NUCLEOTIDE SEQUENCE HOMOLOGY WITHIN THE GENOME OF *DROSOPHILA MELANOGASTER*[1]

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THERE are many facets to the study of gene variability. The determination of patterns and rates of divergence of genes in related organisms affords a comparison with phyletic relationships determined by phenotypic traits. On a molecular level, a general approach has been the comparison of polynucleotide sequences by DNA/DNA duplex formation (DOTY *et al.*, 1960) and RNA/DNA hybridization (MCCARTHY and BOLTON 1963) of nucleic acids from different organisms. A more detailed analysis is possible by comparing structures of specific gene products of related organisms (see ZUCKERKANDL and PAULING 1965). A more limited set of relationships is revealed by the analysis of variants at a single locus within a population. Hemoglobin amino acid sequence data have proved useful in estimating the number and relationship of different alleles (see RUCKNAGEL and NEEL 1961). The detection of products of allelic genes extends this analysis to a number of loci (HUBBY and LEWONTIN 1966; HARRIS 1966).

An additional set of relationships may exist among genes within the genome of a single organism. The well-known increase of DNA content per nucleus with organism complexity (see MCCARTHY 1965) has encouraged speculation as to the mechanism by which more complex genomes have been generated through evolution. An important mechanism for the development of new base sequences may involve gene duplication followed by divergence (BRIDGES 1935). Again, the detailed amino acid comparisons of functionally related proteins have provided specific examples of this kind of gene evolution (WALSH and NEURATH 1964). Proteins with overtly different functions but related amino acid sequences have also been reported (BREW, VANAMAN and HILL 1967), and the number of these examples will surely increase. In Drosophila, gene duplications are well-known. STURTEVANT (1925) reported a duplication at the Bar locus, leading to the suggestion that such duplications could be an important evolutionary process. Cytological comparisons of banding patterns in polytene chromosomes of Drosophila have provided other possible examples of gene duplication (BRIDGES 1935).

An approach to the detection of base sequence relationships within a genome utilizes techniques of specific duplex reactions of polynucleotides. Comparison of the rate of formation and the structures of these duplexes, with the correspond-

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ing properties expected for true renaturation, has suggested the presence of large families of related base sequences in mammals (MARTIN and HOYER 1966; BRITTEN and KOHNE 1968). The application of such molecular techniques to the nucleic acids of Drosophila offers an unusual opportunity to analyze cytogenetic phenomena in molecular detail.

In a preceding paper we reported comparisons of nucleotide sequences between several species of Drosophila (LAIRD and MCCARTHY 1968). Similar experiments

