ON THE REDUNDANCY OF DNA COMPLEMENTARY TO AMINO ACID TRANFER RNA AND ITS ABSENCE FROM THE NUCLEOLAR ORGANIZER REGION OF *DROSOPHILA MELANOGASTER*[1]

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MOLECULAR hybridization (HALL and SPIEGELMAN 1961) of labeled ribosomal RNA (r-RNA) with DNA in *D. melanogaster* has revealed (VERMEULEN and ATWOOD 1965; RITOSSA and SPIEGELMAN 1965) that, as in the bacteria (YANKOFSKY and SPIEGELMAN 1962a, b, 1963), approximately 0.27% of the DNA is complementary to r-RNA. This DNA is believed to constitute the ensemble of templates for the transcription of r-RNA and may be referred to as the r-DNA. The amount of r-DNA per haploid Drosophila genome is sufficient to complement at least 130 molecules each of 18S and 28S r-RNA.

Inversions in the X chromosome of *Drosophila melanogaster* are available (SIDOROV 1930; STURTEVANT and BEADLE 1936; MULLER *et al.* 1937) from which one can derive X chromosomes possessing duplications or deletions of a heterochromatic region which includes the nucleolus organizer (NO). With these chromosomes, flies can be obtained which have from one to four doses of the NO region. With DNA from such flies, annealing experiments demonstrated (RITOSSA and SPIEGELMAN 1965) that the amount of r-RNA hybridizable per unit of DNA was directly proportional to the dosage of the NO region per genome. These data indicated then that the DNA sequences complementary to the ribosomal RNA are confined to the segment contained in the deletion or duplication employed.

The existence in bacteria of DNA complementary to amino acid transfer RNA (t-RNA) has been established (GIACOMONI and SPIEGELMAN 1962; GOODMAN and RICH 1962). However, this issue has thus far not been taken up in the higher forms. It is, in principle, readily resolvable by molecular hybridization with suitably labeled and purified t-RNA. The *D. melanogaster* DNA used in the studies on ribosomal RNA permits the performance of the necessary experiments as well as others relevant to the following two questions: (1) What proportion of the *D. melanogaster* genome is complementary to t-RNA? (2) Are the DNA complements of t-RNA localized in the same region as those of the r-RNA?

An answer to the first question is pertinent to possible interpretations of the 130-fold redundancy in ribosomal DNA complements. The second question is of obvious interest and gains particular importance from recent reports of t-RNA

in the nucleolus and the resulting conjecture (BIRNSTIEL, SIRLIN, and JACOB 1965) that it may have a local origin.

As will be seen from the experiments to be reported, DNA complementary to t-RNA can indeed be demonstrated in *D. melanogaster*. Further, measurements of the saturation plateaus indicate a 13-fold average multiplicity for each of the approximately 60 different kinds of t-DNA. Finally, no clustering of t-DNA was detected in the region of the X chromosome which contains the nucleolar organizer.

MATERIALS AND METHODS

(A) *Isotopic labeling:* For isotopic labeling the standard medium of RITOSSA and SPIEGELMAN (1965) was modified to contain 0.5 g of yeast per 10 ml. To each 10 ml were added either 7 mc of H^3-uridine (21 c/mM, Nuclear-Chicago) or 5 to 10 mc of P^{32} (Oak Ridge National Laboratory) after hydrolysis to remove pyrophosphate, and neutralization. The usual RNA employed in the present studies had a specific activity in excess of 50,000 count/min per μg. All radioactive counting was done on samples precipitated and washed on membrane filters and assayed in a Packard liquid scintillation spectrometer (HALL and SPIEGELMAN 1961).

(B) *Extraction and purification of RNA:* The extraction and deproteinization of labeled bulk RNA followed the detailed protocol of RITOSSA and SPIEGELMAN (1965). Since the present study involved competition experiments between ribosomal RNA and "4S" RNA a more rigorous elimination of the latter from the ribosomal RNA was required. For such purposes a one-step isolation of r-RNA from a methylated-albumen-Kieselguhr (MAK) column is not sufficient because of the tendency of early components to "tail" into later ones. The procedure adopted involved prior salt fractionation to separate "4S" from r-RNA followed by sucrose density gradient centrifugation and finally by MAK chromatography.

Total unlabeled RNA from adult flies was precipitated from 0.04 M sodium acetate with 80% alcohol. The precipitate was mixed with 0.01 M Tris, pH 7.6, containing 0.005 M $MgCl_2$ and 2 M NaCl and allowed to stand overnight at 0° to permit solution of the "4S" RNA components. The residue of undissolved r-RNA was collected by centrifugation (20 minutes at 15,000 rpm) and dissolved overnight in the cold in a buffer which was 0.05 M NaCl, 0.0001 M $MgCl^2$, .05 M Na acetate, pH 5.1. The RNA was layered on a sucrose gradient (5 to 18%) according to the details of SCHERRER and DARNELL (1962) and centrifugation was carried out in a SW-25 Spinco rotor at 25,000 rpm 12 hours at 4°C.

