THE COMPARISON OF DIFFERENCES IN REITERATED SEQUENCES BY RNA-DNA HYBRIDISATION

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THE technique of hybridisation has been widely used in the determination of base sequence homologies. The Carnegie Institution group pioneered the application of DNA-DNA hybridisation to the comparison of sequence differences between a wide range of species (HOYER, MCCARTHY and BOLTON 1964), while differences between rat and mouse have been studied by McLAREN and WALKER (1966). Since hybridisation between DNA:DNA and RNA:DNA are essentially similar reactions RNA-DNA hybridisation may be used to detect genetic differences, provided the RNA is sufficiently representative of the sequences we wish to compare.

Recently, BRITTEN and KOHNE (1967) have shown that the DNA of higher organisms includes a substantial fraction of reiterated sequences and that the earlier DNA-DNA comparisons referred to such fractions rather than whole genomes. The interpretation of these sequences is obscure and we need further information about differences between them, especially in related species. The present paper deals with such evidence, which is based on the method of RNA-DNA hybridisation, in which the RNA is synthesised *in vitro* (cRNA) with the aid of DNA-dependent RNA polymerase and appropriate DNA primers.

Although nearest-neighbour analysis shows good agreement between cRNA and the primer DNA (WEISS and NAKAMOTO 1961), it cannot be assumed that all sequences are equally transcribed. If there are species differences in the frequency of preferred sites of transcription, this will enhance apparent genome differences, and, in some situations, this may be an advantage. The use of hybridisation between DNA and cRNA has several attractions which prompted the experiments described here. It is often difficult or excessively labourious to prepare, for DNA-DNA hybridisation, labelled DNA in sufficient quantity and of adequate specific activity from species which may be of particular interest for reiterated sequence comparisons. Subject to the qualifications noted above, the cRNA method is a general one and the RNA may be made with any desired specific activity. Also, convenient techniques, based on the use of membrane filters, have been developed by NYGAARD and HALL (1964), and by GILLESPIE and SPIEGELMAN (1965) for the quantitative measurement of RNA bound to DNA. Finally, the treatment of reaction mixtures with RNAase offers a fairly stringent test of complementarity.

¹ This paper is dedicated to Dr. THEODOSIUS DOBZHANSKY, in recognition of his outstanding contributions to the genetics of natural populations and of evolutionary processes.

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In the present report we deal with the application of this approach to comparisons between *Drosophila melanogaster* and various other forms, including other insects, and also members of the same genus. This exploration was designed to discover the order of discrimination we can obtain with cRNA-DNA hybridisation, and in this respect, the comparisons between the closely related, sibling species *D. melanogaster* and *D. simulans* are of particular interest.

MATERIALS AND METHODS

(i) *Extraction of DNA*: DNA has been extracted either by the method of RITROSSA and SPIEGELMAN (1965) or by the following procedure which proved very satisfactory. Newly emerged flies grown in population cages were collected by anesthesia with carbon dioxide and kept 2–3 days in bottles with Kleenex moistened with a dilute sugar solution before use. 10–15 g were homogenised in 40–50 ml Twest (0.1% Tween 80, culture grade; 0.05 M EDTA, 0.15 M NaCl and 0.05 M Tris-HCl buffer pH 6.8) at 0–4°C with a M.S.E. homogeniser for 10 sec at full speed. The homogenate was filtered through two layers of gauze and the residue suspended in 40 ml Twest, stirred at moderate speed for 10 min, filtered and the process twice repeated. The pooled filtrates were centrifuged for 30 min at $30,000 \times g$ and the combined pellets suspended in 1.5 volume Twest. Concentrated sodium lauryl sulphate was added to 2%. The viscous solution was quickly brought to 60°C, kept at 60°C for 10 min, cooled to room temperature and 1/10 volume saturated Tris solution (pH 8.5) added with gentle stirring, followed by addition of 5M sodium perchlorate to make the solution 1 M. After shaking gently with an equal volume of chloroform-iso-amyl alcohol, 24:1, (MARMUR 1961), the aqueous phase was separated by centrifugation and the DNA spooled out by adding two volumes of ethanol. The spooled DNA was dissolved in 0.1 SSC, brought to SSC (0.15 M sodium chloride, 0.015 M trisodium citrate pH 7.0) and incubated for 4 hrs at 37°C with pancreatic RNAase at a concentration of 150 µg per ml; the RNAase was first boiled in acid SSC, pH 5.0. Preincubated pronase was added to a final concentration of 0.2 mg/ml and the incubation continued for 1 hr. The solution was then made 1% with sodium lauryl sulphate and treated in turn with equal volumes of water-saturated, redistilled phenol and chloroform-iso-amyl alcohol. Glycogen was removed from the aqueous phase by pelleting at 30,000 rpm for 30 min. The DNA was spooled out by addition of isopropanol (MARMUR 1961), washed in 80% ethanol, dissolved in a suitable buffer and dialysed overnight before storage over a little chloroform. The yield was generally 1.2–1.5 mg per 10 g flies.

