ISOLATION AND CHARACTERIZATION OF BACTERIAL RIBOSOMAL RNA CISTRONS

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ABSTRACT The DNA sequences which code for ribosomal DNA have been isolated and purified. The technique used has general application for RNA:DNA hybridization studies and enables the isolation of any gene for which sufficient gene product can be obtained. Experiments with isolated ribosomal RNA cistrons demonstrated that (*a*) the majority of the ribosomal cistrons are similar to one another; (*b*) the cistrons which are similar to one another are virtually identical to one another; (*c*) ribosomal cistrons of different bacterial species are closely related to one another.

INTRODUCTION

In several bacterial species about 0.3% of the DNA has been shown to be complementary to ribosomal RNA (1), which indicates that four to five ribosomal genes are present in each cell. This report describes the isolation of the DNA which is complementary to rRNA. This fraction of DNA is termed the "rR-cistrons" (ribosomal RNA cistrons). This is believed to be the first instance of the virtually complete purification of deoxyribonucleotide sequences characteristic for a specific gene function.

METHODS AND MATERIALS

Growth and Labeling of Cells

Escherichia coli BB *or Proteus mirabilis*-1 were grown overnight at 37°C with aeration in 1 liter of growth medium (1 part nutrient broth plus 25 parts Tris medium [2]) to which 10–20 mc of ^{32}P had been added. The bacteria were harvested and washed once with 1 × SSC (0.15 M NaCl, 0.015 M Na citrate).

Isolation of DNA

Washed bacterial cells were resuspended in a solution of 1 × SSC, 0.03 M EDTA (sodium ethylenediaminetetraacetate), pH 8. To this was added sodium lauryl sulfate and pronase to final concentrations of 1% and 500 μg/ml, respectively. The mixture was then incubated 4–5 hr at 37°C.

DNA was extracted by a modification of the method of Marmur (3). Two phenol extrac-

tions were carried out following which the aqueous phase was brought to 1 M sodium perchlorate and subjected to three further extractions with chloroform octanol (1% octanol by volume). The DNA then was precipitated with two volumes of 95% ethanol and redissolved in 0.1 × SSC. To remove RNA the DNA was treated with 50–100 μg/ml of bovine pancreatic RNase for 1–2 hr at 37°C. The RNase was removed by one phenol extraction followed by chloroform-octanol extraction until no precipitate was visible at the interface.

Following adjustment of the salt concentration to 1 × SSC, the DNA again was precipitated with 95% ethanol and redissolved in 0.1 × SSC. This procedure was repeated three times. For further purification the DNA was precipitated from 1 × SSC with 2.5 volumes of 2-ethoxyethanol and redissolved in 0.1 × SSC. This step was repeated four times and the DNA was then dissolved in 0.01 M PB (phosphate buffer, pH = 6.8, prepared by mixing equimolar amounts of Na_2HPO_4 and NaH_2PO_4). Since DNA in low concentration is not precipitated by 2-ethoxyethanol, the DNA must be concentrated to at least 200–300 μg/ml before precipitation. Precipitation with 2-ethoxyethanol was necessary to eliminate ^{32}P-labeled contaminants. All labeled DNA preparations were examined for acid soluble and alkali labile (0.2 N NaOH, 60°C, 1 hr) radioactivity. No detectable alkali labile fraction was observed in the *E. coli* ^{32}P-DNA preparation while the acid soluble fraction represented 0.5% or less of the total radioactivity. The *Proteus mirabilis* ^{32}P-DNA preparation contained no alkali labile fraction; the acid soluble fraction, however, represented approximately 6% of the total label.

The purified DNA was sheared by passing a solution of it twice through a needle valve with a pressure drop of 50,000 psi. It was then filtered through a Metricel GA-6 (0.45 micron pore diameter) filter (Gelman Instrument Co., Ann Arbor, Mich.). Such filters do not bind DNA.[1] Alkaline sedimentation studies indicated that the average size of this sheared DNA is 300 to 500 nucleotides in length. All DNA used in these experiments was sheared at 50,000 psi.

Further Purification of the DNA on Hydroxyapatite

Hydroxyapatite (Bio-Rad, Biogel HT) was purchased from Calbiochem, Los Angeles, Calif. The amount of DNA which can be adsorbed to hydroxyapatite varies with different batches of hydroxyapatite. The capacity of each new batch should be checked before using. The hydroxyapatite used in these experiments adsorbed about 80 μg of nucleic acid per cubic centimeter of wet-packed hydroxyapatite.

It was possible to remove most of the acid soluble fraction from the *Proteus mirabilis* DNA by passing the DNA (in 0.035 M PB) through a water-jacketed hydroxyapatite column equilibrated to 60°C and 0.035 M PB. At this phosphate ion concentration single- and double-stranded DNA adsorb to hydroxyapatite (4) while the acid soluble fraction of *Proteus mirabilis* passes through the column. The DNA can be recovered by washing the column with 0.14 M PB at 100°C.

Hydroxyapatite also was utilized for final purification of both of the labeled bacterial DNA's. It was necessary to remove any cross-linked DNA and non-DNA radioactive material which would adsorb to hydroxyapatite. A dilute solution of labeled DNA was denatured at 100°C for 3–5 minutes in 0.14 M PB. This solution was immediately cooled to 50°C and passed immediately through a hydroxyapatite column equilibrated to 50°C and 0.14 M PB. The single-stranded DNA passed through the hydroxyapatite column and subsequently was used for isolation of rR-cistrons. Generally, about 0.5% to 1% of the total radioactivity adsorbed to the column.