The fractions corresponding to the 18S and 28S peaks were collected, pooled, equilibrated with 0.1 M NaCl and .05 M phosphate, pH 6.8 and loaded on an MAK column as detailed by YANKOFSKY and SPIEGELMAN (1962a). After a first wash with several bed volumes of the loading buffer and a second with 0.4 M NaCl in 0.05 M phosphate, the r-RNA was eluted with 1.2 M NaCl in .05 M phosphate. It was then dialyzed against 2 × SSC (SSC is .15 M NaCl and .015 M Na citrate, pH 7.0) and stored frozen.

In all cases labeled "4S" RNA was prepared by MAK column chromatography.

(C) *Preparation of DNA:* The DNA was extracted from adults as described previously (RITOSSA and SPIEGELMAN 1965). Occasionally a digestion with pronase (50 μg per ml for 1 hour at 37°C) was included to facilitate the complete removal of basic proteins from the DNA. Purity of the DNA was monitored by optical density measurement at 230, 260, and 280 mμ, analysis for ribose and deoxyribose, and banding in CsCl. Absence of ribonuclease was assured by assay with radioactive RNA for detectable solubilization for periods corresponding to those used in hybridization tests. Complete denaturation of the DNA is an essential requisite, and alkaline denaturation was carried out in 1/100 SSC (SSC is 0.15 M NaCl, 0.1 M Na citrate, pH 7.4) at a DNA concentration of approximately 100 μg/ml. The pH was adjusted to 12.2 with freshly prepared 1 N NaOH, the solution allowed to stand at room temperature for 10 minutes, followed by readjustment of pH to 7.0. Denaturation was complete as monitored by hyperchromicity at 260 mμ and retention on nitrocellulose membrane filters.

(D) *Base composition of RNA:* Base composition was determined by the isotope dilution procedure on RNA uniformly labeled with P[32] (HAYASHI and SPIEGELMAN 1961). Subsequent to alkaline digestion the resulting 2' to 3' nucleotides were separated on columns of Dowex-formate and the relevant fractions assayed for radioactivity and OD at 260 mμ.

(E) *Hybridization and detection of hybrid structures:* The method of GILLESPIE and SPIEGEL-MAN (1965) was used and it involves the following three steps: Step (1) *Irreversible fixation on DNA filters:* Nitrocellulose filters (Schleicher and Schuell, B-6, 27 mm) are first soaked in 6.6 × SSC and 10 ml of the same buffer is passed through the filter. Following this the appropriate amount of DNA, dissolved in 6.6 × SSC at a concentration of 5 to 10 μg per ml, is passed through the filter. The amount of DNA actually retained is always monitored by measuring the optical density of the DNA solution before and after filtration. In many cases the DNA is labeled so that the amount of DNA retained on the filter can be measured as radioactivity. The loaded filters are allowed to dry at room temperature and are then incubated in a vacuum desiccator at 80° for approximately 4 hours. When monitored with radioactive DNA, no detectable DNA (less than 1%) is lost during any of the subsequent steps involving washing, hybridization, or enzyme treatment. Step (2) Hybridization: The hybridization is carried out by immersion of loaded filters in a 2 × SSC solution (3 ml) of the labeled RNA at the desired concentration and temperature for a suitable interval in a stoppered vial. The time required for completion of hybridization under the conditions specified must be determined by preliminary kinetic experiments to insure that the saturation plateau is obtained and kept. It was found that at 0.75 μg of RNA per ml 12 hours incubation at 50°C was more than adequate to attain plateau values. Step (3) *Removal of the unpaired RNA:* The filters are then removed from the hybridization mixture and washed with 2 × SSC on both sides. They are then placed in a solution containing RNAase (free of DNAase) at a level of 20 μg per ml in 2 × SSC and allowed to digest at 30° for 1 hour. After digestion the filters are again removed and washed with 2 × SSC on both sides, dried, and counted.

It is of obvious importance to monitor the degree of contaminating "noise" (unpaired RNA or RNAase core) and this was accomplished by including in the hybridization mixture a heterologous RNA (*E. coli*) carrying a different identifying isotopic label (P[32]). The amount of P[32] found on the filter after Step (3) provides a measure of the "noise" level.

