

*LOCALIZATION OF DNA COMPLEMENTARY TO
RIBOSOMAL RNA IN THE NUCLEOLUS ORGANIZER
REGION OF DROSOPHILA MELANOGASTER**

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The use of hybrid formation between DNA and isotopically labeled RNA¹ combined with RNAase treatment to eliminate unpaired RNA permitted the detection²⁻⁴ in bacteria of sequences in DNA complementary to the two (16S and 23S) homologous ribosomal RNA components. The methods developed with microorganisms sufficed to establish that a similar situation exists in higher plants,⁵ mammals,^{6, 7} and insects.⁸

The proportion (0.3%) of the total genome involved was constant in the bacteria examined and indicated^{3, 4, 9} a multiplicity of sites for each of the two ribosomal components. The densities of the DNA-RNA hybrids suggested³ that the multiple sites were clustered rather than scattered throughout the genome. However, the bacteria were not convenient material for a more detailed attempt at illuminating the relation of these cistrons to each other and to the rest of the genome.

It seemed likely that higher organisms would furnish a better opportunity by permitting the correlation of cytogenetic and cytochemical information with data derived from molecular hybridization. Thus, diverse observations implicate the nucleolus with protein synthesis,¹⁰⁻¹² ribosomes¹³⁻¹⁶ and ribosomal RNA formation,¹⁷⁻¹⁹ the most striking being the absence of ribosomal RNA synthesis in a lethal anucleolate mutant of the aquatic toad, *Xenopus laevis*.²⁰

The available facts are consistent with the hypothesis that identifies the nucleolus as the site of ribosomal RNA synthesis. They do not, however, eliminate the alternative possibilities that it is a repository for RNA synthesized elsewhere or that the nucleolus serves some other indirect function in the assembly of ribosomes. Nevertheless, if we adopt it as a working concept, the notion of a nucleolar location for ribosomal RNA formation leads to some interesting and experimentally testable predictions.

The nucleolus characteristically occupies a specific position (the nucleolar organizer, or "NO," segment) in the chromosome complement. This invariant relation between the nucleolus and a particular chromosomal locus suggests the obvious possibility that the DNA complements which generate the ribosomal RNA strands are confined to the nucleolar organizer region. Confirmation would resolve the question of nucleolar function as well as decide between scattered versus clustered distributions of the multiple ribosomal RNA cistrons.

A direct attack requires a reliable method for isolating pure nucleoli still attached to their chromosomal organizer segments and uncontaminated by other chromatin fragments. Comparative hybridizations could then be carried out between ribosomal RNA and "nucleolar DNA" versus "nonnucleolar DNA." Two attempts^{5, 6} to carry out such experiments have yielded contradictory results and it is apparent from both investigations that neither had available "nucleolar chromatin" of sufficient purity to permit a truly decisive experiment. Under the circumstances, it seemed worth while to consider other experimental approaches which bypass the limitations of physical separations by employing biological devices to achieve the desired end result.

Mutants of *Drosophila melanogaster* are known²¹ which contain inversions involving²²⁻²⁴ the "NO" region on the X-chromosomes and others which contain useful linked markers.^{25, 26} Stocks can be derived from these possessing duplications or deletions of the "NO" region, making it possible to construct²⁷ by suitable crosses strains which have 1-4 doses of the nucleolar organizer region. Hybridization experiments between the DNA derived from these stocks and isotopically labeled ribosomal RNA should, in principle, provide a precise answer to the following question: Are the DNA complements of the ribosomal RNA confined to the "nucleolar organizer" region? An affirmative outcome would be indicated if the amount of RNA hybridizable per unit of DNA is directly proportional to the dosage of "NO" per genome. The absence of proportionality would indicate that the ribosomal RNA cistrons are not localized in the chromatin region contained in the deleted and duplicated regions.

These experiments have been performed and it is the primary purpose of the present paper to present the results. The data indicate that the DNA sequences complementary to ribosomal RNA are confined to the segment contained in the deletion employed. We conclude that the "nucleolar organizer" region of the chromatin contains the cluster of cistrons for each of the two ribosomal RNA components.

Materials and Methods.—(a) *Growth medium:* The various stocks were raised on a medium made by adding the following in grams to 1 liter of H₂O; corn meal (100); sucrose (100), or 100 ml of molasses (Brer Rabbit, Gold Label); fresh baker's yeast (100); agar (10); methyl-p-hydroxy benzoate (2.7). The flies were collected at the adult stage and stored at -14°C for use as a source of DNA.