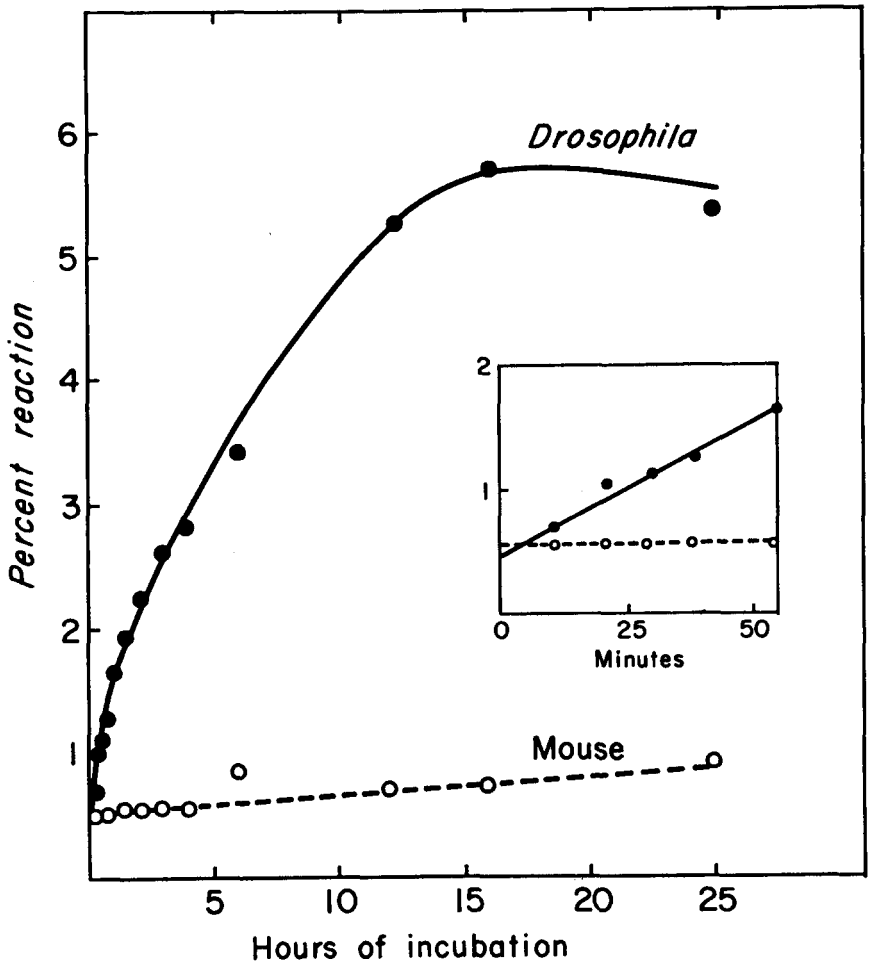

FIGURE 1.—Rate of reaction of labeled Drosophila DNA with filter-bound DNA as a function of time of incubation at 65°C. A series of reaction mixtures, each containing 1 μg of H^3-labeled, denatured *D. melanogaster* DNA and 15 μg of filter-bound, denatured *D. melanogaster* DNA or filter-bound, denatured mouse DNA in a final volume of 0.2 ml 1 × SSC, were incubated at 65°C. At the indicated times filters were removed and washed in 1 × SSC, and the filter-bound H^3 radioactivity was determined by liquid scintillation counting. The percent reaction with homologous Drosophila DNA filters (●—●) and heterologous mouse DNA filters (O—O) is plotted *versus* hours or minutes (inset) of incubation.

discussed below were directed toward defining nucleotide sequence relationships within the genome of *D. melanogaster*.

METHODS AND MATERIALS

Procedures for the labeling and isolation of Drosophila nucleic acids and formation of DNA/DNA duplexes and RNA/DNA hybrids have been outlined previously (LAIRD and MCCARTHY 1968). The determination of the rate of DNA/DNA duplex formation was as follows: a series of reaction vials, each containing filter-bound Drosophila DNA, were equilibrated at the temperature of reaction. 200 μl of 1 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl, 0.015 M sodium citrate) containing 1 μg H^3 labeled *D. melanogaster* pupal DNA (2100 cpm/μg) was added to each vial. At the indicated times a filter was removed and washed twice in 5 ml 1 $\times$ SSC; the filter bound H^3 radioactivity was determined by liquid scintillation spectrometry. Reaction rates were determined from the initial linear slope of the curve, usually from reactions occurring in the first 120 minutes. The prolonged incubations described in Figure 4 were carried out at lower temperatures in aqueous solutions of formamide (BONNER, KUNG and BEKHOR 1967). This reduces the risk of thermal degradation and the leaching of DNA from the membrane filters.

RESULTS

Rate of duplex formation of Drosophila DNA. The reaction of labeled Drosophila DNA with filter-bound DNA was determined as a function of time and of temperature. Figure 1 illustrates the percent of labeled, denatured DNA binding to filter-bound, denatured DNA at 65°C over a 25 hour interval. As a control for heterologous binding, a series of vials containing filter-bound mouse DNA and labeled Drosophila DNA was incubated in parallel. Similar experiments were carried out at various temperatures between 22°C and 75°C. The initial rates of these reactions, expressed as percent labeled DNA bound per hour, are plotted in Figure 2. The rate of the heterologous reaction with *B. subtilis* DNA has been subtracted from that of the homologous Drosophila reaction to give the indicated results. The maximum homologous rate occurs at 50°C, with an indication of a shoulder at 60° to 65°C. The increase with temperature in the ratio of homologous to heterologous rates reflects the increased number of paired bases necessary for duplex stability.

Stability of duplexes formed at different temperatures: The thermal stability of DNA/DNA duplexes is a function of the number of paired bases (BAUTZ and BAUTZ 1964; KOTAKA and BALDWIN 1964). To compare the extent of base pairing relative to that observed in native DNA, Drosophila DNA duplexes were formed by incubating H^3-labeled DNA with filter-bound DNA. The filters were subjected to a temperature gradient and the radioactivity eluted at each temperature was determined. The cumulative percent released is plotted *versus* temperature in Figure 3 for each of five temperatures of duplex formation. At the high temperatures of incubation (60°C and 67°C) the midpoints of the irreversible dissociation ($T_{m,i}$) are 77 and 78°C, respectively, some 4° less than the $T_{m,i}$ expected for perfectly formed duplexes with a percent G+C of 43 (LAIRD and MCCARTHY 1968). The expected stability was estimated from that of *Bacillus subtilis* DNA/DNA duplexes of essentially the same percent G+C. A lower stability is displayed by the Drosophila duplexes formed at 50°C. Two com-

