

EXPERIMENTAL ANALYSIS OF NUCLEOLAR REORGANIZATION

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INTRODUCTION

The transition from telophase to the G_1 period of interphase includes the process of nucleolar reorganization in each of the daughter nuclei, and it has been demonstrated by the classic studies of Heitz (13) and McClintock (17) that only specific regions of particular chromosomes in each karyotype are involved in the formation of new nucleoli. On the other hand, a number of studies revealed the appearance in telophase of material among the chromosomes whose role in the reorganization of the nucleolus is controversial (27, 5, 20).

Biochemical studies carried out on cells in transition from telophase to G_1 have shown that protein and RNA syntheses, which are only slight in the middle stages of mitosis, are rapidly intensified towards the end of telophase, precisely during

the period of nucleolar reorganization (2-4, 21). In line with this finding, it is thought that mitosis plays a role in realigning the transcriptional pattern of the cell cycle.

It was therefore considered that it would be of interest to study nucleolar reorganization under inhibition of either protein or RNA synthesis in order to determine the processes on which nucleologenesis depends.

This was accomplished in *Allium*, where the whole set of ribosomal cistrons are clustered in the nucleolar organizer region (NOR) of one pair of homologous chromosomes, and these give rise to just one pair of nucleoli in the interphasic nuclei.

Recent reports on nucleologenesis (25, 19) were done on different cells where this pattern of nucleolar formation is not followed at all. Unfortu-

nately, the normal karyotypic condition in these cells is unknown.

MATERIALS AND METHODS

The material used was the root meristem of *Allium cepa* L. bulbs. The roots were grown in the dark at $15 \pm 0.5^\circ\text{C}$, in cylindrical glass receptacles, in either tap water which was renewed every 24 h or the treatment solutions, and always with constant air bubbling.

Labeling with Caffeine

0.1% caffeine solution for 1 h was used to label as binucleate those cells that were undergoing cytokinesis since it is known that caffeine selectively inhibits this process on telophasic cells, produces a binucleate population which initiates the interphase at this moment (11), and goes through the whole cell cycle synchronously (10, 12).

Evaluation of Protein Inhibition

Different bulbs were incubated with 0.02 $\mu\text{Ci/ml}$ (10^{-4} M) [^{14}C]leucine (with a specific activity of 344 $\mu\text{Ci/mmol}$). They were considered as control bulbs, while others were incubated for labeling in the same way in solutions containing cycloheximide or anisomycin at different concentrations (10, 5, 1, 0.5, and 0.1 $\mu\text{g/ml}$). 50–60 root tips were removed at the end of 3 h and homogenized with bacteriological grade alumina. At the end of the 6-h period, 50–60 more roots were removed and the same procedure was followed. Each extract was diluted with 0.05 M Tris-hydroxymethyl-amino-methane buffer at pH 7.5, and finally centrifuged for 15 min at 15,000 g in order to pellet the alumina.

The protein content (milligram per milliliter) was determined in an aliquot of the supernate, according to Lowry et al. (16).

Another aliquot was treated for 10 min with 20% trichloroacetic acid plus 0.1 M leucine at room temperature. Afterwards, it was heated for 15 min at $85^\circ\text{--}90^\circ\text{C}$. The extract was then filtered (through a Whatman GF/50 filter), washed with 5% trichloroacetic acid plus 40 mM leucine, and assayed in a gas-flow counter for incorporated radioactivity (counts per minute). The concentration (milligrams) of the [^{14}C]leucine-labeled protein (counts per minute) was also determined.

Anisomycin and cycloheximide (1 $\mu\text{g/ml}$ in each case) showed inhibition of leucine incorporation greater than 85% compared with the controls, after 3 h of treatment.

Choice of RNA Inhibitors Concentration

Cordycepin and ethidium bromide were first evaluated by their cytological action on nucleolar

morphology, since there is a close correlation between inhibition of nucleolar RNA synthesis and nucleolar segregation (23). 10^{-4} M cordycepin and 100 $\mu\text{g/ml}$ ethidium bromide were the minimal concentrations of the drugs producing segregation of nucleolar components in our material.