DNA prepared in this way melted over a range of 60–80°C, with a T_m of 70°C, in agreement with HASTINGS and KIRBY (1966). The GC content, determined from a CsCl buoyant density of 1.70 ± 0.10 g/ml worked out at 40–42% according to the published formulae (ROLFE and MESELSOHN 1959; SUEOKA 1961) and also in agreement with HASTINGS and KIRBY (1966). These authors reported DNA with a $s_{20,w}$ value of 16–18S; with the present procedure, higher values of 30–56S were generally obtained.

DNA from the other insects, *Schistocerca* and *Aedes*, was prepared by this method. T4 DNA was prepared by the method of MANDELL and HERSHEY (1960). The samples of rat and *Xenopus* DNA were donated and had been shown by the donors to be suitable for hybridisation experiments.

All DNA preparations were checked for absorption at 230, 260 and 280 mµ. Protein was determined by the method of LOWRY, ROSEBROUGH, FARR and RANDALL (1951) and the contamination was generally less than 1%. RNA contamination was estimated by comparing absorption at 260 mµ with direct estimates of DNA content either by the diphenylamine reaction (BURTON 1956) or by the fluorometric method of KISSANE and ROBBINS (1958). RNA contamination did not exceed 2% and was generally less than this. All preparations were tested for RNAase activity by incubating labelled cRNA with samples of DNA for several hours at 37°C and only samples which led to no loss of acid precipitable counts were used.

Radioactively labelled DNA was prepared by adding tritiated thymidine (methyl-³H-thymidine), at the rate of 5 µCi per ml to an axenic medium, which consisted of SANG's (1956) syn-

thetic diet, supplemented with dried yeast. Eggs were sterilized and set up as described elsewhere (ROBERTSON 1960). The labelled flies were appropriately diluted with cold material and the DNA extracted by the method of RITOSSA and SPIEGELMAN (1965) up to the phenol step, after pronase treatment, when the extract was purified by equilibrating in CsCl for 5 days in a No. 30 Spinco rotor at 29,000 rpm at 25°C. The sharply-banded DNA was dialysed in suitable buffer, and checked for purity as noted above. Such labelled DNA was pH-denatured and fixed to nitrocellulose membranes (Sartorius MF 50, weight constant) as described by GILLESPIE and SPIEGELMAN (1965), at a level of 1.0–1.3 μ g per membrane.

(ii) *Hydroxyapatite fractionation*: For experiments on the renaturation of DNA, hydroxyapatite, prepared by the method of TISELIUS, HJERTÉN and LEVIN (1956) was used to separate renatured from unrenatured DNA. The DNA was sonicated at level 8 with a Dawe Sonprobe, for a total of 30 sec, heat denatured and then incubated for various times in 0.12 M phosphate at 65°C. DNA concentration, volume, the size and shape of the vessel used for sonication were closely similar in different tests. Fractionation procedure generally followed that described by BRITTEN and KOHNE (1967). The preparation in 0.12 M phosphate was passed through a column of hydroxyapatite held at 60°C, to allow passage of single-stranded DNA, followed by elution of the renatured material with 0.40 M phosphate. The DNA content of the fraction was estimated by the diphenylamine method (BURTON 1956).

For some experiments fractionated DNA was fixed to nitrocellulose membranes and used for hybridisation. In such cases, the sonication was carried out at level 4 for 15 sec. At the usual high level of sonication only 20–25% of the DNA is retained on membranes after slow filtration, while, at the lower sonication level, about 70% is retained.

(iii) *Preparation of cRNA*: RNA polymerase was prepared from *Micrococcus lysodeikticus* by the method of NAKAMOTO, FOX and WEISS (1964); generally Fraction V was used. The preparations were stored at –20°C in 0.01 M Tris-HCl (pH 7.5 at 0°C) in 50% glycerol. RNA synthesis was carried out for 30–40 min at 30°C in 0.01 M Tris-HCl, pH 7.5 at 30°C, 2.5 mM $MnCl_2$, 1.6 mM spermidine, 0.8 mM each of GTP, UTP and CTP and 0.4 mM ATP with a specific activity of 0.93 or 3.52 c/mole. Generally 1–1.3 times as much RNA as primer was obtained. After incubation, RNA was recovered by the procedure described by BISHOP and ROBERTSON (in press); after DNAase and phenol treatment the solution was passed through a column of Sephadex SE-50 to separate the RNA from nucleotides and oligonucleotides.

(iv) *Hybridisation*: Both the methods of NYGAARD and HALL (1964) and of GILLESPIE and SPIEGELMAN (1965) have been used, the first to determine the proportion of RNA bound, when a constant amount of RNA is allowed to react with different amounts of DNA, and the second to measure the amount of RNA bound to a known amount of DNA. When the first method has been used, in which both RNA and DNA are in solution, the DNA was denatured by heating for 10 min at 98–100°C generally in 0.1 SSC, although concentrations up to $1 \times$ SSC have been found satisfactory. After heating, the solutions were quickly cooled. Appropriate mixtures of RNA and DNA were incubated, in $2 \times$ SSC for 15–16 hr at 62°C, in small tubes under a thin layer of paraffin wax. After incubation 10–20 vol of $2 \times$ SSC at 62°C were added and the incubation continued for a further hour when the solution was brought to 37°C and, after removal of the wax, treated with RNAase unless otherwise stated, at 10–12 μ g per ml for 20 min. The solution was then filtered under moderate suction through nitrocellulose filters (Sartorius Membrane filter MF-50 weight constant). The membranes were washed with 100 ml $2 \times$ SSC, dried and counted in a toluene-based scintillation fluid in a scintillation counter.