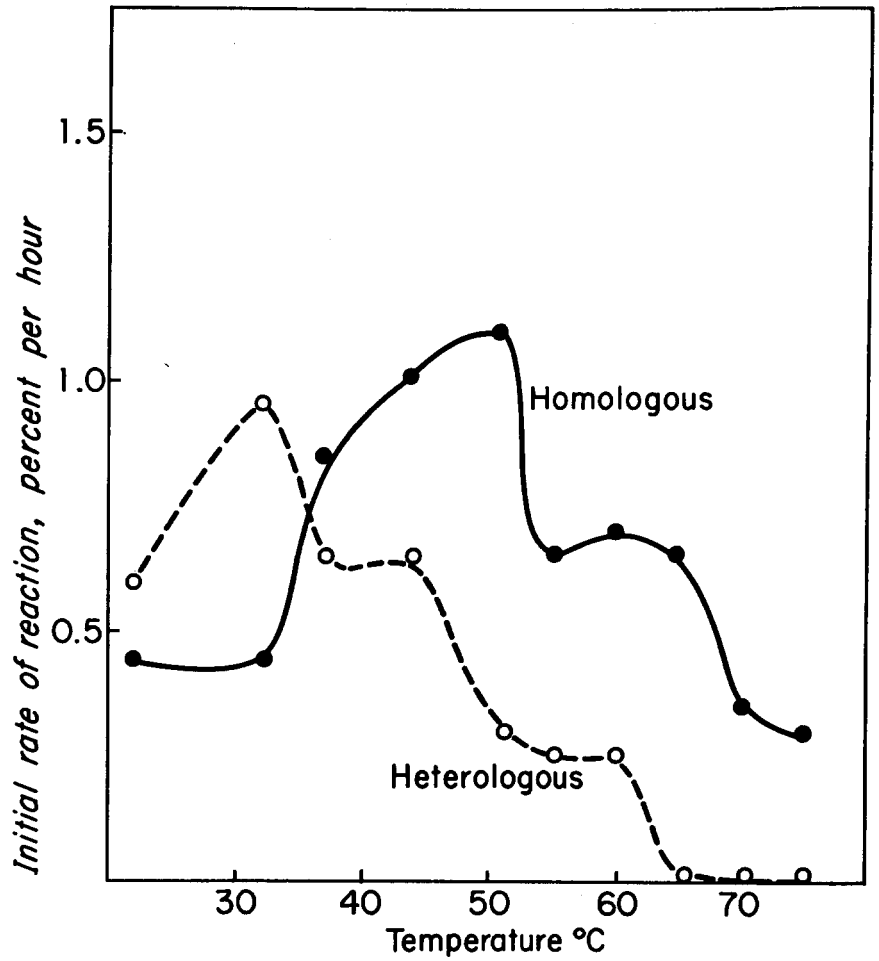

FIGURE 2.—Initial rate of reaction of Drosophila DNA with filter-bound DNA as a function of temperature. A series of reaction mixtures, each containing 1 μg of H^3 labeled *D. melanogaster* DNA (2300 cpm) and 10 μg of filter-bound *D. melanogaster* DNA in a final volume of 0.2 ml 1 × SSC, were incubated at the indicated temperatures. Filters were removed at various times and washed in 1 × SSC; the filter-bound H^3 radioactivity was determined by liquid scintillation counting. The rates of heterologous binding of the labeled Drosophila DNA to 10 μg of *B. subtilis* DNA have been subtracted from the homologous rates.

ponents are present with the majority having a $T_{m,i}$ of about 55°C, and the remainder having a $T_{m,i}$ of 78° to 80°C. Very low stability complexes are formed at incubation temperatures of 22°C and 37°C. The relative rates of homologous and heterologous reactions at various incubation temperatures (Figure 2) indicate that the complexes formed at 50°C and above are predominantly species specific. Thus, the Drosophila-specific complexes have stabilities of either approximately 55°C or 78°C, depending upon the incubation conditions. The extents of reaction for the 60°C and 67°C incubations are 9 and 6% (Figure 3). The sta-