1, 10, 50, and 100 $\mu\text{g/ml}$ of α -amanitin were tested by measuring the lengthening of the interphase in the synchronous binucleate cell population induced by 1 h of caffeine treatment. The time when the first biprophases appeared was recorded in control and treated cells. 10 $\mu\text{g/ml}$ protracted their interphase 1.5 times, whereas 1 $\mu\text{g/ml}$ did not show any action, and 50 and 100 $\mu\text{g/ml}$ protracted the interphase more than 2.4 times.

Cytological Techniques

For studying nucleolar phases the roots were fixed and silver was impregnated according to the technique of Fernández-Gómez et al. (9).

For studying chromosome phases the orcein and squash technique was used (28).

RESULTS

Fig. 1 *a* and *b* are two different focal planes of the same telophase cell in which the prenucleolar bodies appear as silver-impregnated dots following the path of the chromosomes. Incipient nucleoli (arrows) are beginning to reorganize in symmetrical parts of the fresh sister nuclei in the nucleolar organizer regions of each chromosomal complement.

In *Allium*, the normal nuclear condition in interphasic diploid cells is the existence of a pair of nucleoli (Fig. 1 *c*) which may fuse into a single larger nucleolus later in the interphase. Areas of lesser and greater silver affinity appear intermingled in these nucleoli.

Analysis of Nucleolar Reorganization in a Binucleate Population

Caffeine acts at a moment in the cycle, cytokinesis, which coincides with the last part of telophase when nucleolar reorganization also begins to take place. Hence it was considered very convenient to investigate the effects of different inhibitors on the reorganization of the nucleolus in a naturally synchronous cell population labeled as binucleate. The general scheme was:

caffeine for 1 h \rightarrow water (in control)
caffeine + drug for 1 h \rightarrow drug (in the treatments).