(b) *Isotopic labeling*: To achieve a high level of labeling, the standard medium was modified to contain 0.5 gm of yeast per 10 ml, an amount calculated to be the minimum necessary to support the growth of 2–3 gm of larvae, corresponding to approximately 2–3 mg of RNA. To 10 ml of the modified medium 7 mc of H^3 -uridine (21 C/mM, Nuclear-Chicago) were added. In labeling experiments the eggs were collected with a brush, washed, and loaded on wet strips of blotting paper which were laid on the surface of the labeled medium. After 7–8 days of growth, larvae were harvested by Mead's²⁸ method for extraction of the RNA. Final preparations of pure ribosomal RNA had a specific activity of 77,300 cpm/ μ g. All counting was done in a liquid scintillation counter on nitrocellulose membranes¹ permitting assay of P^{32} and H^3 in the same samples.

(c) *RNA extraction and purification*: After repeated washing with water, the larvae were suspended in 10 vol (w/v) of an extraction fluid which contained NaCl (0.1 M), 1% sodium dodecyl sulphate (SDS), and heparin (0.001%). The suspension was homogenized in a glass homogenizer kept at 0°C, following which 1 vol of phenol saturated with 0.1 M NaCl and containing heparin (0.001%) was added and the mixture shaken for 20 min at 0–4°C. This was then followed by a centrifugation and removal of the aqueous layer. The phenol treatment was repeated three more times, the first for 20 min and the others for 10 min each. The phenol was then removed from the aqueous layer with two ether extractions and the ether eliminated by bubbling air through the solution. The RNA was then precipitated by the addition of 2 vol of a cold 80% ethanol containing Na acetate (0.02 M), taken up in a buffer (0.1 M NaCl; 0.05 M phosphate, pH 6.8) and passed through a methylated albumin column as detailed by Yankofsky and Spiegelman.⁴ The two ribosomal RNA components do not separate, the 18S appearing as a shoulder on the leading edge of the 28S peak at about 0.7 M NaCl. The ribosomal fractions were pooled and dialyzed 24 hr at 4°C against 100 vol of $2 \times$ SSC (SSC is 0.15 M NaCl, 0.015 M Na citrate pH 7.4). For analysis in sucrose gradients, the RNA was dissolved in a buffer²⁹ at pH 5.1 containing NaCl (0.05 M), Na acetate (0.01 M), and Mg acetate (0.0001 M). It was dialyzed for 5 hr against the same buffer and layered on a 5–20% sucrose (dissolved in the same buffer) gradient and spun for 12 hr at 25,000 rpm at 4°C in a Spinco SW 25 rotor. The two ribosomal components (28S and 18S) are readily identified and separated.

(d) *Preparation of DNA*: The DNA employed was extracted exclusively from adults suspended in 10 vol (w/v) of the following buffer at pH 7.6, Tris (0.05 M), KCl (0.025 M), Mg acetate (0.005 M), sucrose (0.35 M). Homogenization was carried out in a mortar and the homogenate filtered through 8 layers of gauze. The resulting filtrate was centrifuged for 10 min at 1,000 rpm in the International centrifuge (rotor 253). The pellet was resuspended in 0.15 M NaCl, 2% SDS, and 0.1 M EDTA, and adjusted to pH 8. For each 10 gm of flies, about 35 ml of this medium were used and the resulting suspension was shaken for 10 min at 60°. All subsequent steps followed the procedure detailed by Marmur,³⁰ with the exception that at the first precipitation 1 vol (rather than 2) of cold ethanol was added. This avoided interference by salt precipitation and increased the yield. Fibers were collected on a glass rod, and any flocculent DNA precipitate remaining behind was centrifuged, resuspended in SSC to which 1 vol of ethanol was added yielding fibrous precipitates. The fibrous DNA, dissolved in SSC, was subjected to a 4-hr digestion at 37°C with heated (100°C for 10 min) RNAase (Sigma 5 \times crystallized) at 150 γ /ml. At this stage there was consistent contamination with a polysaccharide detected by model E Schlieren optics in CsCl equilibrium gradients as a hyperfine band with no absorption at 260 $m\mu$. To remove this material, the preparation was digested for 45 min at 37°C with 250 μ g/ml of alpha-amylase (2 \times crystallized, Worthington). This step effectively avoided any subsequent difficulties in the final purification of DNA. The enzymes were removed by adding SDS (1%) and 2 phenol treatments at room temperature followed by 2 successive deproteinizations with chloroform-isoamyl alcohol for 10 min each. The DNA was precipitated by the addition of 2 vol of ethyl alcohol, and the fibers were collected and dissolved in 1/100 SSC. Purity of the final DNA preparations was monitored by optical density measurements at 230, 260, 280 $m\mu$, and analysis for ribose and deoxyribose. It yielded a single peak in a CsCl gradient at a position corresponding to 38% GC. Alkaline denaturation of the DNA was carried out in 1/100 \times SSC at a concentration of 150 μ g/ml. The pH was adjusted to 12.2 with freshly prepared 1 N NaOH, the solution left to stand at room temperature for 10 min, followed by readjusting the pH to 7. Denaturation was complete as measured by either hyperchromicity or adherence to nitrocellulose membrane filters.

