MgCl_2$ to $10^{-2}$ *M*, were treated again with 5 μg/ml RNase at 37°C for 10 min and filtered through a nitrocellulose membrane. The other mixture was run through Sephadex in 2 × SSC; the fractions containing DNA were pooled: one half was treated with 5 μg/ml RNase at 37°C for 10 min and then filtered through a nitrocellulose membrane; the other half was filtered directly. In each case the hybridized RNA was dissociated from DNA and run in a sucrose gradient for 24 hr at 24,000 rpm, 4°C. As a control, an aliquot of 23S $P^{32}$-RNA was incubated at 53°C for 15 hr, then diluted with the buffer used for the elution of hybrid RNA from the membranes, subjected to the same thermal treatment employed in the dissociation steps, and run in a sucrose gradient. The $H^3$-RNA marker added to the elution buffer sedimented in identical position in the 4 sucrose gradients (not shown). The recovered RNA plotted in this graph represents the acid-insoluble radioactivity.

by the acid-insoluble radioactivity recovered in the gradient; furthermore, a second RNase digestion after the gel filtration did not alter the amount and sedimentation properties of the hybridized RNA. However, when the hybrid was isolated in TNC and subjected to a second RNase digestion in the same medium, there was a marked decrease in the average size of the hybridized RNA, as illustrated by the fact that only about 50 per cent of the dissociated RNA was recovered in the gradient as acid-insoluble products, the rest being represented by acid-soluble material.

Independently of the conditions used for isolation of the hybrids, it is apparent that the hybridized RNA sediments more slowly than the input RNA subjected to the same thermal treatment. The lack of any effect of a second RNase digestion on the sedimentation properties of the hybridized RNA isolated in 2 × SSC speaks against the possibility that the difference in sedimentation constant from the input RNA is due to RNase attack on longer polynucleotide stretches systematically hydrogen-bonded with DNA. Likewise, an attack on the dissociated RNA by the RNase accompanying the DNA has to be excluded, as no effect was observed on the amount and sedimentation pattern of the $H^3$-control RNA present in the elution buffer.

Dissociation experiments similar to those discussed above were carried out with the 16S RNA. Also in this case the sedimentation profile of the hybridized RNA did not coincide with that of the input RNA. All experimental evidence spoke against this difference being attributable to events occurring during the isolation of the hybrid or the dissociation of the RNA, and pointed to some restriction operating during the hybridization step itself.

The possibility existed that temperatures of incubation higher than those normally used, while on the one hand favored renaturation of DNA and therefore reduced the efficiency of RNA-DNA hybridization, on the other hand, created conditions necessary for formation of more complete RNA-DNA hybrids by breaking the weak intramolecular hydrogen bonds in denatured DNA and exposing the sites more fully, or by allowing the complete unfolding of the RNA molecules, or by both mechanisms. Appropriate experiments corroborated this hypothesis. As shown in Figure 4, when the hybridization between 23S or 16S RNA and *E. coli* DNA was carried out at 72°C, the dissociated RNA molecules showed a sedimentation profile coinciding with that of the input RNA which had been heated in the same way. By contrast, the very small amount of HeLa 28S $P^{32}$-RNA complexed with *E. coli* DNA at 70°C consisted of very slowly sedimenting pieces.

As concerns the absolute size of the RNA molecules recovered from the hybrids, they were expected to be smaller than the original intact RNA because of the thermal degradation occurring during the incubation and dissociation steps. An indirect estimate of the sedimentation constant has been made by running RNA, having the same thermal history as that used in hybridization experiments, in a sucrose gradient in SSC in the presence of known markers. A more direct estimate has also been made by dialyzing extensively the hybridized and dissociated RNA against 0.1 *M* phosphate buffer, pH 8.5, in order to remove the formaldehyde and the hydroxymethyl groups, and then running it in a sucrose gradient in SSC. On the basis of these runs, the average sedimentation constant of the RNA recovered after hybridization at 72°C has been estimated to be about 8S for the 23S RNA and