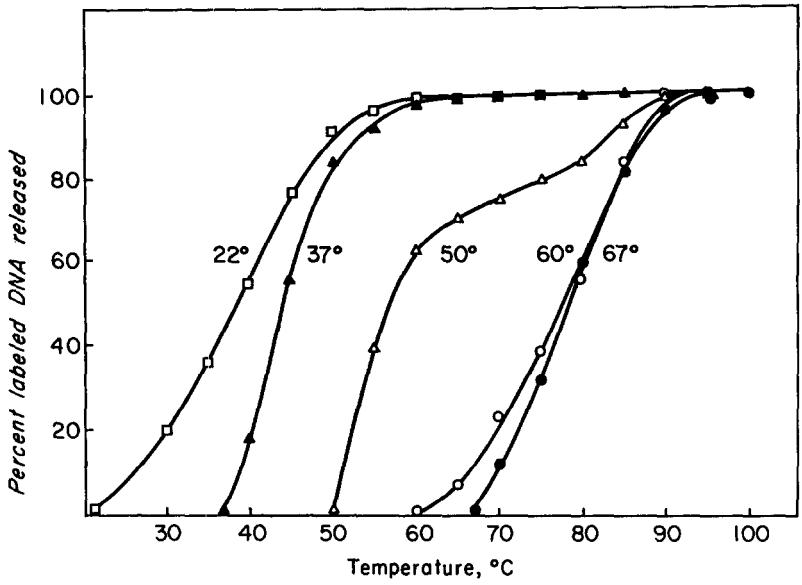

FIGURE 3.—Thermal stability of homologous Drosophila DNA/DNA duplexes formed at various temperatures. 0.4 μg H^3 labeled *D. melanogaster* DNA (680 cpm) was incubated with 40 μg filter-bound Drosophila DNA in 0.2 ml 1 × SSC for 18 hrs at 22, 37, 50, 60 or 67°C. The filters were then washed twice in 5 ml 1.0 × SSC for 3 min at the incubation temperature. The labeled DNA was eluted by incubation of the filters in 5 ml of 1 × SSC for 5 min at temperature increments of 5°C; the eluted DNA was precipitated with 10% trichloroacetic acid in the presence of 200 μg bovine serum albumin and collected by filtration for radioactivity determinations. The percent of input labeled DNA which reacted was 54, 41, 21, 9 and 6%, respectively, for the temperatures indicated above.

bilities of duplexes which are the products of more extensive reaction are described in the next section.

Serial reactions: Partially redundant base sequences react more rapidly than do unique sequences. Thus, the reaction of such molecules predominates in incubations of short duration and low extents of binding. The appropriate experiment, in which such molecules were removed from the reaction mixture by withdrawing the filter after short term incubations with the labeled DNA, is illustrated in Figure 4. The remaining solution DNA was again denatured and additional filter-bound DNA was added for further reaction. This process was repeated, and the extents of reaction and thermal stabilities of the duplexes were determined for each reaction step. This analysis of a large fraction of the total Drosophila DNA allows estimation of the proportion of repeated sequences. The results indicate that after the first 10 to 15% has reacted, the second and third sets of reaction products (15–30% and 30–39%) have the high thermal stability of 82°C expected for perfectly paired duplexes (Figure 4a). Thus, with these relatively restrictive conditions, only about 10% of the Drosophila DNA forms partially-base paired duplexes. A parallel set of incubations with *B. subtilis* DNA

