Pring, D. R., and Thornbury, D. W. (1975), *Biochem. Biophys. Acta 383,* 140–146.
Roberts, B. E., and Paterson, B. M. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 1425–1428.
Roheim, J. R., Knight, R. H., and VanEtten, J. L. (1974), *Dev. Biol. 41,* 137–145.
Shapiro, D. J., and Schimke, R. T. (1975), *J. Biol. Chem. 250,* 1759–1764.
Spirin, A. A. (1963), *Prog. Nucleic Acid Res. Mol. Biol. 1,* 301–345.
Verma, D. P. S., MacLachlan, G. A., Byrne, H., and Ewings, D. (1975), *J. Biol. Chem. 250,* 1010–1026.
Wolf, M. J., Khoo, U., and Seckinger, H. L. (1967), *Science 157,* 556–557.

Base Sequence Complexity of the Stable RNA Species of *Drosophila melanogaster*†

Lee Weber‡ and Edward Berger*

ABSTRACT: The base sequence complexity of *Drosophila* transfer RNA (tRNA), 5S RNA, and 18S + 28S ribosomal RNA was determined by analyzing the kinetics of RNA–DNA hybridization on membrane filters. We find that *Drosophila* tRNA is made up from about 59 basic nucleotide sequences distinguishable by hybridization, suggesting that many of the 99 tRNA species resolved by reverse phase chromatography (RPC-5) are homogenic. In contrast 5S RNA was found to contain a single family of sequences. Either 18S ribosomal RNA (rRNA) alone, or 18S + 28S rRNA together, behaved kinetically as two sequence families, and the possible basis for this unexpected result is discussed.

Drosophila melanogaster is one of the few eukaryotes in which the transfer RNAs for all 20 amino acids have been thoroughly analyzed by reverse phase chromatography (RPC).[1] White et al. (1973a) have shown that *Drosophila* tRNA can be resolved on RPC-5 columns (Pearson et al., 1971) into 99 peaks. The pattern is somewhat more complex than that found in *Escherichia coli,* human, or mouse, where about 56 distinct components are resolved (Gallo and Pestka, 1970). White et al. (1973b) have evidence which suggests that several chromatographically distinct forms of isoaccepting tRNAs essentially have the same nucleotide sequence and are probably products of the same genes. The conversion of one form to another is believed to be mediated by a tRNA modifying enzyme which modifies a single nucleotide. These authors proposed the term "homogenic" to describe tRNAs presumably transcribed from the same genes which are chromatographically distinct as a result of different degrees of post-transcriptional modification.

These findings raise the possibility that other isoaccepting forms of tRNA might also be homogenic. In order to investigate this possibility, the kinetic hybridization technique of Birnstiel et al. (1972) was employed in this study to examine the sequence complexity of 4S RNA of *Drosophila melanogaster.* In this method, the time course of hybridization of a constant large excess of RNA with DNA immobilized on filters is followed at optimum rate temperature (t_{opt}). The kinetics of hybridization are pseudo-first-order with respect to RNA under these conditions, but conveniently can be expressed as a double-reciprocal plot (Bishop, 1969). The saturation value is calculated from the intercept of the linear curve at 1/time = 0. The time taken to reach half of the saturation value ($t_{1/2}$) can then be determined and the reaction expressed in terms of $C_r t_{1/2}$ where C_r is the molar concentration of ribonucleotides in solution. The value $C_r t_{1/2}$ has been shown to be a constant (Birnstiel et al., 1972), which is directly proportional to the kinetic or base sequence complexity of a given RNA. Within certain limits, $C_r t_{1/2}$ is independent of both the degree of fragmentation of the RNA and the amount of complementary DNA on the filter. By comparing the $C_r t_{1/2}$ of a class of RNA with that of a standard RNA of known complexity, it is possible to determine the number of families of gene transcripts which make up the RNA in question.

The $C_r t_{1/2}$ of *Drosophila* 4S RNA was measured and the kinetic complexity was determined relative to that of *Bacillus subtilis* 16S + 23S ribosomal RNA, which has been previously shown to be a kinetically homogeneous RNA (Birnstiel et al., 1972). The results suggest that *Drosophila* 4S RNA is composed of approximately 59 families of sequences coded for by about 590 genes. The complexity of *Drosophila* 5S RNA was also examined by the kinetic hybridization technique and found to be composed of a single family of sequences. *Drosophila* 18S + 28S RNA showed unexpected hybridization properties and a possible molecular basis for these results is discussed.

Materials and Methods

Isolation of RNA from Drosophila Larvae. Tritium-labeled RNA was prepared from 3rd instar larvae of *Drosophila melanogaster* according to Steffensen and Wimber (1971). Specific activities ranged from 2.5×10^4 to 1×10^5 cpm/μg.

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[1] Abbreviations used: rRNA and tRNA, ribosomal and transfer ribonucleic acids; RPC, reverse phase chromatography; EDTA, ethylenediaminetetraacetic acid; OAc, acetate; Tris, tris(hydroxymethyl)aminomethane; UV, ultraviolet; CIB, chromatin isolation buffer; TE buffer, 0.1 M EDTA–0.1 M Tris, pH 8.4; SSC, standard saline citrate.

Similar procedures were also used to prepare unlabeled RNA.

Isolation of RNA from Drosophila Tissue Culture Cells. Schneider's line 2 embryonic *Drosophila* tissue culture cells, a gift from Dr. I. Schneider, were used to label RNA to high specific activity. The cells were grown at 25 °C in Falcon 35 cm^2 bottles in Schneider's modified medium (Gibco) supplemented with 15% fetal calf serum and antibiotics (100 μg/ml streptomycin; 100 IU/ml penicillin). Cells were transferred to fresh medium every 5–6 days.

RNA was labeled as follows: ten cultures were started by suspending the cells from a confluent culture with a Pasteur pipet and seeding 0.15 ml into flasks containing 3 ml of unlabeled medium. The cells were allowed to attach and grow for 24 h at which time the unlabeled medium was withdrawn and replaced with 3 ml of medium containing 50 μCi/ml of [^{3}H]uridine. Labeled medium was prepared by drying 1.5 mCi of [^{3}H]uridine in an air stream, redissolving it in 30 ml of medium, and resterilizing by filtration. The cells were allowed to grow to confluence for 5 days. At this time the labeled medium was carefully withdrawn and replaced with unlabeled medium for an additional 12 h. This medium was then removed and 2 ml of RNA extraction buffer containing 1.1 M NaOAc, pH 5.0, 1.5% sodium dodecyl sulfate, and 10 μg/ml polyvinyl sulfate. The lysates were pooled and extracted with phenol at 0–4 °C. RNA was further purified by chromatography columns of methylated albumin on Kesulguhr (MAK) according to Ritossa et al. (1966). Specific activities ranged from 1.4 to 2.0×10^5 cpm/μg.

