Molecular Hybridization between Rat Liver Deoxyribonucleic Acid and Complementary Ribonucleic Acid

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(Received 19 June 1970)

RNA (cRNA) was synthesized *in vitro* on a template of rat liver DNA and its hybridization with rat liver DNA was studied by using the nitrocellulose-filter method. Sonication of the DNA diminished its apparent capacity to hybridize with RNA by about 50%. This is not due to cross-linkage of DNA molecules, because it could be shown that less than 2% of the sonicated DNA was cross-linked. The effect is due instead to the small size of the sonicated DNA molecules. Below a single-stranded molecular weight of 5×10^5 the DNA showed a progressive loss of capacity to hybridize with decrease in molecular weight. Evidence is presented suggesting that the apparently diminished capacity of the DNA to hybridize is due to loss of hybridized DNA from the membrane filters. When cRNA at concentrations of up to 25 $\mu\text{g/ml}$ is annealed with sonicated total DNA, an apparent hybridization saturation value is found at which about 2.5% of the DNA is hybridized with RNA. Increasing the cRNA concentration tenfold brought about the hybridization of a second component of the DNA approximately equal in amount to the first. The renaturation of rat liver DNA was studied by measuring the fall in the extinction at 260nm and two different components of renaturation were observed within the reiterated fraction of DNA. By hybridizing cRNA with different fractions of rat DNA the two components of the hybridization curve are shown to correspond to the two components of the renaturation curve. The conclusion is drawn that at a cRNA concentration of 250 $\mu\text{g/ml}$ most of the reiterated fraction of rat liver DNA is hybridized after annealing for 16h under standard conditions (0.30M-sodium chloride–30mM-sodium citrate at 65°C). Even with such a high cRNA concentration little or no hybridization of the slowly renaturing DNA fraction occurs. It is suggested that the most highly reiterated DNA component is poorly transcribed *in vitro*.

We found previously (Melli & Bishop, 1969) that only a small proportion of rat liver DNA became hybridized with a large excess of complementary RNA (cRNA*) synthesized *in vitro* with RNA polymerase. It was shown directly that the reactive DNA belonged to the 'reiterated' fraction of rat liver DNA, which renatures more rapidly than the bulk of the DNA. The non-reiterated fraction of the DNA, which consists of sequences represented only once or a few times in each haploid chromosome complement, failed almost completely to hybridize. This is as expected (Bishop, 1969): because of the very large DNA content of the rat genome, very high concentrations of RNA and DNA and/or very long

annealing times would be required before an appreciable proportion of the non-reiterated DNA would hybridize. Over the concentration range of cRNA we used (10–25 $\mu\text{g/ml}$) only the more highly reiterated DNA sequences are expected to become hybridized. However, the RNA saturation curves that we observed gave the appearance of closely approaching an asymptote. If our previous conclusions were correct this apparent saturation value should represent the beginning of a lower rate of increase of the amount of DNA hybridized. In the experiments reported here this prediction has been tested and found to be correct. The more slowly hybridizing component is shown to be a second component of the reiterated DNA fraction.

In our earlier experiments we found indications that the proportion of the DNA hybridized under given conditions depends on the size of the DNA. Before DNA can be fractionated by differential

* Abbreviations: cRNA, RNA synthesized *in vitro* by means of purified RNA polymerase and a DNA primer; Cot, product of the initial DNA concentration (mol of nucleotide/l) and time of annealing (s) (Britten & Kohne, 1966, 1968).

renaturation and treatment with hydroxyapatite it has to be brought to a small size by sonication or other means. Because of the potential importance of this method for the study of DNA-RNA hybridization in higher organisms we have made a more complete study of the effect of DNA size.

MATERIALS AND METHODS

Materials. Ribonuclease A (3×crystallized), deoxy-ribonuclease ('ribonuclease-free'), papain and pronase were purchased from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. A stock solution of ribonuclease was prepared at a concentration of 10mg/ml in 0.30M-NaCl-30mM-sodium citrate, pH 4.5, heated at 100°C for 5 min, chilled and neutralized. The commercial papain solution (25mg/ml) was heated at 72°C for 30 min. Sartorius nitrocellulose membrane filters (MF-50, 3 cm diam.) were from V. A. Howe Ltd., London W.11, U.K. Sephadex SE-50 was from Pharmacia (G.B.) Ltd., London W.13, U.K. Unlabelled nucleoside triphosphates were from Pabst Laboratories, Milwaukee, Wis., U.S.A. [^{14}C]ATP and [^{32}P]P_i were from The Radiochemical Centre, Amersham, Bucks, U.K. Spray-dried cells of *Micrococcus lysodeikticus* were obtained in good condition from Seravac Laboratories (Pty.) Ltd., Maidenhead, Berks., U.K., and hydroxyapatite from Bio-Rad Laboratories, Richmond, Calif., U.S.A.