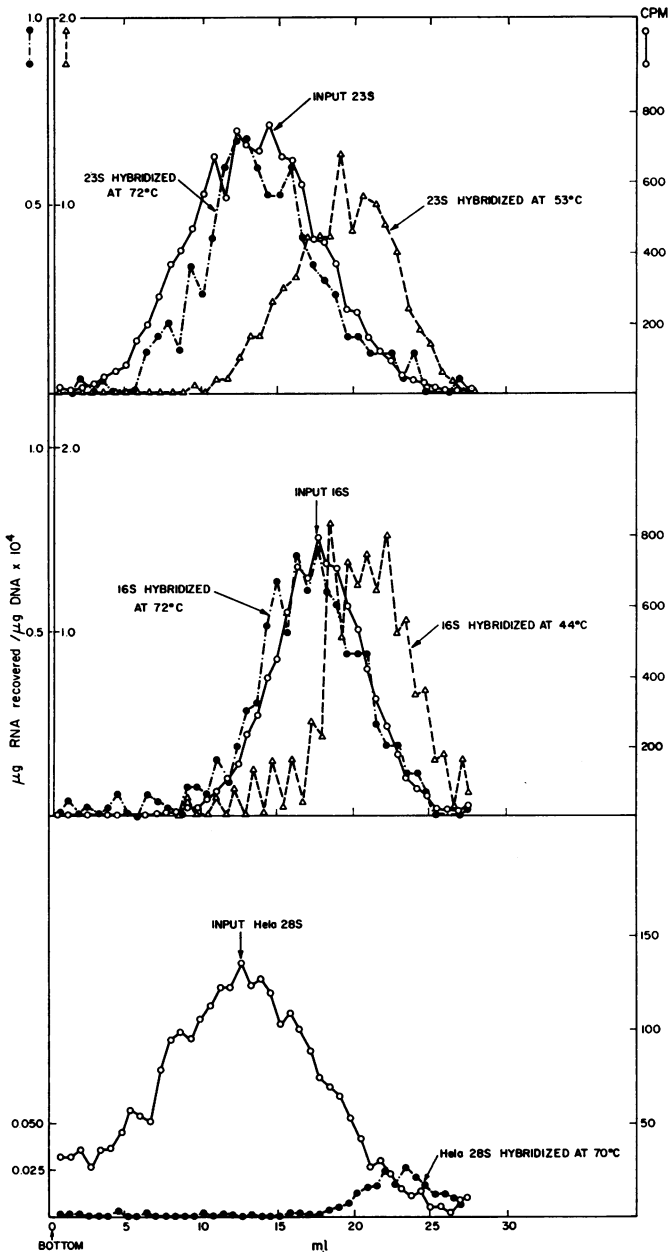


FIG. 4.—Composite diagram showing the sedimentation profile of 23S and 16S *E. coli* RNA and 28S HeLa RNA hybridized with *E. coli* DNA at different temperatures. Two mixtures containing 25 μg/ml DNA and 1.25 μg/ml 23S $P^{32}$-RNA were incubated, one at 72°C for $2^1/_2$ hr, the other at 53°C for 15 hr. Two mixtures with 25 μg/ml DNA and 1.25 μg/ml 16S $P^{32}$-RNA were incubated, one at 72°C for 8 hr and the other at 44°C for 16 hr. The control RNA was incubated in both cases at the higher temperature. One mixture with 25 μg/ml *E. coli* DNA and 2.5 μg/ml HeLa $P^{32}$ 28S RNA was incubated at 70°C for 8 hr. Isolation of hybrids and analysis of hybridized RNA as described in the text. Centrifugation for 40 hr at 24,000 rpm, 4°C.

about 6S for the 16S RNA; that of the RNA recovered after hybridization at lower temperatures has been estimated to be around 4S.

*Relationship between 23S and 16S sites:* It is apparent from Figure 5 that unlabeled 23S RNA competes with $P^{32}$ 23S for sites in DNA to the extent to be expected on the basis of the dilution factor and of the change in position on the saturation curve; on the contrary, there is no appreciable competition by 28S and 18S RNA from a heterologous source. Addition of *E. coli* 16S RNA, on the other

hand, results in a considerable displacement of label from the hybrid (up to 80%). In the reciprocal experiment (Fig. 6), an analogous situation is observed: 100 per cent competition by the homologous RNA subclass, partial competition (up to 60%) by the other RNA component, and no competition by RNA from heterologous source. Several competition experiments carried out at saturating and nonsaturating RNA levels and utilizing different conditions for incubation (53°C, 15 hr, 72°C, $2^1/_2$–8 hr) and isolation of hybrids (filtration through nitrocellulose membranes directly or after Sephadex chromatography) gave results similar to those illustrated above. If due allowance is made for the different saturation levels pertaining to the two RNA subclasses, the fraction of DNA which appears to be complementary to both 23S and 16S, as calculated from the competition of 23S versus 16S and vice versa, is approximately equivalent (respectively, 1.8 and 1.9 $\times$ $10^{-3}$ of the DNA). About 20 per cent of the sequences complementary to 23 S RNA and 45 per cent of those complementary to 16S RNA are specific for these species. The sedimentation analysis of the hybridized and dissociated RNA showed that the segments of 16S which are not displaced by 23S have sedimentation properties very similar to those of the total hybridized 16S and to those of the input RNA subjected to the same thermal treatment as the hybrid.