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[1] Britten, R. J. Personal communication.

RNA Isolation

Ribosomes were isolated from stationary phase bacteria and RNA was isolated from the ribosomes by the phenol procedure (5). *E. coli* 16S and 23S and rRNA subunits purified on a MAK column were purchased from Miles Laboratories (Elkhard, Ind.). A second batch of 16S and 23S rRNA was kindly contributed by Dan Haapola. This preparation also was purified on a MAK column (6).

Conditions for the Formation and Isolation of RNA-DNA Hybrids

Knowledge of the reassociation kinetics of bacterial DNA made it possible to choose conditions for the reaction of rRNA with DNA such that very little DNA:DNA reassociation occurred while all of the DNA complementary to rRNA formed DNA:rRNA hybrids. The hybrids could then be separated from the unreassociated DNA by using hydroxyapatite.

A convenient way to express the exposure of nucleic acids under reassociation conditions is the C_0t (7), which is defined as the product of the nucleic acid concentration (C_0) and the time (t) of the reassociation incubation period. In this report C_0t is conveniently calculated as the product of the nucleic acid concentration expressed as optical density at 260 mμ and the time of incubation expressed in hours, divided by two (C_0t = OD/ 2 × hours) (7). Incubation of a reassociation mixture with an optical density of two for 1 hr results in a $C_0t = 1$. The extent of reassociation of rRNA with DNA complementary to it is controlled by the magnitude of the rRNA C_0t (the product of the concentration of rRNA and the time of incubation). For these experiments a large enough rRNA C_0t is used to ensure complete reaction of all DNA complementary to rRNA.

DNA:DNA reassociation can occur in the incubation mixture as well as rRNA:DNA hybrid formation. In order to minimize the extent of DNA:DNA reassociation the *DNA* C_0t (the product of the optical density of the DNA at 260 mμ and the time span of the reassociation incubation ÷ 2 [7]) is kept very low. It was impractical to utilize a DNA C_0t low enough to completely prevent DNA:DNA reassociation when rRNA was reacted with whole DNA. Consequently the reassociated fraction adsorbed to hydroxyapatite contained both DNA:DNA duplexes and rRNA:DNA hybrids. In order to eliminate DNA which was not complementary to rRNA it was necessary to pass the isolated fraction containing the rRNA:DNA hybrids through two additional cycles of reassociation and isolation of the reassociated fraction. In each cycle of purification the DNA C_0t decreases greatly while the rRNA C_0t is kept constant at a level which ensures complete reaction of the rR-cistrons with rRNA.

All reassociation reactions reported in this communication were performed in 0.14 M PB at 60°C for specified times. Hydroxyapatite fractionation was used to separate RNA:DNA hybrids from the unreassociated DNA. Under the conditions of 0.14 M PB and 60°C, reassociated DNA adsorbs to hydroxyapatite while single-stranded DNA passes through the hydroxyapatite column.[2]

Assay for Radioactivity

^{32}P was assayed by Cerenkov counting (8) in a Packard Tri-Carb 4000 scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.) adjusted for tritium counting. Samples

[2] Kohne, D. E., and R. J. Britten. 1968. Paper in preparation.

can be counted as they are collected without further preparation or the use of scintillation fluid. The samples can be used for further experimentation if necessary.

Isolation of rR-Cistrons

The method used to isolate bacterial rR-cistrons is as follows: (*a*) Mix ^{32}P-labeled (100,000 cpm/μg), sheared, hydroxyapatite purified DNA (10 μg/ml) with unlabeled rRNA (20 μg/ml) in 0.14 M PB.

(*b*) Denature the mixture by heating at 100°C for 3–5 min in a water bath; incubate at 60°C for 1 hr (rRNA C_0t = 0.25). Pass the incubation mixture through a hydroxyapatite column equilibrated to 60°C and 0.14 M PB. Wash all single-stranded DNA through the column. rRNA adsorbs almost completely under these conditions. Recover the reassociated DNA from the column by heating the water-jacketed column to 100°C and washing the RNA and DNA off in 0.14 M PB.

(*c*) Take the material recovered by the 100°C elution of the column and repeat (*b*). The rRNA C_0t should be the same for each cycle.

(*d*) Take the high temperature elution material from (*c*) and bring the solution to 0.2 M NaOH and incubate this solution at 60°C for 1 hr to hydrolyze the RNA. Cool the solution and neutralize the NaOH present. Dilute the solution to 0.035 M PB and pass it through a hydroxyapatite column equilibrated to 60°C and 0.035 M PB. At this phosphate ion concentration DNA adsorbs to hydroxyapatite while ribonucleotides pass through the column. Recover the DNA from the hydroxyapatite column in several milliliters by eluting the column at 100°C with 0.14 M PB.

(*e*) React the ^{32}P-DNA recovered in (*d*) with rRNA (100 μg/ml) for 12 min at 60°C in 0.14 M PB (rRNA C_0t = 0.25) and separate the reassociated fraction on hydroxyapatite and recover it. Again treat the isolated fraction with NaOH and recover as in (*d*). The products of this procedure are purified rR-cistrons.

^{32}P-DNA which did not reassociate in the first cycle (*b*) was reacted again with rRNA and only 0.02% of the ^{32}P-RNA was recovered after two cycles of purification. This indicates that the rR-cistrons were essentially completely removed during the first purification cycle.

