CYTOLOCALIZATION OF RIBOSOMAL CISTRONS
IN PLANT POLYTENE CHROMOSOMES

TOM BRADY and MARY E. CLUTTER. From the Department of Biology, Yale University, New Haven, Connecticut 06520

INTRODUCTION

In both plants and animals the mechanism of ribosomal RNA synthesis is thought to be the same, yet genetic and biochemical evidence points to a dispersed origin along chromosomal DNA in plants (1, 2, 3) and a strict localization of genes coding for ribosomal RNA in the nucleolar organizer regions of chromosomes in animals (4, 5). A precise means of localizing ribosomal cistrons has been made available through cytological molecular hybridization of radioactive rRNA to its complementary DNA in situ (6, 7, 8). This method has been used to show that in several species of insects rDNA is located in the nucleolar organizer regions of chromosomes and that the rDNA extends into the nucleolus proper. We have now used this method to investigate whether ribosomal cistrons in two
species of plants *(Phaseolus coccineus* and *P. vulgaris)* are located only in the nucleolar organizer system as in animals or whether they are widely distributed through the genome.

In the embryonic suspensor of beans of the genus *Phaseolus*, cells contain polytene chromosomes with a ploidy level as high as 4096 (9). There are 22 unsynapsed giant chromosomes per suspensor cell and a single nucleolus attached to heterochromatic regions of two pairs of chromosomes. The chromosome morphology (9), the duration of postmitotic DNA replication (10), and RNA synthesis (11) have been described.

**MATERIALS AND METHODS**

**Preparation of rRNA**

Hypocotyls excised from aseptic 20 hr light-germinated seeds of *P. vulgaris* were incubated in White's medium (12) containing 0.90 mCi/ml of uridine-5-H (SA 29 Ci/micole) for 48 hr at 23°C in white light. Radioactive rRNA was extracted from 0.25 g of hypocotyl tissue by the phenol method of Loening and Ingle (13). DNA was removed by incubation in DNase (400 #g/ml) at 37°C for 1 hr. The RNA was further deproteinized with chloroform and isooamyl alcohol (24:1) and the nucleotides and DNA frag-

![Image](https://example.com/image.jpg)

**Figure 1a** Light micrograph. Feulgen-stained squash preparation of a *P. coccineus* suspensor cell showing all 22 chromosomes. The centromeres and other heterochromatic areas are heavily stained. Nucleolar organizer chromosomes (nc) and intranucleolar chromatin (c) are labeled. X 380.

**Figure 1b** Phase micrograph. Azure B-stained radioautograph of a *P. coccineus* suspensor cell squash preparation after hybridization of the denatured DNA with RNA-H. Silver grains are localized over the organizer regions of four chromosomes. In this figure, only the heavily stained heterochromatic areas are visible. Exposure, 15 days. X 800.
ments were removed with a Sephadex G25 column. The RNA was fractionated on a 5-20% sucrose gradient and the 18 S and 25 S RNA's were pooled. The pooled ribosomal RNA's had a specific activity of 175,000 cpm/µg as determined by scintillation counting of RNA on filter paper disks. Unquenched samples of uridine-3H of equal activity had a counting efficiency of 4.3%. Therefore, the rRNA-3H had a minimum of 3.6 X 10^6 dpm/btg.

Cytological Procedures

Suspensors isolated from seeds and fixed in 3:1 ethanol-acetic acid were squashed, or were dehydrated, embedded in paraffin, and sectioned. Some preparations were stained for DNA with the Feulgen reaction (14), and others were stained metachromatically with azure B at pH 3.6 (15).

Preparations used for cytological hybridization were treated with pancreatic RNase (100 µg/ml in 2 X SSC) for 2 hr, then denatured with 0.07 N NaOH for 2 min at room temperature. Cytological hybridization was carried out according to a modification of the method of Pardue et al. (6), by placing 100 µl of 6 X SSC containing the radioactive rRNA at a concentration of 1.5 µg/ml on each slide and incubating for 12 hr at 66°C. After hybridization the slides were washed in 2 X SSC, treated with pancreatic RNase (20 µg/ml in 2 X SSC) for 1 hr at 37°C, again washed several times, and dehydrated. Dried slides were dipped in Kodak NTB-2 liquid emulsion diluted 1:1 with double-distilled water and were allowed to expose in light-tight boxes at 4°C. Slides were developed in Kodak Ektaflo Developer Type I for 2 min, rinsed in Kodak Ektalflo Stop Bath (1/4 strength), and fixed for 5 min in Kodak Ektalflo Fixer, all at 15°C. After a 15 min wash in running distilled water, slides were stained with azure B at room temperature for 1–2 min and made permanent.

RESULTS AND DISCUSSION

The 22 giant chromosomes of a postmitotic P. coccineus suspensor cell are illustrated in Fig. 1 a. The four nucleolar organizer chromosomes are identified in the figure. Feulgen-positive chromatin strands which penetrate the nucleolus extend from the secondary constrictions of two of the organizer chromosomes (9); similar strands in the other two chromosomes are out of the focal plane. The results of cytological hybridization of rRNA-3H with DNA of the nucleolus-associated chromatin are illustrated in Fig. 1 b. The squash is stained with azure B, and only the densely stained heterochromatic regions of each chromosome are visible here. Silver grains occur in localized groups over the heterochromatic areas of four chromosomes.