DNA purification. Rat liver nuclei were prepared by the method of Chauveau, Moulé & Rouiller (1956). The nuclei were lysed in 1% (w/v) sodium dodecyl sulphate-0.15M-NaCl-10mM-tris-HCl buffer (pH 7.5 at 20°C)-10mM-EDTA (10ml/10g of liver); β -mercaptoethanol was added to 10mM and papain to 1mg/ml, and the lysate was incubated for 16 h at 37°C. The solution was then gently shaken for 10 min at room temperature with an equal volume of redistilled water-saturated phenol, and then centrifuged for 10 min at 10000g at room temperature. The aqueous phase was carefully removed and to it was added an equal volume of ethanol. The DNA was spooled out by means of a glass rod and dissolved in 15mM-NaCl-1.5mM-sodium citrate. Ribonuclease stock solution was added to a concentration of 50 $\mu\text{g}/\text{ml}$ and the solution was kept at 37°C for 3 h. Pronase was then added to a final concentration of 400 $\mu\text{g}/\text{ml}$ and incubation at 37°C was continued for 3 h. Treatment with phenol, recovery of the DNA by spooling from ethanol and ribonuclease-pronase treatment were then performed twice more. Finally the solution was again treated with phenol, spooled from ethanol and dissolved in 15mM-NaCl-1.5mM-sodium citrate. This solution was centrifuged for 18 h at 38000 rev./min at 5°C in the no. 40 rotor of the Spinco centrifuge. This pelleted the DNA, which was then dissolved in 15mM-NaCl-1.5mM-sodium citrate. ^{32}P -labelled DNA was prepared from livers of partially hepatectomized rats (Melli & Bishop, 1969) by the same procedure.

DNA fractionation. DNA was denatured, neutralized and annealed as described by Melli & Bishop (1969), but with variations in the concentrations and time of annealing described in the text. The DNA was fractionated with hydroxyapatite as before (Melli & Bishop, 1969), but with

the omission of the elution with 0.16M-phosphate buffer, which was found to give variable yields.

Breakage of DNA. DNA of different molecular sizes was prepared by stirring solutions containing approx. 25 μg of DNA/ml in 15mM-NaCl-1.5mM-sodium citrate containing 0.2M-NaCl for 1 h at room temperature in a MSE Ato-Mix homogenizer. Constant and reproducible stirring rates were obtained by measuring the current induced in a stationary coil by steel weights attached to the stirring shaft. The rheostat supplied with the instrument was by-passed by turning the speed control full up, and the stirring speed was controlled by connecting the instrument to the mains through a variable transformer.

The smallest DNA size was obtained by sonicating a 25 $\mu\text{g}/\text{ml}$ solution for 1.5 min in an ice bath with a Dawe Sonicator set at position 8. In some experiments the sonicated DNA was incubated overnight at 65°C in 15mM-NaCl-1.5mM-sodium citrate to decrease its size still further.

Measurement of DNA molecular weight. The molecular weight of the separated single strands of the DNA was measured by neutral sucrose-density-gradient sedimentation by the method of Burgi & Hershey (1963) and by using the relationship between molecular weight and sedimentation coefficient given by Studier (1965). A 10-20 $\mu\text{g}/\text{ml}$ solution of ^{32}P -labelled DNA was adjusted to pH 12.3 with M-NaOH. After 10 min at room temperature it was chilled in an ice-bath and neutralized by addition of 0.08 vol. of M-sodium phosphate buffer, pH 6.4. A 0.2 ml portion of this solution was then layered on a 4.8 ml linear 5-20% (w/v) sucrose density gradient containing 0.15M-NaCl and 50mM-tris-HCl buffer, pH 7.5 at 0°C, and centrifuged for 4-9 h at 38000 rev./min at 4°C in a Spinco no. SW 39 rotor. ^{14}C -labelled rRNA from *Xenopus laevis* (10 μg) was added to each sample as a sedimentation marker. At the end of the run 0.2 ml fractions were collected from the bottoms of the tubes directly on to paper filters (1.8 cm-diam. discs of Whatman no. 3 filter paper). These were washed in 5% (w/v) trichloroacetic acid, dried with ethanol and ether (Bollum, 1959) and their radioactivities counted in a two-channel liquid-scintillation counter set to exclude ^{14}C radioactivity from the ^{32}P channel, and with a 5% cross-count of the ^{32}P radioactivity in the ^{14}C channel. The integral distribution of radioactive DNA against fraction number was calculated and plotted, and the weight-average molecular weight was calculated from the sedimentation coefficient of the 50% point of the distribution.

Preparation of ^{14}C -labelled cRNA. cRNA was synthesized and purified as described by Melli & Bishop (1969) by using rat liver DNA and *M. lysodeikticus* RNA polymerase (fraction VI of the preparation described by Nakamoto, Fox & Weiss, 1964).

Preparation of *Proteus mirabilis* ^{32}P -labelled 23S rRNA and ^{14}C -labelled DNA. The procedures described by Bishop (1969, 1970) were followed.

DNA-RNA hybridization. (a) Membrane-filter method. A slight modification of the technique of Gillespie & Spiegelman (1965) was followed (Bishop, 1969). All filters were loaded with denatured DNA at 4°C.

