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# Relations between Nucleolar Morphometric Parameters and Pre-rRNA Synthesis in Animal and Plant Cells

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**Key Words.** Nucleolus · Interphase nucleus · Pre-rRNA synthesis · Morphometry · Quantitative autoradiography

**Abstract.** In order to study if there are differences between cells of the same tissue with one and two nucleoli, nuclear and nucleolar volume, density of tritiated uridine incorporation, amount of DNA per nucleus and intensity of cytoplasmic basophilia were measured in mononucleolated and binucleolated rat epithelial endometrial cells, in onion root meristematic cells and in chick embryo matrix cells of the central nervous system, neuroblasts and neurons. No significant differences in nuclear volume, density of tritiated uridine incorporation and amount of DNA per nucleus were found between cells of the same type with diverse numbers of nucleoli. Binucleolated endometrial cells, matrix cells, and root meristematic cells have biphasic distributions of nucleolar volumes. One peak of this distribution roughly coincides with the nucleolar volume of mononucleolated cells, the other peak corresponds almost to double the volume. As the density of uridine incorporation is the same irrespective of the nucleolar number and volume, the cells with larger nucleolar volumes have higher pre-rRNA synthesis. These cells also have higher amounts of ribosomes in the cytoplasm, as revealed by the photometric study of basophilia. It is concluded that in this population of cells the ribosomal production is regulated to a higher steady equilibrium than in the general population. This difference is not due to polyploidism or to the increased DNA content of G2 phase cells. Binucleolated neuroblasts and neurons have nucleolar volumes similar to those of mononucleolated ones.

## Introduction

Classical studies on the nucleolus in different gland cells demonstrated a relationship between intensity of protein synthesis and nucleolar size [*Gabe and Arvy*, 1961]. Correlated microscopical and microchemical experiments on supraoptic neurons established the existence of a direct relation between nucleolar volume and the RNA content in these cells [*Edström and Eichher*, 1958]. Nucleolar volume and intensity of tritiated uridine incorporation were recently studied in experiments of hormonal inhibition and activation of transcription [*Vázquez Nin* et al., 1979]. However, these findings must be related to the variations of the number of nucleoli. Several small nucleoli are formed during telophasic reconstruction [*McClintock*, 1934]. As they progressively associate during the G1 period, their number diminishes and their volume increases [*Anastassova-Kristeva*, 1977]. Nevertheless, the presence of one, two or more nucleoli is a common observation in liver cells and other differentiated cells that are seldom involved in a mitotic cycle. In the present work, quantitative aspects of solitary and multiple nucleoli are studied in several animal and plant cells, in an effort to elucidate the relationships between the number and volume of nucleoli and the physiological states of the cells.

## Material and Methods

The experiments were performed on rat epithelial endometrial cells, matrix cells, neuroblasts and immature neurons of the spinal cord of the chick embryo in different developmental stages, and in onion root meristematic cells. All tissues were fixed in 2.5% glutaraldehyde in 0.15 *M* cacodylate buffer at pH 7.3 for 1 h at room temperature, then dehydrated in gradual concentrations of ethyl alcohol followed by propylene oxide and embedded in glycide ether 100 (Merck). Three methods of contrast were carried out: uranyl acetate-lead citrate for general studies of the cell

**Table 1.** Nuclear volume ($\mu m^3$) of cells with one and two nucleoli

| | $\bar{X}$ | SD | N | T |
|---|---|---|---|---|
| Epithelial endometrial cells | | | | |
| 1 nucleolus | 83 | 66 | 1,226 | 3.65 |
| 2 nucleoli | 104 | 77 | 159 | |
| Root meristematic cells | | | | |
| 1 nucleolus | 507 | 257 | 485 | 1.19 |
| 2 nucleoli | 533 | 201 | 169 | |
| Matrix cells | | | | |
| 1 nucleolus | 51 | 20 | 37 | 0.59 |
| 2 nucleoli | 48 | 18 | 36 | |
| Multipolar neuroblasts | | | | |
| 1 nucleolus | 108 | 37 | 47 | 2.07 |
| 2 nucleoli | 133 | 59 | 30 | |
| 9-day immature neurons | | | | |
| 1 nucleolus | 275 | 81 | 36 | 2.07 |
| 2 nucleoli | 234 | 103 | 46 | |
| 13-day immature neurons | | | | |
| 1 nucleolus | 285 | 140 | 44 | 0.41 |
| 2 nucleoli | 272 | 124 | 33 | |
| 21-day neurons | | | | |
| 1 nucleolus | 383 | 196 | 45 | 2.05 |
| 2 nucleoli | 301 | 149 | 30 | |