Preparative Gel Electrophoresis of 4S and 5S RNA. Low-molecular-weight RNA prepared by elution from MAK with 0.7 M NaCl was dissolved in E buffer (0.2% sodium dodecyl sulfate, 20 mM NaOAc, 2 mM EDTA, 40 mM Tris-OAc, pH 7.4) containing 5% sucrose at concentrations of about 1 mg/ml. Preparative separation of 4S and 5S RNA was carried out on 0.6 × 12 cm gels of 7.5% acrylamide by a procedure derived from the method of Tartof and Perry (1970). Gels were prepared essentially according to Loening (1967) except that the gels and running buffer contained 0.2% sodium dodecyl sulfate. The gels were prerun at least 12 h at 5 mA per tube with three changes of reservoir buffer. This procedure reduced the amount of UV absorbing material that would elute with RNA to below 0.03 OD_{260} units per cm of gel. The gels swell somewhat during the extensive prerun but this does not seem to affect resolution. Samples of 4S and 5S RNA of 50 to 300 μg were effectively separated on a single gel. Usually 100–150-μl samples were applied to each gel and run at 5 mA per tube at room temperature until brom phenol blue tracking dye in a parallel tube was within 2 cm of the bottom. Gels were then removed from the tubes and the position of the 4S and 5S zones revealed with a Mineralight UV-11 lamp. The appropriate zones were cut out with razor blades and drawn back into clean tubes by suction. A piece of exhaustively cleaned dialysis tubing containing 1 ml of E buffer was secured to the bottom of each tube with a rubber band. The tubes were placed back in the electrophoresis apparatus with the dialysis bags immersed in the lower reservoir buffer. Care was taken to remove any bubbles trapped below each gel. The RNA was quantitatively eluted from the gels into the dialysis bags at 10 mA per tube in 2 h. Analytical gel electrophoresis of the purified preparations of 4S and 5S RNA revealed a single symmetrical peak of radioactivity in each case with no detectable cross contamination. The RNA was then either made up to 6 × SSC by the addition of 20 × SSC or precipitated with ethanol and dissolved in 6 × SSC. Blanks were electroeluted from RNA-free pieces of gel of the same size as those containing 4S or 5S RNA. The material eluted from blank gels always had an $OD_{260} \leq 5\%$ of the comparable RNA-containing gels.

Isolation of 18S + 28S RNA. High-molecular-weight RNA which eluted from MAK between 0.8 and 1.2 M NaCl was further purified on 10–30% sucrose gradients made up in E buffer. Centrifugation was for 18 h at 25 000 rpm in an International SB-110 rotor at 20 °C. The gradients were pumped through a flow cell and fractions containing 18S and 28S RNA were pooled and precipitated with ethanol.

Preparation of ^{3}H-labeled Ribosomal RNA from B. subtilis. Bacillus subtilis, strain 168, were germinated from spores in M9S medium. A glucose-limited (0.04%) overnight culture was prepared from this initial culture. The overnight culture also contained 10 μg/ml unlabeled uridine. The following day, 100 ml of M9S medium containing 2 μg/ml unlabeled uridine was inoculated with 10 ml of the overnight which had reached a limiting OD_{540} of 0.55. After 20 min of agitation at 37 °C, [^{3}H]uridine was added to 10 μg/ml. When the culture had reached an OD_{540} of 0.6, it was diluted with 100 ml of prewarmed M9S containing 220 μg/ml of unlabeled uridine. When the culture had doubled again, Rifampin (Ciba Pharmaceutical) was added to 10 μg/ml. After 20 min the cells were poured over ice and collected by centrifugation at 5000*g*. Ribosomal RNA was then isolated by a modification of the method of Smith et al. (1968). The RNA was centrifuged on 10–30% sucrose gradients made up in E buffer for 20 h at 25 000 rpm in the SB-110 rotor. Fractions containing 16S and 23S RNA were pooled and precipitated with ethanol.

DNA Isolation. High-molecular-weight DNA was isolated from *Drosophila* adults by a procedure designed to eliminate nuclease activity during all preparative procedures (Weber, 1975). Flies collected over several weeks were kept frozen at −20 °C. They were then reduced to a fine powder by pulverization in a mortar kept at dry ice temperature. The frozen powder was poured into a beaker containing 7–10 volumes (w/v) of CIB (chromatin isolation buffer: 0.1 M NaCl, 0.01 M EDTA, 0.03 M Tris-HCl, pH 8.0, 0.01 M mercaptoethanol, and 0.5% Triton X-100) at room temperature. After 3 min of vigorous mixing on a magnetic stirrer, the slurry was further homogenized, on ice, in a Dounce homogenizer. Chitin was removed by a 4-min centrifugation at 500 rpm. Chromatin was then pelleted from the supernatant by centrifugation at 5000*g* for 10 min, and washed once in CIB without Triton X-100. The pellet was resuspended in TE buffer (0.1 M EDTA–0.1 M Tris, pH 8.4) with a Dounce homogenizer using 5 ml per 10 g of starting material. Sodium dodecyl sulfate and Pronase were added to a final concentration of 1% and 250 mg/ml, respectively, and the mixture was incubated at 37 °C for 2 h. Further deproteinization was accomplished by shaking with an equal volume of phenol–Sevag (2 parts redistilled phenol, 1 part 24:1 chloroform–isoamyl alcohol). Following centrifugation the aqueous phase was recovered and reextracted. DNA was recovered by ethanol precipitation. The DNA was then dissolved in 0.1 × SSC and treated with 50 mg/ml ribonuclease A (Sigma) for 2 h at 37 °C, followed by the addition of an equal volume of 0.1 M Tris-HCl, pH 8.0, 200 mg/ml Pronase, and 0.4% sodium dodecyl sulfate. Following 2 additional h of digestion, residual enzyme was extracted with phenol–Sevag, and the DNA was recovered by ethanol precipitation. The average molecular weight of single-stranded DNA recovered by this procedure was 1×10^7, as estimated by alkaline sucrose gradient centrifugation using radioactive T4 phage DNA as internal marker (Weber, 1975). DNA was isolated from *B. subtilis* by the method of Marmur (1961).

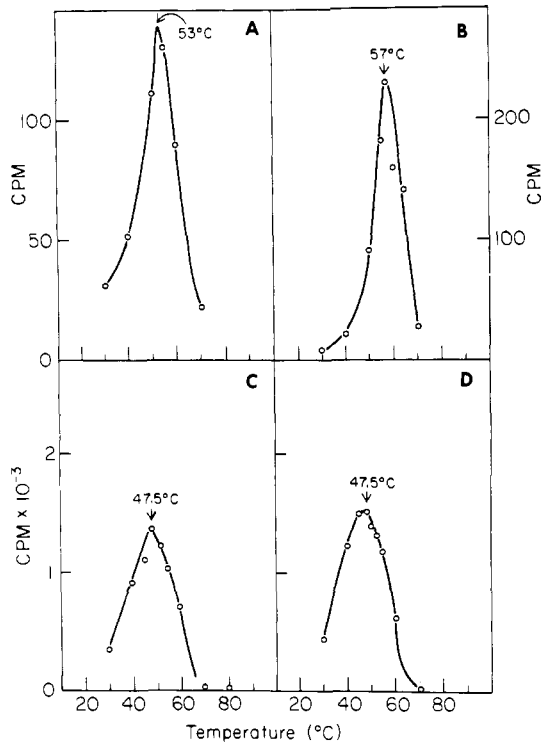

FIGURE 1: Determination of the optimal rate temperatures for hybridization. (A) *Drosophila* 4S RNA at a concentration of 2 μg/ml was hybridized with filters containing 95 μg of DNA for 30 min. The reaction mixture also contained unlabeled 18S + 28S and 5S RNA at 200 and 2 μg/ml, respectively. (B) *Drosophila* 5S RNA at a concentration of 0.05 μg/ml was hybridized with filters containing approximately 100 μg of DNA for 15 min. Unlabeled 18S + 28S RNA was present at 5 μg/ml. (C) *Drosophila* 28S RNA at a concentration of 2 μg/ml was hybridized with filters containing approximately 50 μg of DNA for 60 min. (D) *Drosophila* 18S + 28S RNA at a concentration of 2 μg/ml was hybridized with filters containing approximately 50 μg/ml for 60 min. The extent of RNase-resistant hybridization was determined as described in Materials and Methods.