(F) *The source of the DNA:* The nature and origin of the Drosophila stocks employed in the present experiments may be briefly indicated. One is G-21 of the Oak Ridge National Laboratory and its constitution is: *In(1)sc[4L,8R] γ sc[4+8] cv v f/RA, yf/Y.* The males of this line lack the nucleolus organizer region on the X but carry one on the Y. The corresponding DNA preparations from this stock are designated as ♂ (1). The second is G-31 from the Oak Ridge National Laboratory which has the following relevant constitution: *In(1)sc[S1L,4R], sc[S1] vB/RA,yf/B[S]Y.* The males of this stock carry two NO regions on the X and another on the Y, making three in all. DNA preparations from the males of the stock are therefore designated ♂ (3). The third stock was designed to provide a DNA containing four NO regions per genome. To obtain it males of the G-31 were crossed with wild-type females. The females of the F$_1$ were backcrossed with the males of stock G-31. Advantage was taken of suitable linked markers to select the proper combinations with respect to NO. Females chosen necessarily contain two chromosomes, both of which carry two doses of the nucleolar organizer region. The stock was maintained with males of G-31 which also possess duplicates of NO on their X. DNA preparations derived from the females of this stock are designated ♀ (4).

RESULTS

A. *Preparation and properties of RNA components from* Drosophila melanogaster: The components present in bulk RNA prepared as described in METHODS can be separated by a variety of devices which permit both identification and purification. Figure 1 (top) shows the results of a zonal centrifugation in a linear gradient of sucrose. P[32] labeled ribosomal RNA of *E. coli* was added as known size
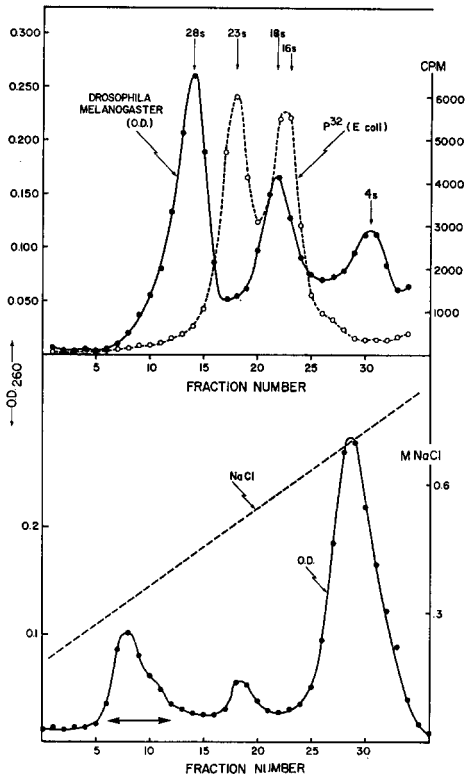
FIGURE 1.—Components in the RNA fraction of *Drosophila melanogaster*.

Top—Suscrose gradient: Bulk RNA (1 mg) purified as described in METHODS (section B) was layered on a preformed linear sucrose gradient (5 to 20%) prepared according to SCHERRER and DARNELL (1962). It was then centrifuged for 12 hours in an SW-25 preparative rotor held at 5°C. Fractions were collected from the bottom for assay of optical density at 260 mμ and radioactivity. Purified P^{32}-labeled ribosomal RNA of *E. coli* was included as a size marker.

Bottom—Columns of methylated albumin coated on Kieselguhr (MAK): Bulk RNA prepared as described in METHODS (section B) is loaded on an MAK column and eluted with a linear NaCl gradient as detailed by YANKOFSKY and SPIEGELMAN (1963).

markers (23S and 16S) permitting ready estimation of the size classes in the Drosophila RNA. The optical density of profile identifies three RNA components of *D. melanogaster*, two corresponding to the ribosomal RNA species (28S and 18S) and the third to the "4S" variety. It is common to find in the higher forms that ribosomal RNA components are somewhat larger than the corresponding 23S and 16S observed in bacteria.

Figure 1 (bottom) shows what occurs when the same sort of material is chromatographed on columns of methylated albumin (MAK). The "4S" varieties separate very well as the first peak (reading from left to right), eluting at about 0.4 M NaCl. The second small peak observed represents contaminating DNA which disappears if the DNAase digestion step is carried out exhaustively. However, its presence introduces no difficulty since it is readily separated from the RNA components by column chromatography.

The third and largest peak represents the combined ribosomal components which do not separate on MAK, a common occurrence with the ribosomal RNA of higher forms. If the third peak is pooled and analyzed in a sucrose gradient, it breaks up into the 28S and 18S ribosomal components. In any event, column fractionation provides a simple method for obtaining "4S" RNA.

Table 1 summarizes the base composition of the three major RNA components of *D. melanogaster* compared to homologous DNA. The 28S and 18S are hardly

distinguishable from each other and are rather DNA-like in their overall composition. However, the "4S" variety is clearly unique and easily identified.