(e) *Hybridization and detection of hybrid structures*: The method of Gillespie and Spiegelman³¹

was used and it involves three steps. *Step 1—Irreversible fixation of DNA to filters:* The denatured DNA is dissolved in a solution of $6.6 \times \text{SSC}$ at a concentration of 5–10 γ/ml . Nitrocellulose filters (Schleicher and Schuell, B-6,27 mm) are first soaked in $6.6 \times \text{SSC}$, and 10 ml of the same buffer is then passed through the filter, following which the appropriate amount of the DNA solution is filtered through. The amount of DNA actually retained is always monitored by measuring the O.D. of the DNA solution before and after the filtration. The loaded filters are allowed to dry at room temperature and then incubated in a vacuum desiccator at 80° for approximately 4 hr. 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 immersing the loaded filter into a $2 \times \text{SSC}$ solution (4 ml) of the labeled RNA at the desired concentration and incubated in a stoppered vial at 65° . In some cases solvents of higher ($4 \times \text{SSC}$) ionic strength were used. The time required for completion of the hybridization must be determined by preliminary kinetic experiments to ensure that the saturation plateau is attained and kept. In the present instance, it was found that a 7–10 hr incubation period was adequate for the combinations tested. *Step 3—Removal of the unpaired RNA:* The filters are removed from the hybridizing mixture and washed with $2 \times \text{SSC}$ on both sides. They are then placed in a solution containing RNAase (free of DNAase) at a level of 20 γ/ml in $2 \times \text{SSC}$ and allowed to digest at 30° for 1 hr. After the digestion, the filters are again removed and washed with 60 ml of $2 \times \text{SSC}$ on both sides, dried, and counted.

It is of obvious importance to monitor the degree of contaminating "noise" and this was accomplished by including in the hybridizing 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. In the experiments to be described, this noise level is negligible.

(f) *Source of DNA:* Four different stocks of *Drosophila* were used in the present studies to prepare DNA containing various proportions of the "NO" region. One is the Standard²¹ Urbana wild type. Individuals of this strain carry one "NO" region on each X and one on each Y chromosome, hence both males and females carry two. The DNA derived from this stock is designated either $\sigma(2)$ or $\varphi(2)$. Another is the G-21 of the Oak Ridge National Laboratory which has the following relevant genetic constitution: $\text{In}(1) \text{sc}^{4L, 8R}, y \text{sc}^{4+8} \text{cv v f/RA,yf/Y}$. The males of this line lack the "NO" region on their X but carry one on the Y. The corresponding DNA preparations are designated in the text as $\sigma(1)$. The third is G-31 from the Oak Ridge National Laboratory which has the following genetic constitution: $\text{In}(1) \text{sc}^{\text{SIL}, 4R}, \text{sc}^{\text{SIVB}}/\text{RA,yf/B}^{\text{SY}}$. The males of this stock carry two "NO" regions on the X and another on the Y chromosome, making three in all. DNA preparations from the males of this stock are, therefore, designated as $\sigma(3)$. The fourth stock was designed to provide a DNA containing 4 "NO" regions per genome. To obtain it, males of G-31 were crossed with wild-type females. The females of the F1 were backcrossed with the males of stock G-31. From these, females were selected exhibiting, because of homozygosity, the genetic markers B,sc^{4+8v} . Here, advantage was taken of the presence of the inversion in the X-chromosome which, by suppressing crossing-over, permits use of the markers linked to "NO" to select the proper combinations with respect to "NO." The females chosen necessarily contain two X chromosomes, both of which carry two doses of the nucleolar organizer region. The stock was maintained with males of G-31 which, of course, also possess duplicates of "NO" on their X. DNA derived from the females of this stock is designated by $\varphi(4)$.

