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Deoxyribonucleic Acid–Ribonucleic Acid Hybridization

ANNEALING AND QUANTITATIVE RECOVERY OF INTACT RIBOSOMAL RIBONUCLEIC ACID MOLECULES FROM HYBRIDS

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A simple and efficient method for hybridization and subsequent recovery of non-fragmented ribosomal RNA from the hybrid is described. The procedure involves annealing of immobilized denatured DNA bound on cellulose nitrate membrane filters to complementary RNA in 50% (v/v) formamide–0·33M-potassium chloride–10mM-tris–hydrochloric acid buffer, pH 7·4, at 33° for 3hr. Under these conditions no detectable changes in the sedimentation coefficients of the input RNA were detected. The RNA can subsequently be recovered quantitatively from the hybrid in intact form by incubating the filters in formamide or in 85% (v/v) dimethyl sulphoxide. The applicability of the method for the evaluation of the absolute size of ribosomal RNA cistrons in *Escherichia coli* DNA and for the determination of the size of messenger RNA molecules is discussed.

Denatured DNA strands may interact with RNA molecules and with denatured DNA from the same or from different species (Marmur, Rownd & Schildkraut, 1963; Hall & Spiegelman, 1961). With DNA from two different species the amount of hybrid formed is a measure of base-sequence analogy. Likewise the extent of the reaction between DNA and RNA reflects similarity in base sequences between the reacting molecules. The detection and measurement of DNA–RNA hybrid molecules demands only radioactively labelled RNA. The method of choice for the detection of DNA–RNA hybrids is that of Gillespie & Spiegelman (1965), which depends on the selective adsorption of DNA–RNA hybrids on cellulose nitrate membrane filters. RNA and double-stranded DNA are not retained. The criterion of hybrid formation is the resistance of the hybrid to ribonuclease.

DNA–RNA hybridization has been applied to the identification of different RNA species from bacterial and, more recently, animal cells. Application of these techniques has yielded important information on the DNA cistrons involved in the synthesis of mRNA* (Kennell, 1968), rRNA (Yankofsky & Spiegelman, 1962), tRNA (Giacomoni & Spiegelman, 1962) and 5s RNA (Morell, Smith, Dubnau & Marmur, 1967) and on the sequential synthesis of mRNA in bacteria infected with bacteriophages (Bolle, Epstein, Salser & Geiduschek, 1968). In all these studies the high specificity of the hybridization technique has been stressed. The DNA–RNA hybridization technique, however, may not be entirely free of shortcomings. The chemical basis of the method has not been clarified, the reactions are not usually quantitative, the resistance of DNA–RNA hybrids to ribonuclease is not absolute and care must be taken to perform the enzyme treatment under special conditions. The most serious objection is that the length of the RNA chains involved in hybrid formation is much shorter than that of the molecules added, this being due to the breakdown of phosphodiester bonds of the input RNA during annealing, which involves prolonged thermal treatment. It has been pointed out that the shorter the chain involved, the higher the hazard of non-specific hybridization (Hadjiolov, 1967), and that with fragmented RNA annealed to DNA the determination of the absolute size of the corresponding DNA cistrons is not feasible (Attardi, Huang & Kabat, 1965). These problems can only be resolved by finding conditions for hybridization and subsequent recovery of intact RNA molecules. The development of such a procedure would confirm or eliminate the objections raised and should allow one to bring the level of site recognition in DNA up to the size of the original intact RNA.

In this paper we describe a simple and efficient method for hybridization of non-fragmented rRNA molecules and a quantitative recovery of intact RNA from the DNA–RNA hybrids.

* Abbreviations: mRNA, messenger RNA; rRNA, ribosomal RNA; tRNA, transfer RNA; SSC, 0·15M-NaCl–0·015M-trisodium citrase, pH 7·0.

MATERIALS AND METHODS

Cells. Escherichia coli B was used.

Growth of bacterial cultures. The cells were grown with agitation at 37° in the glucose–salts medium of Davis & Mingioli (1950) supplemented with vitamin-free casamino acids (casein hydrolysate) (Difco, Detroit, Mich., U.S.A.). This contained (per l.): 7g. of K_2HPO_4, 3g. of KH_2PO_4, 1g. of $(NH_4)_2SO_4$, 0·1g. of $MgSO_4,7H_2O$, 5g. of trisodium citrate dihydrate, 0·2g. of D-glucose and 0·4g. of casamino acids.