With the alternative method, pH-denatured DNA was loaded onto nitrocellulose filters in $6 \times$ SSC, dried and allowed to filter through under gravity, followed by washing with 30–40 ml of $2 \times$ SSC and only very gentle suction. With this procedure DNA retention was 90% or greater. Following the procedure of LANDY, ABELSON, GOODMAN and SMITH (1967) control membranes were treated in the same way, except that DNA was omitted. A pair of membranes, with and without DNA, were rolled up and inserted into narrow tubes, and these were kept *in vacuo* overnight at 4°C. The tubes were then heated *in vacuo* at 80°C to fix the DNA to the membranes. Reaction mixtures were prepared and transferred to the tubes with the membranes, and incubation was carried out as noted above. After incubation, the membranes were extensively

washed on both sides with $2 \times \text{SSC}$, treated with RNAase at a concentration of $20 \mu\text{g/ml}$ for 30 min at 37°C and then again washed extensively with $2 \times \text{SSC}$, before drying and counting.

The RNA bound to DNA was estimated from the difference in counts between the membranes with and without DNA. With this procedure there is a small transference of DNA, about 1–2%, from the loaded to the blank membranes. All comparisons refer to experiments carried out at the same time under identical conditions, and, in addition, when the behaviour of different cRNA preparations has been compared, the same batch of enzyme has been used in their preparation unless stated otherwise.

In double-label experiments, quenching curves were constructed by the channels ratio method (BAILLIE 1960) and the observed values have been converted to equivalent relative efficiencies.

RESULTS

(i) *Properties of the cRNA*: Two types of hybridisation experiment may be carried out to discover the general properties of the hybridisation between cRNA and DNA. In one type the amount of RNA is held constant and the concentration of DNA in the reaction mixture is varied while, in the other, the RNA is varied and the DNA, fixed to a membrane, is held constant. Such tests provide estimates of, respectively, the proportion of the cRNA which can be bound to DNA and the proportion of the DNA which binds to RNA. We deal first with the effects of varying the level of DNA.

Figure 1 shows a typical curve relating the percentage of the RNA bound to the ratio of DNA to RNA in the incubation mixture. The plot of the reciprocals is generally linear and so the maximum estimated level of hybrid formation may be estimated from the intercept of the regression slope on the ordinate. The values vary to some extent between experiments but generally fall between 12 and 15%.

FIGURE 1.—Different concentrations of heat-denatured DNA, prepared from *Drosophila melanogaster*, were annealed for 16 hr at 62°C in 0.30 ml $2 \times \text{SSC}$ in the presence of a constant amount of homologous cRNA at a concentration of $1.3 \mu\text{g/ml}$. General procedure and the recovery of hybrid material are described in the text.

FIGURE 2.—Heat-denatured DNA from *Drosophila melanogaster* and homologous cRNA at concentrations of, respectively, 3 and 5 $\mu\text{g}/\text{ml}$ were annealed for different times under the conditions described in Figure 1.

In a number of determinations of RNAase-resistance it was found that 80–90% of the RNA retained by filtration, after incubation with denatured DNA, was resistant to treatment with RNAase. Thus, although the use of RNAase does not greatly reduce the estimates of the proportion of RNA bound, it reduces the background to unimportant levels.

No increase in hybrid formation has been observed beyond 16 hr at the temperature used. Comparisons are shown in Figure 2 for an incubation mixture containing RNA and DNA at concentrations of, respectively, 5 μg and 3 μg per ml. The regression of the reciprocal of RNA bound on the reciprocal of time is linear. The reaction was more than 50% complete in 2 hr and about 75% complete after 4 hr. The percentage DNA bound to RNA reached a maximum of 0.8% in this test. The relations between rate of hybrid formation and the low concentration of RNA and DNA in the reaction mixture suggest a comparatively high level of reiteration in the sequences which form hybrid.

The proportion of the DNA which can be bound to RNA, in the presence of excess cRNA, which has been synthesised with the aid of homologous primer, was estimated by incubating membranes carrying 1–1.3 μg DNA with different concentrations of RNA. The “coverage” of the DNA may be estimated from the regression of the reciprocal of the percent DNA bound on the reciprocal of the RNA concentration. The reciprocal of the intercept of the ordinate estimates the maximum coverage of the DNA. For hybridisation between *D. melanogaster* DNA and homologous cRNA, this value worked out at 8–9% as shown in Figure 3.

This estimate refers to the total DNA, whereas, *in vivo*, only one strand is transcribed. Symmetry of transcription has been tested by heating cRNA for 10 min at 95°C, to dissociate possible duplexes, followed by incubation in $2 \times \text{SSC}$ for 16 hr at 62°C. At a concentration of 9 μg per ml, the highest used, 5–7% increase in RNAase resistance was observed, from which we infer that symmetrical transcription occurs as expected with *Micrococcus* polymerase (COLVILL, KAN-

FIGURE 3.—Tritium labelled DNA, prepared from *Drosophila melanogaster* and fixed to nitrocellulose membranes at a concentration of approximately $1 \mu\text{g}/\text{membrane}$, was incubated with different concentrations of cRNA which was synthesised with either *melanogaster* (closed circles) or *simulans* DNA (open circles) as template. The other conditions of annealing were as described in Figure 1.