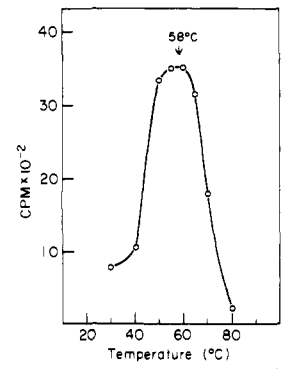

FIGURE 2: Determination of optimal rate temperature for hybridization of *B. subtilis* 16S + 23S RNA at a concentration of 4 μg/ml, hybridized with filters containing 22 μg of DNA for 15 min.

Hybridization Procedure. DNA was loaded on 24-mm B6 filters according to Vincent et al. (1969). Thirteen-millimeter B6 filters were loaded by a similar procedure using a home-made manifold employing stainless steel Swinney filtration units (Millipore).

All RNA preparations were passed through small columns of G-25 Sephadex which had been equilibrated with 6 × SSC to ensure defined salt concentrations.

The hybridization procedure of Birnstiel et al. (1972) was employed. The hybridization medium consisted of equal volumes of 6 × SSC (pH 7.2) and formamide. The formamide (Eastman) was deionized by stirring with a half volume of AG-501 resin (Bio-Rad) for 30 min and filtering. This procedure was necessary in order to neutralize the formamide without altering ionic strength. The 6 × SSC–50% formamide mixture was pH 6.9–7.2 prior to incubation and pH 6.6–6.9 after 12 h at 50 °C. For kinetic studies, the reaction mixture was assembled in a single large vessel and then distributed into separate plastic scintillation vials for each time point. A minimum of 3 ml of hybridization medium was used per vial. DNA filters were soaked in 6 × SSC–50% formamide before use. The hybridization medium was brought to the appropriate temperature and the filters were dropped into the vials after draining off excess medium on filter paper. The vessels were agitated briefly by hand and then nearly filled with prewarmed paraffin oil. The vials were not removed from the water bath during these operations. Each vial usually contained two DNA filters and one blank. The reactions were not agitated in any way during the reaction except as noted. All of the filters in a single vial were withdrawn with forceps at various time intervals and dropped into a large excess of ice cold 6 × SSC. All of the filters from a single experiment were washed and treated with RNase simultaneously. For saturation experiments, where the incubations are carried out for a single time interval, the filters were not usually presoaked and as many as 20 filters were sometimes included in a single vial. Filters were washed and treated with RNase according to the batch procedure of Birnstiel et al. (1972). Filter-bound DNA was determined chemically as described previously (Weber et al., 1976). No loss of DNA occurred during hybridization or washing.

CsCl Fractionation of DNA. CsCl solutions were prepared by mixing 4.2 g of CsCl with 4 ml of DNA solution in 0.1 × SSC in polyallomer centrifuge tubes. The tubes were filled with paraffin oil and centrifuged for 48 h at 42 000 rpm in an International A-321 rotor, at a temperature setting of 20 °C. The tubes were punctured at the bottom with a 26-gauge needle and 25-drop fractions were collected manually. Fractions were diluted with 500 μl of 0.1 × SSC for determination of UV absorbance. Each fraction was then denatured and applied to an individual 24-mm B-6 filter as described by Vincent et al. (1969).

Thermal Dissociation of Hybrids. Hybrids were formed using saturating concentrations of RNA in all cases. The filters were washed as described above, except that RNase treatment was omitted in some cases. When RNase-treated hybrids were studied, the RNase treatment was followed by an incubation in 2 × SSC containing 0.1% diethyl pyrocarbonate for 30 min at room temperature to remove nuclease activity (Birnstiel et al., 1972). The filters were then rinsed exhaustively in 2 × SSC. To determine the thermal stability of the hybrids, the filters were placed in 2 ml of 0.1 × SSC in plastic scintillation vials and heated in a Haake circulating water bath in temperature increments of about 5 °C. The bath was allowed to rise to a given temperature and the incubation was then timed for 5 min. The solution was then rapidly withdrawn from the vial and replaced with 2 ml of fresh 0.1 × SSC. The radioactivity of the released RNA was measured by counting in 3 volumes of Aquasol (New England Nuclear).

Results

A. The Effect of Temperature on the Rate of RNA–DNA Hybridization. In order to compare hybridization rates, the reaction conditions must be optimized for each RNA species examined (Birnstiel et al., 1972). The optimal rate temperature was therefore determined for each type of RNA used in this study. Figures 1 and 2 summarize the results of these experi-

TABLE I: G + C Content and Optimal Rate Temperatures of Various RNA Species.

RNA	G + C Content (%)	t_{opt} 6 × SSC, 50% Formamide (°C)
18S + 28S *Drosophila*	39[b]	47
18S + 28S *Xenopus*[a]	59	64
18S + 28S rabbit[a]	62	62
28S *Drosophila*	38[b]	47
ϕX174 cRNA[a]	42	45
16S + 28S *B. subtilis*	53	58
16S + 28S *B. subtilis*[a]	53	58
5S *Drosophila*	52[b]	57
5S *Xenopus*[a]	57	61
4S *Drosophila*	59[b]	53
4S *Xenopus*[a]	60	54

[a] Data of Birnstiel et al. (1972). [b] Data of Tartof and Perry (1970).

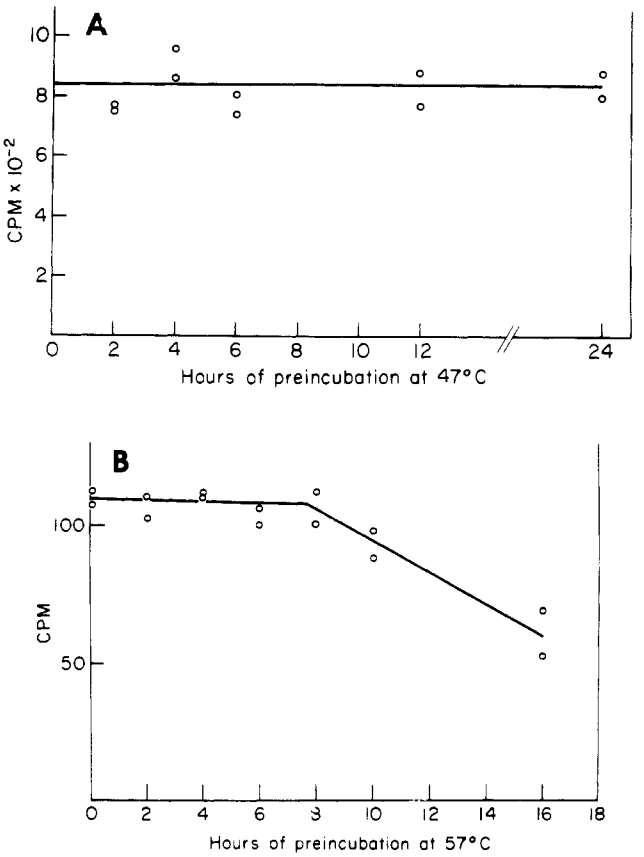

FIGURE 3: The stability of RNA under hybridization conditions. (A) *Drosophila* 18S + 28S at a concentration of 1 μg/ml was preincubated in hybridization medium at 47 °C for the indicated periods of time and then hybridized with filters containing approximately 40 μg of DNA for an additional 2 h. (B) *Drosophila* 5S RNA at a concentration of 0.05 μg/ml was preincubated in hybridization medium at 57 °C for the indicated time periods and then hybridized with filters containing approximately 100 μg of DNA for an additional 15 min. Unlabeled 18S + 28S RNA was present at 100-fold excess. The amount of RNase-resistant hybridization was determined as described in Materials and Methods.

ments. Each experiment was repeated twice, using temperature values in the subsequent series near the apparent optimum found in the initial attempt, and the mean values are plotted. The time of incubation used in each experiment allowed for the hybridization of less than 30% of the saturation value in all cases. The counts per minute hybridized were therefore taken as indications of the initial rates of reaction. The actual RNA concentrations and times of incubation are detailed in the figure legends. Table I summarizes these results and includes temperature optima (t_{opt}) data of some selected RNA species from Birnstiel et al. (1972). The temperature optima of the *Drosophila* RNA species compare with those measured for other eukaryote RNAs when differences in the G + C content are considered. It should be noted that the t_{opt} of 58 °C determined for *B. subtilis* 16S + 23S RNA, the kinetic standard in this work, agrees with the value measured by Birnstiel's group, although it is not clear whether the same ionic conditions were employed.