(b) Liquid annealing of bacterial DNA and rRNA. The conditions were kept as similar as possible to those used in parallel membrane-filter experiments. The ^{14}C -labelled DNA was denatured with alkali and neutralized in the

same way and then brought to 65°C. The ^{32}P -labelled rRNA was adjusted to 0.60 M-NaCl-60 mM-sodium citrate, and also brought to 65°C. Equal volumes of DNA and RNA were then mixed and annealed at 65°C for different times. The concentrations of the DNA and RNA solutions were so adjusted that the concentrations of RNA and the total amount of DNA/ml of annealing mixture were the same as for the parallel membrane-filter experiments. For each time-point two 0.5 ml samples were withdrawn and each was added to 0.1 ml of solution containing 120 μg of pancreatic ribonuclease/ml and 24 units of ribonuclease T1/ml. After 30 min at 37°C samples were precipitated with cold 10% (w/v) trichloroacetic acid in the presence of 50 μg of carrier yeast RNA. After 30 min the samples were collected on Oxoid filters, thoroughly washed with 5% trichloroacetic acid and dried with ethanol and ether.

DNA renaturation. The DNA was sonicated, adjusted to 0.12 M-sodium phosphate and held at 100°C for 10 min. It was then brought quickly to 65°C and held at that temperature. Samples were removed from time to time and diluted with 9 vol. of water. The extinction of the diluted samples at 260 nm was then measured first at 53°C and then at 85°C. The DNA is fully denatured at 85°C in 12 mM-sodium phosphate, so that the second measurement allows a correction to be made for any inaccuracy in sampling.

RESULTS

Effect of sonication of DNA on DNA-RNA hybridization. Melli & Bishop (1969) annealed denatured rat DNA under controlled conditions, and then used hydroxyapatite to separate the renatured reiterated DNA from the single-stranded non-reiterated DNA fraction. To achieve the best separation of the two DNA fractions it is necessary to break the DNA down to a small molecular size, and this was done by sonication. Fig. 1 shows the results of an experiment in which the hybridization of untreated and sonicated total DNA was compared. Under the same conditions the hybridization of the sonicated DNA is about half that of the untreated DNA. This effect of sonication is clearly an important factor when the object of the experiment is to measure the proportion of the DNA that will hybridize with RNA.

Three explanations of the sonication effect seem plausible. (1) Alberts & Doty (1968) have shown that a proportion of bacterial and mammalian DNA is cross-linked. The cross-linked DNA can be denatured, but as soon as the denaturing agent is removed the duplex is re-formed. If sonication increases the number of linkages this would decrease the number of denatured DNA sequences available for hybridization. (2) The rate of DNA renaturation is known to depend on the size of the DNA molecules (Wetmur & Davidson, 1968). If the size of the DNA affects DNA-RNA hybridization in the same way this would produce an effect like that shown in Fig. 1. (3) If there is a tendency for DNA-RNA

Fig. 1. Effect of DNA size on DNA-RNA hybridization. Approx. 1 μg of ^{32}P -labelled DNA was annealed with various concentrations of ^{14}C -labelled cRNA for 16 h at 65°C by the membrane-immobilization method. The three DNA samples were untreated (●), sheared to a single-stranded mol.wt. of 8.9×10^5 (●) and sonicated to a mol.wt. 1.8×10^5 (○).

Fig. 2. Optical 'melting' of a minor DNA component obtained by fractionation of sonicated DNA with hydroxyapatite (see the text). The two profiles show the fraction without further treatment (○) and immediately after alkali denaturation and neutralization (●). At the time of 'melting' the ionic environment of both fractions was 20 mM-NaCl-10 mM-sodium phosphate buffer, pH 6.8 (0.035 g-equiv. of Na^+ /l).

hybrid to be lost from the membrane filters more readily than the DNA that is not hybridized, and if this tendency is increased by sonicating the DNA, the same result would be expected. These possibilities were investigated in detail.

Cross-linked DNA. An experiment was carried

out to measure the proportion of sonicated DNA that is cross-linked. In practice it is very difficult to distinguish between cross-linkage and very rapid renaturation. Our experiment does not do so completely, and therefore provides what is probably an overestimate of the percentage of the DNA that is cross-linked.

Sonicated rat liver DNA at a concentration of 567 $\mu\text{g/ml}$ in 15 mM-sodium chloride–1.5 mM-sodium citrate was adjusted to pH 12.3 at room temperature by adding M-sodium hydroxide. After 10 min the solution was chilled in ice and then neutralized with M-hydrochloric acid. The solution was brought rapidly to 70°C by means of a boiling-water bath, enough M-sodium phosphate buffer (equimolar in Na_2HPO_4 and NaH_2PO_4) was added to make it 0.12M, and it was diluted with an equal volume of 0.12M-sodium phosphate buffer preheated to 70°C. The sample was then fractionated with hydroxyapatite in the usual way. About 3 min elapsed between the neutralization of the DNA and the collection of the first supernatant fraction, which contains most of the single-stranded DNA. Of the total DNA 90% was recovered in the 0.12M-phosphate fraction and 10% in the 0.3M-phosphate fraction.