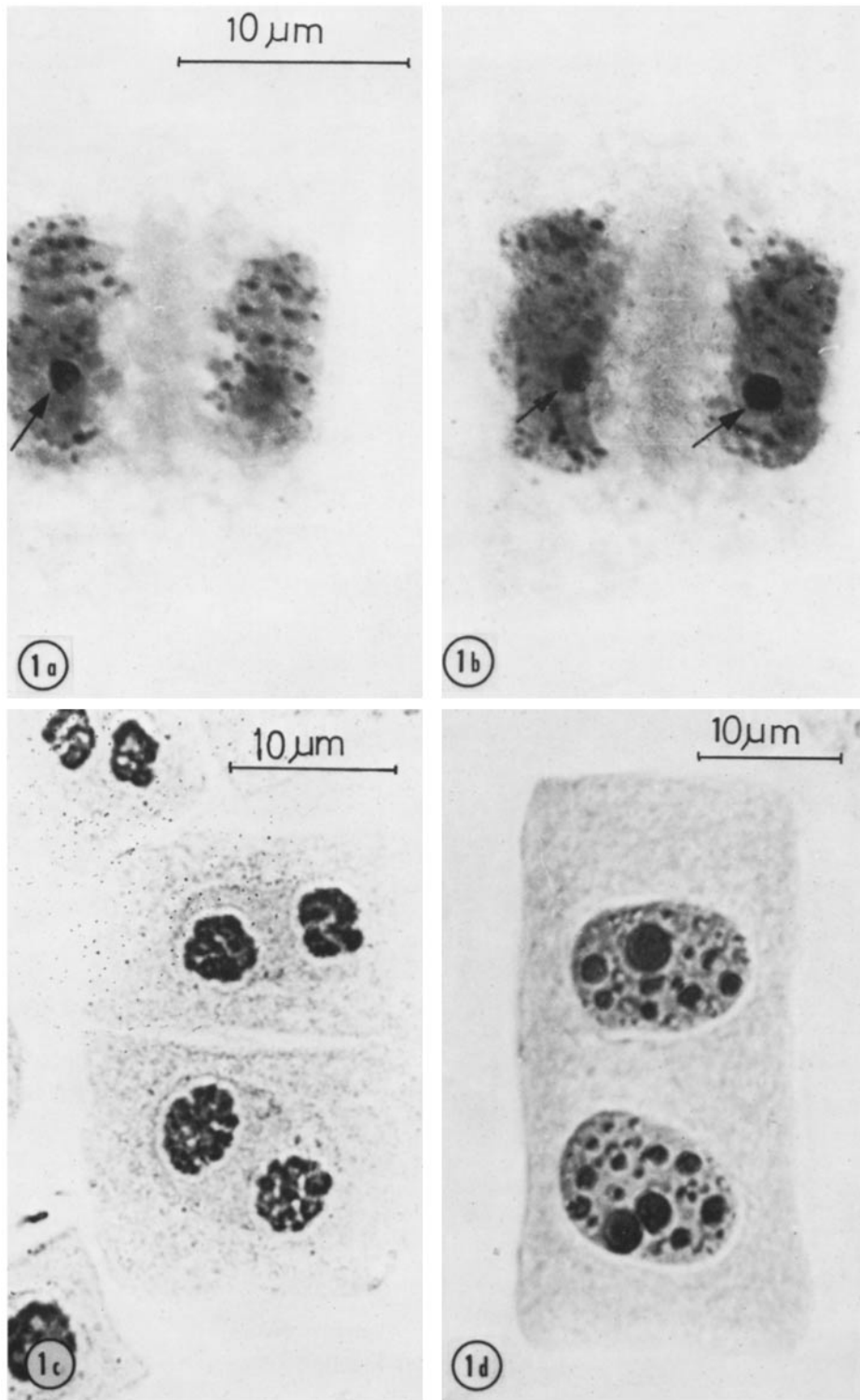


FIGURE 1 Silver impregnation of nucleolar material in untreated meristems. *a* and *b* show different focal planes of the same telophase. The prenucleolar bodies appear as following the path of chromosomes, and incipient nucleoli (arrow) are visible. $\times 3,250$. *c* shows untreated interphase cells. The argyrophilic material is confined to the fully organized nucleoli. Nucleolar components of greater and lesser silver affinity are found intermingled in these nucleoli. $\times 2,350$. *d* shows cells with persistent prenucleolar bodies 12 h in ethidium bromide. $\times 2,050$.

TABLE I
Percent Binucleate Cells with Fully Organized Nucleoli*

Timing (h)	1	2	3	4	5	7	10	12
Control	2.4	34.5	63.3	86.2	99.2	100	100	100
Inhibitors of RNA synthesis								
Ethidium bromide	0	0	0	0	0	0	1.4	0.4
Cordycepin	0	0	0	0	0	0	0	0.7
Inhibitor of extranucleolar RNA polymerase, α -amanitin	0	10.0	48.1	78.2	95.2	100	100	100
Inhibition of protein synthesis								
Cycloheximide	53.0	79.1	82.0	96.8	100	100	100	100
Anisomycin	40.5	73.7	94.1	100	100	100	100	100

* Each figure represents the mean value calculated in above 600 binucleate cells.

The drugs used were cordycepin and ethidium bromide as inhibitors of RNA synthesis (22, 18), α -amanitin as an inhibitor of extranucleolar polymerase (14), and cycloheximide and anisomycin as inhibitors of protein synthesis.

The results of the action of the different drugs tested on nucleolar reorganization are given and compared with the controls in Table I.

Study of this table shows the kinetics of nucleolar reorganization in binucleate cells labeled as binucleate when they are passing through telophase. We see:

(a) 5 h after the end of the 1-h caffeine treatment all binucleate cells in control bulbs have their nucleoli fully organized, 50% of the cells having finished within 2.6 h. From the 1 h, these cells showed the presence either of prenucleolar bodies only, of both prenucleolar bodies and incipient nucleoli, or of fully organized nucleoli.

(b) The blocking of the nucleolar reorganization process induced by inhibitors of RNA synthesis. Even 12 h after caffeine treatment, more than 99% of the binucleate cells remain with scattered prenucleolar bodies (Fig. 1 d). However, coalescence of some prenucleolar bodies seems to occur since there are larger prenucleolar bodies than in the controls (compare Fig. 1 d with control in Fig. 1 a, b).

(c) Delay of nucleolar reorganization during the first 2 h of treatment with α -amanitin, followed by recovery of the normal values in the subsequent 2 h, since 5 h after the 1-h caffeine treatment all binucleate cells have fully organized nucleoli.

(d) A speeding up of nucleolar reorganization when simultaneous protein synthesis is inhibited since more than 90% of the binucleate cells have

organized their nucleoli within 3 h after caffeine treatment.

The appearance of prenucleolar bodies under all treatments was normal in morphology and timing compared to controls even when inhibitors were used for 1 h before caffeine and drug in a parallel experiment.