Results.—(a) Numerical details of a saturation experiment: To exemplify the absolute amounts of materials and radioactivity levels being dealt with, a typical experiment is detailed in Table 1. H^3 -labeled ribosomal RNA at various input levels are incubated with a constant amount of $\sigma(1)$ DNA. The internal "noise" control is provided by including P^{32} -ribosomal RNA of *E. coli* in quantities which approximate the H^3 -marked RNA from *Drosophila*. "Noise" correction is made by subtracting from the H^3 counts the proportion of P^{32} counts observed which survive the purification [section (d), *Methods*] process of step 3, account being taken of difference in specific activities. It is evident that the contamination is negligible compared to the counts hybridized.

(b) *Saturation curves with DNA containing various dosages of the nucleolar organizer*

TABLE 1
DETAILS OF A SATURATION EXPERIMENT

DNA	<i>Drosophila</i> H ³ -RNA	<i>E. coli</i> P ³² -RNA	Cpm-H ³	Cpm-P ³²	H ³ -noise	% of Genome
36.5	0.7	1	3284	10	83	0.113
37.3	1.4	2	4283	11	91	0.145
37.1	2.1	3	4838	15	124	0.164
37.3	2.1	3	4680	16	132	0.157
37.3	2.8	4	4609	20	166	0.154
37.3	3.5	5	4923	23	190	0.164

Incubations were carried out in 4 ml of $2 \times$ SSC at 65°C. The indicated amounts of $\sigma(1)$ DNA were prefixed on the membrane filters as in *Methods*. The specific activity of the H³-RNA of *Drosophila* was 77,285 cpm/ μ g and that of P³²-RNA of *E. coli* was 9200 cpm/ μ g. Removal of irrelevant RNA by washing and RNAase treatment as described in *Methods*. H³-"noise" is calculated from the finally observed P³²-counts corrected for difference in specific activities. All recorded counts are corrected for background. Numbers in the first 3 columns refer to nucleic acid added in μ g.

region: The four types of DNA carrying different proportions of "NO" were subjected to saturation curves and the results obtained are described in Figure 1. A majority of these experiments contained internal "noise" controls, but they are not recorded since they all yielded results identical to those shown in Table 1.

We may first focus our attention on the results with wild type (curve labeled ♀ (2), ♂ (2) DNA) since they settle an issue which could have complicated numerical interpretation of the outcome. It will be noted from section (e) of *Methods* that males of some stocks and females of others were employed to achieve the desired dosage of the "NO" region. Consequently, we had to know whether the nucleolar organizer segments on the X chromosome and the Y chromosomes contribute equally to the observed proportion of complementarity between DNA and ribosomal RNA. For this purpose wild-type male and female DNA were tested in separate hybridization experiments. It is clear that the open circles (♀) and the half-shaded circles (♂) fall on the same curve and approach the same plateau of 0.27 per cent.

Since the XY and XX combinations contribute equivalently to the DNA which is complementary to ribosomal RNA, we can with confidence estimate expected plateaus without regard to sex. We now assume that the plateau attained by the wild type represents a dose of two "NO" segments, from which we can predict the plateaus which should be achieved by the others, *if all the DNA complements of the ribosomal RNA are confined to the "NO" segment*. These predictions are indicated by the solid horizontal lines of Figure 1. The corresponding numerical values are recorded on the right-hand ordinate.

Comparison of the observed and predicted plateau values in Figure 1 suggests that experimental support has been provided for the assertion that the DNA which is complementary to the ribosomal RNA is confined to the region of the nucleolar organizer.

Discussion.—(a) *Nature of the stocks:* The steps involved in the production of the stocks [see *Methods*, (f)] from the original inversion mutants are summarized diagrammatically in Figure 2. The deletion and duplications arise as complementary products of the cross between the two inversions. Thus, the segment appearing as a duplicate in one is equivalent to that deleted in the other. The deletion extends from the proximal break of inversion scute⁴ to the proximal break of inversion scute,⁸ encompassing the nucleolar organizer and the "bobbed" locus.