NER, TOCCHINI-VALENTINI, SARNAT and GEIDUSCHEK 1965) although we cannot yet say how complete this is.

From the general evidence presented by BRITTEN and KOHNE (1967) we might infer, from the rates of hybrid formation and the concentrations of DNA and RNA in the reaction mixtures, that reiterated sequences are responsible for the hybridisation. To provide more direct support for this view, cRNA was incubated with DNA which was either enriched with or deficient in such sequences. The alternative categories of DNA were prepared by hydroxyapatite fractionation after heat-denatured, sonicated DNA had been allowed to renature at $100 \mu\text{g}$ per ml for 6 hr at 65°C . The 0.12M and 0.4M fractions were loaded on nitrocellulose membranes and incubated with homologous cRNA. Figure 4 indicates a low level of binding with the 0.12M fractions, from which the greater part of the rapidly re-naturing DNA has been removed, compared with the high level with the 0.40M fraction. The coverage of the DNA, in the latter case, was higher than is observed when cRNA is hybridised with homologous, unsonicated DNA. Since the 0.40M fraction comprises about 12% of the total DNA, a higher coverage might have been expected. It is possible that the use of sonicated DNA introduces new features, such as loss of some hybrid material from the membranes, leading to an underestimation of the true level of hybridisation. However, there is no doubt

FIGURE 4.—Labelled DNA from *D. melanogaster* was sonicated, denatured, allowed to renature and then separated into unrenatured and renatured fractions of hydroxyapatite fractionation at 0.12 M and 0.40 M phosphate. The alternative fractions were fixed to membranes at 0.30–0.40 $\mu\text{g}/\text{membrane}$ and incubated with different concentrations of cRNA. —●— and —○— represent the RNA bound to the 0.40 M and 0.12 M fractions, respectively.

that the most rapidly renaturing DNA sequences bind RNA much more effectively than the slowly renaturing sequences.

Other tests have shown that the DNA which renatured, under the conditions noted above, during the first 15 min of incubation, formed about twice as much hybrid per μg DNA as DNA which was allowed to renature for a further 6 hr, after the removal of the 15 min fraction. In all cases the level of binding to the 0.12M fraction was very low. We may therefore conclude that the comparisons between genomes described in this paper refer to the highly reiterated fraction of the genome. Also there are indications from the last-mentioned experiment and the formation of hybrid at very low concentrations of DNA and RNA (Figure 2), that the total reiterated fraction of the genome of *Drosophila melanogaster* may be divisible into more and less highly reiterated categories. The relative contribution of these categories to the observed levels of hybridisation will depend chiefly on the concentration of the reacting DNA and/or RNA.

MELLI and BISHOP (1969) have reported that hybridisation between cRNA and homologous rat DNA also involves the reiterated fraction of the genome.

(ii) *The specificity of the reaction:* To determine the level of discrimination, cRNA from *Drosophila melanogaster* was hybridised with homologous DNA and with DNA from T4, *Xenopus* (Amphibia), *Rattus* (Mammalia) and two insects, *Schistocerca gregaria* and *Aedes aegypti*. *Schistocerca* belongs to the order Orthoptera while *Aedes*, like *Drosophila*, is a member of the Diptera, classified in

TABLE 1

Comparison of the relative effects of hybridising D. melanogaster cRNA with DNA from different species

Origin of DNA	Hybridisation
<i>D. melanogaster</i>	100.0
<i>Aedes</i>	5.1
<i>Schistocerca</i>	3.6
<i>Xenopus</i>	0.5
<i>Rattus</i>	1.5
Phage T4	0.0

the Suborder Cyclorrapha. The comparisons were all carried out in replicate at the same time at a high DNA/RNA ratio (200:1). Table 1 shows the values, expressed as percentage of the cRNA which was bound in the homologous reaction between *Drosophila* DNA and cRNA.

No reaction was observed with T4. For the other comparisons, the ratios worked out at less than 2% for *Xenopus* and the rat and only 3.5 and 5.2%, respectively, for *Schistocerca* and *Aedes*, the two insects. The very low level of cross-reaction between *Drosophila* and *Aedes* implies a considerable difference in those sequences which are involved in the hybridisation.

The comparisons were extended to members of the genus *Drosophila*, namely *melanogaster*, *simulans* and *funnebris*. The first two are sibling species which are almost indistinguishable in morphology and whose salivary banding shows only minor differences (PONTECORVO 1942). When *simulans* females are crossed with *melanogaster*, adults of both sexes are produced although the viability of females is low. In the reciprocal cross, only females or nondisjunctional males are obtained (STURTEVANT 1929); the hybrid progeny are sterile. Thus, although the two species are reproductively isolated, they are closely related. *D. funnebris*, on the other hand, is more distantly related and is classified in the subgenus *Drosophila*, while the other two species belong to the subgenus *Sophophora*.