Preparation of radioactively labelled stable RNA. To 100ml. of exponentially growing culture at a density of 2×10^7 cells/ml. [^{3}H]uracil [25μc (0·33μg.)/ml. of medium] (Schwarz BioResearch Inc., Orangeburg, N.Y., U.S.A.) was added. The cells were allowed to grow to a density of about 4×10^8 cells/ml. (the generation time was 55min.), so that only RNA stable for at least one generation contained the isotope. The cells were collected by centrifugation, washed in 10mM-tris–HCl buffer, pH7·4, containing 10mM-magnesium acetate and resuspended in 2·7ml. of the same buffer. Sodium dodecyl sulphate [0·3ml. of a 15% (w/v) solution] was added and the cells were disrupted at 4° for 25sec. in the 20kHz MSE ultrasonic disintegrator with an output of 60w with the 19mm. probe with a 9·5mm. tip. To the disrupted cells an equal volume of freshly distilled 90% (v/v) phenol was added. The mixture with phenol in stoppered glass centrifuge tubes was stirred in the cold for several minutes and centrifuged at 10000*g* for 10min. The upper aqueous layer was carefully collected, twice deproteinized with phenol and freed from phenol by repeated extractions with ether. The dissolved ether was removed by blowing air. The solution was made up to 10mM with respect to Mg^{2+} and incubated with 10μg. of ribonuclease-free deoxyribonuclease/ml. (Worthington Biochemical Corp., Freehold, N.J., U.S.A.). After this incubation the RNA was precipitated with 2 vol. of ice-cold 95% (v/v) ethanol in the presence of 0·1M-NaCl. The product was stored under ethanol at −20°. For the experiments the RNA was dissolved in the desired buffer solutions. Carrier RNA was prepared as described above, except that the cells were not radioactively labelled. In all steps involving RNA, care was taken to avoid contamination with ribonuclease by the exclusive use of dry-heat-sterilized glassware and by wearing plastic gloves.

Preparation of bacterial DNA. This was isolated by a method mainly derived from those of Marmur (1961) and Thomas, Berns & Kelly (1966). A 1l. batch of *E. coli* cells was grown overnight, the cells were collected by centrifugation and washed with standard saline–citrate medium (1×SSC). The washed cells were resuspended in 100ml. of 1×SSC containing 27% (w/v) ribonuclease-free sucrose (Mann Research Laboratories, New York, N.Y., U.S.A.) and 1mg. of Pronase/ml. (B grade, self-digested at 37° for 2hr.; Calbiochem, Los Angeles, Calif., U.S.A.). Solid sodium dodecyl sulphate was added to give a final concentration of 0·2% (w/v), and the mixture was incubated overnight at 37°. To the lysed suspension an equal volume of chloroform–3-methylbutan-1-ol (24:1, v/v) was added and the mixture was gently shaken at room temperature for 20min. The resulting emulsion was separated into three layers by a 15min. centrifugation at 10000*g* in the Servall RC-2 centrifuge. The upper aqueous layer, which contained the nucleic acids, was carefully removed with a wide-tipped pipette and the deproteinization step with chloroform–3-methylbutan-1-ol was repeated twice. After three cycles of deproteinization the nucleic acids were precipitated by gently layering 2 vol. of ethanol on the aqueous phase. The layers were gently mixed with a glass rod and the precipitated DNA fibres were spooled on to the rod. Excess of liquid was drained off the DNA by pressure against a glass surface. The DNA was dissolved in a minimum volume of 0·1×SSC or in 0·016M–KCl–10mM-tris–HCl buffer, pH7·4, by occasional gentle stirring of the solution at 4° overnight or at 37° for 2hr., and adjusted to standard saline–citrate concentration (1×SSC) or to 0·16M-KCl–10mM-tris–HCl, pH7·4, by adding concentrated saline–citrate (12×SSC) or 3·3M-KCl in 10mM-tris–HCl, pH7·4, respectively. A 0·1% (w/v) solution of pancreatic ribonuclease (EC 2.7.7.16) (Sigma Chemical Co., St Louis, Mo., U.S.A.) was heated to 80° and kept at this temperature for 10min. to destroy any deoxyribonuclease activity present. Enough of the cooled solution was added to the DNA solution to bring the final ribonuclease concentration to 25μg./ml. The solution was incubated at 37° for 1hr. After this incubation self-digested Pronase was added to bring the final Pronase concentration to 100μg./ml. and the incubation continued for 1hr. The solution was then shaken as before with an equal volume of chloroform–3-methylbutan-1-ol for 15min., centrifuged and the aqueous layer was removed. The aqueous layer was then deproteinized twice with chloroform–3-methylbutan-1-ol as described. The aqueous layer obtained after the third deproteinization was precipitated with ethanol, the precipitated DNA fibres were spooled on to the rod as described above and the drained DNA was dissolved in 0·1×SSC or in 0·016M-KCl–10mM-tris–HCl buffer, pH7·4. The final product was adjusted to a concentration of 50μg. of DNA/ml. and stored in solution at 4° with the addition of a few drops of chloroform.

Denaturation of DNA. Native DNA preparations (50μg./ml.) were denatured by boiling the solution for 20min. and instantaneous cooling to 0° by immersing the heated DNA solution in an acetone–solid CO_2 bath. The denatured DNA solution was adjusted to 2×SSC or to 0·33M-KCl–10mM-tris–HCl, as described above.