The 0.3M-phosphate fraction, which contains the double-stranded DNA, was exhaustively dialysed at 4°C against 10 mM-phosphate buffer. To estimate how much of the total DNA was actually in the double-stranded form at this stage its optical 'melting' profile was determined (Fig. 2). The sample showed 28% hyperchromicity. Since native rat liver DNA shows 38% hyperchromicity, this indicates that 70% of the 0.3M-phosphate fraction, or 7% of the total DNA recovered from hydroxy-

apatite, was in the double-stranded form. This 7% should consist both of cross-linked DNA and of DNA that renatured during the hydroxyapatite fractionation step. To estimate more closely the amount of cross-linked DNA, a sample of the dialysed 0.3M-phosphate fraction was again denatured with alkali and neutralized in ice. The sample was then brought rapidly to 60°C in a boiling-water bath and a determination of its optical 'melting' profile was begun immediately (Fig. 2). While the temperature was being brought from 60 to 70°C (which took 20 min) a significant fall in the extinction was observed. This is presumed to be due to renaturation. If we take the first measured point as the point of comparison, the overall rise in the extinction was 7%. This should be due to cross-linked DNA, which renatures immediately on neutralization, together with the DNA that renatured during the time (30 s) required to bring the sample to 60°C. Thus less than 18% of the 0.3M-phosphate fraction, corresponding to less than 2% of the total DNA, is cross-linked. This is essentially in agreement with the value of 1% found by Alberts & Doty (1968) for calf thymus DNA and by Walker & McLaren (1969) for mouse and rat DNA.

Even if the cross-linkage of the sonicated DNA were as much as 2%, this could not explain the 50% loss of hybridizability observed.

Effect of DNA size. A series of DNA samples of different molecular size was prepared by stirring and sonication, and the weight-average single-stranded molecular weight of each sample was measured. A portion of each was denatured and loaded on membrane filters and then annealed with cRNA under standard conditions. The

Table 1. *Effect of DNA size on DNA-RNA hybridization*

The ^{32}P -labelled DNA was degraded by stirring or sonication and its single-stranded molecular weight (weight average) determined as described in the text. The DNA was loaded on membrane filters (approx. 1 μg /filter) and annealed for 16 h at 65°C in 0.3M-NaCl–30 mM-sodium citrate with cRNA at the concentration shown. Percentage hybridization is calculated by taking the hybridization of the untreated DNA as 100%.

Treatment	$10^{-5} \times$ Single-strand mol. wt.	Concn. of cRNA ($\mu\text{g/ml}$)	Hybridization (μg of RNA/ μg of DNA)	Hybridization (%)
None	—	26.3	0.043	(100)
None	—	25.5	0.043	(100)
Stirred	8.9	25.5	0.041	95
Stirred	7.9	25.5	0.051	118
Stirred	5.8	26.3	0.045	104
Stirred	4.0	26.3	0.038	88
Stirred	3.6	26.3	0.037	86
Sonicated	2.6	25.5	0.035	81
+5 h at 65°C	2.1	26.3	0.024	56
+18 h at 65°C	2.1	26.3	0.022	51
Sonicated	1.8	26.3	0.020	47
+2 h at 65°C	1.6	26.3	0.018	42
+40 h at 65°C	1.3	26.3	0.017	40

Fig. 3. Effect of DNA size on DNA-RNA hybridization. DNA of various single-stranded molecular weights was annealed for 16 h at 65°C with cRNA at either 25.5 or 26.3 $\mu\text{g/ml}$. The control was untreated DNA. For further details see the legend to Table 1.

saturation curves obtained with DNA of mol.wt. 8.9×10^5 and 1.8×10^5 are shown in Fig. 1, compared with untreated DNA (mol.wt. approx. 10^8). Hybridization of the DNA of mol. wt. 8.9×10^5 was indistinguishable from the control, but hybridization of the DNA of mol.wt. 1.8×10^5 was depressed by about 50%. The complete series of experiments is summarized in Table 1 and Fig. 3. Diminished hybridization is evident at a molecular weight of 5×10^5 , and falls rapidly to 40% with the smallest size used. The shape of the curve suggests that the hybridization would be even less with DNA of still smaller size.

The rate constant of DNA renaturation depends on the size of the DNA (Wetmur & Davidson, 1968): the smaller the molecular size the lower the rate of renaturation. In the present case the DNA is immobilized on nitrocellulose membrane filters, and over the entire range of DNA sizes the size of the DNA molecules is much greater than that of the RNA molecules. Therefore we would not expect that the DNA size would affect the basic rate constant of the reaction. Nevertheless it is important to test this possibility because, as we show below, saturation curves obtained with rat DNA are very deceptive, and a lower hybridization value might simply reflect a lower rate of reaction. To do this we studied the effect of DNA size on the hybridization of bacterial DNA and rRNA. In this case the

reacting RNA and DNA sequences are rather homogeneous (Kohne, 1968), so that the approach to the saturation value is meaningful (Bishop, Robertson, Burns & Melli, 1969). The results showed that when the size of the DNA was decreased by sonication to a molecular weight of 220 000, hybridization was decreased by 30% (see below). There is thus no reason to suppose that the results observed with rat liver DNA do not represent a real loss of hybridization, independent of reaction rate.

In the experiments with rat liver DNA we observed that an increasing loss of DNA from the membrane filters paralleled the loss in hybridization. Our results are corrected for DNA recovery, so that an equal loss of hybridized and non-hybridized DNA should have no effect. A preferential loss of hybridized DNA would, however, produce the sort of effect observed. This could be tested by annealing the DNA and RNA together in solution. However, this test is difficult to perform because the cRNA is self-complementary, and forms a ribonuclease-resistant duplex whether DNA is present or not. We therefore used the hybridization of rRNA with bacterial DNA to test this, comparing the hybridization observed by the membrane method with the hybridization observed by annealing RNA and DNA together in solution.