For these comparisons cRNA was prepared with DNA from either *melanogaster* or *simulans*. Each type was hybridised with each of the alternative types of DNA and also with *funnebris* DNA. Figures 5 (a) and (b) show the relations between the RNA bound at corresponding DNA/RNA ratios in the experiments with, respectively, *melanogaster* and *simulans* cRNA. There is a striking difference between the sibling species, while the cross-reaction between *funnebris* DNA

FIGURE 5.—cRNA prepared with either *D. melanogaster* (a) or *D. simulans* (b) DNA as template was incubated at 1.9 μg per ml in the presence of either homologous DNA (black circles) or heterologous DNA from the sibling species (open circles) or *D. funnebris* DNA (crosses). Incubation conditions and recovery of hybrid material were as described in Figure 1.

TABLE 2

Intragenetic comparison of the relative effect of hybridising melanogaster or simulans cRNA with heterologous DNA

Origin of the DNA	Relative hybridisation	
	<i>melanogaster</i> cRNA	<i>simulans</i> cRNA
<i>D. melanogaster</i>	100	36
<i>D. simulans</i>	34	100
<i>D. funebris</i>	10	10

and the cRNA of either of the sibling species is very low. The maximum of hybridisation for the *melanogaster* and *simulans* comparisons were estimated from the plot of the reciprocal of the percentage RNA bound on the reciprocal of the DNA/RNA ratio and these values have been tabulated as a proportion of the level of hybridisation in the corresponding homologous reactions (Table 2). In these terms, *funebris* DNA binds either cRNA only about a tenth as well as does either homologous DNA. For the two sibling species, the heterologous reactions result in only about 40% of the hybridisation which is obtained in the homologous reactions. Thus, in spite of the close taxonomic affinity of *melanogaster* and *simulans*, there appear to be substantial differences in the sequences which take part in the DNA-RNA hybridisation.

To provide further evidence of difference between the sibling species, the amount of *melanogaster* or *simulans* cRNA which binds to a known amount of

FIGURE 6.—cRNA prepared with DNA template, derived from either *D. melanogaster* (closed circles) or *D. simulans* (open circles) was incubated at a concentration of 3 $\mu\text{g}/\text{ml}$ with labelled *melanogaster* DNA, fixed to membranes at a concentration of approximately 1 $\mu\text{g}/\text{membrane}$, for different periods of time. Conditions were, otherwise, as described in Figure 1.

labelled *melanogaster* DNA was determined at different times at a low RNA concentration and also for 16 hr with different concentrations of RNA. Figure 6 shows the time course of hybridisation between DNA, fixed to membranes, and homologous or heterologous RNA at a concentration of $5\mu\text{g}$ per ml. As noted earlier, the reaction is virtually complete by 8 hr, by which time the coverage of the DNA amounts of 1.68% by weight for *melanogaster* RNA and only 0.80% for *simulans* RNA. The ratio between these two values is 0.48.

In the other test, illustrated in Figure 3, in which variable concentrations of RNA were hybridised to standard amounts of DNA, the maximum coverage, estimated from the intercept of the ordinate by the regression of the reciprocal of coverage upon the reciprocal of RNA concentration, worked out at 4.1% for *simulans* RNA compared with 8.9% for the homologous, *melanogaster* RNA, as noted earlier. The ratio between these two values is 0.46.

Thus the alternative estimates of the differences between the genomes of the sibling species agree quite well. They suggest that 40–50% of the sequences, which hybridise with cRNA under our conditions, are sufficiently different to prevent RNAase-resistant duplex formation when the RNA and DNA are heterologous in origin.

(iii) *The proportion of the DNA due to reiterated sequences:* The interpretation of species differences in reiterated sequences, either by DNA-DNA or RNA-DNA hybridisation, has to take account of possible differences in the fraction of the genome made up of families of reiterated sequences. To discover whether such gross differences were involved in the present comparisons, DNA of *Schistocerca*, *Aedes*, *Drosophila melanogaster* and *D. funebris* was sonicated at the high level, heat denatured and incubated for sixteen hours at similar concentrations at 60°C ; the C_{ot} value was 38 (BRITTEN and KOHNE 1967). After renaturation the mixtures were passed through hydroxyapatite columns, held at 60°C in 0.12M phosphate. The retained DNA was then eluted stepwise with 0.20, 0.28 and 0.40M phosphate. The proportion of the total DNA eluted at the different molarities is shown in Figure 7. The higher the molarity of elution the more exact will be the degree of duplex formation. The elution profiles therefore afford some indication of frequency differences in levels of complementarity, while the total DNA eluted at 0.20M and above will be mostly reiterated DNA. Under the conditions used, we expect part of the reiterated DNA and a fraction of the single-copy DNA to renature, but the greater part of the renatured DNA will be due to the former. Of course, hydroxyapatite fractionation will overestimate the true level of duplex formation and the extent to which this is so will probably vary according to the frequency and homogeneity of the sequences in different reiterated families.