Immobilization of denatured DNA on cellulose nitrate membrane filters. This was done essentially by the method of Gillespie & Spiegelman (1965). Denatured DNA solutions in 2×SSC or in 0·33M-KCl–10mM-tris–HCl buffer, pH7·4, were passed through cellulose nitrate membrane filters (Millipore type HA, 25mm. radius, 0·45μm. pore width) presoaked in 2×SSC or in 0·33M-KCl–10mM-tris–HCl buffer, and washed with 10ml. of the appropriate buffer. Filters carrying 100, 50, 20, 10 and 2μg. of immobilized DNA were prepared. A large number of Millipore membrane filters without DNA were treated in a similar manner. These were used for the estimation of non-specific binding of RNA to filters in the hybridization reaction. The filters with bound DNA and those without DNA were dried overnight at room temperature and then at 80° for 4hr. over KOH in an evacuated desiccator. The immobilized denatured DNA samples irreversibly bound on the dry cellulose nitrate membrane filters were stored over KOH in an evacuated desiccator at 4°. The amount of DNA bound to the filters was determined as described by Pigott & Midgley (1968).

Hybridization of DNA with RNA. Procedure (A). Hybridization of DNA with RNA was carried out essentially

as described by Gillespie & Spiegelman (1965). Immobilized heat-denatured DNA bound on cellulose nitrate membrane filters was placed in scintillation vials and incubated with various amounts of radioactively labelled stable RNA in 0·5ml. (final vol.) of 2×SSC at 66° for 20hr. The filters were removed from the hybridization fluid and each side was washed with 10ml. of 2×SSC by suction filtration. The filters were then immersed in 1ml. of 2×SSC containing 25μg. of heat-treated ribonuclease/ml. and kept at 37° for 1hr. After treatment with ribonuclease, the vials were chilled and the filters were again washed, each side with 60ml. of 2×SSC. Finally the filters were dried and counted.

Procedure (B). Cellulose nitrate filters with bound heat-denatured DNA were incubated with various amounts of radioactively labelled stable RNA in 30% (v/v) formamide (British Drug Houses Ltd., Poole, Dorset) in 1×SSC (final vol. 0·5ml.). Incubation was at 4° for 20hr. The filters were then treated as described above.

Procedure (C). Cellulose nitrate filters with bound heat-denatured DNA were incubated with various amounts of radioactively labelled stable RNA in 50% (v/v) formamide–0·33M-KCl–10mM-tris-HCl buffer, pH7·4 (final vol. 0·5ml.). Incubation was at 33° for 3hr. The filters were removed from the annealing mixture and each side was washed with 10ml. of 0·33M-KCl–10mM-tris–HCl buffer, pH7·4. The filters were incubated in 1ml. of the above buffer containing 25μg. of heat-treated ribonuclease/ml. at 37° for 1hr. The filters were then again washed, each side with 60ml. of the 0·33M-KCl–10mM-tris–HCl buffer. Finally the filters were dried and counted. In experiments in which the ribonuclease step was omitted, the filters were washed, each side with 100ml. of 0·33M-KCl–10mM-tris–HCl buffer.

Incubation of RNA without DNA filters. Radioactively labelled stable RNA (250μg.) from *E. coli* was incubated with membrane filters without DNA under the following conditions: (1) in 0·5ml. of 2×SSC at 66° for 20hr.; (2) in 0·5ml. of 30% (v/v) formamide–1×SSC at 4° for 3hr. and 20hr.; (3) in 0·5ml. of 50% (v/v) formamide–0·33M-KCl–10mM-tris–HCl buffer, pH7·4, at 33° for 3hr. After the incubation the nucleic acids in the solution were precipitated by adding 2vol. of ice-cold ethanol. After standing at −20° for 2hr. the precipitated nucleic acids were collected by centrifugation and dissolved in the minimum volume of 10mM-tris–HCl buffer, pH7·4, containing 1mM-EDTA and 0·1M-NaCl, again precipitated with ethanol and stored under ethanol at −20°. For sedimentation analysis, the RNA was recovered by centrifugation, dissolved in the above buffer and adjusted to give 15000–30000c.p.m./0·1ml.

Recovery of RNA from DNA–RNA hybrids. Procedure (1). Six cellulose nitrate membrane filters with 50μg. of immobilized heat-denatured DNA were each annealed with 250μg. of radioactively labelled stable RNA in 50% (v/v) formamide–0·33M-KCl–10mM-tris–HCl buffer, pH7·4 (final vol. 0·5ml.), at 33° for 3hr. as described above. Three filters were treated with ribonuclease and the remaining filters were used as such. All filters were washed, each side with 100ml. of 0·33M-KCl–10mM-tris–HCl buffer, pH7·4. The washed dry filters were immersed in 0·5ml. of 10mM-tris–HCl buffer, pH7·4, containing 1mM-EDTA and 0·1M-NaCl. Then 3ml. of dimethyl sulphoxide (Fluka A.-G., Buchs SG, Switzerland), kept under alum–sodium silicate beads (molecular sieve type 4A, 4–8 mesh; British Drug Houses Ltd.), was added and the mixture was incubated at 37° for 18min. The filters were then removed from the incubation mixture, dried and counted for remaining radioactivity. Non-radioactive carrier RNA (200μg.) was then added to all samples as internal standard for 23s and 16s rRNA and 4s tRNA. The nucleic acids in the solution were recovered by two ethanolic precipitations as described above. The final product was dissolved in 0·1ml. of 10mM-tris–HCl buffer, pH7·4, containing 1mM-EDTA and 0·1M-NaCl.