In solution there is competition between DNA renaturation and DNA-RNA hybridization. To minimize the effect of DNA renaturation as far as possible we used a high relative concentration of complementary RNA sequences. The ratio of total RNA to DNA was 1:4. Taking into account the fact that 23S rRNA hybridizes with about 0.3% of *Proteus* DNA (Kohne, 1968), the ratio of RNA and DNA sequences complementary to the sense strand of the ribosomal DNA is 1:(4×0.003) or about 80:1. The amount of DNA-RNA hybrid formed in each experiment was estimated by treating the reaction mixture with ribonuclease and then collecting the cold-trichloroacetic acid-insoluble ribonuclease-resistant material on Oxoid cellulose acetate filters. To decrease the amount of non-hybridized RNA that survived this treatment to an acceptable level it was found necessary to use ribonuclease T1 in addition to pancreatic ribonuclease. This led to a loss in the amount of hybridized RNA recovered. For this reason the absolute extents of hybridization observed are not meaningful. However, for the present purpose it is sufficient to compare the relative extents of hybridization observed with untreated and sonicated DNA.

As mentioned above, sonication of the bacterial DNA diminished the hybridization found by the membrane method (Fig. 4a). With liquid annealing there was in fact a slight increase in hybridization

Fig. 4. Effect of DNA size on the hybridization of bacterial DNA with rRNA: comparison of membrane and liquid methods. (a) 4 μ g of untreated (■) or sonicated (○) denatured *Proteus mirabilis* ^{14}C -labelled DNA was annealed with 1 μ g of purified ^{32}P -labelled rRNA/mg in 0.3M-NaCl-30mM-sodium citrate at 65°C by the membrane method. (b) The same experiment was carried out in solution with 4 μ g of DNA/ml and 1 μ g of RNA/ml.

after sonication (Fig. 4b). This may be due to the decreased renaturation rate of sonicated DNA. Whatever the reason, this experiment shows that the loss of hybridization brought about by sonicating the DNA is an artifact peculiar to the membrane method.

Optical renaturation of rat DNA. To provide a basis for comparison with DNA-RNA hybridization, the renaturation of sonicated rat liver DNA was followed by measuring the fall in extinction at 260nm. The renaturation was carried out in 0.12M-sodium phosphate (0.18g-equiv. of Na^+ /l) at 65°C, which is 19–20°C below the 'melting' temperature, T_m , of rat liver DNA. These conditions are comparable with those used here for DNA-RNA hybridization, namely 0.30M-sodium chloride-30mM-sodium citrate (0.36g-equiv. of Na^+ /l) at 65°C. The higher salt concentration increases the T_m of DNA by 5°C (Marmur & Doty, 1962). This is equal to the decrease in T_m of a DNA-RNA hybrid compared with the corresponding DNA duplex (Chamberlin & Berg, 1963), so that we are studying the formation of the two types of duplex at temperatures that are the same number of degrees below their respective T_m values.

The denatured DNA was annealed at high concentration, and at different times samples were removed, diluted, and adjusted to 12mM-sodium phosphate. The extinction of each sample at 53°C measures the hyperchromicity due to the remaining single-stranded DNA quite accurately, because at 53°C in 12mM-sodium phosphate single-stranded DNA shows little or no hypochromism. At 85°C the DNA is completely denatured, so that the difference $E_{260(85)} - E_{260(53)}$ gives the extinction due to single-strandedness. In Fig. 5 we plot $(E_{260(85)} - E_{260(53)})/E_{260(85)}$ as a measure of renaturation. An

Fig. 5. Optical renaturation of rat liver DNA. The left-hand ordinate shows the proportional loss of hyperchromicity, the right-hand ordinate the percentage renaturation estimated from this.

appropriate conversion to percentage renaturation may be made by multiplying the ordinate values by 380 (right-hand scale). The abscissa scale is the log Cot scale used by Britten & Kohne (1968).

Three components of renaturation were observed (Fig. 5), which appear to correspond with the 'fast', 'intermediate' and 'slow' components of DNA renaturation discovered by Britten & Kohne (1968). The slow component was half renatured at a Cot value of 4000. *Escherichia coli* DNA annealed under the same conditions became half-renatured at a Cot value of 4. The ratio of 10^3 between these values is close to that expected from the analytical complexity of the two types of DNA (Britten & Kohne, 1968; Wetmur & Davidson, 1968). The most rapidly renaturing fraction made up about 15% of the total DNA, and became half renatured at a Cot value of 0.0025, corresponding to a reiteration frequency of close to 2×10^6 . The intermediate fraction made up about 10% of the total DNA, and was half renatured at a Cot value of about 1, corresponding to a reiteration frequency of about 4000.

Presentation of DNA-RNA hybridization results. The RNA sequences synthesized *in vitro* may be assumed to reflect broadly the frequency distribution of the DNA sequences of the template. We therefore expect that the pattern of hybridization between DNA and cRNA will show major components of different frequency similar to those found in DNA renaturation experiments.

