IN SITU DEMONSTRATION OF DNA HYBRIDIZING

WITH CHROMOSOMAL AND NUCLEAR SAP RNA

IN CHIRONOMUS TENTANS

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ABSTRACT

Cytological hybridization combined with microdissection of *Chironomus tentans* salivary gland cells was used to locate DNA complementary to newly synthesized RNA from chromosomes and nuclear sap and from a single chromosomal puff, the Balbiani ring 2 (BR 2). Salivary glands were incubated with tritiated nucleosides. The labeled RNA was extracted from microdissected nuclei and hybridized to denatured squash preparations of salivary gland cells under conditions which primarily allow repeated sequences to interact. The bound RNA, resistant to ribonuclease treatment, was detected radioautographically. It was found that BR 2 RNA hybridizes specifically with the BR 2 region of chromosome IV. Nuclear sap RNA was fractionated into high and low molecular-weight RNA; the former hybridizes with the BR 2 region of chromosome IV, the latter in a diffuse distribution over the whole chromosome set. RNA from chromosome I hybridizes diffusely with all chromosomes. Nucleolar RNA hybridizes specifically with the nucleolar organizers, contained in chromosomes II and III. It is concluded that the BR 2 region of chromosome IV contains repeated DNA sequences and that nuclear sap contains BR 2 RNA.

INTRODUCTION

The newly synthesized, heterogeneous, nuclear RNA (H RNA) in eukaryotic cells is to a large extent degraded to acid-soluble products within the nucleus shortly after its synthesis (1, 2). Some of the surviving molecules are probably transported to the cytoplasm (3). Their possible role as mRNA in protein synthesis has been considered but not finally proven (4).

H RNA synthesis in the salivary gland cells of *Chironomous tentans* takes place in the chromosomal puffs (5, 6), which are interpreted as the morphological expression of differential genome transcription (7). The presence of large puffs, named Balbiani rings, has been correlated with the appearence of specific salivary proteins in the gland sectetion of two species of Diptera (8, 9).

For this reason mainly, a mRNA function has been ascribed to Balbiani ring RNA (9, 10).

The Balbiani ring 2 (BR 2) of *C. tentans* can be isolated by means of microdissection, and its RNA content analyzed separately from chromosomal and nuclear sap RNA (6). The characteristics of nuclear sap H RNA are particularly interesting in view of the recent suggestion that a selective degradation of H RNA takes place on the chromosomes (11). Since RNA from nuclear sap, chromosomes, and BR 2 readily hybridizes with filter-bound homologous DNA,[1] cytological hybridization ap-

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[1] Lambert, B., B. Daneholt, J.-E. Edström, E. Egyházi, and U. Ringborg. Manuscript in preparation.

peared to offer a way to localize the complementary DNA sequences for the different RNA fractions. Knowledge about this localization would permit conclusions about the identity of nuclear sap H RNA.

MATERIALS AND METHODS

Animals

Mature, fourth-instar larvae of *C. tentans* were used. They were either from a laboratory stock originally obtained from Tübingen, Germany, or were freshly collected locally. The salivary glands were removed and immediately transferred to the appropriate media for RNA labeling or preparation of tissue squashes. The somatically paired salivary gland polytene chromosomes are designated I–IV for the pairs. Chromosomes II and III each carry a nucleolus. The small chromosome IV exhibits three large RNA-synthesizing puffs, the Balbiani rings (BR 1, 2, and 3), of which BR 2 is usually the largest.

Preparation of Radioactive RNA

INCUBATION OF GLANDS, FIXATION, AND MICRODISSECTION: Six salivary glands were transferred to 50 μl of modified Cannon's insect medium (12). Incubation was carried out in the presence of tritiated cytidine and tritiated uridine (100 μCi each, 27–29 Ci/mmole, The Radiochemical Centre, Amersham, England) for 90 min in sealed watch glasses at 18°C (the cultivating temperature of the living larvae). Incubated glands were fixed for 30 min in a mixture of ethanol, formaldehyde, and acetate buffer (13), rinsed 3 times for 10 min each in 70% ethanol, and finally transferred to a buffered ethanol-glycerol solution (13). The fixatives were kept at +4°C. Glands were microdissected in an oil chamber as described by Edström and Daneholt (14). Nuclear sap and the Balbiani ring 2 from about 200 cells were isolated for each experiment.

ISOLATION AND FRACTIONATION OF RNA: The pooled samples of nuclear sap and BR 2 were digested separately in microdroplets of a solution, predigested for 30 min at 37°C, containing 5 mg sodium dodecyl sulfate (SDS) and 1 mg pronase (B grade, Calbiochem, Los Angeles, Calif.) per ml of 0.02 M Tris-HCl, pH 7.4. The samples were dissolved within 15 min, but the digestion was continued for 3 hr at 37°C.

After digestion, the droplets were absorbed into small pieces of filter paper (Munktell, 1F) which retained the RNA during the ensuing washing and fractionation procedures (15). The filter papers were subsequently cleaned of adhering liquid paraffin with chloroform, transferred to 70% ethanol containing 0.2 M potassium acetate, and stored overnight at +4°C. This treatment removes the pronase and SDS from the filter papers, while the RNA is precipitated (15).

RNA was extracted from the filter papers containing BR 2 RNA by repeated washings in distilled water. The extract was adjusted to 2 times SSC (standard saline citrate is 0.15 M NaCl and 0.015 M Na-citrate) and used for hybridization or electrophoresis. Filter papers containing nuclear sap RNA were extracted in salt solution so as to separate H RNA from low molecular weight RNA. The filter paper was first transferred to 5 μl of 10 times SSC and shaken gently for 5 min at room temperature. The saline solution containing low molecular weight RNA was then removed, diluted to 2 times SSC, and used for hybridization or electrophoresis. The H RNA remaining in the filter paper was eluted with distilled water, made to 2 times SSC and used for hybridization or electrophoresis.