Procedure (2). Hybridization and subsequent processing of the filters including ribonuclease treatment, were as described above. The washed and dried filters with DNA–RNA hybrids were each immersed in 3ml. of formamide. Incubation was at 25° for 30min. The filters were removed, dried and their radioactivities counted and 200μg. of carrier RNA was added to each sample. The nucleic acids were recovered from formamide by adding 0·1vol. of 10% (w/v) NaCl and 2vol. of ice-cold ethanol, and processed as described above.

Procedure (3). Hybridization was as described above. The filters with hybrids were immersed in 0·1×SSC and heated at 85° for 3–4min. The eluted RNA was recovered by ethanolic precipitation.

Determination of DNA and RNA. Concentrations of nucleic acids were calculated from measurements of E_{260} in silica cuvettes of 1cm. light-path in the Beckman DU spectrophotometer. The extinction coefficient was taken as $E^{1\%}_{1cm.}$ 200.

Measurement of radioactivity. In all experiments the samples were counted in a three-channel Tri-Carb liquid-scintillation spectrometer (model 3310; Packard Instrument Co. Inc.). In the filter method, hybrids were detected by the retention of radioactive RNA–DNA complexes on the filter. The total input of RNA (μg.) was estimated from its radioactivity on a Whatman no. 1 filter paper and its specific radioactivity. In our experiments radioactivities of labelled RNA on filter paper and in the form of a hybrid complex on cellulose nitrate membrane filter were counted with the same efficiency ±10%. Washed filters containing no DNA were incubated with the same quantities of labelled stable RNA as in the experiments proper and subjected to identical hybridization and washing procedures. The radioactivities of the filters were counted and served as blanks for the calculation of the degree of RNA–DNA hybrid formation, i.e. for the correction of the results for non-specific RNA binding. Radioactivities of samples from sucrose-density-gradient centrifugations were counted in the form of ethanol-insoluble material on filter papers.

Counting was done in 10ml. of a scintillation fluid containing 0·4% of 2,5-diphenyloxazole and 0·01% of 1,4-bis-(5-phenyloxazol-2-yl)benzene in toluene.

Sedimentation of RNA in linear sucrose gradients. Linear sucrose gradients (5–20%, w/v) in 10mM-tris–HCl buffer, pH7·4, containing 1mM-EDTA and 0·1M-NaCl were used. For analytical purposes, gradients of volume 4·4ml. were run in the Spinco model L ultracentrifuge (SW 39 rotor). Samples (0·1ml.) of RNA in 10mM-tris–HCl buffer, pH7·4, containing 1mM-EDTA and 0·1M-NaCl were centrifuged in sucrose gradients at 4° for 4·5hr. From each gradient 24–30 fractions (0·15ml.) were collected. Each fraction was made up to 1ml. with water and the E_{260} and the radioactivity were determined.

RESULTS

A method for hybridization and elution of intact RNA molecules should meet the following requirements. In all steps the size of the input RNA molecules should remain unaltered. Conformational changes that the RNA molecules undergo during annealing to and dissociation from homologous DNA segments should be reversible so that the RNA after isolation from the hybrid should be recognizable by its original sedimentation coefficient. The elution of RNA from the hybrid should be quantitative.

Annealing conditions. RNA molecules having extensive secondary structure will not hybridize until their own 'melting' temperature is approached (Gillespie & Spiegelman, 1965), or their secondary structure is sufficiently disrupted by formamide at low ionic strength (Bonner, Kung & Bekhor, 1967). The fate of rRNA after incubation under the above hybridization conditions is illustrated in Figs. 1(*a*) and 1(*b*). When the rRNA was heated at 66° for 20hr. it became extensively degraded and sedimented at about 8s (Fig. 1*a*). Incubation of rRNA in 30% (v/v) formamide–1 × SSC at 4° for 3hr. resulted in an almost quantitative disappearance of the 23s component. The rRNA now sedimented as a single peak at 16s with a slight shoulder at 23s (Fig. 1*b*). Figs. 1(*c*) and 1(*d*) show that after incubation of rRNA in formamide alone and in 1 × SSC the 23s component of rRNA remained unaltered. Hence concerted action of formamide and SSC was required for the conversion of the 23s RNA into the 16s RNA. We propose that this conversion is brought about by citrate after the 23s RNA has lost its secondary structure in the presence of formamide. This explanation is based on the observation of Rodgers (1966), who showed that 23s rRNA of *E. coli* could be converted into 16s RNA at low ionic strength by removal of Mg^{2+}. We have therefore attempted to replace the SSC in the annealing solution. An incubation mixture was