*Discussion.*—The results described in this paper suggest that, under defined temperature conditions, regular and complete hydrogen bonding occurs in the hybridization between *E. coli* ribosomal RNA and homologous DNA. These results, together with the stringent specificity pattern of the interactions of ribosomal RNA with different DNA's, give strong support to the conclusion that the

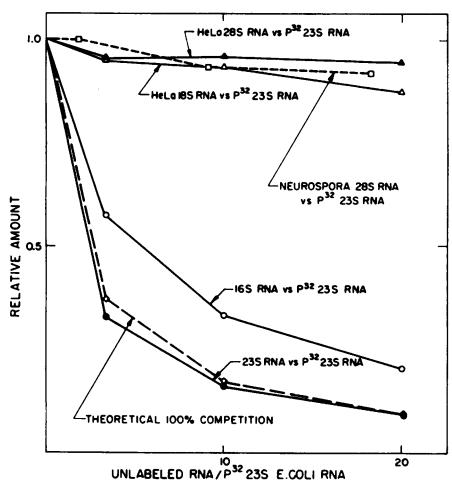


FIG. 5.—Capacity of different RNA's to compete with 23S RNA for sites in DNA. Each mixture contained 5 $\mu$g/ml DNA, 0.1 $\mu$g/ml 23S $P^{32}$-RNA and a variable amount of cold RNA. Incubation was at 53°C for 15 hr; the hybrids were isolated by direct filtration through nitrocellulose membranes. Each value is the average of triplicate determinations and is corrected for the background given by nonannealed mixtures (1–2% of maximum level). Ratios on the abscissa are expressed on a weight basis.

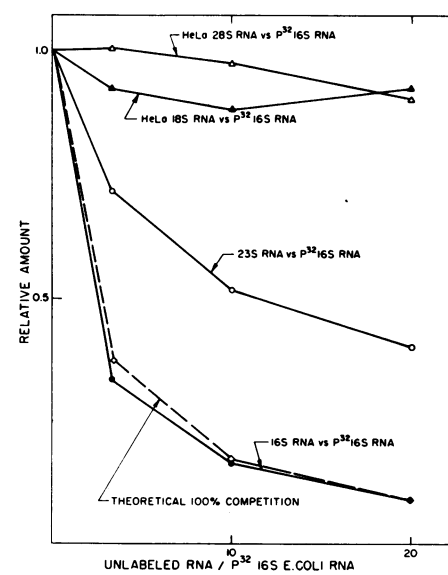


FIG. 6.—Capacity of different RNA's to compete with 16S RNA for sites in DNA. Each mixture contained 5 $\mu$g/ml DNA, 0.1 $\mu$g/ml 16S $P^{32}$-RNA, and a variable amount of cold RNA. Conditions of incubation and isolation of hybrids as in Fig. 5.

hybridization detected in these experiments does occur at the level of ribosomal RNA sites in DNA. In view of the data of base composition analysis and of the specificity tests, it seems justifiable to extend this conclusion to the incomplete hybrids obtained at suboptimal temperatures (40–53°C).

The total amount of sites specific for the two ribosomal RNA subclasses which has been detected here corresponds to a fraction of the DNA of about $4 \times 10^{-3}$, which is close to that found by Yankofsky and Spiegelman for *B. megatherium*.[3] That the two ribosomal RNA subclasses must have at least partially distinct genetic origin is indicated by the lack of complete identity of sequences, as shown in the competition experiments. On the other hand, the extensive cross hybridization observed here suggests an evolutionary relationship between the two ribosomal RNA species. This overlapping of sequences introduces an element of uncertainty in interpreting the data in terms of amount of sites in DNA pertaining to each RNA subclass. If a figure of $2.8 \times 10^9$ daltons is used for the total DNA content of *E. coli*[14] and if the inequality of G and C and A and U in the hybridized RNA is taken as evidence that only one of the DNA strands is copied *in vivo*, one can estimate that the *E. coli* DNA contains the equivalent of 1–6 stretches complementary to 23S RNA and, correspondingly, the equivalent of 16–7 stretches complementary to 16S RNA. It is obvious that there is more than one site per cell for at least the 16S ribosomal RNA component. The significance of this multiplicity is at present obscure.

As concerns the absolute size of the ribosomal RNA sites in *E. coli* DNA identified in these experiments, the average length estimated (by using Gierer's[15] or Spirin's[16] equation for conversion of S values of hybridized RNA to molecular weights) is of the order of 350 nucleotide pairs for the 23S component and 200 nucleotide pairs for the 16S. These are obviously minimum estimates because of the limitations inherent in the experimental procedure employed, as discussed above. It is hoped that when conditions are found for hybrid formation and dissociation which do not involve the thermal treatment used here, it will be possible to bring the level of site recognition in DNA up to the size of the original intact RNA.

We wish to thank Drs. J. Bonner and R. C. Huang for their gift of T4 DNA and pea embryo DNA, and Dr. A. G. DeBusk and E. Brandt for the Neurospora DNA. The capable assistance of Sandra Kiddy is gratefully acknowledged.

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## *ERRATUM*

In the article entitled "On the Stability of Stochastic Dynamical Systems," by Harold J. Kushner, which appeared in the January issue of Volume 53, pages 8–12, the line above equation (5) should read " . . . $0 \leqq V(x) \leqq r$, . . . ."

Reference 3, which was available to the author at the start of the work, should read " . . . to appear in *J. Differential Equations*, April 1965; see also *Notices Am. Math. Soc.*, A.M.S. 64T-233 (April 1964)."