A method for analysing hybridization results was described by Bishop *et al.* (1969). The method was based on consideration of simple RNA mixtures in

which all sequences are equal in concentration. A mathematical model of DNA-RNA hybridization was developed and used to investigate the effects of RNA sequence frequency distributions on the results obtained. The situations considered were of the type that would be produced by differential transcription of DNA sequences that were themselves equal in frequency. Under these circumstances the method was found to be insensitive to RNA sequences present in concentrations 100-fold lower than the mean sequence concentration. Here we consider the different situation in which the DNA sequences themselves differ in frequency, and transcription is equal. To illustrate the difference between the two situations we may think of an RNA population half of the sequences of which are 100-fold more concentrated than the remainder. In the first case the more concentrated sequences made up about 99% of the total RNA, while in the present case they make up 50%.

Two specific models were examined. (1) Half of the total RNA (and DNA) consists of 100-fold reiterated sequences, and the remainder is non-reiterated. (2) Half of the total RNA (and DNA) consists of 1000-fold reiterated sequences, and the remainder is non-reiterated.

The methods tested were: (a) a double reciprocal plot of the type described by Bishop *et al.* (1969); (b) a plot of hybridization against the logarithm of RNA concentration; (c) a plot of the logarithm of hybridization against the logarithm of RNA concentration. Of these, plots (a) and (b) were found to be useful (Fig. 6). Plot (a) shows two components with different slopes corresponding to the two frequency components of the RNA. Plot (b), which is analogous to the Cot plot of Britten & Kohne (1968), also shows the two components clearly.

Effect of RNA concentration on DNA-RNA hybridization. Melli & Bishop (1969) showed that only a small part of rat liver DNA became hybridized in 16 h at cRNA concentrations of up to 25 $\mu\text{g}/\text{ml}$. An example of this type of experiment is shown in Fig. 1. Because of the complexity of rat liver DNA, we expect that with a tenfold increase in the cRNA concentration other components of hybridization will be detected. This was tested by using sonicated total DNA (which was annealed for 5 h at 65°C but not fractionated) and the results shown in Fig. 7. At a cRNA concentration of 25 $\mu\text{g}/\text{ml}$ about 3% of the DNA became hybridized. At a cRNA concentration of 270 $\mu\text{g}/\text{ml}$ the amount of DNA hybridized increased to 5%.

The same results are shown plotted by the double-reciprocal method in Fig. 8. Two components are seen, and these extrapolate to values of 2.6 and 5.5% hybridization respectively. Heavily sonicated DNA was used in this experiment, and

Fig. 6. Methods for presenting hybridization results in a case where the DNA contains a reiterated fraction and the RNA is a perfect complementary copy of the DNA. Half of the total DNA consists of sequences reiterated 100 ($1 + 10^2$) or 1000 ($1 + 10^3$) times. (a) Double-reciprocal plot. (b) Plot of hybridization against the logarithm of RNA concentration.

we have shown that the apparent hybridization of such DNA is only half that of untreated DNA. Bishop *et al.* (1969) showed that the hybridization of untreated DNA falls short of the complementarity between DNA and RNA, again twofold. Overall, then, a correction factor of about 4 is required in this case, suggesting that 10% of the DNA (4×2.6) is complementary to the faster-renaturing component, and 11% [$4 \times (5.5 - 2.6)$] to the more slowly renaturing component. There is some uncertainty in the precise value of the correction factor, so that these values are only approximations. It should be noted, however, that they correspond quite closely in relative magnitude to the two components of DNA renaturation observed under comparable conditions (Fig. 5).

Fractionation of DNA after controlled renaturation. After partial renaturation of DNA the renatured fraction can be considerably enriched by treatment

Fig. 7. Hybridization of total rat liver DNA with cRNA. The ^{32}P -labelled DNA was sonicated, denatured and annealed at 65°C for 5 h in 0.12M-sodium phosphate buffer, pH 6.8, but not fractionated. It was then dialysed against 15mM-NaCl-1.5mM-sodium citrate, denatured and loaded on to membrane filters (approx. $1\mu\text{g}/\text{filter}$) in the usual way. Hybridization was in 0.3M-NaCl-30mM-sodium citrate at 65°C for 16 h.

Fig. 8. Hybridization of total rat liver DNA with cRNA. This is a double-reciprocal plot of the results shown in Fig. 7.

with hydroxyapatite (Britten & Kohne, 1968; Flamm, Walker & McCallum, 1969). Previously we showed that cRNA at low concentrations hybridized preferentially with such a fraction (Melli & Bishop, 1969). The conditions used in those experiments (Cot values about 100) were such that the entire reiterated fraction of the DNA was enriched (see Fig. 5). To identify the components of the hybridization curve shown in Figs. 7 and 8 the renatured fraction of sonicated rat liver DNA was isolated after annealing to a Cot value of 0.6 and to a Cot value of 21 (see Fig. 9). Each DNA

Fig. 9. Recovery of DNA in the hydroxyapatite 0.3M-phosphate fraction after renaturation. Sonicated rat liver DNA was alkali-denatured and annealed in 0.12M-sodium phosphate buffer, pH 6.8 (0.18g-equiv. of Na^+/l) for 5 h at 65°C at the DNA concentrations shown. The annealed DNA was fractionated with hydroxyapatite. The ordinate shows the percentage of each DNA sample recorded in the 0.3M-phosphate fraction. The calculated Cot values were 0.6, 1.8, 3.6, 6, 12 and 21 for the six samples from left to right.

fraction was then annealed with a series of RNA concentrations. The results are shown in Fig. 10 in the form of a semi-logarithmic plot, together with the results of hybridizing total sonicated DNA in the same way. The DNA isolated after annealing to a Cot value of 0.6 is very much enriched in the first component. The DNA isolated after annealing to a Cot value of 21 is relatively more enriched in the second component. These are the results expected if the first and second components of DNA-RNA hybridization (Figs 6 and 10) correspond to the first and second components of DNA renaturation (Fig. 5).