(B) *Conditions for interpretable hybridizations:* It is imperative to bear in mind that our purpose is to determine the amount, if any, of DNA complementary to the amino acid transfer RNA. It is likely that the corresponding t-DNA will represent a *smaller* fraction of the total than either the r-DNA or the DNA employed to generate translatable messages (m-DNA). A difficulty is thereby introduced which must not be ignored in designing the necessary experiments. Thus, appropriate calculations from the base compositions recorded in Table 1 reveal that the "4S" RNA cannot be grossly contaminated with either of the other two cellular RNA components. One might be tempted to conclude that the "4S" fraction can consequently be safely employed in saturation hybridizations to determine plateau values for t-RNA. However, the logic of such experiments should warn against acceptance of such conclusions. A minor RNA impurity can cause complete confusion if it occupies a much larger proportion of the DNA in the hybrid structure. Conversely, even massive contamination can be tolerated if the contaminant is complementary to a DNA segment which is small compared to the relevant RNA.

In the case which is our immediate concern, it is obvious that the presence of either m-RNA or r-RNA in the labeled "4S" fraction could generate uncertainties in attempts at estimating t-DNA. It is necessary, therefore, to decide initially whether impurities exist and if they do, to identify the nature of the non-t-RNA components. Once identified, the participation in the assay of the unwanted RNA can be eliminated by swamping the reaction with unlabeled counterpart.

We have available a very simple device to decide whether the "4S" fraction is contaminated primarily with r-RNA or m-RNA. If r-RNA is the principal contaminant, saturation curves on DNA from flies carrying different doses of the "NO" region should yield plateaus which obey the "NO" rule (RITOSSA and SPIEGELMAN 1965). In particular, the RNA/DNA ratio (in percent) in the hybrid should approximate 0.135 per NO region in the genome.

On the other hand, if the principal contaminant is m-RNA, no such response to this particular genetic manipulation will be observed, and furthermore, the

TABLE 1

Base composition of the RNA fractions of D. melanogaster

Fraction	Cytosine	Adenine	Uracil(thymine)	Guanine
28S	17.5	30.4	30.1	22.0
18S	17.8	28.1	31.0	23.1
4S	25.9	20.2	24.6	29.3
DNA	20.0	30.0	30.0	20.0

P^{32}-labeled RNA was prepared as described in METHODS and fractionated on sucrose density gradients according to SCHERRER and DARNELL (1962) to yield the 18S and 28S fractions. The "4S" fraction was isolated from a MAK column in Figure 2. The separated RNA fractions were incubated with carrier unlabeled RNA (3 mg of bulk *E. coli* RNA) and the resulting 2'-3'-nucleotides separated on Dowex-formate. The determination of the base composition from the P^{32} content and OD at 260 mμ followed the procedure of HAYASHI and SPIEGELMAN (1961). The base composition of the DNA was determined in an analytical ultracentrifuge by equilibrium density centrifugation using DNA of *Pseudomonas aeruginosa* as an internal marker. The numbers given represent molar ratios.

(C) *The number and distribution of t-RNA cistrons:* We now describe two kinds of experiments designed to determine the amount of t-DNA and its relation to the nucleolar organizer region. For the latter purpose DNA preparations carrying one, two, three, and four doses of the nucleolus organizer region per genome were used.

Consider the situation where DNA's of differing NO content are challenged with a saturating amount of labeled "4S" RNA in the presence of increasing levels of unlabeled r-RNA. The amount of hybridized "4S" RNA should descend to a plateau as the contaminating r-RNA is competitively eliminated from the reaction. If the t-DNA is clustered in the NO region, the plateaus attained should be proportional to the NO dosage. If, on the other hand, the t-DNA segments are located somewhere else in the genome, the results should be independent of the NO content and all the curves should descend to the same plateau. It is obvious that in experiments of this nature, the unlabeled r-RNA employed must be free of contaminating t-RNA. It was for this reason that the three-step purification described in METHODS (B) was used. The data obtained in this sort of an experiment are shown in Figure 3. It is clear that the plateau approached is independent