Thermal Chromatography with Hydroxyapatite[2]

The incubation mixtures were passed through a hydroxyapatite column equilibrated to 60°C and 0.14 M PB. After washing all of the single-stranded DNA off the column the temperature of the column was raised in 5°C steps. After each 5°C temperature rise the column was washed once with 20 ml of 0.14 M PB. After the 0.14 M PB wash at 100°C, 20 ml of 0.4 M PB were passed over the column to elute any remaining DNA. The column effluents (20 ml) were collected in a standard glass counting vial and assayed for radioactivity by Cerenkov counting. When necessary, the optical density (260 mμ) of the same solution was measured in a spectrophotometer.

Hydroxyapatite Determination of Reassociation Kinetics[2]

Nucleic acids in 0.14 M PB were denatured at 100°C for 3–5 min and incubated at 60°C for specified times. At the end of the incubation period the sample (or a fraction of it) was passed through a hydroxyapatite column equilibrated to 60°C and 0.14 M PB. After washing single-stranded DNA from the column, the DNA adsorbed to hydroxyapatite could be recovered by thermal elution at 100°C in 0.14 M PB, or high salt (0.4 M PB) elution at 60°C. DNA recovered by high salt elution retains its double-stranded form. The amount of DNA in the reassociated and unreassociated fractions was then measured.

RESULTS

Table I presents data which show that in the presence of *E. coli* rRNA the quantity of *E. coli* DNA adsorbing to hydroxyapatitie did not fall below 0.27% during the cycles of purification described in Methods. Where no rRNA was present the fraction of *E. coli* DNA recovered fell to 0.01% after only three cycles of purification. The reaction of chicken rRNA with the *E. coli* DNA resulted in a level of recovery of DNA almost identical to the value obtained when rRNA was absent. This demonstrates the specificity of the reassociation reaction. Control or blank values were determined for each different ^{32}P-DNA preparation and varied from 0.01% to 0.02% of the input radioactivity.

TABLE I

	Cycle	^{32}P-DNA C_0t	rRNA C_0t	Per cent of original input ^{32}P-DNA adsorbed
A	1	0.08	0.25 (*E. coli* RNA)	2.37
	2	0.0008	0.25 " "	0.307
	3	1.5×10^{-5}	0.25 " "	0.276
	4	1.5×10^{-5}	0.25 " "	0.265
B	1	0.08	0	2.1
	2	0.0008	0	0.029
	3	7×10^{-6}	0	0.01
C	1	0.033	0.3 (chicken RNA)	1.1
	2	0.0002	0.3 " "	0.038
	3	9×10^{-6}	0.3 " "	0.017

Data showing the fraction of original input ^{32}P-*E. coli* DNA adsorbing to hydroxyapatite when reacted (*A*) with *E. coli* rRNA, (*B*) in the absence of any rRNA, (*C*) with chicken rRNA. The reassociation reactions were performed as described in Methods.

Table II shows the quantity of DNA recovered with increasing rRNA C_0t. These "saturation" experiments indicate that the quantity of DNA complementary to rRNA under these conditions is about 0.27% for *E. coli* and about 0.35% for *Proteus mirabilis*. The value for the fraction of *E. coli* DNA which is complementary to rRNA agrees well with the values obtained by other investigators using the reaction of *labeled rRNA* with *unlabeled DNA* (1).

The data in Table III show that isolated rR-cistrons from *E. coli* and *Proteus mirabilis* reassociate almost completely with their respective DNA's and rRNA's. Further, 94% of the *E. coli* rR-cistrons reassociates with a mixture of MAK purified 16S and 23S *E. coli* rRNA subunits. This high percentage of reaction indicates that little if any DNA which codes for 5S or transfer RNA is present in the rR-cistron preparation. The 6% of the radioactivity which did not reassociate has not been characterized. About 3% of the *E. coli* rR-cistron radioactivity adsorbed to hydroxyapatite when reacted with a rRNA preparation which was first hydrolyzed in

0.2 N NaOH for 1 hr at 60°C. Approximately 3% of the rR-cistron radioactivity also adsorbs to hydroxyapatite when the preparation is denatured and passed through hydroxyapatite in the absence of rRNA.

Assuming that only complete chromosomes existed in these cells at the time of DNA isolation, *E. coli*, with a DNA content of 2.8×10^9 daltons (9) contains enough DNA complementary to rRNA for about five separate rR-cistrons per chromosome. *Proteus mirabilis* also has a DNA content of about 2.8×10^9 daltons[1] and apparently contains about six separate rR-cistrons per chromosome. These numbers are calculated using values of 1.1×10^6 and 0.55×10^6 daltons for the 23S and 16S rRNA subunits (10). Moore and McCarthy (11) have reported that 0.18% of *E. coli* DNA is complementary to 23S rRNA. This is enough DNA for five separate copies of the nucleotide sequence coding for 23S rRNA. This value and data reported here and elsewhere (1) suggest that the number of 16S cistrons is equal to the number of 23S cistrons.

TABLE II

	rRNA C_0t	Per cent of original input DNA recovered (corrected for blank)
E. coli	0	0.00 (blank = 0.01)
	0.12	0.274
	0.25	0.266
	0.5	0.261
Proteus mirabilis	0	0.00 (blank = 0.018)
	0.11	0.36
	0.11	0.35
	0.38	0.36

The fraction of original input ^{32}P-DNA adsorbed to hydroxyapatite with increasing rRNA C_0t. Each value represents the fraction adsorbed after three cycles of purification. The approximate one-half C_0t for the reaction of rRNA with rR-cistrons is about 0.008.