ELECTROPHORESIS: Samples of 2–5 μl were withdrawn from the RNA solutions obtained after elution of the filter papers. To these samples was added 10–25 μg of *Escherichia coli* RNA as carrier. Electrophoresis was carried out in 7.5% polyacrylamide gels for an appropriate separation of low molecular weight RNA from H RNA. For the separations of unfractionated RNA, the pronase-SDS digest was diluted with 20 μl electrophoresis buffer containing 20 μg *E. coli* RNA, and applied to a 2% agarose gel. Electrophoretic conditions, gel slicing, and activity measurements were in accordance with earlier descriptions (15, 16), with one exception: the treatment of RNA extracts for 5 min at 55°C before electrophoresis was omitted in these experiments because it causes degradation.[2]

Cytological Hybridization

PREPARATION OF TISSUE SQUASHES: The salivary glands were fixed for 15 sec in ethanol/acetic acid (3/1, vol/vol) and then transferred to 45% acetic acid where they were allowed to swell for 3 min. The gland cells were then removed from the gland secretion with the aid of two insect needles under a dissecting microscope. About 25 gland cells were isolated in 2 min while still being kept in the 45% acetic acid and were squashed under a cover slip on a gelatinized slide. The slide was dipped into liquid nitrogen, and the cover slip was rapidly removed with a scalpel. Slides were subsequently fixed for 10 min in 3/1 ethanol/acetic acid, transferred to 70% ethanol, and, if necessary, stored in this medium at +4°C overnight. The further treatment of the tissue squashes followed essentially the

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[2] Daneholt, B. Manuscript in preparation.

descriptions by Pardue et al. (17) and Wimber and Steffensen (18). Before denaturation the preparations were treated at room temperature for 60 min with pancreatic ribonuclease ("RAF," Worthington Biochemical Corp., Freehold, N. J., 20 µg/ml in 2 times SSC, preheated at 90°C for 5 min, and rapidly chilled), and then extensively washed in 2 times SSC and phosphate buffer. Chromosomes were denatured *in situ* with 90% formamide in 0.1 times SSC at 62°C for 2.5 hr. The slides were immediately transferred to ice-cold 70% ethanol, extensively washed in 2 times SSC at +4°C, and used for hybridization without drying.

INCUBATION WITH RADIOACTIVE RNA: The radioactive RNA in 2 times SSC was heated for 5 min at 100°C in order to degrade the large molecules to smaller fragments. This procedure gives reproducible results, one of which is shown in Fig. 1. The thermally degraded molecules migrate between 4S and 20S, with a broad peak around 10S. For hybridization, 10 µl of the heated, radioactive RNA in 2 times SSC was applied to each squash preparation which was then immediately covered with a cover slip (12 X 12 mm). The different RNA preparations were used in approximately the same concentrations. The specific activities of chromosomal and nuclear-sap RNA after 90 min labeling have been determined by using a modified application of Edström's microtechnique for small amounts of RNA (19). A mean value of about 2 cpm/µµg for chromosomal H RNA was obtained from a large number of previous analyses. The RNA concentrations used in the present investigation were calculated from this value. Between 1500 and 3000 cpm were added to each slide, corresponding to approximately 0.75–1.5 X 10^{-3} µg of RNA.

Incubations were performed in sealed Petri dishes at 62°C for 4 hr. The cover slips were then rapidly removed by dipping the slides into ice-cold 2 times SSC. After several changes of 2 times SSC the preparations were treated with 100 µg pancreatic ribonuclease per ml 2 times SSC (preheated as described above) for 60 min at 37°C. Extensive washes in 2 times SSC preceded ethanol dehydration and air drying.

RADIOAUTOGRAPHY: The dried slides were covered with Kodak AR 10 stripping film which was floated on a 0.001 M acetic acid solution. The emulsion was rapidly dried under a blower, and slides were exposed at +4°C in light-tight boxes, provided with desiccator. Exposure times are indicated in the figure legends. Kodak D-19b was used for development for 5 min at room temperature, and fixation was carried out in Kodak F24. The slides were rinsed in 0.001 M acetic acid for 15 min and stained in toluidine blue (0.1% toluidine blue in 0.05 M acetate buffer, pH 5.2, for 1.5 min) before dehydration and mounting in Entellan (Merck Chemical
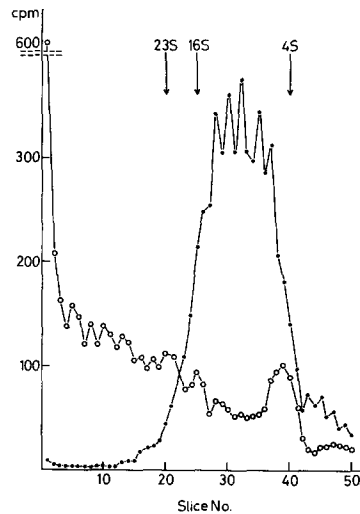
FIGURE 1 The effect of heat treatment of H RNA. Glands were incubated for 90 min with tritium-labeled nucleosides, fixed, and microdissected. RNA was liberated by pronase-SDS digestion from the chromosomes and dissolved in 2 times SSC. Half of the solution was analyzed without further treatment; the other half was heated for 5 min at 100°C. Both samples were electrophoresed simultaneously on a 2% agarose gel with 20 µg of *E. coli* RNA for optical density reference. Untreated RNA, o—o; treated RNA, ●—●.

Div., Merck and Co., Inc., Rahway, N. J.). The photographs were taken with a Leitz Orthoplan photomicroscope.

RESULTS

Electrophoresis of Labeled RNA from Nuclear Sap and Balbiani Ring 2

Extensive analyses of the molecular size distribution and kinetics of labeling of newly synthesized RNA in the nuclear sap and BR 2 have been presented earlier (6, 15). Although (pre)ribosomal RNA has been detected on the chromosomes and in the nuclear sap (12), after short labeling times its quantitative share of chromosomal and nuclear sap H RNA is negligible as in the present analyses (less than 5%, cf. Fig. 2). Fig. 2 shows the electrophoretic separations of nuclear sap RNA and BR 2 RNA after 90 min labeling of the glands in vitro.

Low molecular weight RNA which amounted to around to 30–50% of the total labeled RNA in nuclear sap (Fig. 2) was separeted from H RNA by a salt fractionation technique, and the two fractions were used separately for hybridization. Fig. 3
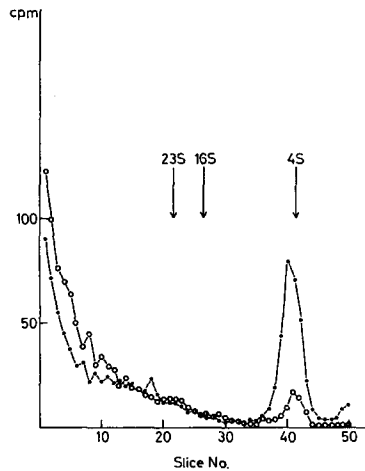
FIGURE 2 Electrophoresis of RNA from nuclear sap and Balbiani ring 2. Glands were incubated for 90 min with tritium-labeled nucleosides, fixed, and microdissected. Nuclear sap and BR 2 from 25 cells were pooled and the RNA liberated by pronase-SDS digestion for 90 min at 37°C. Electrophoresis was in 2% agarose with 20 µg of *E. coli* RNA for optical density reference. Nuclear sap RNA, ●—●; BR 2 RNA, ○—○.

shows that more than 90% of high molecular weight RNA is excluded from the salt fraction, which contains more than 85% of the low molecular weight RNA.