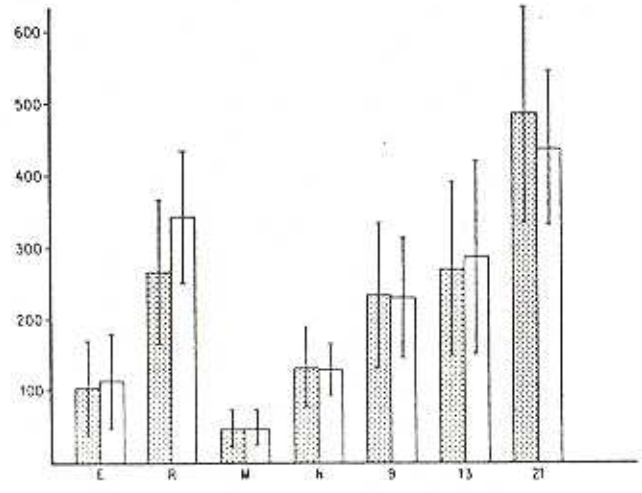


Fig. 1. Nuclear volume of cells with two nucleoli (dotted columns) and with three nucleoli (open columns). Ordinate is calibrated in cubic micrometers. In abscissa, E = endometrial epithelial cells; R = root meristematic cells; M = matrix cells; N = neuroblasts; 9,13 and 21 = neurons of chick embryos of 9, 13, and 21 days of incubation.

structures. *Bernhard's* [1969] uranyl acetate-EDTA-lead citrate for ribonucleoprotein (RNP) particles, and phosphotungstic acid procedure for chromatin [*Vázquez Nin* et al., 1973]. For autoradiography the cells were labelled with 5-[$^3$H]-uridine (New England Nuclear) of specific activity 23 Ci/Mm at 100 $\mu$Ci/ml. Small pieces of endometrium of adult rats in estrus were incubated for labelling, as reported previously [*Echeverría* et al., 1985]. Onion roots were immersed in aqueous solutions of tritiated uridine. Ilford L4 emulsion was applied by the method of the platinum loop [*Haase and Jung*, 1964]. After 3 or 6 months of exposure, the autoradiograms for electron microscopy were developed in a Phenidon (Geigy)-containing solution [*Lettré and Paweletz*, 1966] with gold latensification [*Salpeter and Bachmann*, 1964]. Those autoradiograms intended for light microscopy were developed with D-19 (Kodak) after 1 month of exposure. After development, RNP structures were contrasted for electron microscopy according to *Fakan and Bernhard* [1973]. Toluidine blue-borax was used for general staining in light microscopy. Quantitative analyses were carried out by the hypothetical grain analysis method [*Blackett and Parry*, 1973; *Parry and Blackett*, 1976] using a Hewlett-Packard 9825 microcomputer interfaced with a graphic digitizer and a plotter. The program calculating the hypothetical point sources, the hypothetical grain distribution and comparing it with the real one was developed by us. Pictures were taken at a fixed magnification (8,400 ± 100) in an EM 9S (Carl Zeiss) electron microscope and enlarged to × 28,500. Areas were measured using the graphic digitizer of the microcomputer. Light-microscopic studies were performed on 2-to 8-µm-thick sections of material fixed and embedded for electron microscopy, stained with toluidine blue-borax, the Feulgen method for DNA or with the toluidine blue-molybdate method for ribonucleoprotein [*Pearse*, 1968] and mounted in synthetic resin. Nucleolar diameters were measured on the slices in an Axiomat (Carl Zeiss) provided with an internal graticule at × 3,200 or in a standard microscope (Carl Zeiss) with an intermediate magnification lens (Optovar) and a micrometric ocular with a vernier at × 1,600. Pictures intended for densitometric measurements were taken at × 800 on Technical Pan film (Kodak) in and Amplival (Carl Zeiss, Jena) photomicroscope using the complementary color for each type of staining. Pictures of nuclei with different numbers of nucleoli were made with the same film. All films with pictures to be compared were developed at the same time in the same solution. The illuminating lamp was powered with regulated current and exposure time was kept constant. Transmittance was measured in an Ortec 4310 scanning densitometer provided with a 20-µm aperture and a paper recorder. Linear records were obtained on the whole image of the cell. However, only the nucleus was measured in Feulgen preparations and only the cytoplasm in those stained with toluidine blue-molybdate. Some preparations were demounted and poststained in order to establish the number of nucleoli in each cell. The area under the curve was measured with a planimeter and the results were expressed in relative, arbitrary units.