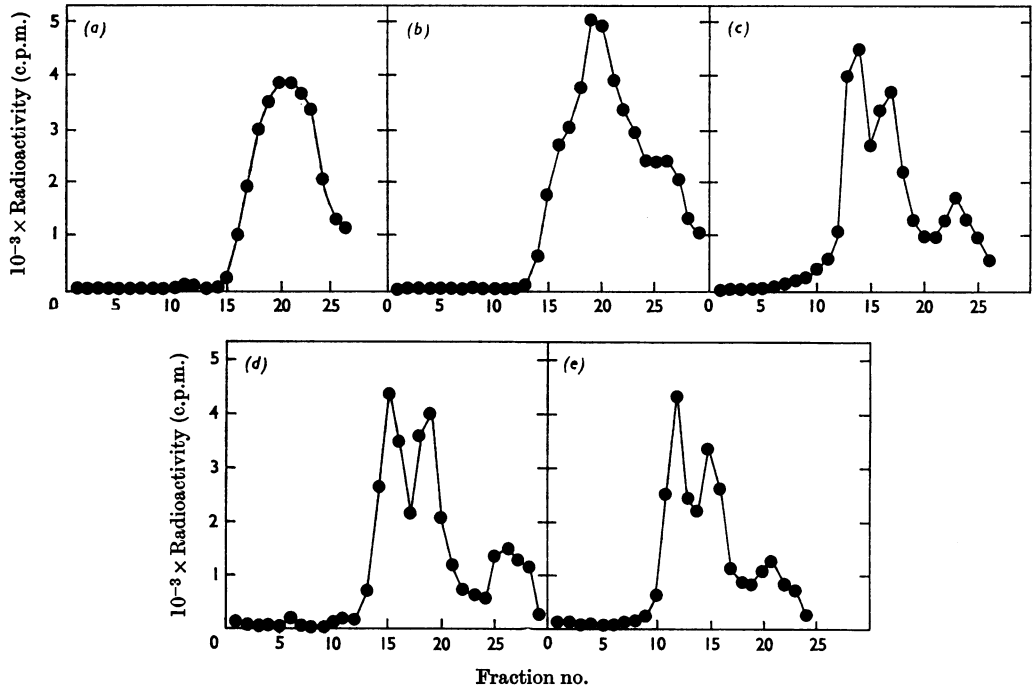

Fig. 1. Sedimentation analyses of radioactively labelled stable *E. coli* RNA after incubation under various annealing conditions. The RNA was incubated in: (*a*) 2 × SSC at 66° for 20hr.; (*b*) 30% (v/v) formamide–1 × SSC at 4° for 3hr.; (*c*) 30% (v/v) formamide at 4° for 3hr.; (*d*) 1 × SSC at 4° for 3hr.; (*e*) 50% (v/v) formamide–0·33M-KCl–10mM-tris–HCl buffer, pH7·4, at 33° for 3hr. After incubation the RNA was recovered by two ethanolic precipitations, dissolved in 10mM-tris–HCl buffer, pH7·4, containing 1mM-EDTA and 0·1M-NaCl. Samples (0·1ml.) were sedimented in sucrose gradients (5–20%, w/v) in the same buffer at 4° for 4hr. at 37 000rev./min. in the Spinco model L ultracentrifuge (SW39 rotor). The gradient was fractionated and the radioactivity was determined as described in the Materials and Methods section.

finally adopted in which standard SSC was replaced by 0·165 M-potassium chloride buffered with tris, and formamide concentration was increased to 50% (v/v). Incubation of rRNA in the system consisting of 50% (v/v) formamide–0·33 M-potassium chloride–10 mM-tris–hydrochloric acid buffer, pH 7·4, at 33° for 3 hr. caused no detectable change in the sedimentation behaviour of the input RNA. Sucrose-density-gradient analysis of *E. coli* stable RNA after incubation in the above system is illustrated in Fig. 1(*e*).

DNA–RNA hybridization in the formamide–potassium chloride–tris system. From the results of the experiments described above it follows that, when the two subspecies of rRNA are incubated in a system consisting of 50% (v/v) formamide–0·33 M-potassium chloride–10 mM-tris–hydrochloric acid buffer, pH 7·4, at 33° for 3 hr., their original sedimentation coefficients remain unaltered. Thus preliminary conditions for hybridization of intact RNA were at hand. Next we carried out experiments to determine whether RNA molecules will hybridize under these conditions and, if they do, to compare the hybridization efficiency and the saturation curve in this system with those observed after thermal annealing and after incubation in the 30% (v/v) formamide–SSC system.