Figure 7 shows striking differences between species. The lowest estimate of total renatured fraction was provided by *Drosophila melanogaster* (18%), *D. funebris* was next with a value of 34%. The other insects had much higher values, namely 44% and 60% for *Schistocerca* and *Aedes*, respectively. In addition, the elution profiles differ. Both *Aedes* and *Schistocerca* have a much higher proportion eluting at intermediate molarities (0.20M and 0.28M). The contrast between *melanogaster* and *funebris* has been observed in a number of independent ex-

FIGURE 7.—DNA from different species was sonicated, heat denatured and allowed to renature for 16 hrs at 130 $\mu\text{g}/\text{ml}$ at 65°C, and then fractionated by passing through hydroxyapatite columns at 60°C at 0.12 M, followed by stepwise elution at the higher molarities indicated. The amounts of DNA were estimated by the diphenylamine reaction.

periments, in which the renatured DNA has been eluted at 60°C with 0.12M and 0.4M phosphate after 6 hr incubation. The 0.40M fraction is generally about twice as great for *funebria*. Similar comparisons for *melanogaster* and *simulans* for different times of incubation have failed to show any difference between them.

Just how much hydroxyapatite fractionation overestimates the true level of renaturation is unknown but a two-fold overestimation may not be too wide of the mark. If this is so, then the reiterated fraction of the genome would work out roughly at about 10% for *melanogaster* and *simulans*, approximately twice this value for *funebria* and appreciably higher values for *Aedes* and *Schistocerca*. Experiments are in progress to arrive at more precise values. However it is obvious that gross differences in the proportion of the genome which is reiterated, as well as differences in the frequency of levels of reiteration, pose problems for the interpretation of DNA-DNA or RNA-DNA hybridisation.

DISCUSSION

These experiments support the view that the cRNA which binds to DNA, under our conditions, is in most cases, complementary to the reiterated sequences of the genome and that the species differences in levels of DNA-RNA hybridisation refer predominantly or almost exclusively to that fraction. The demonstration by MELLI and BISHOP (1969) that hybridisation between *in vitro* RNA and homologous rat DNA involves the reiterated fraction of the genome, suggests that this relationship is generally true for this kind of system. It cannot be assumed that the observed differences are equally representative of the single-copy sequences and this question is being studied in other experiments. It is a reasonable assumption that the products of transcription are sufficiently representative of the primer DNA, including the reiterated sequences, to permit certain general inferences. Since the maximum hybridisable DNA of *D. melanogaster* was estimated at about 9% in experiments in which a fixed amount of DNA was incubated with different concentrations of cRNA, this value provides an estimate of the reiterated fraction of the genome.

The possibility of detecting hybrid formation at extremely low concentrations of both DNA and RNA, after a few hours incubation, suggests the presence of a highly reiterated fraction, possibly analogous to what has been reported for some mammals (BRITTEN and KOHNE 1967). In experiments in which the DNA/RNA ratios include high values, it is likely that the hybrid material will include both the more and less highly reiterated fractions, and probably some nonreiterated sequences as well. Also, if we assume that estimates of renaturation by hydroxyapatite fractionation are about twice as high as the true values, we arrive at a similar figure.

It is unlikely that the fraction of cRNA bound to excess DNA is seriously influenced by competing DNA-DNA interaction. BOLTON *et al.* (1965) found little evidence for renaturation in denatured mouse DNA of high molecular weight, after incubation for 18 hr at 60°C, and also reported that 60% of denatured mouse DNA fragments could be bound to DNA networks formed by pre-annealing high molecular weight DNA in free solution. Since the *Drosophila* DNA also has a high molecular weight (45×10^6 to 110×10^6 daltons), similar networks are probably also formed and provide favourable conditions for hybridisation with RNA.

When cRNA is allowed to react with DNA from different forms, the levels of hybridisation show internal consistency; the closer the relationship, the higher the level of hybridisation. However, the level of binding between *Drosophila* cRNA and the DNA from another member of the Cyclorrapha, namely *Aedes*, is remarkably low. The relations between the taxonomic scale of difference and the primary difference in base sequence may vary. Indeed BOLTON (1965) claimed that the diversity within the plant family Leguminosae is equivalent, in terms of DNA-DNA hybridisation, to the diversity between different orders of mammals. There is little doubt that gross differences in the proportion of the

genome accounted for by reiterated sequences will favour the detection of differences by hybridisation.

A possible explanation of such low levels of cross-reaction between *Drosophila* and *Aedes*, or between members of the same genus, is that transcription is restricted so that only or mostly the sequences peculiar to a given species are transcribed. But if this were so we should expect a much higher level of hybridisation of cRNA with homologous DNA than is observed. Also, *a priori*, it is improbable that transcription will be biased in this particular way.

The differences detected by RNA-DNA hybridisation may be compared with the DNA-DNA hybridisation experiments recently reported by LAIRD and MCCARTHY (1969). On the basis of competition with unlabelled DNA, they reported approximately 20% and 75% differences between *melanogaster* and, respectively, *simulans* and *funebis* compared with our figures of about 50 and 90%. The alternative approaches agree in showing a well-defined difference between the sibling species and also in the greater difference between members of the different subgenera. The apparently higher level of discrimination in the RNA-DNA experiments may be due to the treatment of the reaction mixture with a high concentration of RNAase, which provides a more stringent test of complementarity. Since double-labelling was not used in LAIRD and MCCARTHY's tests, it is not clear what proportion of the genome was involved in their comparisons.