B. The Stability of RNA under Hybridization Conditions. The rate of hybridization is measured from double-reciprocal plots of the approach to saturation (Bishop, 1969). Therefore, the stability of the RNA during the reaction is an important consideration. Since the concentration of the RNA in solution drives the reaction, the effect of RNA breakdown to nonhybridizable material would be to gradually lower the effective concentration during the course of the reaction which would result in erroneously low saturation values and consequently increase the apparent rate of the reaction. The problem of RNA instability can be minimized by using high concentrations of RNA. This increases the reaction rate and allows for shorter times of incubation. However, in practice more accurate and reproducible results can be obtained by using lower RNA concentrations and longer incubation where the sampling error of individual time points is minimized.

The stability of 18S + 28S *Drosophila* RNA under hybridization conditions was examined and the results are shown in Figure 3. Preincubation of this RNA at its t_{opt} in hybridization medium for up to 24 h had no significant effect on the rate of subsequent hybridization.

This same type of experiment was repeated with *Drosophila* 5S RNA where the higher t_{opt} and the smaller size of the molecule might make the reaction more susceptible to the effects of RNA degradation. Figure 3 shows that a decrease in initial hybridization rate did not occur until after 8 h of incubation. Since thermal hydrolysis and depurinization of the RNA should not have been factors in this amount of time at 57 °C, we attribute this decrease to trace nuclease activity. The results of these stability experiments were considered when designing subsequent experiments to measure hybridization rates. The hybridization of 4S and 5S RNA was limited to 4 h or less.

C. Purity of the RNA Preparations. All the labeled RNA preparations used in this work were found to be less than 0.2% resistant to hydrolysis with 0.2 N NaOH and were at least 98% susceptible to digestion with 10 μg/ml RNase A in 0.1 × SSC. The spectral ratios at 230, 260, and 280 nm taken in 10 mM Tris (pH 7.0) were those of a pure nucleic acid (260/230 = 2.3, 260/280 = 2.0). The spectral ratios of 4S and 5S RNA were taken before electrophoretic purification because of a UV-absorbing impurity from the gel which distorted the spectrum of eluted RNA.

The labeled RNA preparations were shown to be free from heterogeneous RNA contaminants by performing standard saturation experiments as shown in Figure 4. Increasing amounts of RNA were hybridized to constant amounts of DNA for fixed incubation times. The attainment of a flat saturation plateau, above which no more hybrid would form, was taken as an indication of the lack of other hybridizable material. Low concentration of contaminating sequences would

TABLE II: The Complexity of RNA Sequences and the Reiteration of DNA Sites.

RNA Hybridized (Anal. Complexity, daltons × 10^6)	cDNA (%)	No. of Genes	$t_{1/2}$ at 3 μg/ml (min)	Mean $C_r t_{1/2}$ × 10^3 (mol s/l.)	Kinetic Complexity (daltons)	No. of Families (mean and range of values)
16S + 23S *B. subtilis* rRNA (1.6)	0.65	8	59-71	38.6	Kinetic Standard	1
5S *Drosophila* RNA (0.038)	0.006	180	1-1.5	0.71	2.9×10^4	1 ± 0.3
4S *Drosophila* tRNA (0.025)	0.013	590	53-63	35.8	1.48×10^6	59 ± 5
18S + 28S *Drosophila* rRNA (2.1)	0.42	226	177-189	110.0	4.5×10^6	2.2 ± 0.1
28S *Drosophila* rRNA (1.4)	0.30	242	113	67.8	2.8×10^6	2.0

be expected to increase the level of hybridization as the input of RNA is increased. The presence of contaminating sequences in an initial preparation of *B. subtilis* ribosomal RNA, prepared without the use of rifampin, was detected by this type of control.

Drosophila 4S and 5S RNA prepared by a number of methods has been reported to be contaminated with 18S + 28S degradation products (Ritossa et al., 1966; Tartof and Perry, 1970; Quincey, 1971). Initial experiments indicated that the 4S and 5S preparations used in the work also contained some contaminating sequences which could be eliminated from the reaction by the addition of a 100-fold excess of unlabeled 18S + 28S RNA. The addition of unlabeled 4S RNA had no effect on the hybridization of ^{3}H-labeled 5S preparations. Including an equal concentration of unlabeled 5S RNA with the only ^{3}H-labeled 4S preparation so tested had no significant effect on hybridization. However, unlabeled 5S RNA was included in all reactions with ^{3}H-labeled 4S RNA as a precaution against the presence of 5S RNA degradation products. Saturation experiments were not carried out with 4S RNA due to the difficulty of obtaining large amounts of this RNA. Nonetheless, the saturation values obtained from the rate experiments using 4S RNA (Table II) were similar to those reported from standard saturation experiments (Ritossa et al., 1966; Tartof and Perry, 1970).

A control experiment was performed to determine whether mitochondrial RNA was present in our labeled preparations. The results are illustrated in Figure 5. The *Drosophila* RNA preparations were hybridized to a complete range of *Drosophila* DNA fractions taken from CsCl gradients. These gradients separated DNA essentially on the basis of G + C content. The hybridization of all the RNA classes occurred within main DNA peak with no significant hybridization at the density of 1.68 g/ml where *Drosophila* mitochondrial DNA bands (Polan et al., 1973; Bultman and Laird, 1973). The *Micrococcus luteus* DNA included in each gradient as a density marker also failed to bind any RNA.

D. The Base Sequence Complexity of Drosophila RNA. The kinetics of hybridization of each *Drosophila* RNA class and the *B. subtilis* standard were followed at t_{opt}. Data are expressed as double-reciprocal plots of the approach to saturation (Bishop, 1969). The saturation values were calculated from the intercept at 1/time = 0 (i.e., $t = \infty$) from plots of 1/cpm vs. $1/t$ (not shown). All experiments were then normalized by plotting 1/% saturation vs. $1/t$ and the results are illustrated in Figure 6. The time taken to reach 50% of the saturation value ($t_{1/2}$) was calculated from a regression line drawn through the points by the method of least-squares. The RNA concentrations used in each experiment are given in the individual figure legends. The total amount of RNA used in each experiment was at least 80 times the amount of complementary DNA. The $t_{1/2}$ for each RNA species was calculated at 3 μg/ml using the inverse relationship between $t_{1/2}$ and concentration (Bishop, 1969). The average value of $C_r t_{1/2}$ was then calculated, taking the molar concentration of nucleotide to be 300 g/l. These results are summarized in Table H.