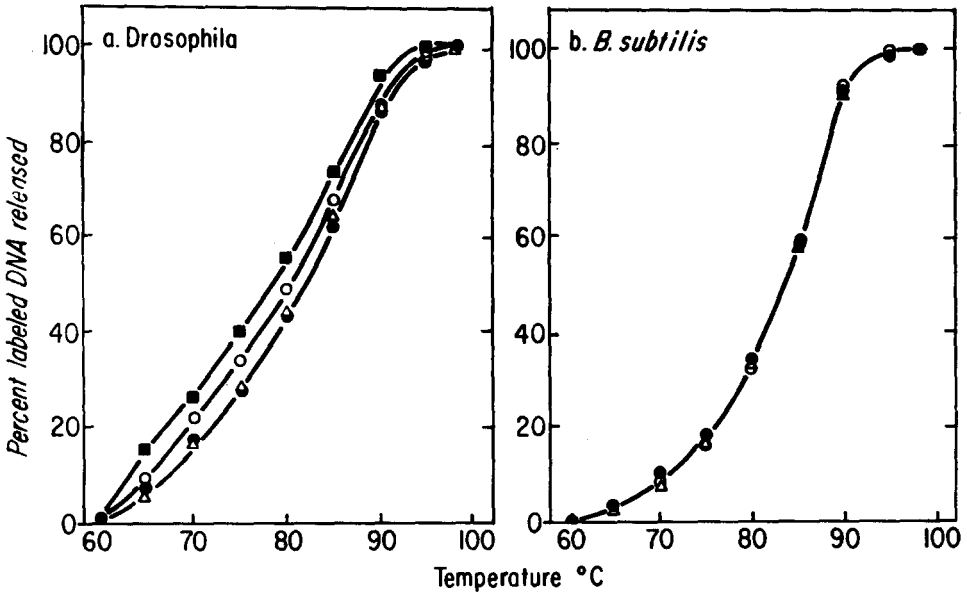

FIGURE 4.—Thermal stabilities of serial reaction products. (a) H^3-labeled, denatured Drosophila DNA (0.5 μg, 5600 cpm) was incubated with 70 μg filter-bound Drosophila DNA in 0.2 ml 2 × SSC, 40% (v/v) formamide at 40°C under mineral oil. After 4 hrs or 10 hrs, the filters were removed, washed 3 min in 2 × SSC, 40% formamide at 40°C, and 3 min in 1 × SSC at 40°C. The 10 hr incubation mixture was heated to 85°C to denature the labeled DNA in solution and 70 μg of filter-bound Drosophila DNA was added to the vial. After 24 hrs, the process was repeated and a third incubation continued for a further 46 hrs. The thermal stabilities of these four sets of duplexes were determined as described in the legend to Figure 3. The percent of the initially added labeled DNA which reacted at each step was 6% for the 4 hr incubation (■, $T_{m,i} = 78°C$); 14% for 10 hrs, (○, $T_{m,i} = 80°C$); 16% for 24 hrs, (●, $T_{m,i} = 82°C$); and 9% for 46 hrs, (△, $T_{m,i} = 82°C$). (b) H^3-labeled, denatured *Bacillus subtilis* DNA (0.5 μg, 3050 cpm) was incubated with 60 μg filter-bound *B. subtilis* DNA in 0.2 ml 2 × SSC, 40% formamide, at 40°C under mineral oil. After 10 hrs the filters were removed; further incubations for 24 hrs and 46 hrs were carried out as described in (a). The percents of labeled DNA bound were 19% (10 hrs, ○), 16% (24 hrs, ●), and 10% (46 hrs, △). The $T_{m,i}$ was 83.5%.

(Figure 4b) containing only unique base sequences (McCARTHY 1967) resulted in high thermal stability duplexes for each fraction of the reacting DNA.

Stability of ribosomal RNA/DNA hybrids: The reaction of 28S rRNA with the rRNA cistrons offers the opportunity to investigate whether similar relationships exist within a specific group of genes. H^3-labeled 28S Drosophila RNA was hybridized at different temperatures to filter-bound Drosophila DNA. Thermal stabilities were determined by dissociating the hybrids at increasing temperatures. The results are plotted in Figure 5 and indicate $T_{m,i}$'s of 77° to 78°C, in 0.5 × SSC, for the hybrids formed at 55°C, 60°C and 67°C. This $T_{m,i}$ is that expected for 40% G+C rRNA/DNA hybrids (MOORE and MCCARTHY 1967) and suggests that well-paired hybrids are formed even when the requirements for stability are lowered by decreasing the temperature of formation.