Thermal Stability of Reassociated DNA

The thermal stability of reassociated DNA indicates the accuracy of base-pair matching between component strands of DNA (12). Native DNA is assumed to have perfect base-pair matching. Since the thermal stability of reassociated and native DNA are virtually the same (12), perfect or near-perfect base-pair matching should exist also between component strands of the reassociated DNA.

Hydroxyapatite has been utilized to measure the thermal stability of native (13) and reassociated DNA (12, 14). Fig. 1 presents a hydroxyapatite thermal elution profile for *E. coli* rRNA:DNA hybrids. These hybrids were formed by allowing

radioactive rRNA cistrons to reassociate with a mixture of unlabeled 16S and 23S rRNA subunits. The Tm of the rRNA-DNA hybrids (Fig. 1) is about 10°C lower than the Tm of reassociated bulk *E. coli* DNA measured under the same conditions (Fig. 2, dashed curve). *Proteus mirabilis* rRNA:DNA hybrids have a similar Tm. Other investigators also have reported RNA:DNA hybrids to have a lower thermal stability than double-stranded DNA of the same base composition (11, 15, 16).

TABLE III

Source of rR-cistrons	Per cent reaction of rR-cistrons with		Mixture of purified 16S and 23S rRNA subunits
	Homologous rRNA	Homologous DNA	
E. coli	96	96.5	94
Proteus mirabilis	96	96.5	—

The extent of reassociation of preparations of ^{32}P-rR-cistrons with various preparations of unlabeled nucleic acids. The rRNA C_0t for these reactions was greater than 0.2. The DNA C_0t utilized in each case was sufficient for the reassociation of greater than 95% of the unlabeled DNA. All reactions were carried out at 60°C in 0.14 M PB. *E. coli* rRNA subunits were purchased from Miles Laboratories (Elkhard, Ind.). Similar results were obtained using an alternate source of 16S and 23S rRNA subunits.

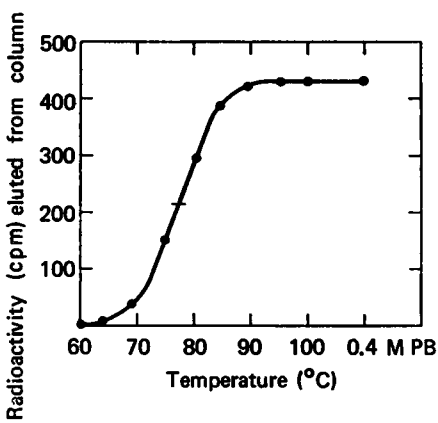

FIGURE 1 Thermal stability profile of RNA:DNA hybrids formed between a mixture of 16S and 23S rRNA subunits and ^{32}P-labeled rR-cistrons. The rRNA subunits were purchased from Miles Laboratories. A rRNA C_0t of 0.3 (120 μg rRNA/ml incubated for 12 min at 60°C in 0.14 M PB) was utilized for this reaction. 94% of the rR-cistrons reassociated with the rRNA. The thermal stability profile was performed as described in Methods.

Fig. 2 shows the thermal stability profile of reassociated *E. coli* rR-cistrons and bulk DNA. The Tm of the rR-cistrons is about one to two degrees lower than the Tm of the bulk DNA. A variety of factors influence the thermal stability of these reassociated rR-cistrons. *E. coli* rRNA, and hence its DNA complement, has a slightly greater guanine-cytosine content than bulk *E. coli* DNA (17). Reassociated rR-cistrons (55% guanine-cytosine) should have a Tm one to two degrees higher

than bulk DNA (52% guanine-cytosine) (18). This assumes, however, that perfect base-pair matching occurs between component strands of the reassociated rR-cistrons as it does for bulk *E. coli* DNA (12). Occurrence of base-pair mismatches in the reassociated rR-cistrons would reduce the Tm about 0.5 to 1°C per 1% mismatched bases (19, 20, 21). If the multiple rR-cistrons were not identical but were similar enough so that each cistron could react with another cistron's complement, the thermal stability of the reassociated rR-cistrons would be lower than that expected from their guanine-cytosine content.

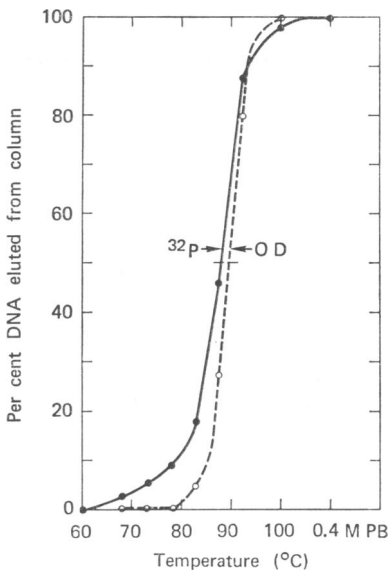

FIGURE 2 Thermal stability profile of reassociated unlabeled bulk *E. coli* DNA (dashed line) and reassociated radioactive *E. coli* rR-cistrons (solid line). A mixture of rR-cistrons (0.023 μg) and unlabeled bulk DNA (530 μg) was denatured and incubated in 0.5 ml for a DNA C_0t of 306. 94% of the radioactivity and 96% of the bulk DNA adsorbed to hydroxyapatite.