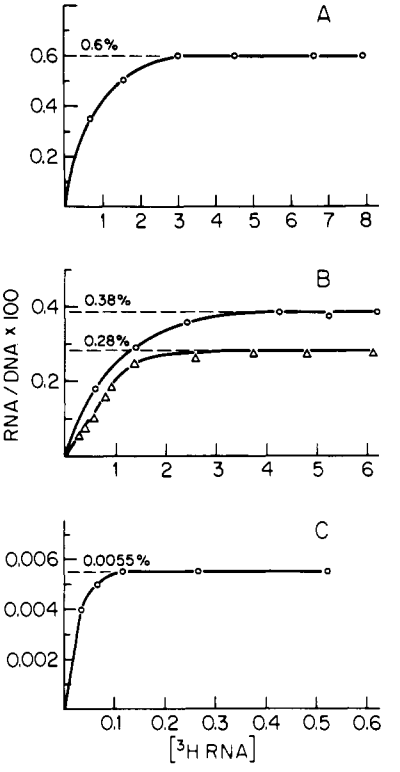

FIGURE 4: The saturation of homologous DNA sites with various RNA preparations. Hybridizations were performed in 3 ml of hybridization medium using increasing concentrations of ^{3}H-labeled RNA. (A) *B. subtilis* 16S + 23S RNA was hybridized with filters containing 16 μg of DNA for 20 h at 58 °C. (B) *Drosophila* 28S (Δ—Δ) or 18S + 28S (O—O) RNA was hybridized with filters containing approximately 40 μg of DNA for 20 h at 47 °C. (C) *Drosophila* 5S RNA was hybridized with filters containing approximately 95 μg of DNA for 12 h at 57 °C. A 100:1 ratio of unlabeled 18S + 28S RNA to ^{3}H-labeled 5S RNA was maintained in each reaction. The amount of DNA on each filter and the amount of RNase-resistant hybridization was determined as described in Materials and Methods. Points indicate the average of duplicate determinations.

The kinetic or base sequence complexity of each class of RNA was calculated relative to *B. subtilis* 16S + 23S RNA, which was assumed to have a base sequence complexity in daltons equal to its molecular weight of 1.6×10^6 (Birnstiel et al., 1972). Birnstiel's group has shown that, under optimum

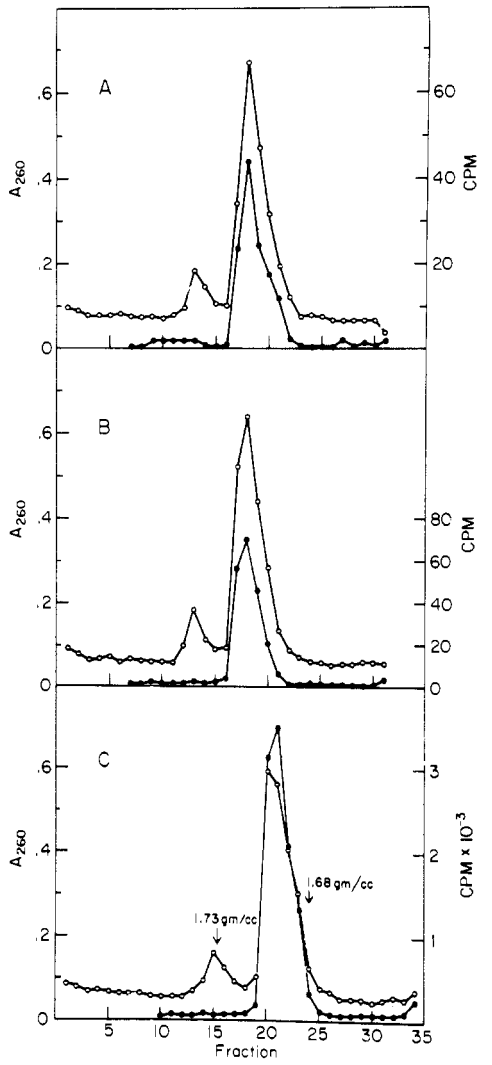

FIGURE 5: CsCl gradients containing approximately 40 μg of *Drosophila* DNA together with about 20 μg of *M. luteus* DNA as a density marker were centrifuged to equilibrium and fractionated, and each fraction was loaded onto an individual filter as described in Materials and Methods. The filters were then hybridized for 12 h at t_{opt} with: (A) *Drosophila* 4S RNA isolated from 3rd instar larvae at a concentration of 2.0 μg/ml; (B) *Drosophila* 5S RNA at a concentration of 0.1 μg/ml (unlabeled 18S + 28S RNA was present at 10 μg/ml); (C) *Drosophila* 18S + 28S RNA at a concentration of 4 μg/ml.

conditions for hybridization, $C_rt_{1/2}$ is directly proportional to the base sequence complexity of the RNA hybridized. The slope relating these two parameters was calculated to be 2.41×10^{-8} mol s/l. daltons from the hybridization of *B. subtilis* RNA under our conditions. Inserting the values for $C_rt_{1/2}$ found in this work provides the kinetic complexity of each class of *Drosophila* RNA. The number of independently hybridizing families composing each class was calculated by dividing the kinetic complexity by the analytical complexity of molecular weight. For *Drosophila* 4S RNA a kinetic complexity which suggests 59 independently hybridizing families of sequences was calculated. The values for all the RNA classes studied are enumerated in Table II.

The absolute rate of hybridization of *B. subtilis* 16S + 23S RNA was found to be slower than that reported by Birnstiel et al. (1972), by a factor of about three. This RNA had a $C_rt_{1/2}$ value of 12×10^3 mol s/l. in their hands, while in this study the average value of four experiments (Figure 6) is 38.7×10^3 mol s/l. The $C_rt_{1/2}$ value for *Drosophila* 5S RNA in this present study is also about threefold greater than the value for *Xenopus* 5S RNA reported by Birnstiel et al. (1972). This large discrepancy in rates might result from the use of a hybridization medium with a lower ionic strength, in the present study, or from various technical differences in methodology. In our experiments 6 × SSC–50% formamide contains 0.495 M Na^+, while in the earlier work the ionic strength may have been double this value. Increasing ionic strength in this range would be expected to speed up the reaction rate (Wetmur and Davidson, 1968). Birnstiel's group employed 13-mm filters and carried out the hybridization reaction with constant agitation, while we used 24-mm filters without agitation. In order to test whether these procedural differences could affect the rate of reaction, the $t_{1/2}$ for the saturation of DNA sites by *Drosophila* 18S + 28S RNA at 4 μg/ml was measured using 13-mm filters, with constant agitation in a rotary shaker water bath. In a simultaneous control experiment, the $t_{1/2}$ was measured by the usual procedure using 24-mm filters without shaking. The results are shown in Figure 6e. The reaction rate is, indeed, almost doubled when measured using the 13-mm filters and constant agitation. Since the same reaction mixture was used in both experiments and the amount of DNA on both types of filters was about the same, these results suggest that the reaction is influenced by agitation and/or some property of the filter. These factors probably can account for the discrepancy between the absolute reaction rates measured in this study and those reported by the Birnstiel group. Since all of the filters used in this present work were from the same lot and all of the reactions were carried out without shaking, it is assumed that the *relative* rates of reaction of the different RNA species tested were not influenced by these factors.