Although DNA-DNA and RNA-DNA hybridisation can reveal striking differences in the reiterated sequences of closely related species (see also McLAREN and WALKER's (1968) comparisons between species of rodents), the quantitative interpretation of such differences in terms of base sequence changes poses some formidable problems. The same sequence may be subject to different levels of reiteration in different species so that its probability of hybridisation and relative contribution to the total hybrid material which is formed may be different. And this may be true even though the total reiterated fractions of the genomes are the same. In addition, of course, there are the effects of base substitution which progressively destroy the resemblance between corresponding sequences. There will be great variation in just how precisely particular sequences are reiterated. The level of effective similarity in hybridisation experiments will be influenced by how many successive base pairs must be complementary to form stable hybrid in the particular conditions of the experiment. WALKER (1968) has suggested a figure of 20–30 pairs. At present such estimates are tentative and the real value may be higher. Thus in RNA-DNA or DNA-DNA hybridisation experiments, the observed differences represent the summation of differences in complex frequency distributions of sequences.

These considerations also apply to the measurement of rates of renaturation by hydroxyapatite fractionation or to monitoring the change in optical density. In either case, the observed changes represent the summation of hydrogen bonding between more or less complementary sequences which probably span a wide range of frequency. A discriminating analysis of the relative contribution to rates of change in terms of the underlying frequency distribution of reiterated sequences presents a problem which has yet to be solved.

The difficulties of interpretation do not end there. BRITTEN and KOHNE's (1967) interpretation of the origin of reiteration implies some resemblance between members of particular families of reiterated sequences for historical reasons, insofar as they have arisen by "saltatory replication". This implies a corresponding functional affinity. On the other hand, WALKER (1968) has argued that the range of possible primary structures of proteins is limited and that there are sets of recurring amino acids in unrelated proteins and hence sets of recurring nucleotide sequences to provide the basis for different families of reiterated sequences. In this situation the historical affinity within such families would be highly attenuated and apparently similar sequences could be functionally unrelated. The two interpretations are not mutually exclusive and both functionally related and unrelated sequences may contribute indistinguishably to apparent reiteration. However, in the second alternative, it does seem a little difficult to account for the striking differences between species in the proportion of the genome which is reiterated, while it is not immediately obvious why recurring sequences of amino acids should be conspicuously scarce in bacterial proteins.

Nevertheless, the striking fact remains that the populations of reiterated sequences are apparently subject to rapid evolutionary divergence. Whether this represents merely the product of mutation pressure and the duration of genetic isolation or whether some form of natural selection actively promotes such divergence is open to speculation and future study.

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SUMMARY

Hybridisation between DNA and synthetic, complementary RNA, prepared with the aid of RNA polymerase extracted from *Micrococcus lysodeikticus* has been investigated as a method for the evaluation of genome differences. The general properties of the hybridisation reaction are described. Fractionation of re-natured DNA by hydroxyapatite and the incubation of different fractions with synthetic RNA showed that only, or mostly, the reiterated fraction of the genome was involved in the RNA-DNA hybridisation.—Synthetic RNA transcribed from the DNA of *Drosophila melanogaster* was used as a basis for comparing genomes and determining the level of discrimination between reiterated sequences. Cross-reaction between cRNA made from a template of *D. melanogaster* DNA and DNA from unrelated forms like phage T4, *Rattus* and *Xenopus* was nil, or extremely low. With insects such as *Schistocerca* and the more closely related fellow-member of the Diptera, *Aedes*, the reaction was, respectively, about 3.5 and 5%

of the values obtained in the homologous reaction.—Intragenetic comparisons included the sibling species *Drosophila melanogaster* and *simulans* and also *funnebris*, which is placed in a different subgenus. Hybridisation between either *melanogaster* or *simulans* cRNA and the heterologous DNA was 40–50% as effective as either of the homologous reactions. The cross-reaction with either cRNA and *funnebris* DNA was only about 10% as effective. Alternative estimates of differences between the sibling species based on estimates of the proportion of *melanogaster* DNA which was bound to either homologous cRNA at different concentrations of RNA, or with incubation for different times, led to similar conclusions.—Stepwise elution from hydroxyapatite of renatured DNA from different species showed substantial differences in the fraction of the genome accounted for by reiterated sequences and in the elution profiles.—The discrimination achieved by these methods favours their application to the study of differences in reiterated sequences generally, especially in comparisons between closely related forms.—Problems of interpretation are discussed.