The hybridization reaction was carried out over a wide range of RNA/DNA input ratios. In one set of experiments the hybrids were treated with ribonuclease and in another set the ribonuclease step was omitted. Immobilized denatured *E. coli* DNA bound on cellulose nitrate membrane filters was placed in scintillation vials and incubated with various amounts of radioactively labelled stable RNA from *E. coli* under the following conditions: (*a*) in 2 × SSC at 66° for 20 hr.; (*b*) in 30% (v/v) formamide–1 × SSC at 4° for 20 hr.; (*c*) in 50% (v/v) formamide–0·33 M-potassium chloride–10 mM-tris–hydrochloric acid buffer, pH 7·4, at 33° for 3 hr. After annealing, the filters were processed by using the procedures described in the Materials and Methods section. The types of hybrid formed are shown in Fig. 2. From the results of the experiments the following conclusions were drawn. Under all annealing conditions studied saturation of homologous DNA with stable RNA species in hybrids resistant to ribonuclease occurs when approx. 0·5% of the DNA is occupied. This value is comparable with previous estimates of the total number of sites specific for rRNA and tRNA (Yankofsky & Spiegelman, 1962; Giaconomi & Spiegelman, 1962). The fraction of RNA that can hybridize with a given homologous DNA is not only a function of the RNA/DNA input ratios, but is strongly influenced by annealing conditions. At any given RNA/DNA ratio the highest hybridization efficiency was found after thermal annealing, the next highest after annealing in the 50% formamide–0·33 M-potassium chloride–10 mM-tris–hydrochloric acid buffer and the lowest after annealing in the 30% formamide–1 × SSC system. Thus at saturation point the input RNA in the form of complexes with DNA, by thermal treatment, annealing in 50% formamide–0·33 M-potassium chloride–10 mM-tris–hydrochloric acid buffer and in 30% formamide–1 × SSC, was 1% (RNA/DNA ratio 0·3:1), 0·16% (RNA/DNA ratio 3:1) and 0·01% (RNA/DNA ratio 20:1) respectively.

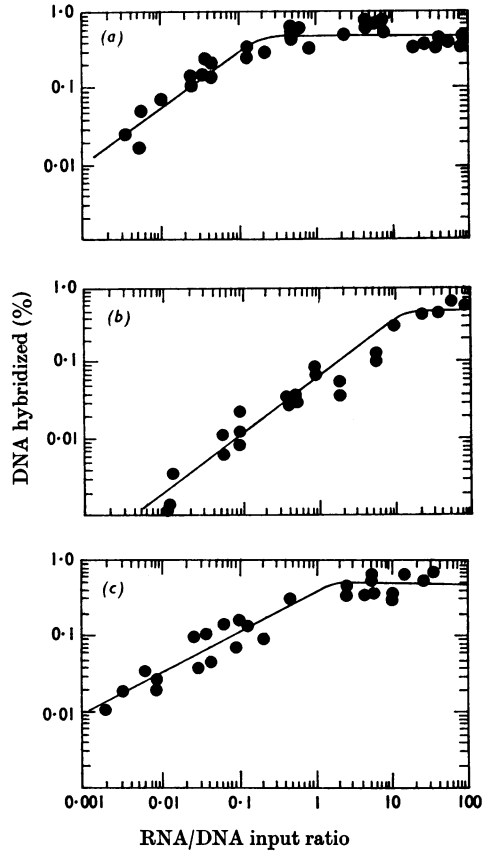

Fig. 2. Titration of immobilized denatured *E. coli* DNA bound on cellulose nitrate membrane filters with RNA of *E. coli* containing radioactivity only in stable RNA. The percentage of DNA hybridized is plotted against the RNA/DNA input ratio. Hybridization was in (*a*) 2 × SSC at 66° for 20 hr.; (*b*) 30% (v/v) formamide–1 × SSC at 4° for 20 hr.; (*c*) 50% (v/v) formamide–0·33 M-KCl–10 mM-tris–HCl buffer, pH 7·4, at 33° for 3 hr. The reaction vials contained filters with the following amounts of DNA: at RNA/DNA ratios 0·001–0·01:1, 100 μg.; at RNA/DNA ratios 0·01–0·5:1, 50 μg.; at RNA/DNA ratios 0·5–7·5:1, 20 μg.; at RNA/DNA ratios 7·5–20:1, 10 μg.; at RNA/DNA ratios 20–100:1, 2 μg.

Similar experiments on hybridization in 50% formamide–0·33 M-potassium chloride–10 mM-tris-hydrochloric acid buffer, pH 7·4, in which the ribonuclease step was omitted showed that the treatment with ribonuclease was essential to obtain reproducible values of saturation of genes for stable RNA. When the ribonuclease step was omitted, the amount of radioactively labelled RNA retained on the filters was 2–3 times that retained on the filters after digestion with the enzyme. In eight experiments the average value for the fraction of DNA in the form of complexes with RNA at saturation was 1·1%, but it was as high as 2·5% in one and as low as 0·6% in another experiment.

Recovery of intact RNA from DNA–RNA hybrids. The hybridization reaction was carried out at an RNA/DNA ratio 5:1 in the system consisting of 50% (v/v) formamide–0·33 M-potassium chloride–10 mM-tris–hydrochloric acid buffer, pH 7·4, at 33° for 3 hr. After annealing the filters were washed, by using the procedures described in the Materials and Methods section, and divided into two batches. The hybrids in one batch were digested with ribonuclease; in the other batch the ribonuclease step was omitted. The RNA from the hybrids was eluted by several procedures causing the disruption of the secondary structure of polynucleotides and interfering with the stacking of bases: (1) incubation in 85% (v/v) dimethyl sulphoxide at 37° for 18 min.; (2) incubation in formamide at 25° for 30 min.; (3) heating at 85° for 3–4 min. in 0·1 × SSC and quick cooling. Radioactively labelled RNA was eluted from the filters almost quantitatively by all the procedures used. More than 90% of the hybridized RNA was recovered after ethanolic precipitation of the eluate from filters that were not subjected to digestion with ribonuclease. In experiments in which the hybrids were treated with ribonuclease the radioactivity retained on the filters after elution was also less than 10%. However, only 20–50% of the RNA originally in the form of complexes with DNA was recovered by ethanolic precipitation. These results show that ribonuclease digested 50–80% of the RNA originally bound in the form of hybrid. Sedimentation analysis of RNA eluted from hybrids by incubation in formamide is illus-