In order to interpret properly the data of Fig. 2 a correction must be made for the fact that the rR-cistrons are slightly damaged during the isolation procedure. ^{32}P-labeled bulk *E. coli* DNA, when put through the same processes of boiling, column fractionation and alkaline hydrolysis, had a Tm two to three degrees lower than the Tm of untreated bulk DNA with which it was reassociated. If these data are used to adjust the data of Fig. 2, the corrected Tm of the rR-cistrons is slightly higher than the Tm of bulk *E. coli* DNA. Such a high thermal stability indicates that perfect or nearly perfect base-pair matching is present in the reassociated rR-cistrons. The present data cannot rule out, however, the existence of a small amount (1% or less) of base-pair mismatching.

Whole DNA from *Proteus mirabilis* has a guanine-cytosine content of about 39% (18) while its rRNA contains about 54% guanine-cytosine (17). Thermal stability studies similar to those described above for *E. coli* show that reassociated *Proteus mirabilis* rR-cistrons exhibit a Tm 2–3°C higher than whole DNA (Fig. 3). This suggests that these isolated rR-cistrons have a higher guanine-cytosine content than

does whole DNA. The high thermal stability of the rRNA cistrons once again indicates that nearly all bases have paired properly with their complementary bases during reassociation. Again, the existence of a small amount of base-pair mismatching cannot be ruled out.

Since multiple rR-cistrons are known to exist in each bacterial cell the possible heterogeneity of rRNA has been an open question of evident importance. Isolation of these rR-cistrons has made it feasible to examine the possibility of rRNA heterogeneity. The thermal stability results presented here indicate that the population of rR-cistrons in a cell does not show the nucleotide sequence divergence typical of a "family" of repeated nucleotide sequences present in higher organism DNA's (12). The rR-cistrons in bacterial cells should be either (*a*) completely different from one

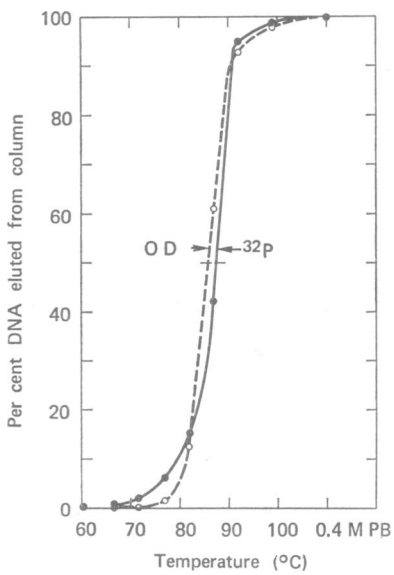

FIGURE 3 Thermal stability profile of reassociated unlabeled *Proteus mirabilis* bulk DNA (dashed line) and reassociated radioactive *Proteus mirabilis* rR-cistrons (solid line). A mixture of rR-cistrons (0.014 μg) and bulk DNA (400 μg) was denatured and then incubated in 0.3 ml for a C_0t of 277. 96% of both the radioactivity and bulk DNA adsorbed to hydroxyapatite.

another; (*b*) all identical, or nearly identical, to one another; or (*c*) a situation intermediate between (*a*) and (*b*) where, for example, three of the rRNA cistrons have identical nucleotide sequences and two are different. Reassociation kinetic studies make it possible to partially discriminate among these alternatives. These experiments are described in the next section.

Kinetics of Reassociation of rRNA Cistrons

Virtually all nucleotide sequences in bacteria occur only once per cell (12). If the five separate rR-cistrons of *E. coli* each represent a different nucleotide sequence, occurring once per cell, such cistrons would be expected to reassociate at the same rate as bulk *E. coli* DNA. If, however, these separate rR-cistrons represent one nucleotide sequence repeated five times in each cell, they would be expected to re-

associate five times faster than bulk *E. coli* DNA. Intermediate cases, while more complex in nature, would fall between the two extremes. If, for example, four similar and one different rR-cistrons were present they would reassociate somewhat less than four times faster than bulk *E. coli* DNA.