E. The Base Sequence Complexity of Drosophila Ribosomal RNAs. The kinetic complexity of *Drosophila* 18S + 28S RNA was about 4.5×10^6 daltons (Table II), a value slightly more than *twice* the value expected for molecules with a combined analytical complexity of 2.1×10^6 daltons. Table II also shows that 28S RNA alone behaved kinetically as two sequences. The simplest interpretation of these results is that *Drosophila* 18S + 28S RNA is transcribed from two independent sets of rRNA cistrons, sharing little or no homology. This possibility is highly unlikely, however, since a thorough fingerprint analysis of *Drosophila* 28S and 18S rRNA did not suggest extensive base sequence heterogeneity (Maden and Tartof, 1974). Birnstiel et al. (1972) reported that *E. coli* 16S + 23S rRNA hybridized slower, by a factor of 1.7, than expected for a single family of sequences. This has been attributed to a cross hybridization between 23S and 16S cistrons which share common base sequences (Attardi et al., 1965; Mangiarotti et al., 1968). The poor locus specificity of hybridization is reflected by a decrease in hybridization rate. Aside from *Drosophila, E. coli* is the only reported rRNA which fails to hybridize as a single sequence family.

Two lines of evidence suggest that the unusual hybridization properties of *Drosophila* rRNA are not a result of cross hybridization between 18S and 28S cistrons, or poor specificity of hybridization. The experiment shown in Figure 4B demonstrates that *Drosophila* 28S rRNA hybridizes with only about two-thirds of the DNA sites bound by a mixture of 28S + 18S rRNA. This is the value predicted on the basis of their molecular weights assuming no homology. Similar results have been obtained by Tartof and Perry (1970) using less stringent hybridization conditions. Since these experiments examined only RNase-resistant hybrids, they do not rule out the possibility that some cross hybridization does occur in the form of RNA-sensitive structures. This possibility was examined di-

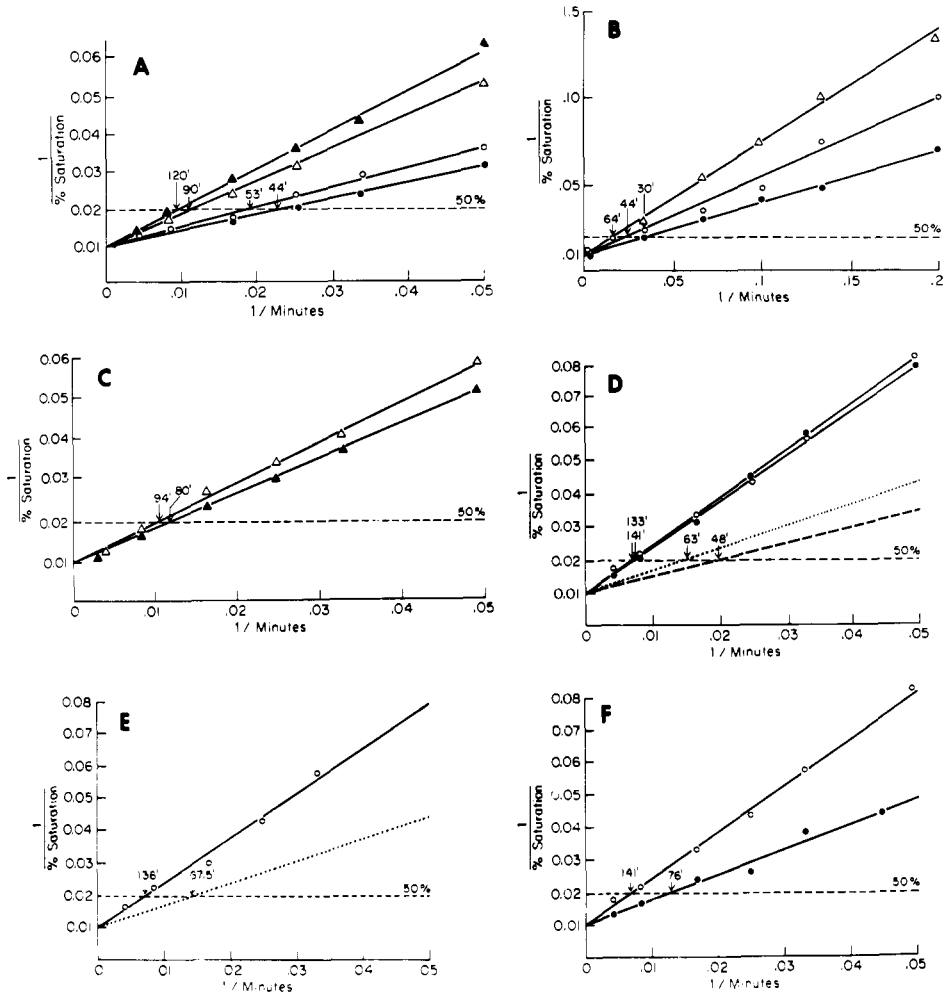

FIGURE 6: Double-reciprocal plots of the hybridization of various RNAs with homologous DNA. RNase-resistant hybridization was measured as described in Materials and Methods. Each point represents the average of two filters. (A) *B. subtilis* 16S + 23S RNA. (△—△, ▲—▲) Two independent experiments using an RNA concentration of 2 μg/ml and filters containing 20 μg of DNA. The total reaction volume for each time point was 10 ml. (○—○, ●—●) Two independent experiments using RNA concentration of 4 μg/ml and filters containing 16 μg of DNA. The total volume used for each time point was 5 ml. (B) *Drosophila* 5S RNA isolated from 3rd instar larvae. (△—△) A 5S RNA concentration of 0.05 μg/ml with volumes of 15 ml per point. (○—○, ●—●) Independent experiments using a 5S RNA concentration of 0.1 μg/ml with volumes of 10 ml per point. Filters contained 96 μg of DNA. Unlabeled 18S + 28S RNA was present at concentrations 100-fold in excess of ^{3}H-labeled 5S RNA. (C) *Drosophila* 4S RNA isolated from tissue culture cells. (△—△, ▲—▲) The hybridization of two different preparations of 4S RNA at concentrations of 2 μg/ml. Incubation volumes were 3 ml. The reaction mixtures contained unlabeled 5S and 18S + 28S RNA at 1 and 200 μg/ml, respectively. Filters contained approximately 100 μg of DNA. (D) *Drosophila* 18S + 28S RNA isolated from 3rd instar larvae. (○—○, ●—●) Two independent experiments using an RNA concentration of 4 μg/ml and filters containing 20 μg of DNA. The reaction volume for each time point was 4 ml. Dashed line is a theoretical curve for the hybridization of *B. subtilis* 16S + 23S RNA at 4 μg/ml based on the results shown in A. Dotted line represents a theoretical curve for the hybridization of a molecule with a kinetic complexity of 2.1 × 10^6 daltons at 4 μg/ml. (E) *Drosophila* 28S RNA isolated from 3rd instar larvae. (○—○) The RNA concentration was 2.5 μg/ml with volumes of 4 ml per point. Filters contained 20 μg of DNA. Dotted line represents a theoretical curve of the hybridization of a molecule with a kinetic complexity of 1.4 × 10^6 daltons at 2.5 μg/ml. (F) The effect of agitation and/or filter size on the rate of hybridization. *Drosophila* 18S + 23S RNA at a concentration of 4 μg/ml in 4 ml reaction volumes was hybridized with either 13-mm filters containing 16 μg of DNA with constant agitation (●—●), or 24-mm filters containing 20 μg of DNA without agitation (○—○) as in Figure D.

rectly by determining the thermal stability of hybrids formed at different temperature in the absence of RNase treatment. If cross hybridization were occurring, it should be accentuated at lower reaction temperatures where poorly matched hybrids are more stable. This would be reflected by a reduction in the thermal stability of the hybrids. Figure 7 demonstrates that, in the case of both *B. subtilis* and *Drosophila,* only well-paired hybrids are formed even at suboptimal temperatures. This result does not support the notion that incomplete homology between 18S and 28S rRNA is responsible for the unexpected hybridization rates.