## Results

Nuclear volumes of cells with one and two nucleoli are very similar (table 1). In fact, the largest percentage increase in nuclear volume (23%) was found in endometrial epithelial cells with two nucleoli. In all the types of cells studied, the nuclear volume of the few of them with three nucleoli does not differ significantly from those with two (fig. 1). The nucleolar volume of cells with one nucleolus

**Table II.** Volume ($\mu m^3$) of solitary nucleolus and the sum of nucleolar volumes of cells with two nucleoli

| | $\bar{X}$ | SD | N | T |
|---|---|---|---|---|
| Epithelial endometrial cells | | | | |
| 1 nucleolus | 2.5 | 1.9 | 1,907 | 4.57 |
| 2 nucleoli | 3.1 | 1.9 | 257 | |
| Root meristematic cells | | | | |
| 1 nucleolus | 14.2 | 7 | 322 | 7.72 |
| 2 nucleoli | 20.6 | 9 | 116 | |
| Matrix cells | | | | |
| 1 nucleolus | 2.8 | 1.6 | 77 | 3.37 |
| 2 nucleoli | 4.1 | 2.4 | 37 | |
| Multipolar neuroblasts | | | | |
| 1 nucleolus | 4.6 | 2.4 | 47 | 0.20 |
| 2 nucleoli | 4.7 | 1.7 | 39 | |
| 9-day immature neurons | | | | |
| 1 nucleolus | 4.3 | 2.1 | 38 | 0.97 |
| 2 nucleoli | 4.7 | 1.5 | 45 | |
| 13-day immature neurons | | | | |
| 1 nucleolus | 10.2 | 7.9 | 44 | 1.28 |
| 2 nucleoli | 8.3 | 2.8 | 29 | |
| 21-day neurons | | | | |
| 1 nucleolus | 6.7 | 4 | 35 | 0.17 |
| 2 nucleoli | 6.5 | 5.3 | 30 | |

is smaller than the sum of the volumes of both nucleoli of those cells that have two, in endometrial cells, in onion root meristematic cells, and in matrix cells, but not in neuroblasts or in neurons (table II). The distributions of nucleolar volumes of cells with one and two nucleoli in the endometrial epithelium (fig. 2, 3), meristematic cells (fig. 4, 5), and matrix cells (fig. 6, 7) demonstrate that there are two populations among binucleolate cells, those with a nucleolar volume similar to that of the cells with one nucleolus, and those with almost the double nucleolar volume. The total nucleolar volume of meristematic cells, neuroblasts and neurons with three nucleoli does not differ from that of cells with two nucleoli (fig. 8).

Comparison of the numerical density of the silver grains per unit area of nucleoli demonstrates no differences between the density of labelling of solitary and multiple nucleoli in endometrial and meristematic root cells (table III).

In order to estimate the amount of ribosomes, the intensity of the toluidine blue staining at pH 3 was measured on the cytoplasm of root meristematic cells with one and two nucleoli. The cells with two nucleoli have significantly more cytoplasmic basophilia than those with only one (table IV).

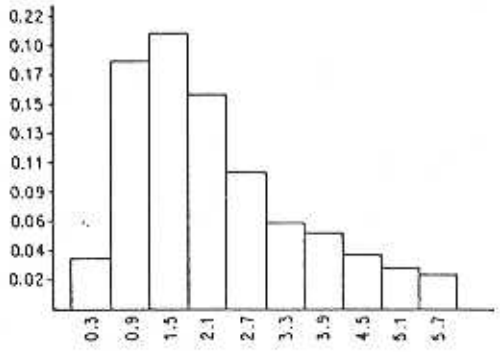


**Fig. 2.** Distribution of the nucleolar volumes of the endometrial epithelial cells with one nucleolus. The ordinate shows the relative frequency. The abscissa shows the mid-points of each class in cubic micrometers.