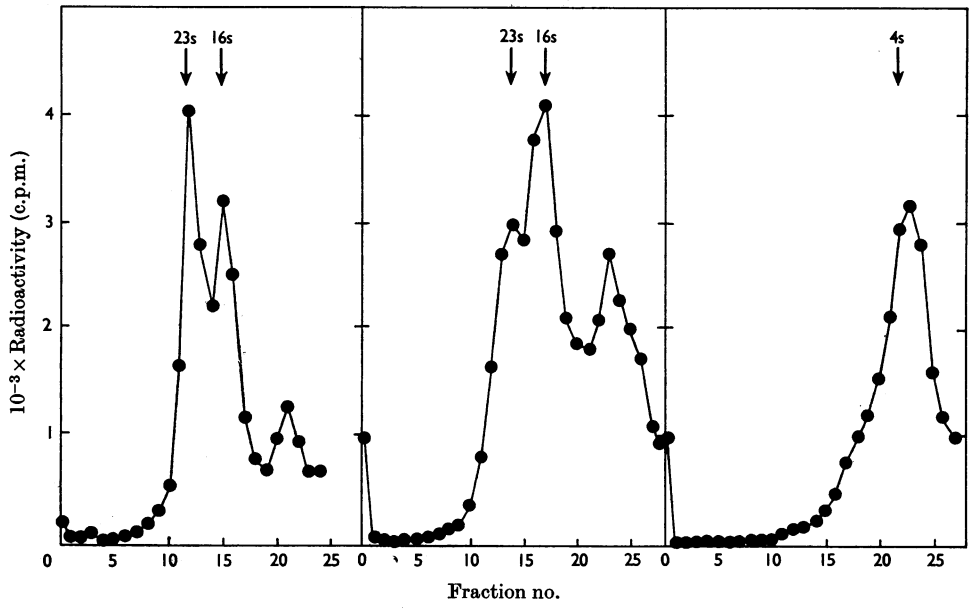

Fig. 3. Sedimentation analysis of radioactively labelled stable RNA of *E. coli* after elution from DNA–RNA hybrids. Radioactively labelled stable RNA (250 μg.) was hybridized to 50 μg. of DNA bound on a cellulose nitrate membrane filter in a system of the following composition: 50% (v/v) formamide–0·33 M-KCl–10 mM-tris–HCl buffer, pH 7·4, at 33° for 3 hr. The filters with DNA–RNA hybrids were incubated in formamide (final vol. 3 ml.). Non-radioactive RNA carrier was mixed with all samples as internal standard for 23 s and 16 s rRNA and 4 s tRNA. Their positions are shown by arrows in each figure. The incubation was at 25° for 30 min. The nucleic acids were recovered from formamide by ethanolic precipitation. Samples of RNA (0·1–0·3 ml.) in 10 mM-tris–HCl buffer, pH 7·4, containing 1 mM-EDTA and 0·1 M-NaCl were sedimented in sucrose gradients as described in the legend to Fig. 1. (*a*) RNA subjected to hybridization and elution procedures in the presence of filters without DNA (control); (*b*) RNA from hybrids not treated with ribonuclease; (*c*) RNA from hybrids treated with ribonuclease.

trated in Fig. 3. Fig. 3(*a*) shows the sedimentation behaviour of stable RNA from *E. coli*, incubated in formamide in the presence of a cellulose nitrate membrane filter without DNA and recovered from formamide by ethanolic precipitation (control sample). Here the RNA is resolved into three peaks, the 23s and 16s rRNA and 4s tRNA. The last constituted about 20% of the total input RNA. The sedimentation analysis of RNA eluted from a DNA–RNA hybrid not digested with ribonuclease and recovered by ethanolic precipitation is presented in Fig. 3(*b*). Only about 10% of the rRNA became converted into slowly sedimenting chains during elution under the specified conditions, bringing the total RNA sedimenting at about 4s to 30%. The bulk of rRNA eluted from the filter sedimented at the original 23s and 16s. Results similar in all respects to those described above were obtained in experiments in which the RNA was eluted from filters by incubation in 85% (v/v) dimethyl sulphoxide and recovered by ethanolic precipitation. It is noteworthy that in the rRNA recovered from the hybrids the ratio of 16s to 23s material was higher than in the control samples. This may indicate that there was some retention of the 23s RNA on the filter, preferential breakdown of a fraction of 23s RNA during elution or higher efficiency of hybridization of the 16s RNA. The sedimentation analysis of stable RNA recovered from filters treated with ribonuclease (i.e. from ribonuclease-resistant hybrids) is presented in Fig. 3(*c*). Here the ethanol-precipitable RNA sediments as a single broad peak at about 3s. Since under these conditions the carrier RNA is also degraded to chains sedimenting at about 3–4s, it follows therefore that the ribonuclease, a basic protein that tends to adsorb on cellulose nitrate filters, is subsequently eluted from the filter and continues to digest the RNA recovered by ethanolic precipitation. Not shown here is the sedimentation analysis of RNA eluted from hybrids by thermal treatment. In this case the RNA was recovered in the form of short oligonucleotides sedimenting at 3–4s.