BR 2 RNA was not fractionated since the low molecular weight RNA amounts to less than 10% of the total labeled RNA after a 90 min incubation (Fig. 2).

Cytological Hybridization with High Molecular Weight, Nuclear-Sap RNA

About 3000 cpm of nuclear sap H RNA in 10 µl 2 times SSC were added to each denatured squash preparation and incubated for 4 hr at 62°C. Fig. 4 shows representative micrographs of the results after 2 and 9 wk of radioautographic exposure. The BR 2 area is obviously labeled in all chromosomes IV. Only little lable is seen in the remainder of the chromosome set. The nucleolar organizer regions (Fig. 4 *a*) and Balbiani rings 3 (Fig. 4 *b*) also contain considerably less grains than BR 2. After longer exposure times the BR 2 area is labeled heavily (Fig. 4 *c*) The grain density over the chromosome set is increased and higher than the background. The grains show no regular localization, although bands of grains may be seen coin-

ciding with the chromosome bands. The nucleolar organizer regions are not more heavily labeled than the chromosomes elsewhere.

Cytological Hybridization with Low Molecular Weight, Nuclear-Sap RNA

Low molecular weight, nuclear-sap RNA was added to tissue squashes in amounts equivalent to the amounts used for H RNA hybridization. After 6 wk exposure a weak and diffuse labeling of the whole chromosome set can be seen (Fig. 5). Nucleolar organizers are generally without grains. Most of the chromosomes IV exhibit a higher grain density in the BR 2 region (Fig. 5 *b*), while some do not show any particular localization of grains. This irregular increase of label in the BR 2 region is small compared to the high grain density obtained in the same region with nuclear-sap H RNA and may therefore be attributed to small amounts of H RNA present in the salt fraction (cf. Fig. 3). The formation of hybrids between low
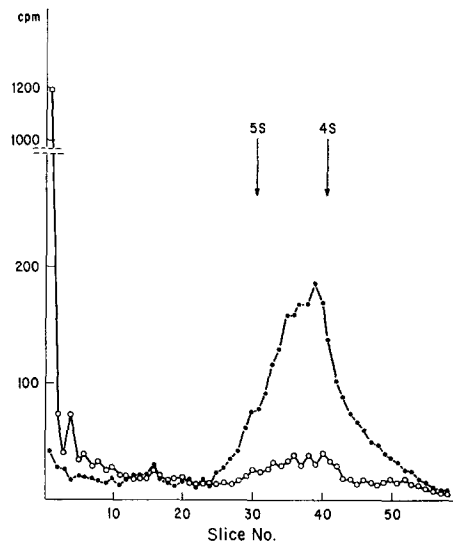

FIGURE 3 Salt fractionation of nuclear sap RNA. Glands were incubated for 90 min with tritium-labeled nucleosides, fixed, and microdissected. Nuclear saps from 200 cells were pooled and digested in a drop of pronase-SDS solution, and the digest was taken up into a piece of filter paper. After the pronase and SDS were washed off, low molecular weight RNA was first solubilized in 5 µl of 10 times SSC, and then H RNA in distilled water. Equal portions from the two fractions were electrophoresed in 7.5% polyacrylamide gels with *E. coli* RNA for optical density reference. Salt eluate, ●—●; distilled water eluate, ○—○.
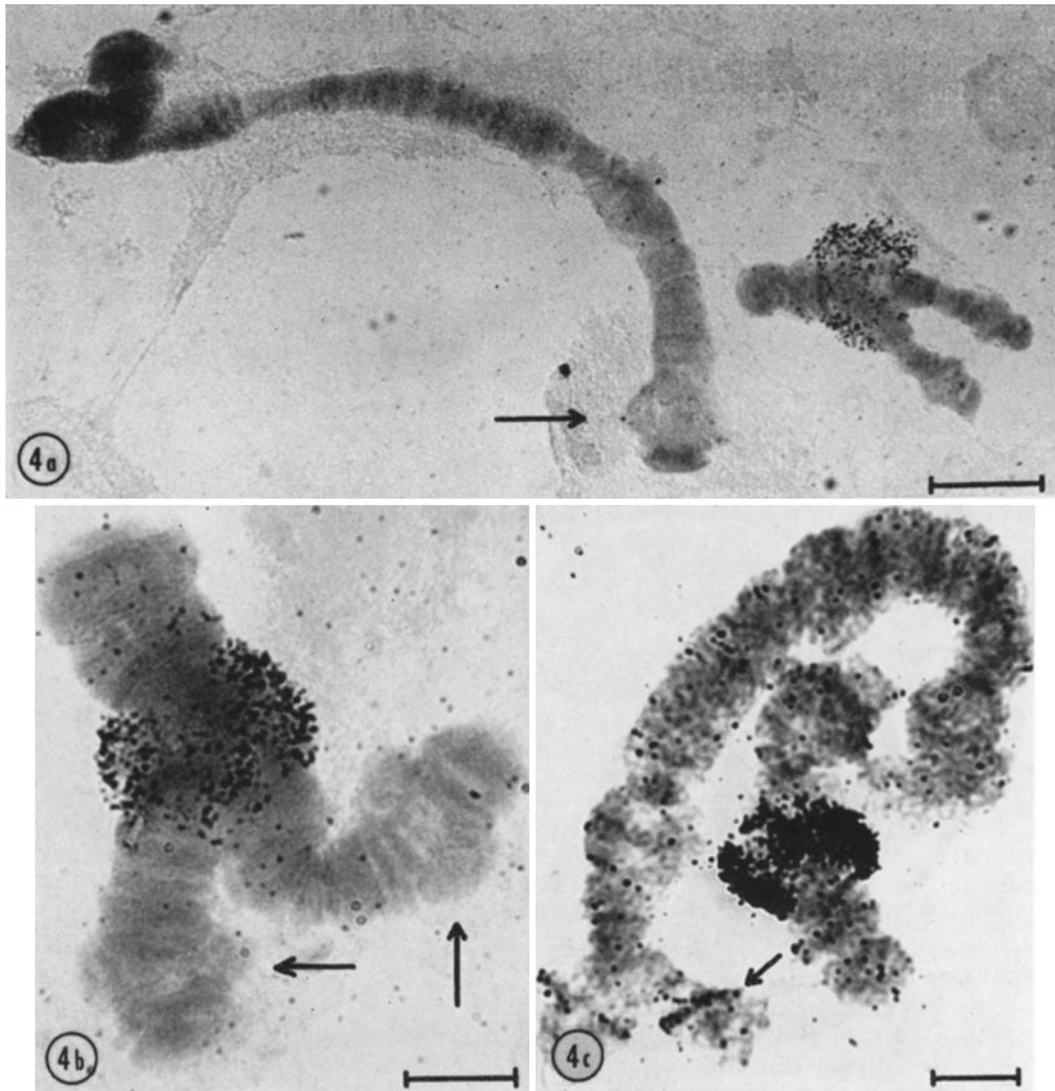
FIGURE 4 Radioautograph after hybridization with high molecular weight RNA from nuclear sap. Denatured tissue slides were challenged for 4 hr at 62°C with 10 μl of 2 times SSC containing 3000 cpm of H RNA, obtained after salt fractionation (cf. Fig. 3). After ribonuclease treatment the slides were subjected to radioautographic exposure for 2 wk (a and b) or 9 wk (c). (a) The arrow indicates the nucleolar organizer region of chromosome II. Chromosome IV, far to the right, is partially unpaired and shows label in the BR 2 region. The bar represents 25 μ. (b) Chromosome IV with grains confined to the BR2 region. Arrows point to BR 3 of the unpaired homologous chromosome segments. The bar represents 10 μ. (c) Chromosome IV and chromosome II. Arrow indicates the position of the nucleolar organizer. The bar represents 10 μ. (a) × 600, (b) × 1500, (c) × 1100.