The experiments described indicate that all of the DNA complementary to

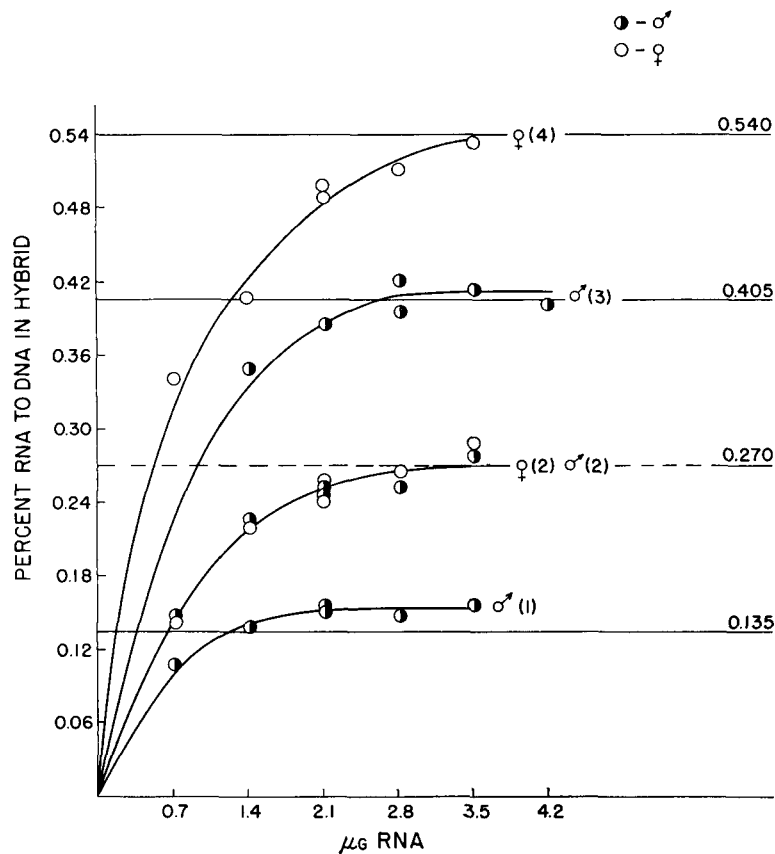


FIG. 1.—Saturation levels of DNA containing various dosages of nucleolar organizer region. Dosage of “NO” is indicated by the number in parentheses. The dotted horizontal line at 0.270 is assumed to be a correct estimate for a dosage of 2, and the solid horizontal lines represent predicted plateaus for dosages of 1, 3, and 4, respectively. Numerical values of the plateaus are given on the right. Replicate determinations are indicated by multiple points at the same input levels. All hybridizations and subsequent RNAase and washing treatments were carried out as described in *Methods*.

ribosomal RNA is confined to the relatively small region involved in the deletion employed. The cytological facts establish that the nucleolus is invariantly associated with the “NO” segment. In view of these observations, arguments on whether the relevant complementary DNA is in fact located precisely at the “NO” locus or at some neighboring site within the deleted segment are not likely to generate a particularly useful dialogue.

It would have been interesting to test a DNA completely lacking in nucleolar organizer. With *Drosophila*, death occurs rather early in the development of stocks homozygous for the deletion, making it difficult to obtain DNA uncontaminated with cytoplasmic DNA of maternal origin. The anucleolate mutant of *Xenopus laevis*²⁰ ultimately may furnish more suitable material, providing it is a deletion and not an operator mutation.

(b) *The plateau value*: It should be evident that the successful completion of the experiments described required the use of a hybrid detection method which

would be comparatively "noise"-free and accurate enough to estimate 25 per cent differences at levels involving 0.1 per cent of the input DNA. The method³¹ used involves hybridization with DNA previously fixed irreversibly to nitrocellulose membranes. It combines the advantages of RNAase treatment¹ to eliminate noise, DNA immobilization^{32, 33} to avoid DNA-DNA interaction, and the convenience of membrane filters.³⁴ Since each hybridization curve of Figure 1 acts as a quantitative control for the others, the nature of the experiments provided a useful challenge of the quantitative adequacy of the method which was successfully met. Further, the value of 0.27 per cent for the wild type is in agreement with an earlier⁸ independent estimate carried out by a somewhat different procedure. It must be emphasized that all such plateau measurements are more likely to be below than above the true value.