DISCUSSION

Silver impregnation, the technique used here (9), contrasts a proteinic nucleolar component that concentrates preferentially in the fibrous part of the nucleolus as shown by parallel cytochemical and ultrastructural studies in normal and experimental segregated nucleoli (8).

Our experiment shows that the prenucleolar bodies are not immediate products of the transcriptional activity of any part of the postmetaphasic chromosomes. They appear independently of any simultaneous protein synthesis as well. They could represent the remnants of the prophase nucleoli, for Fan and Penman (7) showed that there are particles containing 45 and 32 S preribosomal RNA associated with the metaphase chromosomes, and that they only differ from the nucleolar 45 S component in their unexpected stability under RNA synthesis inhibitors.

Are the Prenucleolar Bodies Incorporated in the Reorganization Nucleolus?

Chouinard (1), in a study of the nucleolus during mitosis in *Allium cepa*, states that the only structural component left of the disaggregated nucleolus at late prophase is the nucleolar organizing region of the nucleolar chromosome. On

the other hand, only circumstantial evidence of the incorporation of prenucleolar bodies into the nucleolus in formation has been accumulated till now. These proofs are (a) the vanishing of prenucleolar bodies during nucleogenesis, (b) the cytochemical and ultrastructural similarities between prenucleolar bodies and nucleoli (26), and (c) the continuity of fibrillogranular material external to the chromosomes with the surface of the growing nucleolus (15).

The perfect timing correlation observed between disappearance of prenucleolar bodies and growing of the nucleoli when nucleolar reorganization was experimentally accelerated or delayed (by protein or RNA synthesis inhibitors, respectively) seems to show conclusively that prenucleolar bodies are truly incorporated into the nucleolus in formation. Phillips' finding that new nucleoli do actually integrate RNA synthesized before mitosis, as well as the proof earlier commented upon transportation of nucleolus-like material on metaphase chromosomes (7), is in line with our results.

Factors Involved in Nucleolar Reorganization

The experimental research in this study has shown that nucleolar reorganization is a process fully dependent on simultaneous RNA synthesis. This is so in our material and most probably is the rule in eukaryotic cells. Phillips (19) found similar results in human tumor cells, but in two other cell lines, under RNA inhibition, there was re-formation of the nucleoli. But the normal karyotypic condition of these cell lines are not sufficiently known to explain such a discrepancy. It well could be that nonclustered ribosomal cistrons lead to the coexistence of numerous and small nucleoli. For this reason, it would be difficult to distinguish between the appearance of nucleolar components on nonribosomal DNA segments and the final tiny nucleoli. This seems to be the case in *Amoeba* where nucleolar material remains dispersed along its whole cell cycle, and nucleolus-like bodies appear when simultaneous RNA synthesis is inhibited (25).

The possibility that a non-nucleolar RNA is produced that is specific for the activation of transcription of ribosomal cistrons was investigated by means of α -amanitin. This seems not to be the case.

This inhibitor slows down the initiation of nucleolar reorganization in the first 2 h of treatment and then speeds up the process subsequently so the normal rate of nucleolar reorganization is attained after the 4th h.

These results agree with those of Jacob et al. (14), who report that the administration of α -amanitin in vivo inhibits the incorporation of [14 C]orotic acid into both nucleolar and nucleoplasmic (extranucleolar) RNA within 1 h. However, the inhibition of ribosomal RNA (rRNA) synthesis in the nucleolus was reversed after 3 h, whereas the synthesis of nonribosomal RNA remained inhibited at this time.

With respect to the role of protein synthesis in nucleogenesis our results showed an increase in the rate of nucleolar reorganization compared with the normal course of the process. Prenucleolar bodies seem to group themselves together to form fully developed nucleoli at almost double the rate at which they do so in untreated cells.

There are contradictory reports on the action of protein synthesis inhibitors on rRNA synthesis. Fakan (6) postulates that this inhibition uncouples 45 S rRNA processing and hence back-inhibits rRNA transcription. Our data could better be explained if protein synthesis inhibition increases rRNA synthesis as has been reported (see Stenram, 24). Direct measurement of nucleolar synthesis and comparison with the nucleolar reorganization rate in our experimental conditions will provide us with a sound knowledge of the function of ribosomal genes.

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