These chromosomes are attached to the nucleolus which is not visible in the figure. There are no silver grains over other areas of any of the chromosomes.

The relationship of the nucleolus to the nucleolar organizer chromosomes is shown more clearly in Fig. 2, an azure B-stained squash. Three of the four organizer chromosomes are attached to the nucleolus, the fourth having broken off during preparation. After rRNA-DNA hybridization, silver grains are distributed over the surface of the nucleolus above chromatin strands, thereby demonstrating the presence of rDNA in the nucleolus proper (Figs. 3 a, 3 b). No other regions in the nucleus were labeled, and diploid cells of the embryos were not labeled. Similar results were obtained with serial sections of P. coccineus suspensors.
as well as with squashes of the giant chromosomes of *P. vulgaris* suspensors.

Our observations on both serial sections and squash preparations show that the loci for rDNA are the nucleolar organizer regions of the two pairs of chromosomes attached to the nucleolus. After exposure periods of 7-95 days, silver grains occur only over these regions. This evidence is similar to that found in insects (6) and suggests that sites of synthesis for rRNA are the same in plants and animals. This does not completely exclude the possibility that some ribosomal cistrons may exist in other parts of the chromosomes. The rDNA of diploid cells did not hybridize in our experiments; therefore, it is possible that any unreplicated rDNA in the giant chromosomes would not be detected by this method.

Recently, Avanzi et al. (16) have hybridized rRNA-\(^3\)H from *Vicia faba* (100,000 dpm/\(\mu\)g) to heat-denatured DNA of polytene chromosomes of *P. coccineus* suspensor sections on filters. After exposing radioautographs of their preparations for up to 90 days, they found silver grains widely distributed over chromosomes and concluded that this indicated a wide distribution of ribosomal cistrons throughout the genome. Our data give no indication of a dispersed origin for ribosomal cistrons. It is possible that the difference between our results and theirs lies in the rRNA used.

Avanzi et al. (16) used RNA derived from plants of a different genus from the material used for the annealing. Ours was derived from plants of the same genus and for part of the experiment the same species. Previous work has demonstrated that bean chloroplast rRNA appears as a discrete band in acrylamide gels (13), or shoulder in sucrose gradients (17), on the light side of each of the cytoplasmic rRNA fractions, 25 S and 18 S. This

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**Figure 3** Radioautograph of a *P. coccineus* suspensor cell squash preparation after rRNA-\(^3\)H-rDNA hybridization. Silver grains are distributed over the heterochromatic attachment regions of two chromosomes (arrows) and over the numerous chromatin strands which penetrate the nucleolus. Exposure, 25 days. Fig. 3 a. Light micrograph. Heterochromatic regions of the chromosomes are heavily stained with azure B. The nucleolus treated with RNase does not bind the dye. Fig. 3 b. Phase micrograph of the same area to show a fragment of the nucleolus, a highly lobed structure in *Phaseolus*. The chromosome at the lower left broke away from the nucleolus during the squashing procedure. \(\times 800\).
organellar rRNA constitutes from 20-30% (13) to 86% (10) of the total RNA in light-grown plants. We were careful to remove this contaminant by always using cuts made on the heavy side of the rRNA peaks as shown in Fig. 4. Avanzl et al. (16) did not indicate that they attempted to remove this component from the RNA that they used for hybridization.

At certain times during development of Phaseolus suspensor cells nucleolus-like bodies occur, and they have been thought to originate at numerous sites on the chromosomes (19). The absence of rRNA-rDNA hybridization except at the organizers and the presence of most of the nucleolus-like bodies near the organizer regions suggests that these bodies originate at these sites only. We have found no concentration of silver grains over these bodies. They, therefore, differ from the micronucleoli of Scara and Rhynchosciara many of which contain material which hybridizes with rRNA (6). We conclude that the ribosomal cistrons of Phaseolus are located in the nucleolar organizer regions and that hybridization of rRNA with intranucleolar chromatin is evidence for an extensive organizer network extending from the nucleolar organizer region of the chromosome into the nucleolus. That we find no evidence of hybridization in the nucleolus-like bodies may simply mean that they do not contain hybridizable rDNA. Thus, it appears that previous conclusions about the location of the synthesis of nucleolar material in plant cells are in error; and there is strong evidence based on in situ hybridization that ribosomal cistrons in both plants and animals are localized within the nucleolar organizer regions of chromosomes and that, at least in some species, this region extends into the nucleolus.

SUMMARY

rRNA-3H (3.6 x 10^6 dpm/µg) extracted from Phaseolus vulgaris hypocotyls was hybridized in situ to its complementary DNA in the polytene chromosomes of the beans Phaseolus vulgaris and P. coccineus. Radioautographs revealed that ribosomal cistrons are localized in the nucleolar organizer regions of four of the giant chromosomes.

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