Calculations based on this conclusion are shown in Table 2. The product of the percentage of the total DNA recovered after hydroxyapatite treatment and the enrichment observed in hybridization with a given RNA component gives the percentage of the corresponding DNA component recovered in the hydroxyapatite fraction. The value for the recovery of the DNA corresponding to the first RNA component after renaturation to a Cot value of 0.6 is 53%. This is at first sight inconsistent with correspondence of the DNA and RNA components, because the first DNA component is completely renatured at this Cot value (Fig. 5). However, we

have shown that hydroxyapatite fractionation of renatured DNA is imperfect (Melli & Bishop, 1969). Non-renatured DNA is recovered in the 'renatured' fraction and renatured DNA in the 'non-renatured' fraction. In the light of our previous results, 53% recovery of renatured DNA is roughly what is expected. After renaturation to a Cot value of 21, the recovery of DNA corresponding to the first RNA component is increased to 60%. Of the DNA corresponding to the second component of the RNA, 30% is recovered after renaturing to a Cot value of 0.6, but this increases to 44% after renaturing to a Cot value of 21. Thus these results are consistent with the interpretation that the first and second components of DNA-RNA hybridization (Fig. 10) correspond to the first and second components of DNA renaturation (Fig. 5).

Fig. 10. Hybridization of reiterated DNA fractions and total rat liver DNA. cRNA at various concentrations was annealed with ^{32}P -labelled DNA ($1\mu\text{g}/\text{sample}$) for 16h at 65°C . The DNA was sonicated and annealed but not fractionated (○, see Fig. 7) or fractionated after annealing to a Cot value of 0.6 (●) or 21 (■) (see Fig. 9).

An apparent inconsistency in the results is the shift in the cRNA concentration that gives 50% hybridization of the first component, from about $1\mu\text{g}/\text{ml}$ with total DNA to $3\mu\text{g}/\text{ml}$ with the DNA renatured to a Cot value of 0.6. This is attributable to scarcity of complementary RNA sequences when the enriched fraction is being hybridized. If 10% of the cRNA is complementary to the first component of the DNA, $1\mu\text{g}$ of cRNA/ml contains only $0.1\mu\text{g}$ of these sequences/ml. When the DNA is enriched fourfold, $1\mu\text{g}$ of DNA contains $0.4\mu\text{g}$ of the first component. This difficulty does not arise with the second component, since hybridization is only apparent at cRNA concentrations in excess of $50\mu\text{g}/\text{ml}$.

DISCUSSION

The finding that the molecular size of the DNA may critically affect the apparent extent of DNA-RNA hybridization is, as far as we are aware, entirely new. Its importance here is that it explains the loss of total hybridization previously observed in fractions isolated after renaturation and hydroxyapatite treatment (Melli & Bishop, 1969). During the annealing period there is breakage of the DNA (see Table 1). In experiments involving pre-annealing of sonicated DNA it is therefore important to adopt the practice of pre-annealing all DNA samples for the same period of time. In general our results show that any experiments involving the hybridization of degraded DNA must be interpreted with caution. We have shown that the effect is found when the membrane-immobilization method (Gillespie & Spiegelman, 1965) is used, but not when the DNA and RNA are annealed in solution and the hybrid is collected by trichloroacetic acid precipitation after ribonuclease treatment. We have not investigated the effect of DNA size when the method of Nygaard & Hall (1964) is used.

We estimate that the two reiterated fractions of

Table 2. *Recovery of DNA complementary to components of the RNA after controlled renaturation and fractionation*

The recovery of DNA after renaturation is shown in Fig. 9. Enrichment for hybridization is calculated from the results shown in Fig. 10. A complete description is given in the text.

Cot value for renaturation	DNA recovered (%)	Hybridization with first RNA component		Hybridization with second RNA component	
		Enrichment	Enrichment × recovery	Enrichment	Enrichment × recovery
0.6	12	4.4	53	2.5	30
21	20	3	60	2.2	44
Total DNA	(100)	(1)	(100)	(1)	(100)

rat liver DNA become renatured to 50% at Cot values of about 0.0025 and 1 (i.e. $Cot_{1/2} = 0.0025$ or 1). Taking 4000 as the $Cot_{1/2}$ of the slowly renaturing fraction of mammalian DNA we can calculate mean reiteration frequencies of 2×10^6 and 4×10^3 for the fast and intermediate fractions respectively (Britten & Kohne, 1968).