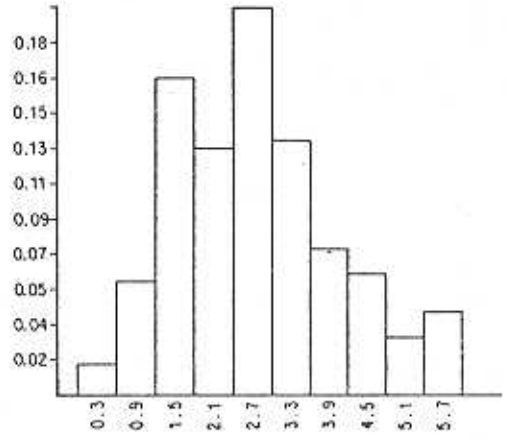


**Fig. 3.** Distribution of the total nucleolar volumes of endometrial epithelial cells with two nucleoli. Ordinate and abscissa as in figure 2.

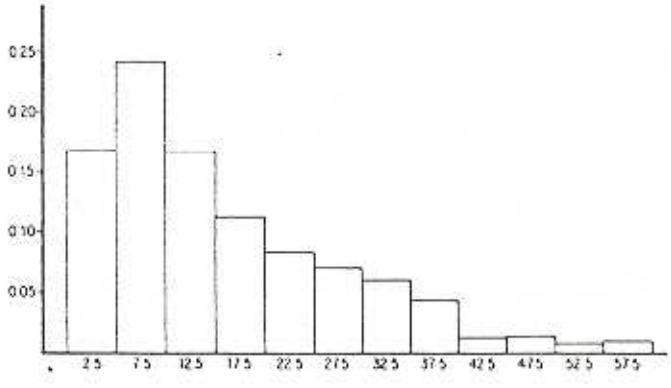


**Fig. 4.** Distribution of nucleolar volumes of root meristematic cells with one nucleolus. Ordinate and abscissa as figure 2.

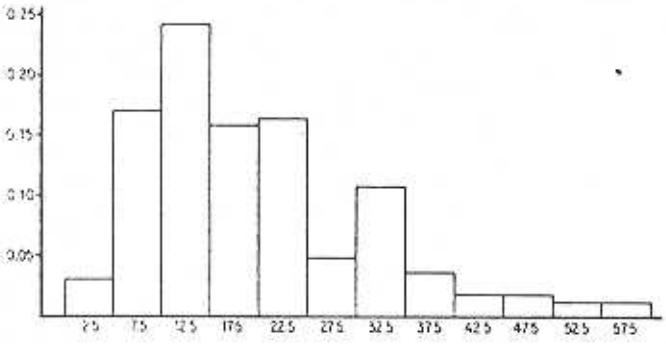


Fig. 5. Distribution of total nucleolar volumes of root meristematic cells with two nucleoli. Ordinate and abscissa as in figure 2.

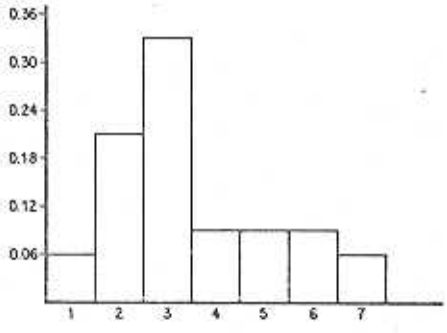


Fig. 6. Distribution of nucleolar volumes of matrix cells of the central nervous system with one nucleolus. Ordinate and abscissa as in figure 2.

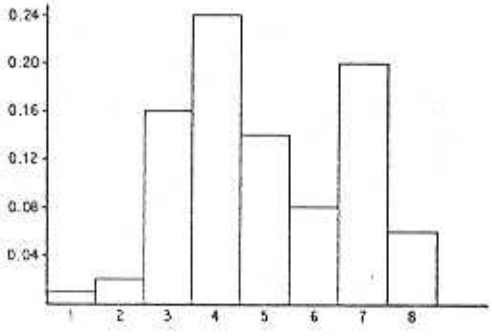


Fig. 7. Distribution of total nucleolar volumes of matrix cells of the central nervous system with two nucleoli. Ordinate and abscissa as in figure 2.