The multiplicity of ribosomal cistrons can be estimated from the saturation plateau and the DNA content of a haploid genome which lies between 0.2 and 1×10^{-12} gm.^{35, 36} Conversion of the lower³⁵ value to equivalent molecular weights yields 1.2×10^{11} daltons which must be divided by two for our purpose since available evidence³⁷⁻³⁹ indicates that RNA is found complementary to only one of the two DNA strands in any given region. Thus a wild-type haploid genome contains 1.6×10^8 daltons of DNA complementary to ribosomal RNA, which is equivalent to about 100 stretches for each of the two ribosomal RNA components. If the higher estimate of the genome is taken, this number becomes 500. It is interesting to note that plateau values clustering around 0.3 per cent of the genome for the ribosomal RNA cistrons have been found for several bacteria,^{3, 4} a higher plant,⁵ and now for an insect.

(c) *Implications of the findings:* The experiments reported here locate all the DNA complementary to both 18S and 28S ribosomal components in the region of the nucleolar organizer. They therefore decide the issue in favor of a clustered rather than a disperse distribution. The data further specify a location of the relevant DNA templates which supports the notion that the nucleolus is the site of ribosomal RNA synthesis. The fact that approximately 0.3 per cent of the DNA is set aside for this purpose in genomes varying over several orders of magnitude raises an interesting problem. It may, however, be worth pointing out that this may turn out to be a simple numerical consequence of supply and demand. Ribosomal RNA constitutes the bulk of cellular RNA (85%) and in bacteria the corresponding cistrons must turn out as much RNA per generation as all the other cistrons put together. If this is general, and no cistron can be made to work more than

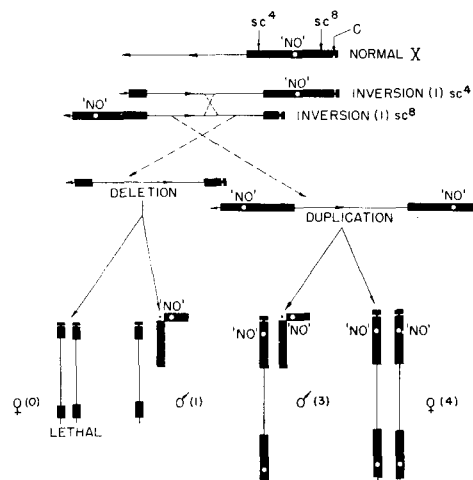


FIG. 2.—Origin and construction of stocks. Symbols identify nucleolar organizer (NO), centromere (C), scute⁴ (*sc⁴*), and scute⁸ (*sc⁸*). A cross between the two inversions yields the desired deletion and duplication from which the various stocks can be developed as described in *Methods*.

300 times faster than the average, about 0.3 per cent of any genome would have to be set aside for ribosomal cistrons. The availability of the *Drosophila* material makes possible a host of potentially informative experiments along lines similar to those described here. Among these are the interrelation of 18S and 28S cistrons, the location of the transfer-RNA cistrons, and others. Finally, we may note that it is a source of some satisfaction to be able to record here an example illustrating the successful union of classical genetic material and the more recent preoccupations with "molecular matching."

Summary.—Experiments were designed to see whether DNA complementary to ribosomal RNA was confined to the nucleolar organizer (NO) region. DNA was prepared from four stocks of *Drosophila melanogaster* carrying 1, 2, 3, and 4 doses of the "NO" segment and hybridized to isotopically labeled ribosomal RNA. The results obtained support the following conclusions. (1) The wild-type genome saturates at 0.27 per cent of the DNA indicating that it contains approximately 200 sites per diploid set for each of the two ribosomal components. (2) The nucleolar organizer regions on the X and Y chromosomes contribute equally to the proportion of DNA complementary to ribosomal RNA. (3) The proportions of the DNA found to be complementary to ribosomal RNA in the different stocks correspond to that predicted from the genetic constitution and the assumption that all the DNA complements of ribosomal RNA are confined to the nucleolar organizer locus. (4) By identifying the "NO" segment as the site of the required DNA templates the data support the assertion that the nucleolus is the site of ribosomal RNA synthesis.

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