An inspection of the melting profile in Figure 7B indicates a biphasic transition, introducing the possibility that distinct regions of the rRNA–DNA hybrid have higher and lower G + C contents than the average.

Discussion

The Base Sequence Complexity of Drosophila 4S RNA. The results shown in Table II indicate that 0.013% of *Drosophila* DNA is complementary to 4S RNA. This estimate is in good agreement with the value of 0.015% determined by Ritossa et al. (1966) and Tartof and Perry (1970) and suggests that there are about 590 transfer RNA genes per haploid genome. The slight difference in saturation values might reflect differences between the 4S RNAs of the embryonic tissue culture cells used in this study and 3rd instar larvae used in the earlier work, or differences between *Drosophila* stocks.

A complexity of about 14.7 × 10^6 daltons would be expected for all 590 tRNA cistrons combined (590 × 2.5 × 10^4 daltons). However, 4S RNA shows a kinetic complexity of only 1.48 × 10^6 daltons (Table II) or only one-tenth the complexity ex-

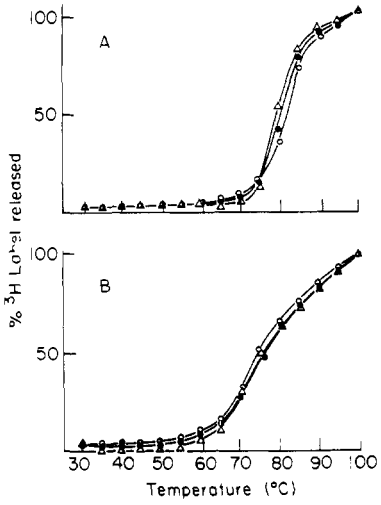

FIGURE 7: The thermal stabilities of hybrids formed at optimum and suboptimum temperatures. Filters containing 20 μg of DNA were hybridized with 10 μg/ml of homologous RNA for 16 h. (A) *B. subtilis* 16S + 23S hybrids; (Δ—Δ) formed at 58 °C; (●—●) formed at 53 °C; (○—○) formed at 48 °C. (B) *Drosophila* 18S + 28S hybrids; (Δ—Δ) formed at 47 °C; (●—●) formed at 42 °C; (○—○) formed at 37 °C. RNase treatment was omitted and hybrids were dissociated as described in Materials and Methods.

pected for 590 unique sequences. Therefore, these results suggest that *Drosophila* 4S RNA is encoded for by about 59 families of genes, which are repeated an average of 10 times per haploid genome.

These results may be compared with the complexity of unfractionated tRNA from *E. coli* and *Xenopus* (Birnstiel et al., 1972). *E. coli* has about 60 tRNA cistrons as measured by hybridization (Zehavi-Willner and Comb, 1966; Brenner et al., 1970; Birnstiel et al., 1972) or about 1.5×10^6 daltons of complementary DNA. *E. coli* 4S RNA exhibits a kinetic complexity of only $0.7-0.8 \times 10^6$ as might be expected for only about 30 families of sequences. In *Xenopus,* there are 6500–8000 tRNA cistrons, yet 4S RNA shows a kinetic complexity of only 1.1×10^6 daltons which suggest only 43–44 families of sequences (Birnstiel et al., 1972; Clarkson and Birnstiel, 1973). These studies all would seem to indicate that tRNA is encoded for by a restricted number of sequences despite the multiplicity of the tRNA cistrons found in higher organisms.

The kinetic complexity of *Drosophila* 4S RNA suggests that there are fewer basic tRNA sequences than indicated by the 99 chromatographically distinct amino acid accepting forms (White et al., 1973a) and is consistent with the interpretation that many chromatographically distinct forms of tRNA arise from posttranscriptional modifications of a smaller number of basic tRNA sequences. There are several factors, however, which could affect the complexity values determined for tRNA. Birnstiel's group (Birnstiel et al., 1972; Clarkson and Birnstiel, 1973; Clarkson et al., 1973a) points out that a few tRNA species in disproportionately high concentration would accelerate the overall reaction and thus lead to an underestimate of the true sequence complexity. Similarly tRNAs present at very low concentration, or totally absent, in our cell line would lead to an underestimate. There is no objective way of assessing this bias.

Birnstiel et al. (1972) and Clarkson et al. (1973a) also point out that some tRNA sequence heterogeneity may not be large enough to be detected by hybridization, again leading to an underestimate of complexity. However, Clarkson et al. (1973a) have shown that the kinetic hybridization approach can distinguish between the two isoaccepting forms of methionyl-tRNA in *Xenopus* and that the presence of all of the other tRNAs has no effect on the hybridization of these species. Furthermore, methionyl- and valyl-tRNA hybridize to different densities of CsCl fractionated DNA (Clarkson et al., 1973b). These observations, plus the kinetic data on leucyl- and aspartyl-tRNA (Clarkson and Birnstiel, 1973), suggest that cross hybridization between similar but nonidentical tRNA sequences does not seem to be a problem in *Xenopus*. In *Drosophila,* lysyl-5 tRNA has recently been shown to hybridize in situ with a single small chromosomal region (Grigliatti et al., 1974), while unfractionated 4S RNA hybridizes with a multitude of sites (Steffensen and Wimber, 1971), suggesting that hybridization can distinguish between tRNAs. A recent catalog of all available tRNA sequence data (Sodd and Doctor, 1974) reveals that few isoaccepting tRNAs isolated from the same organism do have primary sequences which differ by only one or two bases, and the nature of these substitutions suggests that they occur at the genetic level and are not posttranscriptional modifications. These would not be distinguishable by hybridization. Again, the relative frequency of such minor gene differences and their effect on the complexity determination are not known.

Finally, the posttranscriptional modification of tRNA bases should reduce the efficiency of its hybridization and lead to an overestimate of sequence complexity. Transfer RNA has a much higher percentage of modified bases than any other cellular RNA (Söll, 1971). The chemical nature of many of the base modifications in tRNA (Söll, 1971; Zachau, 1972) would predict that they would interfere with base pairing during hybrid formation. Certainly, reduced ring structures such as dihydrouridine cannot base pair and are, in fact, nonplanar structures. Bases with more complex modifications bearing large side chains or additional ring structures (e.g., N^6-(Δ^2-isopentenyl)adenosine) may also sterically interfere with helix formation involving adjacent nucleotides. Since the total content of altered bases in tRNA can be as high as 20% (Staehelin, 1971) and base mispairing reduces the rate of hybrid formation (Sutton and McCallum, 1971; Bonner et al., 1973), tRNA molecules might be expected to hybridize more slowly than comparable sequences without extensive modifications. The magnitude of the rate reduction due to base modification cannot be estimated since the exact nature and extent of all the modifications are not known. It should be noted that studies using deaminated DNA (Bonner et al., 1973) show that a rate reduction of approximately 50% is affected by deamination of 10% of the bases. This would be equivalent to only eight mispaired bases in a typical tRNA–DNA hybrid.

The physiological roles of the multiple forms of tRNA are not clear. The Wobble hypothesis (Crick, 1966) predicts that the genetic code could be translated by a few as 31 different tRNAs; yet all organisms thus far examined show at least 56 chromatographically separable species (Gallo and Pestka, 1970). Certain isoaccepting tRNAs have different coding properties in vitro (Weisblum et al., 1962, 1965, 1967; Von Ehrenstein and Dais, 1963; Gonano, 1967). However, in yeast one phenylalanyl-tRNA may translate more than one codon but two seryl-tRNA may translate the same codon (Söll et al., 1966). The two seryl-tRNAs found in yeast are reported to have the same anticodon but to differ in other parts of the primary sequence (Zachau et al., 1966). Specific tRNAs have been proposed to have other functions besides acting as amino acid acceptors. In prokaryotes, histidyl-tRNA is involved in the regulation of the histidine operon (Brenner and Amers, 1971). In higher organisms, there is evidence that a certain

isoaccepting form of tyrosyl-tRNA inhibits the enzyme tryptophan pyrrolase in the *vermilion* mutant of *Drosophila* (Jacobson, 1971; Twardzic et al., 1971). In addition, it has been suggested that different tRNAs may have a role in the regulation of protein synthesis at the translational level (Sueoka and Kano-Sueoka, 1964; Ilan, 1969).