molecular weight RNA and BR 2 DNA is, however, an open possibility.

It is difficult to rule out the possibility that the diffuse grain distribution over the chromosomes, observed after hybridization with both H RNA and low molecular weight RNA, is unspecific, because the compact structure of the chromosomes may protect trapped RNA from ribonuclease digestion, which nuclear sap or cytoplasm, used as background labeling reference, does not. However,

the control hybridization with nucleolar RNA did not show this diffuse chromosomal grain distribution, nor did the undenatured squash preparations incubated with radioactive RNA (cf. Results, Control Experiments, below). At present, we therefore find it more likely that the diffusely distributed grains do reflect specific DNA/RNA interactions.

Cytological Hybridization with Balbiani Ring 2 RNA

Although the previous results demonstrate the presence in the nuclear sap of an H RNA fraction which hybridizes with the BR 2 region of chromosomes IV, they prove neither that all the H RNA in the nuclear sap is of BR 2 origin nor that all the H RNA synthesized in BR 2 is recovered in the nuclear sap. The cytological hybridization technique is less suited for interpretations of quantitative differences or similarities. However, a crude estimate of the relative size of a hybridizable RNA fraction

in proportion to the total RNA added can be obtained from the grain density of the radioautographs, if the RNA concentration and the exposure time are taken into account. The micrographs of Fig. 6 show the results obtained after hybridization with BR 2 RNA, which are similar to those obtained after hybridization with nuclear sap H RNA, (cf. Fig. 4). The BR 2 areas of the chromosomes IV exhibit a high grain density (Fig. 6 *b*, *c*), and few grains above background can be seen in the nucleolar organizers (Fig. 6 *a*) or elsewhere in the chromosomes. This and other results (11, footnote 2) show that a large percentage of the nuclear sap RNA is derived from the RNA of BR 2.

Control Experiments

Tissue squashes, treated similarly but undenatured, were included in each experimental series, as were normally treated slides incubated without radioactive RNA. Both types of slide showed only a weak and homogeneous background labeling, slightly lower than that observed in experimental preparations (not shown).

It might be argued that the expanded morphology of the Balbiani rings, especially BR 2, makes the DNA in these sites more easily available for denaturation and subsequent hybridization, while the high DNA concentration in the more compact chromosomal bands might promote DNA renaturation and hence prevent hybridization. In a

series of experiments run in parallel to those presented here, we have therefore challenged squash preparations with nucleolar RNA and chromosomal RNA.[3]

Fig. 7 shows the result when tissue squashes were hybridized with RNA from chromosomes I only, in order to exclude contamination from BR 2 and nucleoli. No increase of label in the nucleolar organizer regions compared to the chromosomes could be seen, while usually a higher grain density in the BR 2 regions was obvious (Fig. 7). The latter result can be explained by the presence of small amounts of nuclear sap in the chromosomes after isolation. From a comparison with the nuclear sap RNA hybridization (cf. Fig. 4), it may be calculated that less than 5% nuclear sap RNA contaminating the chromosomal RNA would give rise to a labeling in the BR 2 region comparable to that of Fig. 7. However, the existence of chromosomal RNA sequences similar to DNA in BR 2 can not be excluded. Still there is an obvious quantitative and qualitative difference between the results obtained with RNA from BR 2 and from chromosome I, indicating that hybridization with chromosomal DNA outside the BR 2 region does take place under present conditions, if the slides are challenged with chromosomal, non-BR 2, RNA.

Nucleolar RNA, on the other hand, hybridizes specifically with the nucleolar organizer regions of chromosomes II and III (Fig. 8). Only few grains can be detected in the BR 2 region, or elsewhere in the chromosomes.

DISCUSSION

The use of cytological hybridization to localize chromosomal DNA sequences has been successful for ribosomal RNA (17, 20) and 5 S RNA (18). The main obstacle for an extended application of the technique has been the difficulty of obtaining a chemically and functionally characterized RNA with a specific activity high enough for demonstration of the DNA/RNA hybrids in a single cell.

One way of solving this problem has been to transcribe RNA in a cell-free system, from physicochemically defined DNA fractions (21–25). However, if information is wanted about differential gene activity as expressed in the composition of newly synthesized RNA, in vivo synthesized RNA has to be used.