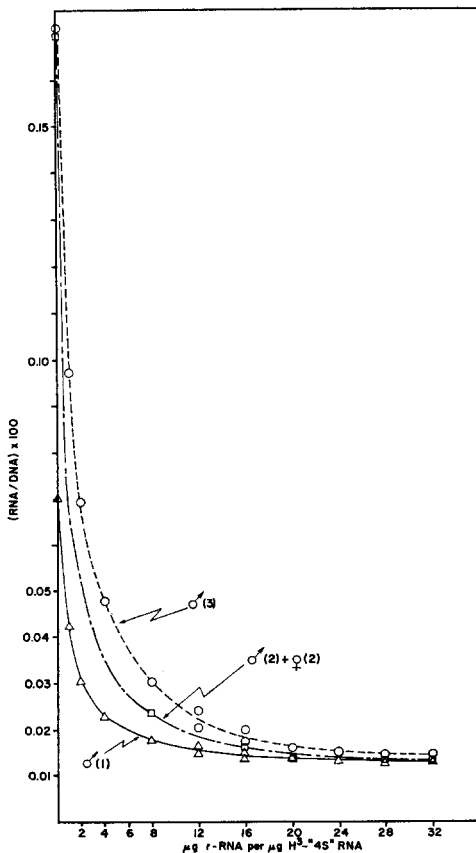FIGURE 3.—Competitive replacement of a component in the labeled "4S" RNA with unlabeled r-RNA.

Each filter contained 50 μg of Drosophila DNA of the indicated NO content and was immersed in 3 ml of 2 × SSC containing 3 μg of H^3 "4S" RNA (90,500 count/min per μg) and the indicated amount of purified unlabeled r-RNA. After 12 hours at 50°C the filters were removed and prepared for counting as described in METHODS.

of the genetic constitution, all curves converging asymptotically to a value of 0.015%.

It should be noted that a comparison of the numbers obtained in the absence of r-RNA (Figure 2) with the plateau values (Figure 3) provides a measure, *not of the contamination with r-RNA, but of the comparative lengths of t-DNA and r-DNA segments.* Recalling that each reaction mixture contains 4 μg of labeled "4S" RNA, it is evident from the response to 1 μg of r-RNA that the major component in the preparation is t-RNA and not r-RNA.

The level of contamination with r-RNA varies from one preparation to another and the amount of competing unlabeled r-RNA required must be individually determined on each "4S" preparation. With this known, a direct determination of plateau values can be made by performing the usual saturation curves with increasing amounts of "4S" RNA. These were carried out with H³-labeled "4S" RNA at 50°C for 12 hours. To monitor internal "noise," P³²-labeled ribosomal RNA of *B. megaterium* was included in the reaction mixture. To exemplify in detail the absolute amounts of material and the radioactivity levels being dealt with, a typical experiment is detailed in Table 2. Noise correction is made by subtracting from the tritium counts the proportion of P³² counts which survives the purification of Step (3) in the hybridization procedure (METHODS, Section C), account being taken of the difference in specific activities of the control and Drosophila RNA. Examination of Table 2 shows, that compared to the tritium counts hybridized, the "noise" level can be ignored.

Two series of saturation experiments carried out with two different "4S" RNA preparations and a variety of DNA's are summarized in Figure 4. It is evident that the saturation curves yield the same answer provided by the competition experiments of Figure 3. The five kinds of DNA examined all yield the same plateau value, there being no discernible effect of increasing the NO content from 1 to 4 per genome. The three independent determinations (Figures 3 and 4) of the proportion of t-DNA per diploid genome yielded values of 0.015, 0.013,

TABLE 2

Numerical details of a saturation experiment

DNA on filter (μg)	H³ "4S" RNA (μg)	P³²-r-RNA (μg)	H³- count/min	P³² count/min	"Noise" (P³² counts × 4.1)	Corrected count/min per 100 μg	% DNA hybridized
70.0	1	1	445	17	69	537	0.0100
73.2	2	2	594	12	49	744	0.0139
75.6	3	3	730	12	49	900	0.0169
75.6	4	4	793	28	114	898	0.0168
74.6	5	5	734	28	114	831	0.0156
74.6	6	6	757	26	106	872	0.0163

Filters containing ♂ (3) DNA were incubated for 12 hours at 50°C in 2×SSC containing increasing amounts of Drosophila H³ "4S"-RNA. In addition the incubation mixture contained P³² *B. megaterium* r-RNA and unlabeled Drosophila r-RNA (8 μg for each μg of H³-4S-RNA). The H³-"4S" RNA had 53,191 count/min per μg and the P³²-r-RNA assayed at 12,600 count/min per μg. After the incubation, the filters were prepared for counting as described in METHODS (section C).
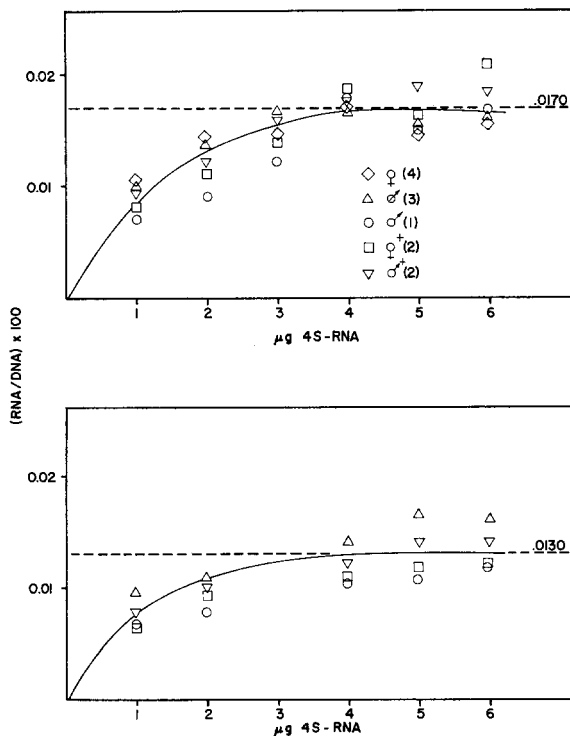
FIGURE 4.—*Saturation curves of t-RNA on types of DNA.*