While *Drosophila* 5S rRNA hybridized as a single kinetic family, 18S + 28S rRNA or 28S rRNA alone, had a kinetic complexity suggesting two independently hybridizing families. Since combined genetic and biochemical studies make this an unlikely possibility (Maden and Tartof, 1974), it seems more probable that the hybridization properties are a consequence of an unusual primary and secondary structure of the rRNA. *Drosophila* tRNA has an overall G + C content of about 39% (Tartof and Perry, 1970), whereas the rRNAs of most higher eukaryotes contain nearly 60% G + C. All eukaryotic rRNAs share some common sequences as judged by interspecific hybridization (Sinclair and Brown, 1971). Furthermore, double-stranded loops several hundred nucleotides long, and rich in their G + C content, are structural characteristics of the 28S rRNAs of higher organisms studied (Wellauer and Dawid, 1973). The presence of such loops in *Drosophila* rRNA might have a greater effect on hybridization than in the rRNA of other eukaryotes with higher overall G + C contents. At the low t_{opt} for *Drosophila* rRNA hybridization, which reflects its low overall G + C content, regions of high G + C content might retain considerable secondary structure, thus slowing down the rate of hybrid formation.

References

Attardi, G., Huang, P. C., and Kabat, S. (1965), *Proc. Natl. Acad. Sci. U.S.A. 54,* 185.

Birnstiel, M. L., Sells, B. H., and Purdom, J. F. (1972), *J. Mol. Biol. 63,* 21.

Bishop, J. O. (1969), *Biochem. J. 113,* 805.

Bonner, T., Brenner, D., Neufeld, B., and Britten, R. (1973), *J. Mol. Biol. 81,* 123.

Brenner, D., Fournies, M., and Doctor, B. P. (1970), *Nature (London) 227,* 448.

Brenner, M., and Amers, B. N. (1971), in Metabolic Pathways, Greenberg, D. M., and Vogel, H. J., Ed., New York, N.Y., Academic Press, p 349.

Bultman, H., and Laird, C. D. (1973), *Biochim. Biophys. Acta 299,* 196.

Clarkson, S. G., and Birnstiel, M. L. (1973), *Cold Spring Harbor Symp. Quant. Biol. 38,* 451.

Clarkson, S. G., Birnstiel, M. L., and Purdom, J. F. (1973b), *J. Mol. Biol. 79,* 411.

Clarkson, S. G., Birnstiel, M. L., and Serra, V. (1973a), *J. Mol. Biol. 79,* 391.

Crick, F. H. C. (1966), *J. Mol. Biol. 19,* 548.

Gallo, R. C., and Pestka, S. (1970), *J. Mol. Biol. 52,* 195.

Gonano, G. (1967), *Biochemistry 6,* 977.

Grigliatti, T. A., White, B. N., Tener, G. M., Kaufman, T. C., and Suzuki, D. T. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 3527.

Ilan, J. (1969), *Cold Spring Harbor Symp. Quant. Biol. 34,* 784.

Jacobson, K. B. (1971), *Nature, (London) New Biol. 231,* 17.

Loening, U. E. (1967), *Biochem. J. 102,* 251.

Maden, B., and Tartof, K. (1974), *J. Mol. Biol. 90,* 51.

Mangiarotti, G., Apirion, D., and Silengo, L. (1968), *Biochemistry 7,* 456.

Marmur, J. (1961), *J. Mol. Biol. 3,* 585.

Pearson, R. L., Weiss, J. F., and Kelmers, A. D. (1971), *Biochim. Biophys. Acta 228,* 770.

Polan, M. L., Friedman, S., Goll, J. G., and Gehring, W. (1973), *J. Cell Biol. 56,* 580.

Quincey, R. U. (1971), *Biochem. J. 123,* 227.

Ritossa, F. M., Atwood, K. C., and Spiegelman, S. (1966), *Genetics 54,* 663.

Sinclair, J. H., and Brown, D. D. (1971), *Biochemistry 10,* 2761.

Smith, I., Dubnov, D., Morell, P., and Marmur, J. (1968), *J. Mol. Biol. 33,* 123.

Sodd, M. A., and Doctor, B. P. (1974), *Methods Enzymol. 29E,* 741.

Söll, D. (1971), *Science 173,* 293.

Söll, D., Jones, D. S., Ohtsuka, E., Faulkner, R. D., Lehrmann, R., Hayatsu, H., Khorana, H. G., Cherayll, J. D., and Bock, R. M. (1966), *J. Mol. Biol. 19,* 556.

Staehelin, M. (1971), *Experientia 27,* 1.

Steffensen, D. M., and Wimber, D. E. (1971), *Genetics 69,* 163.

Sueoka, N., and Kano-Sueoka, T. (1964), *Proc. Natl. Acad. Sci. U.S.A. 52,* 1535.

Sutton, W. D., and McCallum, M. (1971), *Nature (London), New Biol. 232,* 83.

Tartof, K. D., and Perry, R. P. (1970), *J. Mol. Biol. 51,* 171.

Twardzic, D. R., Grell, E. H., and Jacobson, K. B. (1971), *J. Mol. Biol. 57,* 231.

Vincent, W. S., Halvorson, H. O., Chen, H. R., and Shin, D. (1969), *Exp. Cell Res. 57,* 240.

Von Ehrenstein, G., and Dais, D. (1963), *Proc. Natl. Acad. Sci. U.S.A. 50,* 81.

Weber, L. (1975), Ph.D. Thesis, Biology Department, University of Connecticut.

Weber, L., Berger, E., Vaslet, C., and Yedvobnick, B. (1976), *Genetics* (in press).

Weisblum, B., Benzer, S., and Holley, R. W. (1962), *Proc. Natl. Acad. Sci. U.S.A. 48,* 1449.

Weisblum, B., Cherayil, J. D., Bock, R. M., and Söll, D. (1967), *J. Mol. Biol. 28,* 275.

Weisblum, B., Gonano, F., Von Ehrenstein, G., and Benzer, S. (1965), *Proc. Natl. Acad. Sci. U.S.A. 53,* 328.

Wellauer, P. K., and Dawid, I. B. (1973), *Cold Spring Harbor Symp. Quant. Biol. 38,* 525.

Wetmur, J. G., and Davidson, N. (1968), *J. Mol. Biol. 31,* 349.

White, B. N., Tener, G. M., Holden, J., and Suzuki, D. T. (1973a), *Dev. Biol. 33,* 185.

White, B. N., Tener, G. M., Holden, J., and Suzuki, D. T. (1973b), *J. Mol. Biol. 74,* 635.

Zachau, H. G. (1972), in The Mechanism of Protein Synthesis and Its Regulation, Bosch, L., Ed., Amsterdam, North-Holland Publishing Co., p 173.

Zachau, H. G., Dutting, D., Feldman, H., Melchers, F., and Karau, W. (1966), *Cold Spring Harbor Symp. Quant. Biol. 31,* 417.

Zehavi-Willner, T., and Comb, D. G. (1966), *J. Mol. Biol. 16,* 250.