The absolute amounts of hybridization observed, and the results of hybridizing RNA with fractions of the DNA both suggest strongly that the two observed components of DNA-RNA hybridization correspond to the two major reiterated components of the DNA. It is of interest to see whether the observed rates of hybridization are consistent with results obtained under similar conditions with micro-organisms. Bishop (1969) derived the following approximate general equation to describe DNA-RNA hybridization under the conditions used here: $1/r = 73C/ht + 1/r_s$, where r and r_s represent respectively observed hybridization and hybridization saturation estimated by extrapolation to infinite time (μg of RNA hybridized/ μg of DNA), C is the analytical complexity of the system under study ($10^{-8} \times$ daltons of DNA/haploid genome), h is the RNA concentration ($\mu\text{g}/\text{ml}$) and t is the time (h). To incorporate reiteration frequency the frequency, R , is made a divisor of C , and $1/r = 73C/Rht + 1/r_s$. When the reaction is half complete, $1/r = 2/r_s$. The equation was derived by using homogeneous systems (microbial as opposed to eucaryotic DNA), and for these $1/r_s \sim 3$ for cRNA (Bishop, 1969). Hence $R = 24.3C/ht$, and taking $C = 18000$ for rat DNA, $R = 4.4 \times 10^5/ht$. We make the initial assumption that the cRNA components are at the same relative frequencies as the corresponding DNA components, and write $x = h/h'$ where h is the concentration of the RNA component in question and h' is the overall RNA concentration. Then $x = 0.15$ for the fast component and $x = 0.1$ for the intermediate; h' is approx. $1.4 \mu\text{g}/\text{ml}$ for half-reaction of the first component of hybridization and $70 \mu\text{g}/\text{ml}$ for the second. From $R = 4.4 \times 10^5/xh't$ ($t = 16\text{h}$) we calculate $R = 1.3 \times 10^5$ for the first component, and $R = 4000$ for the second. Comparing these with the calculated DNA reiteration frequencies of 2×10^6 and 4000, we find the RNA reiteration frequency of the first component is lower by the factor 15, whereas the second component shows very good agreement.

With an annealing time of 16h and a low cRNA concentration, there is a danger that very rapidly hybridizing components of the RNA may become so depleted that the rate of hybridization is significantly decreased. We have suggested this as an explanation of the altered shape of the curves found where cRNA is annealed with DNA fractions enriched for reiterated sequences. To test the validity of the estimates of R we need to anneal

higher concentrations of cRNA with the DNA for shorter times. A suitable experiment with the faster annealing fraction has already been described (Fig. 1 of Melli & Bishop, 1969): total rat liver DNA was annealed with $22 \mu\text{g}$ of cRNA/ml for different times. The time for half-reaction was about 1h. From $R = 4.4 \times 10^5/xh't$, $R = 1.2 \times 10^5$. Thus, with the assumption that $h = 0.15h'$, 1.3×10^5 is a reasonable estimate of R for this fraction.

Disparate reiteration frequencies calculated from DNA renaturation and DNA-RNA hybridization results may be explained in several different ways. (1) DNA-RNA hybridization with the use of ribonuclease is probably a more stringent technique than DNA renaturation measured by optical methods: poorly matched base-paired sequences that contribute a loss of hyperchromicity in renaturation experiments may be digested by ribonuclease and lost in hybridization experiments. This explanation would require, in addition, the rather surprising assumption that the more rapidly reacting sequences are the more grossly mismatched. (2) The relationships that homogeneous systems obey in hybridization are not obeyed by the reiterated fractions of the mammalian system. However, the reiteration frequencies calculated from DNA renaturation are based on a similar assumption. (3) The assumption that the reiterated DNA fractions are transcribed *in vitro* to the same extent as the non-reiterated DNA may be incorrect. If so, we may calculate the proportional transcription, p , of the most highly reiterated fraction, by setting $R = pR'$ where R' is the reiteration frequency calculated from DNA renaturation, and letting $h = p \times h'$. Then $p = (4.4 \times 10^5/R' \times h't)^{1/2}$. This gives $p = 0.35$ for the fast component. That is to say, the results we have observed may be explained by assuming that, *in vitro*, the fast renaturing DNA component is transcribed ten times less frequently than the slow component.

As yet, we have not devised a method of distinguishing clearly between these possibilities. We consider, however, that the third is the most likely. Further, unpublished experiments with cRNA synthesized on a template of denatured rat DNA tend to support it. This RNA reaches the same early hybridization plateau as cRNA transcribed from the native template (Melli & Bishop, 1969) but in time-course experiments the plateau value is reached more rapidly (Melli & Bishop, unpublished work). This is the result we expect if the denatured template is transcribed less selectively than the native template.

In general, these results bear out our previous conclusion that at readily attainable cRNA concentrations only reiterated fractions of the DNA of higher organisms become hybridized. The evidence given above suggests strongly that both fast- and

intermediate-renaturing fractions of rat liver DNA become hybridized at cRNA concentrations of about 250 $\mu\text{g/ml}$. Only the fast-renaturing fraction becomes hybridized at cRNA concentrations of up to 30 $\mu\text{g/ml}$. Since both nuclear and cytoplasmic RNA from rat liver were effective competitors of cRNA at 30 $\mu\text{g/ml}$ (Melli & Bishop, 1969) it would seem that this fraction of rat liver DNA is transcribed, at least to some extent, in living tissue.

We are indebted to Mr S. S. Mathur, Miss Melville Richardson, Miss Patricia Wallace and Miss Linda Paton for technical assistance. *Xenopus laevis* rRNA was kindly provided by Dr M. Birnstiel. Financial support was provided by the Science Research Council. For part of the time during which this work was carried out the authors were members of the Medical Research Council Epigenetics Research Group.

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