Hydroxyapatite has been utilized to determine the rate of reassociation of rR-

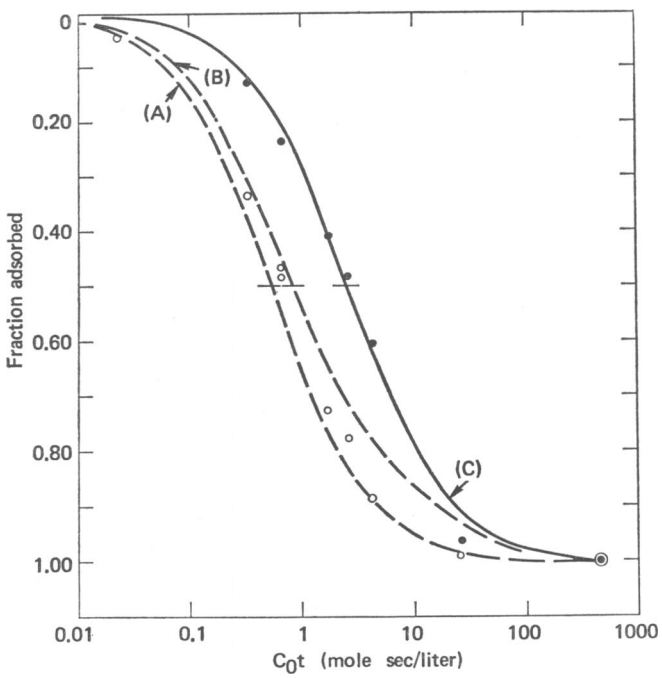

FIGURE 4 Reassociation kinetics of a mixture of radioactive *E. coli* rR-cistrons and *E. coli* unlabeled bulk DNA. Open circles represent the reassociation of ^{32}P-rR-cistrons while the closed circles represent the reassociation of bulk DNA. Curves (*A*) and (*B*) represent the theoretical second-order reaction curves calculated for situations where: (*A*) all of the rR-cistrons are identical, (*B*) four of the rR-cistrons are identical and one is different. The curve (*C*) drawn through the bulk DNA reassociation points (closed circles) is the theoretical second-order reaction curve calculated for a homogeneous DNA with a C_0t at one-half reassociation equal to that of the bulk DNA. Each point represents a separate incubation mixture which was denatured and incubated for a specific C_0t before passing it through hydroxyapatite. The lowest unlabeled DNA to rR-cistron ratio used for any incubation mixture was 6500/1. Incubation mixtures with a ratio of 30,000/1 or greater were used for the majority of points. Several different bulk DNA concentrations (18–880 μg/ml) were utilized for these experiments. Reassociation of the rR-cistrons was followed by monitoring the radioactivity. Reassociation of bulk DNA was followed by assaying for optical density at 260 mμ. The maximum observed reassociation of the radioactivity was 95.5% while that of the bulk DNA was 95.5%. At the C_0t utilized for this point (C_0t = 484) all of the DNA present in the reaction mixture should have reassociated (12). At zero time of incubation about 1.7% of the rR-cistron radioactivity adsorbed to hydroxyapatite. The data points on this curve have been corrected for the zero time adsorption and for the radioactive and optical density material which is incapable of reassociating.

cistrons relative to the rate of reassociation of bulk bacterial DNA. For these experiments radioactive rR-cistrons were mixed with nonradioactive whole bacterial DNA and the kinetics of reassociation of the two fractions determined. Measuring the reassociation kinetics of the mixture of rR-cistrons and bulk DNA should control the many variables which affect the rate of reassociation (12, footnote 2). It therefore should be possible to obtain a reasonably accurate value for the relative

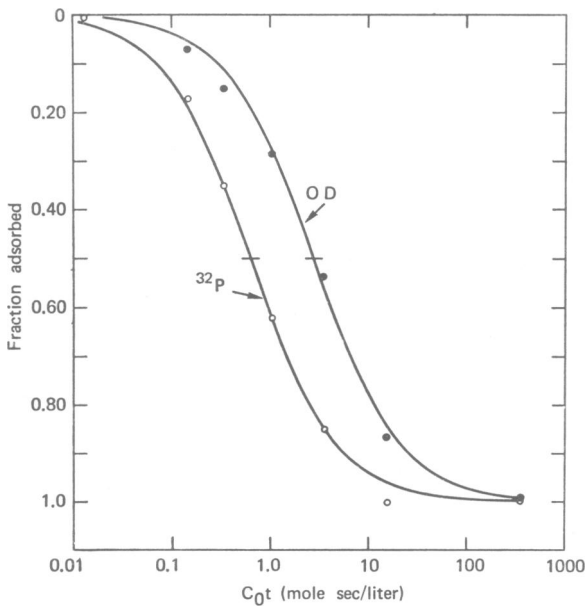

FIGURE 5 Reassociation kinetics of a mixture of radioactive *Proteus mirabilis* rR-cistrons and unlabeled *Proteus mirabilis* bulk DNA. Each point represents a separate incubation mixture which was denatured and incubated for a specific C_0t before passing it through hydroxyapatite. The curves drawn through these points are ideal second-order reaction curves. The lowest ratio of unlabeled DNA to rR-cistrons used for any incubation mixture was 1000/1. Incubation mixtures with a ratio of 20,000/1 or greater were used for the majority of points. Several different bulk DNA concentrations (8–1240 μg/ml) were utilized for these experiments. Reassociation of the rR-cistrons was measured by monitoring the radioactivity. Reassociation of bulk DNA was followed by assaying for optical density at 260 mμ. The maximum observed reassociation of the radioactivity DNA and bulk DNA was 96.5%. At zero time of incubation about 2% of the rR-cistron radioactivity adsorbed to hydroxyapatite. The data points on this figure have been corrected for zero time adsorption and for the fact that not all of the radioactive or optical density material is capable of reassociating.

rates of reassociation. The reassociation kinetics data have been plotted using the C_0t method of presentation (7, 12).

Fig. 4 shows the reassociation kinetics of *E. coli* rR-cistrons and bulk *E. coli* DNA. The curves drawn are for theoretical second-order reactions. The experimental points for the bulk DNA closely follows the ideal curve. *E. coli* rR-cistrons reassociate

about 3.6 times faster than bulk *E. coli* DNA. Fig. 5 presents a similar study on the reassociation of *Proteus mirabilis* rR-cistrons with unfractionated *Proteus mirabilis* DNA. In this case the rR-cistrons reassociate about 4.4 times more rapidly than bulk DNA.

The rate of reassociation of the rR-cistrons indicates that a large majority of the cistrons are sufficiently alike to reassociate together to form a stable double-stranded product. Thermal stability studies show that very nearly perfect base-pair matching exists between the component strands of the reassociated rR-cistrons. These results strongly indicate that the majority of the rR-cistrons in both *E. coli* and *Proteus mirabilis* are very similar to one another and that a high degree of homogeneity exists in the rRNA gene family.