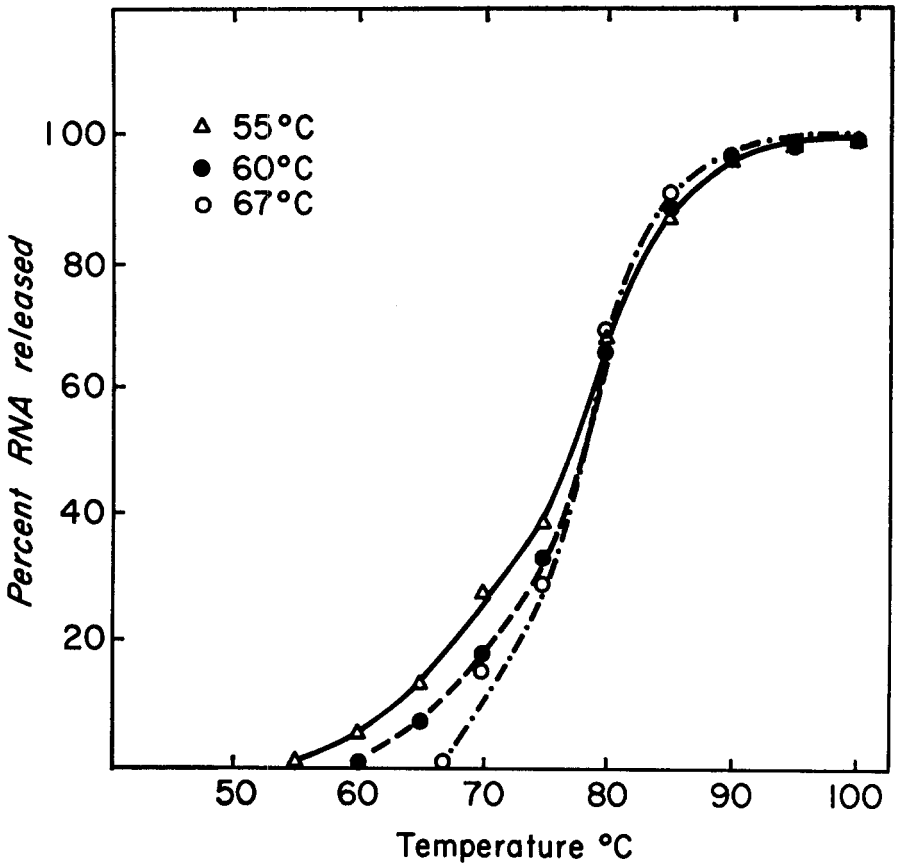

FIGURE 5.—Temperature stability of Drosophila 28S rRNA/DNA hybrids formed at different temperatures. 40 μg of filter-bound Drosophila DNA was incubated with 1 μg (1500 cpm) of H^3 labeled 28S rRNA in 0.2 ml 2 × SSC for 30 hrs at 55°C, 60°C or 67°C. The filters were then washed twice in 5 ml 0.5 × SSC for 3 min at the incubation temperature. The hybridized RNA was eluted by incubation of the filters in 5 ml of 0.5 × SSC for 5 min at temperature increments of 5°C; the eluted RNA was precipitated and counted as described in the legend to Figure 3. The ratio of RNA to DNA in the initial hybrids was 0.27%, 0.24% and 0.17% for the 55°C, 60°C and 67°C incubation conditions, respectively.

Experiments of GILLESPIE and SPIEGELMAN (1965) demonstrated the utility of RNase treatment in eliminating partially base-paired RNA/DNA hybrids. Resistance to enzyme digestion therefore provides an alternative assay for the fidelity of base-pairing. Figure 6 illustrates parallel saturation curves of DNA with rRNA in which one of each pair of hybrids was treated with RNase. In agreement with RITOSSA and SPIEGELMAN (1965) we find plateau values of 0.2% to 0.3% of the DNA saturated. Even at levels of RNA 20-fold above saturation of the rRNA cistrons, only 25–30% of the labeled 28S rRNA is released by RNase treatment, and this proportion does not change appreciably once saturation is reached. These results, together with the data on the thermal stability of

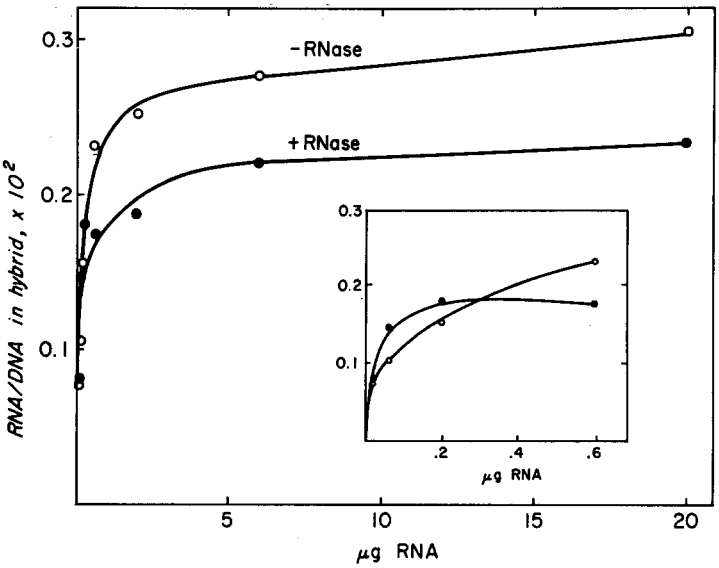

FIGURE 6.—Saturation of Drosophila DNA with H^3 labeled 28S rRNA. 10 μg of filter-bound *D. melanogaster* DNA was incubated in 0.2 ml 2 × SSC at 60°C for 18 hrs with increasing amounts of H^3-labeled *D. melanogaster* 28S rRNA (1500 cpm/μg). Filters were removed from parallel reaction vials, incubated for 20 min in 2 ml 2 × SSC at 37°C in the presence or absence of 5 μg/ml RNase, and washed 5 min at 60°C in 10 ml 2 × SSC.

the rRNA/DNA hybrids, suggest either that the nucleotide sequences of rRNA cistrons are so similar to one another as to form well-paired intercistronic hybrids, or so different that the rRNA molecules do not cross-react with DNA representing other ribosomal RNA cistrons.