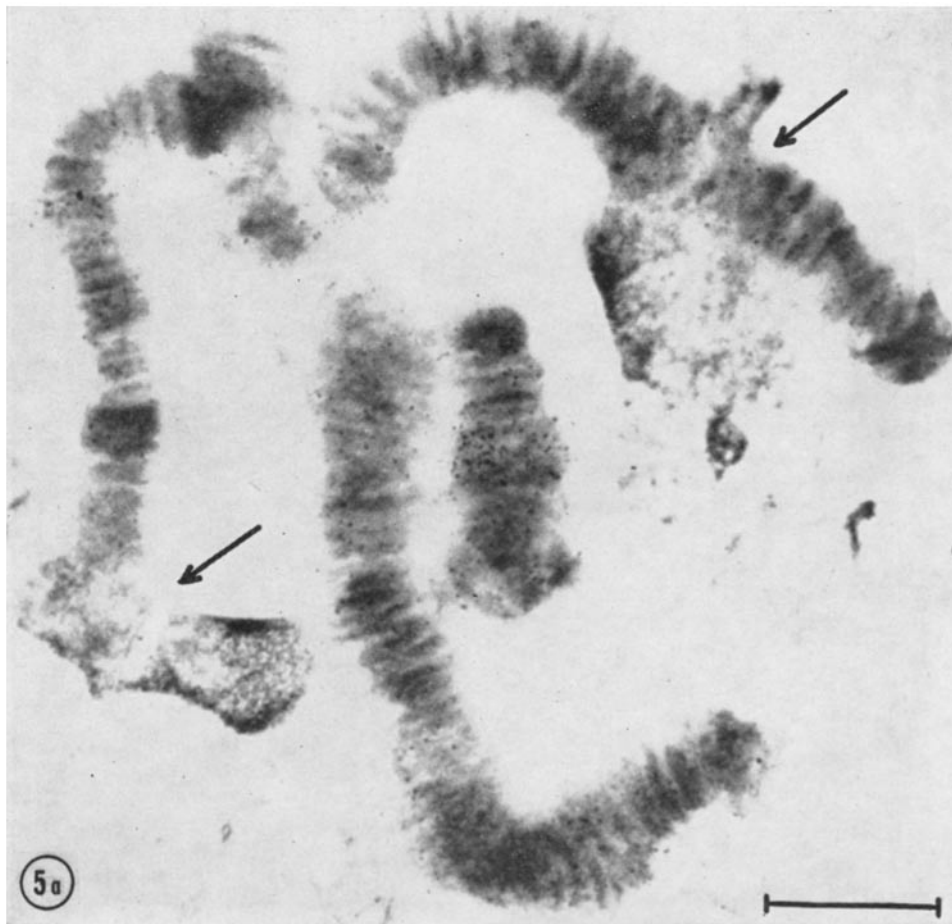[3] Lambert, B., and L. Wieslander. Manuscript in preparation.