Top—70–100 μg of the three kinds of DNA were fixed to individual nitrocellulose filters and these incubated for 12 hours at 50°C in 3 ml 2×SSC in the presence of increasing amounts of H^3-labeled Drosophila "4S" RNA (53,191 count/min per μg). In addition, for each μg of "4S" RNA of Drosophila the incubation mixture contained 1 μg of P^{32}-labeled *B. megaterium* r-RNA and 8 μg unlabeled Drosophila r-RNA. At the conclusion, the filters were prepared for counting as described in METHODS (section E). After normalization of the specific activities, P^{32} was subtracted from the H^3 count/min to correct for "noise."

Lower—Same as the upper except that 24 μg of unlabeled r-RNA was used to quench the contaminant in this H^3 "4S" RNA preparation which had a specific activity of 90,500 count/min per μg.

and 0.017%. Considering the technical complexities and the small segment of DNA being measured, the agreement would appear to be satisfactory.

It will be noted that in both experiments of Figure 4, DNA preparations from wild-type males and females were examined separately with no detectable difference evident. In any event, the complete independence of t-DNA content and NO dosage exhibited by the data of Figures 3 and 4 discourages localizing any significant proportion of the t-DNA in the sc^4-sc^8 region which contains the nucleolar organizer.

DISCUSSION

(A) *Nature of the RNA involved in the hybridization tests:* The "4S" RNA used was characterized by chromatography and sedimentation in sucrose gradients. The base composition (Table 1) shows clearly that neither r-RNA nor m-RNA can be major components. The analysis of the "4S" fraction by hybridization (Figure 2) and the competition experiment with r-RNA (Figure 3) indicate

the presence of r-RNA fragments to the extent of about 20%. We have seen that these fragments are readily eliminated as radioactive participants by the simple expedient of adding adequate amounts of unlabeled purified r-RNA. The constancy of the plateaus achieved in Figure 3 demonstrate that the fractionation procedure has indeed removed virtually all t-RNA from the r-RNA used.

The validity of the experiments described and the conclusions drawn demand only that the majority of the residual 80% of the "4S" RNA which hybridizes in the presence of excess r-RNA is, in fact, t-RNA. There exist no compelling reasons for doubting this conclusion. The available literature contains no evidence for an unknown RNA which is present in amounts approaching that of either r-RNA or t-RNA. We feel certain, therefore, that our t-DNA estimates are correct to at least within a factor of two.

(B) *Origin of the DNA types employed:* The differences among the genomes to be assayed for t-DNA can be understood from the manner in which the deletion and duplication-bearing X chromosomes originated (Figure 5). Both were recovered as crossover products from the heterozygote of the long inversions *In(1)sc⁴* and *In(1)sc⁸* (or the similar *In(1)sc*^{S1}). The left break of *In(1)sc⁴* is just to the right of *sc*, while the right break is in the X heterochromatin (Xh) to the left of the nucleolus organizer. The left break of *In(1)sc⁸* is just to the left of *sc*, while the right break is in Xh to the right of the nucleolus organizer. (*In(1)sc*^{S1} has its right point of breakage similarly to the right of NO but has its left point of breakage just to the right of *sc*). Thus, one crossover product carries a duplication for a segment of Xh, including NO and the *bobbed* locus, while the complementary product is deficient for the same segment of Xh. We will refer to the chromosome with the Xh duplication as the *sc⁸-sc⁴* chromosome, and to the chromosome deficient for the Xh segment as the *sc⁴-sc⁸* chromosome. The segment between the right breaks of the inversions will be referred to as the *sc⁸-sc⁴* segment (Figure 6). Measurements of the *sc⁸-sc⁴* and *sc⁴-sc⁸* chromosomes at oogonial metaphase show that the *sc⁸-sc⁴* segment is about 25% of the X-chromosome length; that is, a little under 5% of the total length of a haploid chromosome set at this stage. In salivary chromosomes, the *sc⁸-sc⁴* segment extends from 19F to 20D1 on BRIDGES' (1938) map, and so constitutes a much smaller proportion of the total length in polytene chromosomes than in mitotic chromosomes. For a thorough discussion of the cytogenetics of Xh and the Y chromosome, see COOPER (1959).