The dashed curves on either side of the rR-cistron reassociation kinetic data (Fig. 4) are the theoretical second-order curves calculated for the reassociation of rR-cistrons when: (*a*) all are identical; (*b*) four are identical and one differs. The present data cannot discriminate between these and other alternatives. Measurements of the kinetics of reassociation of rRNA and rR-cistrons should make it possible to discriminate between the various possibilities. Such studies are now in progress.

Other investigators have studied the question of the heterogeneity of rRNA in bacteria. Aronson and Holowczyk (22), Doi and Igarashi (23), and Gould (24) have reported data which suggest that heterogeneous rRNA might be present in a bacterial cell. In each case, however, heterogeneity of the rRNA was only one of several possible interpretations of their data.

Comparison of rRNA Cistrons in Different Bacterial Species

Isolation of rR-cistrons of *E. coli* made possible the direct comparison between these rR-cistrons and those which exist in other species of bacteria. This was accomplished by reassociating small quantities of labeled *E. coli* rR-cistrons with large amounts of unlabeled DNA from other bacterial species. A time period long enough to ensure greater than 90% reassociation of the unlabeled DNA was used. Reassociated DNA then was separated from single-strand DNA by using hydroxyapatite. Thermal stability profiles of both reassociated bulk DNA and reassociated rR-cistrons were obtained by thermally eluting DNA off hydroxyapatite and assaying for radioactivity and optical density at 260 mμ. Thermal stability profiles for the unlabeled DNA served as a useful internal marker for determining the relative Tm of the DNA:DNA "hybrid" molecules. The bacterial species examined showed over 90% relatedness between their rR-cistrons. In addition, thermal stability profiles indicated a high precision of base-pair matching between component strands of the interspecies DNA:DNA hybrids (Figs. 2, 6, 7). Considerably less relatedness between these bacterial species was detected when the degree of relatedness between the whole DNA's was measured under incubation conditions identical to those used

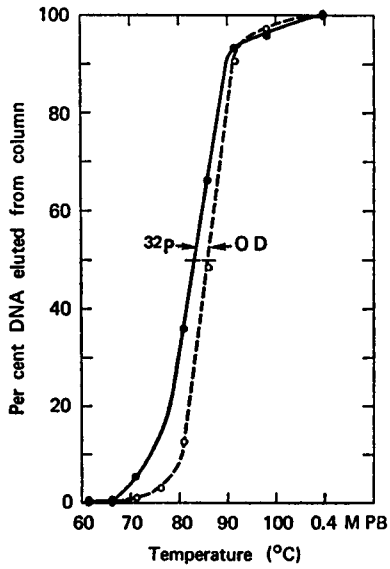

FIGURE 6 Thermal stability profile of DNA:DNA interspecies "hybrids" formed between ^{32}P-labeled *E. coli* rR-cistrons and unlabeled *Proteus mirabilis* bulk DNA. A mixture of rR-cistrons (0.006 μg) from *E. coli* and *Proteus mirabilis* bulk DNA (400 μg) was denatured and then incubated in 0.5 ml for a C_0t of 1350. 92% of the radioactivity and 97% of the bulk DNA adsorbed to the hydroxyapatite. The per cent relatedness of *E. coli* rR-cistrons to those of *Proteus mirabilis* is about 93% when normalized to the extent of reassociation of *E. coli* rR-cistrons with *E. coli* DNA.

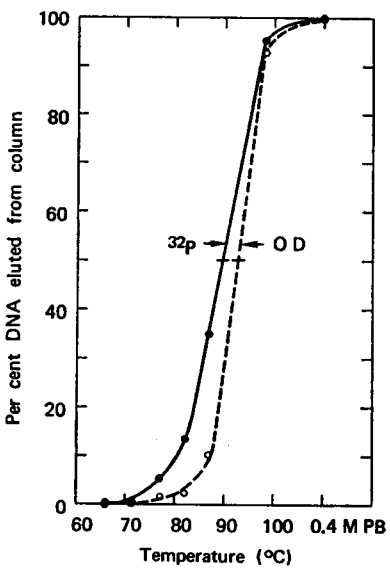

FIGURE 7 Thermal stability profile of DNA:DNA interspecies "hybrids" formed between ^{32}P-labeled *E. coli* rR-cistrons and unlabeled *Salmonella typhimurium* bulk DNA. A mixture of rR-cistrons (0.007 μg) from *E. coli* and unlabeled *Salmonella typhimurium* bulk DNA (360 μg) was denatured and then incubated in 1 ml for a C_0t of 511. 92% of the radioactivity and 97% of the bulk DNA adsorbed to the hydroxyapatite. The per cent relatedness of *E. coli* rR-cistrons to those of *Salmonella* is 96% when normalized to the extent of reassociation of *E. coli* rR-cistrons with *E. coli* DNA.

here. There is about 40% relatedness between *Salmonella typhimurium* and *E. coli* DNA's (25) while only about 4–6% relatedness can be detected between the DNA's of *E. coli* and *Proteus mirabilis*.[3] Further, thermal stability studies indicate that the nucleotide sequences which are held in common between these species are similar

[3] Brenner, D. J. 1968. Personal communication.

but not identical. The Tm of interspecies DNA:DNA hybrids formed between whole DNA's of *Salmonella typhimurium* and *E. coli* is about 9.0°C below that expected for perfect base-pair matching, while the Tm of *E. coli-Proteus mirabilis* DNA:DNA hybrids is about 14°C lower than that of reassociated *E. coli* DNA.