FIGURE 5 Radioautograph after hybridization with low molecular weight, nuclear-sap RNA. 3000 cpm of low molecular weight RNA, obtained in the salt eluate (cf. Fig. 3), were hybridized to denatured tissue squashes. Exposure for 6 wk. Other conditions as in legend to Fig. 4. (a) Whole chromosome set. Arrows indicate nucleoli of chromosome II (left) and chromosome III. The bar represents 25 μ. (b) Close up of Fig. 4 a showing chromosome IV. The bar represents 10 μ. (a) $\times$ 920, (b) $\times$ 1500.
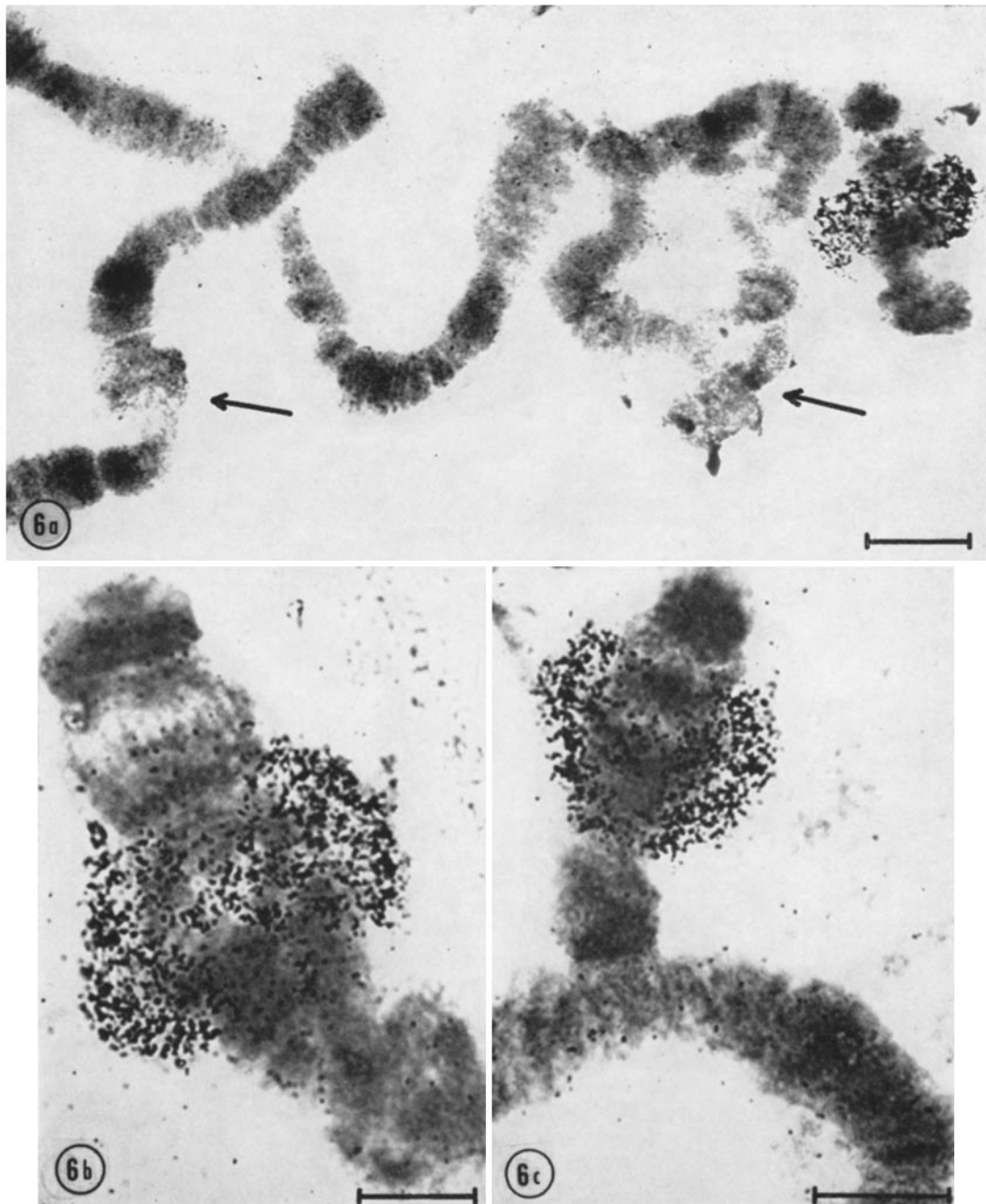
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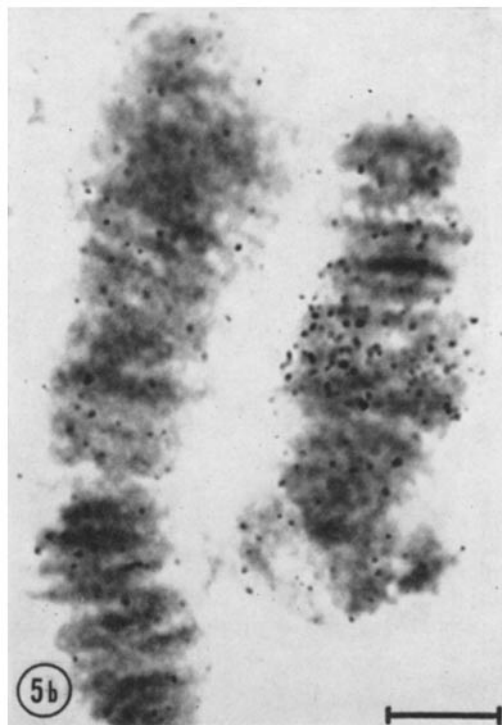
FIGURE 6 Radioautograph after hybridization with BR 2 RNA. 1500 cpm of unfractionated BR 2 RNA were used. Exposure for 6 wk. Other conditions as in legend to Fig. 4. (a) Whole chromosome set. Arrows indicate the positions of the nucleoli of chromosome III (left) and chromosome II. Chromosome IV with strongly labeled BR 2 region is far to the right. The bar represents 25 μ. (b) Chromosome IV with label confined to BR 2. The bar represents 10 μ. (c) Chromosome IV and part of chromosome I. The bar represents 25 μ. (a) × 600, (b) × 1700, (c) × 800.
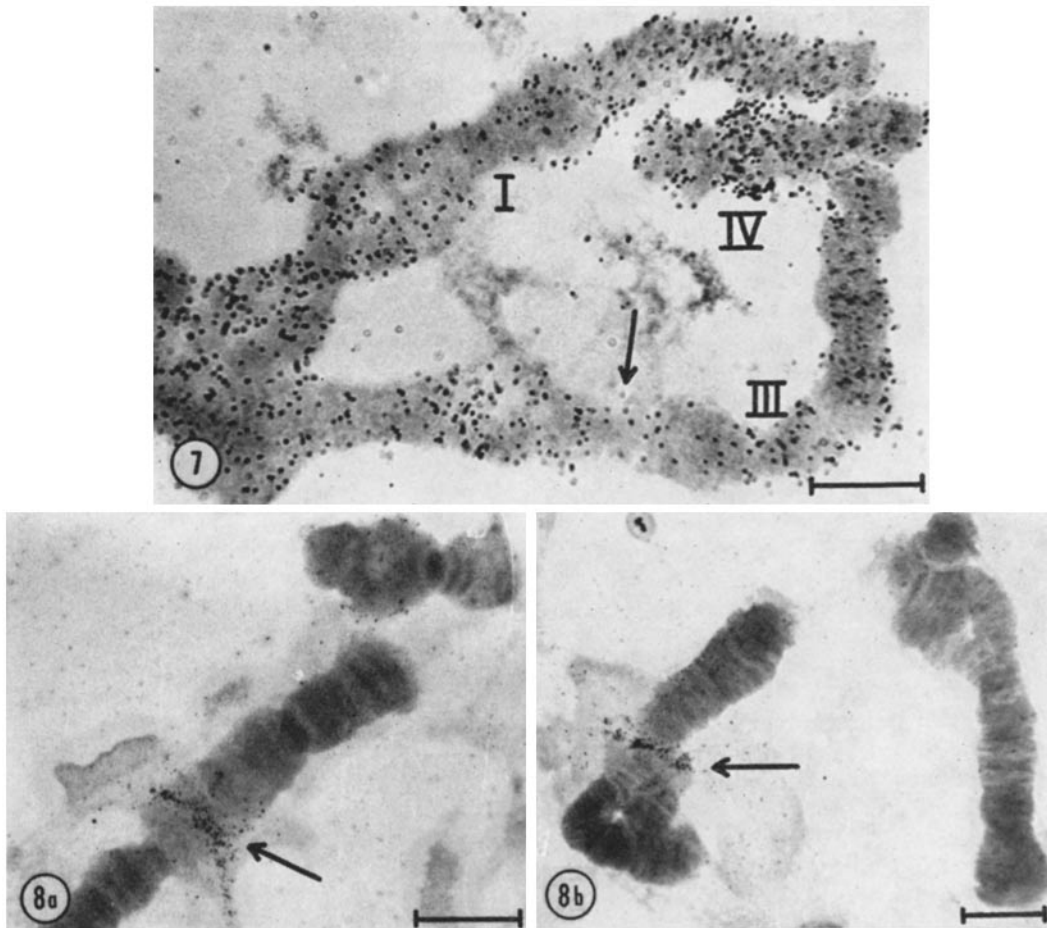
FIGURE 7 Radioautography after hybridization with 2000 cpm of RNA from chromosome I and exposure for 8 wk. Other conditions as in legend to Fig. 4. The chromosomes are indicated by numbers. The arrow indicates the position of the nucleolar organizer. The bar represents 25 μ. × 600.