LITERATURE CITED

- BAILLIE, L. A., 1960 Determination of liquid scintillation counting efficiency by pulse height shift. *Internatl. J. Appl. Radiation Isotopes* **8**: 1–27.
- BISHOP, J. O., and F. W. ROBERTSON, *In vitro* transcription of bacteriophage T4 DNA. *Biochem. J.* (in press).
- BOLTON, E. T., 1965 Plant nucleic acids. *Carnegie Inst. Wash. Yearbook* 314–342.
- BOLTON, E. T., R. J. BRITTEN, D. B. COWIE, R. B. ROBERTS, P. SZAFRANSKI, and M. J. WARING, 1965 Plant nucleic acids. *Carnegie Inst. Wash. Yearbook* 313–348.
- BRITTEN, R. J., and D. E. KOHNE, 1967 Nucleotide sequence repetition in DNA. *Carnegie Inst. Wash. Yearbook* 78–106.
- BURTON, K., 1956 A study of the condition and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J.* **62**: 315–323.
- COLVILL, A. J. E., L. C. KANNER, G. P. TOCCHINI-VALENTINI, M. T. SARNAT and E. P. GEIDUSCHEK, 1965 Asymmetric RNA synthesis *in vitro*: heterologous DNA-enzyme systems; *E. coli* RNA polymerase. *Proc. Natl. Acad. Sci. U.S.* **53**: 1140–1147.
- GILLESPIE, D., and S. SPIEGELMAN, 1965 A quantitative assay for DNA-RNA hybrids with DNA immobilized on a membrane. *J. Mol. Biol.* **12**: 829–842.
- HASTINGS, J. R. B., and K. S. KIRBY, 1966 The nucleic acids of *Drosophila melanogaster*. *Biochem. J.* **100**: 532–539.
- HOYER, B. H., B. J. MCCARTHY, and E. T. BOLTON, 1964 A molecular approach in the systematics of higher organisms. *Science* **144**: 959–967.
- HOYER, B. H., E. T. BOLTON, B. J. MCCARTHY, and R. B. ROBERTS, 1965 The evolution of polynucleotides, pp. 581–590. In: *Evolving genes and proteins*. Edited by V. BRYSON and H. J. VOGEL. Academic Press, N.Y.
- KISSANE, J. M., and E. ROBBINS, 1958 The fluorimetric measurement of deoxyribonucleic acid in animal tissues with special reference to the central nervous system. *J. Biol. Chem.* **233**: 184–188.
- LAIRD, C. D., and B. J. MCCARTHY, 1969 Magnitude of interspecific nucleotide sequence variability in *Drosophila*. *Genetics* **60**: 303–322.
- LANDY, A., J. ABELSON, H. M. GOODMAN, and J. D. SMITH, 1967 Specific hybridisation of

- tyrosine transfer ribonucleic acids with DNA from a transducing bacteriophage ϕ 80 carrying the amber suppressor gene su_{111} . J. Mol. Biol. **29**: 457-471.
- LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL, 1951 Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**: 265-275.
- MANDELL, J. D., and A. D. HERSHEY, 1960 A fractionation column for analysis of nucleic acid. Analytical Biochem. **1**: 66-77.
- McLAREN, A., and P. M. B. WALKER, 1966 Discriminating power of rodent deoxyribonucleic acid on incubation in agar. Nature **211**: 486-490. ——— 1968 The comparison of closely related rodents by DNA/DNA annealing. Genet. Res. **12**: 117-124.
- MARMUR, J., 1961 A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J. Mol. Biol. **3**: 208-218.
- MELLI, M., and J. O. BISHOP, 1969 Hybridisation between rat liver DNA and complementary RNA. J. Mol. Biol. **40**: 117-136.
- NAKOMOTO, T., C. F. FOX, and S. B. WEISS, 1964 Enzymatic synthesis of ribonucleic acid 1. Preparation of ribonucleic acid polymerase from extracts of *Micrococcus lysodeikticus*. J. Biol. Chem. **239**: 167-174.
- NYGAARD, A. P., and B. D. HALL, 1964 Formation and properties of RNA-DNA complexes. J. Mol. Biol. **9**: 125-142.
- PONTECORVO, G., 1943 Viability interactions between chromosomes of *Drosophila melanogaster* and *Drosophila simulans*. J. Genet. **45**: 51-66.
- RITOSSA, F. M., and S. SPIEGELMAN, 1965 Localization of DNA complementary to ribosomal RNA in the nucleolus organizer region of *Drosophila melanogaster*. Proc. Natl. Acad. Sci. U.S. **55**: 737-745.
- ROBERTSON, F. W., 1960 The ecological genetics of growth in *Drosophila* 1. Body size and development time on different diets. Genet. Res. **1**: 228-304.
- ROLFE, R., and M. MESELSON, 1959 The relative homogeneity of microbial DNA. Proc. Natl. Acad. Sci. U.S. **45**: 1039-1043.
- SANG, J. H., 1956 The quantitative nutritional requirements of *Drosophila melanogaster*. J. Exptl. Biol. **33**: 45-72.
- STURTEVANT, A. H., 1929 The genetics of *Drosophila simulans*. Carnegie Inst. Wash. Publ. **399**: 1-62.
- SUEOKA, N., 1961 Variation and heterogeneity of base composition of deoxyribonucleic acids: a computation of old and new data. J. Mol. Biol. **3**: 31-40.
- TISELIUS, A., S. HJERTÉN and Ö. LEVIN, 1956 Protein chromatography on calcium phosphate columns. Arch. Biochem. Biophys. **65**: 132-155.
- WALKER, P. M. B., 1968 How different are the DNAs from related animals? Nature **219**: 228-232.
- WEISS, S. B. and T. NAKAMOTO, 1961 On the participation of DNA in RNA biosynthesis. Proc. Natl. Acad. Sci. U.S. **47**: 694-697.