These data suggest that rR-cistrons apparently have been strongly conserved during bacterial evolution and that the great majority of nucleotide sequences have diverged at a much faster rate than the rR-cistrons. Such a conclusion, while not new information, serves to confirm in an independent manner the previous findings of several other investigators (11, 26, 27, 28).

The thermal stability data indicate that some divergence has occurred between the rR-cistrons of bacterial species. This is most striking in the case of the comparison of *E. coli* rR-cistrons with those of *Proteus mirabilis* (Fig. 6). Moore and McCarthy have more extensive data concerning this point (11). Their data were obtained by reacting radioactive rRNA from one species with unlabeled DNA from other species and measuring the thermal stability profiles of the rRNA:DNA hybrids formed.

SUMMARY AND CONCLUSION

The isolation of bacterial rR-cistrons made it possible to study the heterogeneity of rRNA in bacterial cells. Kinetic measurements of the reassociation of rR-cistrons with bulk DNA and the thermal stability characteristics of the reassociated rR-cistrons indicated that the majority of rR-cistrons in a cell are very similar to one another.

The direct comparison of the rR-cistrons of *E. coli*, *Proteus mirabilis* and *Salmonella typhimurium* showed that the rR-cistrons of these bacteria are very similar and apparently have been strongly conserved.

These experiments further demonstrate the usefulness of the process of reassociation for studying nucleic acids. The basic technique of reacting labeled DNA with unlabeled RNA has broad application for RNA:DNA hybrid studies. It can be used to purify any specific DNA sequence for which sufficient product RNA can be obtained. Although specific messenger RNA fractions are very difficult to isolate, rRNA and transfer RNA are readily available. In addition, the method provides a very powerful tool for fractionating DNA on the basis of the expression of that DNA during the life cycle of a cell or an organism.

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REFERENCES

1. SPIEGELMAN, S., and S. A. YANKOFSKY. 1965. *In* Evolving Genes and Proteins. V. Bryson and H. J. Vogel, editors. Academic Press, Inc., New York.

2. BOLTON, E. T., and B. J. MCCARTHY. 1962. *Proc. Natl. Acad. Sci. U.S.* **48**:1390.
3. MARMUR, J. 1961. *J. Mol. Biol.* **3**:208.
4. BERNARDI, G. 1965. *Nature.* **206**:779.
5. BOLTON, E. T. 1966. *In* Procedures in Nucleic Acid Research. G. L. Cantoni and David R. Davies, editors. Harper and Row, New York.
6. OTAKA, E., H. MITSUI, and S. OSAWA. 1962. *Proc. Natl. Acad. Sci. U.S.* **48**:425.
7. BRITTEN, R. J., and D. E. KOHNE. 1966. *Carnegie Inst. Wash. Year Book.* **65**:78.
8. CLAUSEN, T. 1968. *Anal. Biochem.* **22**:70.
9. CAIRNS, J. 1963. *Cold Spring Harbor Symp. Quant. Biol.* **28**:43.
10. KURLAND, C. G. 1960. *J. Mol. Biol.* **2**:83.
11. MOORE, R. L., and B. J. MCCARTHY. 1967. *J. Bacteriol.* **94**:1066.
12. BRITTEN, R. J., and D. E. KOHNE. 1968. *Science.* **161**:529.
13. MIYAZAWA, Y., and C. A. THOMAS. 1965. *J. Mol. Biol.* **11**:223.
14. WALKER, P., and A. MCLAREN. 1965. *Nature.* **208**:1175.
15. CHAMBERLIN, M., and P. BERG. 1964. *J. Mol. Biol.* **8**:297.
16. BOLTON, E. T., and B. J. MCCARTHY. 1964. *J. Mol. Biol.* **8**:201.
17. MIDGLEY, J. E. 1962. *Biochim. Biophys. Acta.* **61**:513.
18. MARMUR, J., and P. DOTY. 1962. *J. Mol. Biol.* **5**:109.
19. KOTAKA, T., and R. L. BALDWIN. 1964. *J. Mol. Biol.* **9**:323.
20. BRITTEN, R. J. 1965. *Carnegie Inst. Wash. Year Book.* **64**:322.
21. BAUTZ, E. 1965. *In* Evolving Genes and Proteins. V. Bryson and H. J. Vogel, editors. Academic Press, Inc., New York.
22. ARONSON, A. I., and M. A. HOLOWCZYK. 1965. *Biochim. Biophys. Acta.* **95**:217.
23. DOI, R. H., and R. T. IGARASHI. 1966. *J. Bacteriol.* **92**:88.
24. GOULD, H. 1968. Symposium on Sero and Chemotaxonomy at the Birmingham University, Birmingham, England. In press.
25. BRENNER, D. J., and D. B. COWIE. 1968. *J. Bacteriol.* **95**:2258.
26. DOI, R. H., and R. T. IGARASHI. 1965. *J. Bacteriol.* **90**:384.
27. DUBNAU, D., I. SMITH, P. MORELL, and J. MARMUR. 1965. *Proc. Natl. Acad. Sci. U.S.* **54**:491.
28. TAKAHASHI, M., M. SAITO, and Y. IKEDYA. 1967. *Biochim. Biophys. Acta.* **134**:124.