FIGURE 8 Radioautography after hybridization with nucleolar RNA. 15000 cpm of nucleolar RNA were used. Exposure for 10 days. Other conditions as in legend to Fig. 4. Chromosome II and chromosome IV (a), and chromosome I and chromosome III (b). The arrows indicate label in the center of the nucleoli. The bars represent 25 μ. (a) × 560, (b) × 440.

The RNA synthesis in the salivary glands of C. tentans incubated in vitro is similar to the RNA synthesis during in vivo conditions (11–13). Since in vitro incubations yield nuclear RNA with high specific activity which can be isolated from different nuclear compartments by means of microdissection, these techniques in combination with cytological hybridization open a way for the study of the genetic origin of newly synthesized RNA isolated from different chromosomal regions and nuclear sap.

The reliability of the cytological hybridization technique can be inferred from the results of independent techniques. It has been demonstrated by biochemical methods that the ribosomal cistrons are located in the nucleolar organizers (26, 27). When applied to tissue squashes or sections, ribosomal RNA hybridizes only with the nucleolar organizers (17, 28–30). In the present work it was shown that nucleolar RNA selectively hybridizes with the two nucleolar organizer regions of the C. tentans genome. The 5S cistrons of Drosophila melano-

gaster were shown by biochemical techniques to be located outside the nucleolar organizers (31, 32). In the *in situ* hybridization work of Wimber and Steffensen (18), the 5S cistrons were located in a single defined chromosomal region.

In original reports on cytological hybridization (28–30) the label observed in the radioautographs was convincingly shown to be due to the formation of DNA/RNA hybrids. The specificity test described in the work of Henning et al (21) demonstrated that RNA transcribed in vitro from one subspecies of Drosophila hybridized only to its homologous counterpart in a partially unpaired hybrid chromosome. The control experiments performed in the present investigation showed that denaturation is a prerequisite for the hybridization to occur, and that RNA from chromosome I and RNA from BR 2 show different preferential localizations when hybridized to identical genomes. Thus there is ample evidence for the specificity and reliability of cytological hybridization when used for the localization of complementary DNA sequences in the genome.

The concentrations of RNA and the incubation time used in this investigation permit repeated nucleotide sequences to hybridize, but probably exclude most of the unique sequences from interacting (33). The following discussion therefore primarily concerns RNA which is complementary to repeated DNA sequences.

Hybridization with RNA from chromosome I gives rise to labeling of all chromosomes (Fig. 7). This result is consistent with a widespread distribution of repeated DNA sequences in the polytene chromosomes. The existence of such sequences may be inferred from results of cytological hybridization with synthetic RNA, transcribed from main band DNA (21, 23–25) and satellite DNA (21), of various Drosophila species. The labeling over the chromosomes, observed after hybridization with approximately the same amounts of nuclear sap H RNA, is much weaker (Fig. 4). Since it is not known if cytological hybridization takes place under conditions of DNA excess, the difference in labeling between chromosomal and nuclear sap RNA may be explained by a large pool of unlabeled chromosomal RNA existing in the nuclear sap. There is, however, no support for the latter assumption as judged by the microelectrophoretic separations of nuclear sap RNA (34). The most probable explanation for the difference in labeling is therefore that newly synthesized RNA, other than BR 2 RNA, is present in the nuclear sap in lower concentrations than in the chromosomes. This interpretation is in accord with the results of Daneholt and Svedhem (11), who showed that nuclear sap H RNA has a base composition different from that of total chromosomal H RNA, but similar to that of H RNA from BR 2. In the present work it was shown that the grain density in the BR 2 region of chromosome IV after hybridization with nuclear sap RNA is similar to the grain density obtained in the same chromosomal region after hybridization with BR 2 RNA. An interpretation based on a comparison of grain densities must be considered tentative. Nevertheless these results support the base ratio analysis, again indicating that BR 2 RNA predominates in the nuclear-sap H RNA.

The present results are therefore consistent with the previously suggested possibility (11), that a selective degradation of H RNA takes place on the chromosomes, or shortly after the delivery of H RNA to the nuclear sap, while BR 2 RNA largely escapes degradation and appears in the nuclear sap.

The presence in the nuclear sap of RNA complementary to unique or low frequency DNA sequences cannot be excluded, nor can the presence of small amounts of RNA complementary to extensively repeated DNA sequences widely spread throughout the genome. The last possibility could in fact explain the diffuse labeling on the chromosomes, observed after hybridization with all types of chromosomal and nuclear sap RNA.

Repeated DNA sequences of eukaryotes constitute "families" of different nucleotide complexity and redundancy (33). Results from biochemical hybridization experiments indicate that part of these repeated sequences is transcribed, but the transcription sites and the functions of the transcription products are not known, except for the ribosomal cistrons. Lack of specificity in hybridization experiments involving repeated sequences has been explained by cross-reaction taking place between sequences of different families (35). Recently Kedes and Birnstiel (36) reported specific hybridization with 9S mRNA, suggesting that the genes for the histones of sea urchin, and several other eukaryotes, are reiterated and closely clustered. Our present work may be taken to indicate that DNA complementary to BR 2 RNA contains repeated sequences, largely confined to the BR 2 region of chromosome IV. If the Balbiani

rings of Chironomus species produce the mRNA for the salivary polypeptides (9, 10), it becomes of interest to investigate the informational content of the Balbiani ring DNA. Recent results by Grossbach (personal communication) indicate that the different salivary polypeptides have very similar amino acid composition. Accordingly, sequences coding for different polypeptides may be similar enough, not to be discriminated between, under the conditions of cytological hybridization, which may give the false impression of a high redundancy. At present, however, a function for Balbiani ring RNA, other than coding for the salivary polypeptides, can not be excluded.