DISCUSSION

The need for a method of hybridization and subsequent elution of intact RNA molecules has become increasingly apparent in recent years. There are several inherent limitations in the hybridization procedure involving prolonged thermal treatment, all resulting from the extensive degradation of the input RNA during annealing. The use of degraded RNA for hybridization does not allow the determination of the absolute size of different cistrons or of mRNA molecules. In addition, the use of fragmented RNA has been criticized on the grounds that under these conditions there is a possibility of non-specific hybridization.

This problem was resolved in the present study by avoiding the use of elevated temperatures during annealing and elution of RNA molecules. Efficient hybridization of intact RNA molecules was achieved by incubating immobilized denatured *E. coli* DNA bound on cellulose nitrate membrane filters with radioactively labelled stable RNA of *E. coli* in a system consisting of 50% (v/v) formamide–0·33M-potassium chloride–10mM-tris–hydrochloric acid buffer, pH 7·4, at 33° for 3hr. The hybridized RNA could then be quantitatively eluted in non-fragmented form by incubating the filters in formamide or in 85% (v/v) dimethyl sulphoxide.

It was hoped that the method of hybridization and elution of intact RNA molecules would make it possible to determine the absolute size of various DNA cistrons. This expectation was not realized. The results of our experiments showed that DNA cistrons for rRNA were not hybridized to the entire length of homologous RNA molecules, even when intact RNA chains were annealed, but rather to fragments of two to five rRNA molecules. This was borne out by the finding that at the saturation point digestion with ribonuclease degraded 50–80% of the intact rRNA, originally in the form of complexes with DNA, to material not precipitable by ethanol. The reason for the failure of the entire intact rRNA molecule to hybridize to homologous DNA is not immediately apparent. One possible explanation could be that at saturation point (RNA/DNA ratio 5:1) about 1000 rRNA molecules compete for a single homologous cistron, resulting in partial hybridization of several molecules to the same DNA cistron. The loose ends of such partially hybridized RNA molecules would remain sensitive to the action of ribonuclease. If this explanation is correct one may expect that, at a high DNA/RNA ratio with the DNA sites in a great excess over the hybridizing rRNA molecules, entire RNA chains will hybridize to the corresponding DNA cistrons.

With regard to the size of RNA molecules eluted from a hybrid after digestion with ribonuclease, it should be pointed out that it bears no relation to the length of the RNA originally in the form of complexes with DNA in the form of a ribonuclease-resistant hybrid. This is due to the fact that ribonuclease used for elimination of unpaired RNA is at first adsorbed on and subsequently eluted from the filter and continues to digest the eluted RNA. Thus for the analysis of RNA eluted from hybrids treated with ribonuclease it is essential to remove the enzyme from the filter before elution.

The results of titration of cistrons for rRNA and tRNA with intact or extensively fragmented homologous RNA species showed that in all cases

the ribonuclease resistant hybrid occupied approx. 0·5% of the DNA. This finding seems to alleviate the objection that the use of fragmented RNA in hybridization decreases the specificity of the reaction, since in all instances, even when intact RNA molecules were used, only segments of the molecule were involved in hybrid formation.

It is hoped that the method of hybridization and elution of intact RNA molecules may greatly aid studies on the size and molecular weight of total and individual mRNA molecules. In general, sucrose-density-gradient centrifugations and methylated albumin columns have been used for size determinations (reviewed by Singer & Leder, 1966). Unfortunately, the results obtained cannot be regarded as trustworthy, since all the procedures used for the extraction of RNA in these studies brought about a selective degradation of pulse-labelled RNA by mechanical shear. The determination of the size of mRNA from sedimentation analysis of non-fragmented pulse-labelled RNA is not feasible, since under these conditions the entire pulse-labelled RNA, comprising the newly synthesized rRNA and mRNA, sediments as two peaks, one at 23s and the other at 16s (Fry & Artman, 1968). The use of methylated albumin columns for the determination of the size of mRNA is unreliable. mRNA molecules have been found to form complexes with rRNA molecules on the columns at sodium chloride concentrations needed for elution of the latter (Asano, 1965).

Isolation of intact bacterial mRNA molecules for further analysis should become feasible by annealing non-fragmented pulse-labelled RNA extracted by the procedure of Fry & Artman (1968) to DNA saturated with non-radioactive stable RNA or to DNA extracted from defective bacteriophages carrying specific cistrons of bacterial origin, and subsequent elution of intact RNA from the hybrid.

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