DISCUSSION

These experiments are based on the expectation that duplex strand formation can occur between single-stranded DNA molecules representing different genetic sites that have similar but not identical base sequences. Such cross-reactions increase rates of reassociation since these depend on the concentration of reacting sequences (BRITTEN and KOHNE 1968). The duplexes formed between strands which are not perfectly complementary will be of lower thermal stability. Thus, quantitation of rates of reassociation of denatured DNA, and characterization of the reformed duplexes, will provide information concerning the relationships among sequences within a genome.

Experiments based on these principles have been reported for DNAs from a number of different organisms (MARTIN and HOYER 1966; MCCARTHY 1967; BRITTEN and KOHNE 1968). These indicate that, if attention is focused on sequences very similar to one another, bacteriophage and bacterial genomes are simple in the sense that denatured DNA sequences react only with their exact complements upon renaturation. On the other hand, the genomes of higher

organisms, in particular mammals, are complex in that a given sequence can reassociate with a large number of similar sequences. An extreme example is offered by the mouse satellite DNA containing up to 500,000 related base sequences (WARING and BRITTEN 1966).

These questions may be approached through study of duplex reactions between sheared, denatured DNA, and filter-bound DNA. The detailed results using the technique have been summarized elsewhere for bacteriophage T4, *Bacillus subtilis*, and mouse DNAs (MCCARTHY 1967). The data presented here for Drosophila DNA show that at high temperatures and with limited reactions, duplexes are formed with a mean stability of 78°C, about 4°C lower than expected for renatured DNA, suggesting that these Drosophila DNA structures are not perfectly base-paired. Duplexes with stability comparable to that of native DNA are observed when these initially-reacting sequences are removed from the incubation mixture (Figure 4). Five to fifteen percent of renatured Drosophila DNA is of lower stability, while the majority is indistinguishable from native DNA. This implies that about 10% of the base sequences in the Drosophila genome are partially redundant. These conclusions are substantiated by optical studies of the renaturation of Drosophila DNA (LAIRD and MCCARTHY in preparation).

Different structures, distinguishable by their further reduced stability, are formed at 50°C (Figure 3). Similar complexes are observed with *B. subtilis* and mouse DNA (MCCARTHY 1967). These duplexes are at least partially species specific since lower extents of reaction are displayed with DNA from very distantly related organisms. An interpretation offered for these phenomena in *B. subtilis* and mouse (MARTIN and HOYER 1966; MCCARTHY 1967) is that these duplexes are formed between very distantly related sequences, so dissimilar as to be unstable at high temperatures. With *B. subtilis* and Drosophila DNAs there seems to be a discontinuity between these low stability and high stability complexes, while mouse DNA duplexes exhibit a continuum of stabilities when reaction temperatures are varied. Possibly such distant base sequence homologies result from high rates of divergence of duplicated sequences necessitated by recombination restrictions operating in bacteria (THOMAS 1966) and some eucaryotes.

The number of elements within a family of interacting sequences may be estimated from considerations of reaction rates. Under similar conditions, DNA from organisms with small genomes renatures more rapidly than does DNA from organisms with large genomes (MARMUR and DOTY 1961) since in the former case a given complementary sequence is present at a higher relative concentration. A similar inverse correlation with size of the genome is evident from the variation of the rate of reaction of different DNAs in solution with immobilized DNA. Although the initial rates are functions of the concentration of DNA in solution and the amount in DNA fixed to the filter (MCCARTHY 1967) the real relationship is complicated by the immobilization of one of the interacting components, and the competing reassociation reaction in solution. This relationship must therefore be determined empirically rather than from simple theoretical considerations. In Figure 7, data obtained with bacteriophage, bacterial and