REFERENCES

1. Harris, H. 1963. Nuclear ribonucleic acid. *Progr. Nucl. Acid Res.* 2:20.
2. Darnell, J. E. 1968. Ribonucleic acids from animal cells. *Bacteriol. Rev.* 32:262.
3. Darnell, J. E., G. N. Pagoulatos, U. Lindberg, and R. Balint. 1970. Studies on the relationship of mRNA to heterogeneous nuclear RNA in mammalian cells. *Cold Spring Harbor Symp. Quant. Biol.* 35:555.
4. Sherrer, K., G. Spohr, N. Granboulan, C. Morel, J. Grosclaude, and C. Chezzi. 1970. Nuclear and cytoplasmic messenger-like RNA and their relation to the active messenger RNA in polyribosomes of HeLa cells. *Cold Spring Harbor Symp. Quant. Biol.* 35:539.
5. Pelling, C. 1964. Ribonukleinsäure-Synthese der Riesenchromosomen. *Chromosoma.* 15:71.
6. Daneholt, B., J.-E. Edström, E. Egyházi, B. Lambert, and U. Ringborg. 1970. RNA synthesis in a Balbiani ring in *Chironomus tentans.* *Cold Spring Harbor Symp. Quant. Biol.* 35:513.
7. Beermann, W. 1966. Differentiation at the level of the chromosomes. *In* Cell Differentiation and Morphogenesis. North Holland Publishing Co., Amsterdam. 24.
8. Baudisch, W., and R. Panitz. 1968. Kontrolle eines biochemischen Merkmals in den Speicheldrüsen von *Acricotopus Lucidus* durch einen Balbiani-ring. *Exp. Cell Res.* 49:470.
9. Grossbach, U. 1969. Chromosomen-Aktivität und biochemische Zell-differenzierung in den Speicheldrüssen von Camptochironomus. *Chromosoma.* 28:136.
10. Beermann, W. 1964. Control of differentiation at the chromosomal level. *J. Exp. Zool.* 157:49.
11. Daneholt, B., and L. Svedhem. 1971. Differential representation of chromosomal H RNA in nuclear sap. *Exp. Cell Res.* 67:263.
12. Ringborg, U., and L. Rydlander. 1971. Nucleolar-derived ribonucleic acid in chromosomes, nuclear sap, and cytoplasm of *Chironomus tentans* salivary gland cells. *J. Cell Biol.* 51:355.
13. Ringborg, U., B. Daneholt, J.-E. Edström, E. Egyházi, and B. Lambert. 1970. Electrophoretic characterization of nucleolar RNA from *Chironomus tentans* salivary gland cells. *J. Mol. Biol.* 51:327.
14. Edström, J.-E., and B. Daneholt. 1967. Sedimentation properties of the newly synthesized RNA from isolated nuclear components of *Chironomus tentans* salivary gland cells. *J. Mol. Biol.* 28:331.
15. Egyházi, E., B. Daneholt, J.-E. Edström, B. Lambert, and U. Ringborg. 1969. Low molecular weight RNA in cell components of *Chironomus tentans* salivary glands. *J. Mol. Biol.* 44:517.
16. Daneholt, B., J.-E. Edström, E. Egyházi, B. Lambert, and U. Ringborg. 1969. Chromosomal RNA synthesis in polytene chromosomes of *Chironomus tentans.* *Chromosoma.* 28:399.
17. Pardue, M. L., S. A. Gerbi, R. A. Eckhardt, and J. G. Gall. 1970. Cytological localization of DNA complementary to ribosomal RNA in polytene chromosomes of Diptera. *Chromosoma.* 29:268.
18. Wimber, D. E., and D. M. Steffensen. 1970. Localization of 5 S RNA genes on Drosophila chromosomes by RNA-DNA hybridization. *Science (Washington).* 170:639.
19. Edström, J.-E. 1964. Microextraction and microelectrophoresis for determination and analysis of nucleic acids in isolated cellular units. *Methods Cell Physiol.* 1:417.
20. Gerbi, S. A. 1971. Localization and characterization of the ribosomal RNA cistrons in *Sciara coprophila.* *J. Mol. Biol.* 58:499.
21. Hennig, W., J. Hennig, and H. Stein. 1970. Repeated sequences in the DNA of *Drosophila* and their localization in giant chromosomes. *Chromosoma.* 32:31.

22. ECKHARDT, R. A., and J. G. GALL. 1971. Satellite DNA associated with heterochromatin in *Rhynchosciara. Chromosoma.* 32:407.

23. JONES, K. W., and F. W. ROBERTSON. 1970. Localization of reiterated nucleotide sequences in *Drosophila* and mouse by in situ hybridization of complementary RNA. *Chromosoma.* 31:331.

24. RAE, P. M. M. 1970. Chromosomal distribution of rapidly reannealing DNA in *Drosophila melanogaster. Proc. Nat. Acad. Sci. U. S. A.* 67:1018.

25. GALL, J. G., E. H. COHEN, and M. L. POLAN. 1971. Repetitive DNA sequences in *Drosophila. Chromosoma.* 33:319.

26. BROWN, D. D., and J. B. GURDON. 1964. Absence of ribosomal RNA synthesis in the anucleolate mutant of *Xenopus laevis. Proc. Nat. Acad. Sci. U. S. A.* 51:139.

27. RITOSSA, F. M., and S. SPIEGELMAN. 1965. Localization of DNA complementary to ribosomal RNA in the nucleolus organizer region of *Drosophila melanogaster. Proc. Nat. Acad. Sci. U. S. A.* 53:737.

28. GALL, J. G., and M. L. PARDUE. 1969. Formation and detection of RNA-DNA hybrid molecules in cytological preparations. *Proc. Nat. Acad. Sci. U. S. A.* 63:378.

29. JOHN, H. A., M. L. BIRNSTIEL, and K. W. JONES. 1969. RNA-DNA hybrids at the cytological level. *Nature (London).* 223:582.

30. BUONGIORNO-NARDELLI, M., and F. AMALDI. 1970. Autoradiographic detection of molecular hybrids between rRNA and DNA in tissue sections. *Nature (London).* 225:946.

31. TARTOF, K. D., and R. P. PERRY. 1970. The 5 S RNA genes of *Drosophila melanogaster. J. Mol. Biol.* 51:171.

32. QUINCEY, R. V. 1971. The number and location of genes for 5 S ribonucleic acid within the genome of *Drosophila melanogaster. Biochem. J.* 123:227.

33. BRITTEN, R. J., and D. E. KOHNE. 1968. Repeated sequences in DNA. *Science (Washington).* 161:529.

34. RINGBORG, U., E. EGYHÁZI, B. DANEHOLT, and B. LAMBERT. 1968. Agarose-acrylamide composite gels for microfractionation of RNA. *Nature (London).* 220:1037.

35. PAGOULATOS, G. N., and J. E. DARNELL, JR. 1970. Fractionation of heterogeneous nuclear RNA: rates of hybridization and chromosomal distribution of reiterated sequences. *J. Mol. Biol.* 54:517.

36. KEDES, L. H., and M. L. BIRNSTIEL. 1971. Reiteration and clustering of DNA sequences complementary to histone messenger RNA. *Nature (New Biology) (London).* 230:165.