(C) *t-DNA and the NO region:* The previous experiments (RITOSSA and SPIEGELMAN 1965) with r-RNA showed that the r-DNA is confined to the *sc⁸-sc⁴* segment and to the Y chromosome, which also bears a nucleolus organizer and an amount of r-DNA equal to that in the *sc⁸-sc⁴* segment. Together with other evidence (EDSTROM *et al.* 1961; PERRY 1962; BROWN and GURDON 1964; Mc-CONKEY and HOPKINS 1964) associating the nucleolus with r-RNA formation, those experiments indicate that NO is the site of r-DNA. In the present experiments, we make use of DNA from flies with the same chromosome formulae as in those just mentioned. The flies are designated ♂ (1), ♂ (3), and ♂ (4). The relevant components in ♂ (1) are Y and *sc⁴-sc⁸*; in ♂ (3), Y and *sc⁸-sc⁴*, and in
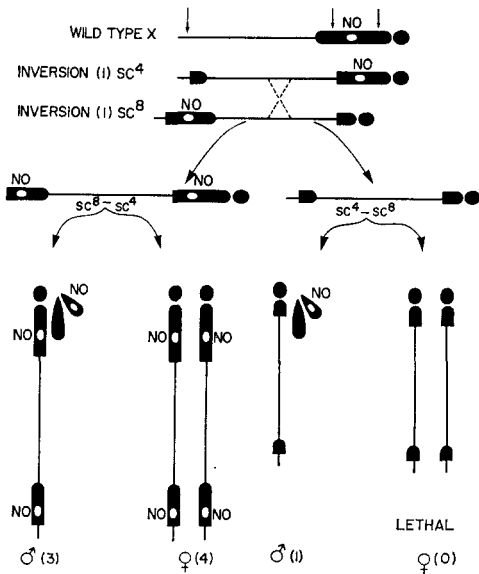
FIGURE 5.—Origin and construction of stocks. Symbols identify Nucleolar Organizer (NO), Centromere (C), scute⁴ (sc⁴), and scute⁸ (sc⁸). A crossover between the two inversions yields the desired deletion and duplication from which the desired chromosome complements can be constructed.

$♀$ (4) two sc^8-sc^4 chromosomes. Thus, the comparison of saturation plateaus provides a strong test for clustering within the sc^8-sc^4 segment, a difference by a factor of four between $♂$ (1) and $♀$ (4) being expected if the t-DNA is all within the segment. However, if the t-DNA is largely in X outside the segment, but not in Y, the saturation plateaus for males and females should be different and this was shown not to be the case (Figure 4). Other distributions of the t-DNA are more or less indistinguishable in the present experiments since they would depend on the relative amounts in the autosomes as compared with X and Y.

In any event, the experiments clearly show that DNA of fly genomes possessing one, two, three, and four NO regions do not exhibit any detectable differences in t-DNA content. This outcome stands in striking contrast to the similar experiments with r-RNA hybridization in which the r-DNA content was directly proportional to the dose of NO. We conclude that, unlike the r-DNA, very little—possibly none—of the t-DNA is in the nucleolus organizer or the sc^8-sc^4 segment. A further conclusion is justified that the X chromosome alone does not contain a preponderance of the t-DNA. Finally, since each kind of t-DNA is required for
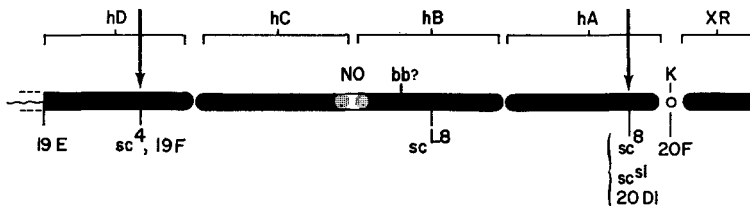

FIGURE 6.—A diagram of the relevant regions of the X chromosome. The region between the arrows is the sc^8-sc^4 segment. (After COOPER 1959).

genetic translation, any t-DNA in the Y chromosome must also be represented elsewhere.