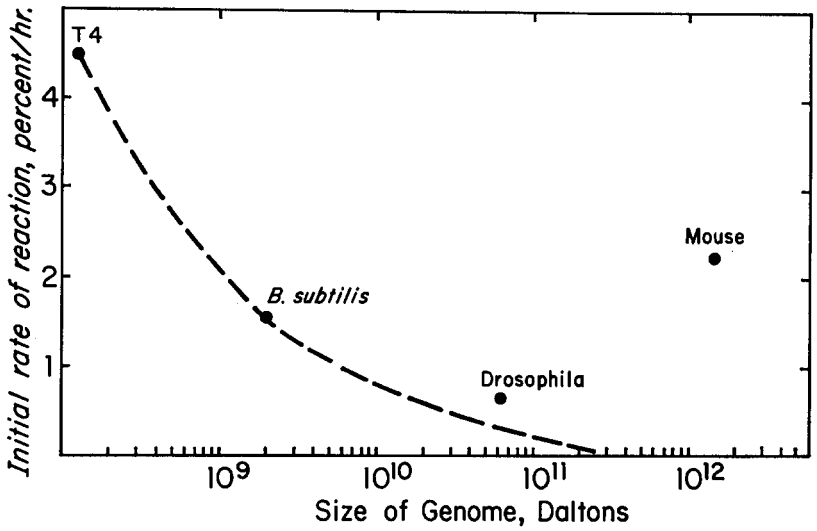

FIGURE 7.—Initial rates of duplex formation by the DNA of various organisms. The initial rate of reaction at 65°C is plotted *versus* the molecular weight of the genome (haploid value). The Drosophila data are taken from Figure 2. Data for T4, *B. subtilis* and mouse DNAs are taken from MCCARTHY (1967). The curve is an extrapolation from empirically determined reaction rates for simple DNAs.

mammalian DNAs (MCCARTHY 1967), are compared with the Drosophila data. Initial rates of reaction at 65°C are plotted against the genome size. The reference curve is an extrapolation from empirically determined reaction rates for simple genomes (LAIRD and MCCARTHY in preparation). These data suggest that the degree of partial redundancy of base sequences in Drosophila DNA is considerably lower than that present in mouse DNA. On the other hand, the partially mispaired duplexes formed by Drosophila DNA strongly imply some degree of intragenome homology. Preliminary estimates based on more detailed comparisons with DNA representing a nonredundant genome suggest an average size of 50 for the families of related sequences in Drosophila (LAIRD and MCCARTHY in preparation).

The set of 100–200 cistrons for Drosophila ribosomal RNA exhibit a different and more restricted kind of sequence relationship. The high thermal stability of the rRNA/DNA hybrids, and their relative insensitivity to ribonuclease, imply a high fidelity of base pairing. Moreover, structures of the same high stability are formed over a temperature range of 12°C and the thermal stability is close to that expected for 40% G+C rRNA/DNA hybrids (MOORE and MCCARTHY 1967). In these respects the properties of Drosophila rRNA/DNA hybrids differ from those of mammalian rRNA/DNA hybrids, but are analogous to those of bacteria. In the latter case, highly-paired, enzyme resistant structures are also formed over a range of temperatures by *E. coli* rRNA (MOORE and MCCARTHY 1968). In experiments with Drosophila rRNA, the relatively high rate of reaction (RITOSSA and SPIEGELMAN 1965; LAIRD and MCCARTHY in preparation), suggests

that hybrids can be formed between an rRNA molecule and a DNA sequence different from its original template. The high stability and the rapid rate of hybridization imply that these rRNA cistrons have very similar nucleotide sequences. A more precise quantitation of the base sequence differences among these interacting molecules can, in principle, be made through a knowledge of the relationship between percent of mispaired bases and $T_{m,i}$ as discussed for DNA of various species of Drosophila (LAIRD and MCCARTHY 1968). Preliminary experiments suggest that a $\Delta T_{m,i}$ of one degree results from about 1.5 percent unpaired bases (BAUTZ and BAUTZ 1964; KOTAKA and BALDWIN 1964). This intercistronic conservation is superimposed upon the high degree of interspecific rRNA sequence homology (LAIRD and MCCARTHY 1968).

It will be of interest to determine whether duplicated sequences are, in general, localized or broadly distributed throughout the genome. An example of strictly contiguous related genes is provided by the clustered ribosomal RNA cistrons (RITOSSA and SPIEGELMAN 1965). Other localized nucleotide sequence duplications may be represented by the doublet banding patterns in polytene chromosomes (BRIDGES 1935). Larger and more dispersed banding pattern similarities, perhaps resulting from a combination of duplication and translocation, were also visualized by BRIDGES. Furthermore, nonhomologous pairing of chromosomes (SANDLER and NOVITSKI 1956) may be associated with a distribution of related base sequences among different chromosomes.

SUMMARY

Intragenome nucleotide sequence homology in *D. melanogaster* has been investigated by DNA/DNA duplex formation. Rates of reaction and characterization of the duplex products suggest the presence of a limited number of closely related sequences. Similar studies with ribosomal RNA genes suggest that a conservative relationship exists among these duplicated cistrons.

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