Our results are consistent with the conclusions of PERRY (1962) from experiments with L cells in which partial inhibition by actinomycin-D prevented synthesis of nucleolar RNA, but allowed synthesis of a 4S component at least some of which was probably t-RNA, in extranucleolar parts of the nucleus. Similarly, in agreement is the finding of BROWN and GURDON (1964) that lethal homozygotes of the anucleolate mutant of *Xenopus laevus* can synthesize "4S" RNA, but not r-RNA.

(D) *Multiplicity of t-DNA cistrons and its implications:* The DNA content of the haploid *D. melanogaster* genome has been estimated microspectrophotometrically by RUDKIN (1965) to be 0.2×10^{-12} g which corresponds to an equivalent molecular weight of 1.2×10^{11} daltons. When this value is multiplied by the average saturation plateau of 0.015% we obtain about 1.8×10^7 for the mol wt equivalent of the total t-DNA. With 60 different kinds of t-DNA, the average quantity of a given kind is about 3.7×10^5 daltons. This divided by the mol wt of t-RNA, 2.5×10^4, gives an average of 12.5 templates per haploid set for the transcription of each kind of t-RNA. As already suggested (YANKOFSKY and SPIEGELMAN 1963; RITOSSA and SPIEGELMAN 1965; VERMEULEN and ATWOOD 1965) for the case of r-DNA, the redundancy of t-DNA may be related to a requirement at certain times of a peak rate of t-RNA synthesis that exceeds the maximum possible transcription rate of a single template. Concurrent transcription of an appropriate number of templates would meet such a requirement.

Note that the comparative redundancy obtained for the two types of RNA is in agreement with this interpretation. The equivalent molecular weight for one set of ribosomal RNA's is about 2×10^6 and the corresponding number for one complete set (i.e., 60) of s-RNA's is not very different, being 1.5×10^6. In cells one finds, in general, ten times as much r-RNA as t-RNA. Therefore, if a 130-fold redundancy of r-DNA satisfies the template requirements of r-RNA, a multiplicity of 13 should do for t-RNA, a value in remarkable agreement with the estimate of 12.5 from the saturation plateaus with t-DNA.

The possession of multiple copies, far in excess of two for each type of t-RNA, would make it difficult to employ suppressor mutations which function via mutations in t-RNA anti-codons to correct corresponding codon mistakes in structural cistrons. The efficiency with which this type of suppression would operate would decrease as the t-RNA redundancy exceeds two per haploid set. Thus, while the t-RNA type of suppression is common in the bacteria it may occur in higher forms only where low levels of suppression are sufficient.

The question will arise whether the redundancy of the r-DNA and t-DNA is built into the mitotic chromosomes, or instead represents local elaboration of DNA segments in some manner disproportionate to the remainder of the genome. This possibility is not supported by experiments which compared the amounts of r-DNA in DNA from such sources as spermatozoa, liver, etc., of the chicken (RITOSSA, BLEYMAN, and SPIEGELMAN 1966).

Assuming that the redundancy is chromosomal, the demonstrated multiplicity

of both r-DNA and t-DNA has a bearing on the question of multistrand chromosome models. A multistranded chromosome would have for any DNA region a redundancy, calculated by the method used here, at least as great as the number of strands the chromosome comprises. Any amount in excess of that number necessarily represents DNA segments repeated in the same strand. Thus, the multiplicity of a specific segment of DNA provides an upper limit for the strandedness of the chromosome. Since the redundancy of t-DNA is much less than that of r-DNA, we can conclude that the redundancy of r-DNA is largely in the form of linear repeats rather than multiplicity of strands.

(E) *A possible interpretation of the t-DNA cistrons:* Finally, we mention a conjecture, to be developed in detail elsewhere, (ATWOOD and WHITE 1966) which locates the t-DNA cistrons at the genetic loci of the dominant markers known as Minutes. The Minutes form a phenotypically homogeneous class with an estimated number of about 55 members. Ten to 15% are in X, and the rest distributed among the autosomes. They are most frequently caused by deletions, suggesting that each locus has linear redundancy in the presence of which point mutations are largely ineffective. The 15-fold redundancy of t-DNA is consistent with the foregoing interpretation of the Minutes. Further, a deletion in any one of them should lead to a similar phenotypic effect. Finally, if they are scattered, there should be in the neighborhood of 60 such loci. The phenotype— delayed development, small bristles, homozygous lethality—is not inconsistent with a general retardation of genetic translation.

SUMMARY

Experiments are described which establish that approximately 0.015% of the DNA of *Drosophila melanogaster* is complementary to the amino acid transfer RNA (t-RNA). This number leads to about a 13-fold redundancy for each of the approximately 60 t-RNA species. Hybridizations with DNA from stocks carrying one, two, three, and four doses of the nucleolus organizer region established that the t-DNA cannot be detected in the region of the genome which has been shown to contain the complete cluster of DNA complementary to the two ribosomal RNA components.

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