Table III. Incorporation of tritiated uridine in nucleoli: number of silver grains per unit area of nucleolus over the background

| | $\bar{X}$ | SD | N | T |
|---|---|---|---|---|
| Epithelial endometrial cells | | | | |
| 1 nucleolus | 2.27 | 1.48 | 56 | 0.27 |
| 2 nucleoli | 2.14 | 1.44 | 21 | |
| Root meristematic cells | | | | |
| 1 nucleolus | 24.53 | 11.44 | 34 | 0.74 |
| 2 nucleoli | 20.82 | 7.47 | 34 | |

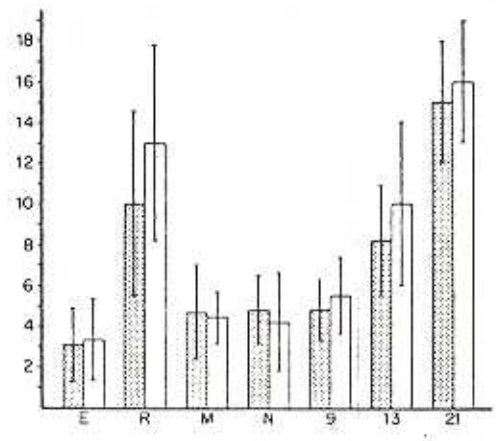


Fig. 8. Nucleolar volumes of cells with two nucleoli (dotted columns) and with three nucleoli (open columns). Ordinate and abscissa as in figure 1.

Table IV. Cytoplasmic basophilia of onion root meristematic cells with one and two nucleoli: arbitrary units of the area under the part of the transmittance curve over the background

| | $\bar{X}$ | SD | N | T |
|---|---|---|---|---|
| Cells with 1 nucleolus | 0.157 | 0.069 | 28 | 2.943 |
| Cells with 2 nucleoli | 0.229 | 0.103 | 27 | |

Table V. Comparative measurements of DNA per nucleus in Feulgen-stained preparations: arbitrary units of the part of the area of the transmittance curve over the background

| | $\bar{X}$ | SD | N | T |
|---|---|---|---|---|
| Endometrial epithelium | | | | |
| 1 nucleolus | 21 | 6 | 13 | 0.29 |
| 2 nucleoli | 22 | 7 | 13 | |
| Root meristem | | | | |
| 1 nucleolus | 10 | 3.8 | 17 | 0.45 |
| 2 nucleoli | 9.5 | 4.5 | 17 | |
| Matrix cells | | | | |
| 1 nucleolus | 168 | 68 | 21 | 1.389 |
| 2 nucleoli | 145 | 28 | 14 | |
| Multipolar neuroblasts | | | | |
| 1 nucleolus | 162 | 28 | 28 | 0.704 |
| 2 nucleoli | 163 | 39 | 16 | |

The estimation of the DNA per nucleus in cells with one and two nucleoli shows no difference between them (table V).

## Discussion

A very important fact in the interpretation of the results is the absence of differences in the density of incorporation of tritiated uridine between solitary and multiple nucleoli with diverse nucleolar volumes. The changes in the numerical density of the silver grains per unit area in pictures at the same magnification obtained from sections of the same thickness, are the correct estimations of the changes of the density of labelling per unit volume [*Underwood*, 1970]. Thus, there is a direct relation between the total nucleolar volume and the total synthesis of pre-rRNA in each cell type. Tritiated uridine incorporation has been found distributed all over the fibrillar zone of the nucleolus [*Granboulan and Granboulan*, 1965; *Mirre and Stahl*, 1981]. In nucleoli with mixed granular and fibrillar components, as those of the cells studied in the present work, the nucleolar organizer is dispersed in the nucleolar volume, as the extended chromatin found inside the nucleolus by the PTA method [*Vázquez Nin* et al., 1973]. Furthermore, studies of the changes in nucleolar volume during experimental modifications of the rate of tritiated uridine incorporation demonstrated that they vary in parallel, although the changes in volume lag some hours behind the alterations of incorporation [*Vázquez Nin* et al., 1978, 1979]. This retardation may be explained by the time required to increase the content in RNA and protein of the nucleolus to such an extent as to make the augmentation of volume measurable. This is supported by the finding that the volume of the nucleolus is proportional to its content of RNA and protein [*Lin*, 1955]. However, the number of nucleoli per cell was seldom taken into account. The sum of the individual nucleolar volumes of every nucleolus in each cell was analyzed in erythroblasts in different stages of maturation, and correlates well with the changes in RNA synthesis during the differentiation of these cells [*Sasaki* et al., 1983].

Binucleolated cells in endometrial epithelium, root meristem, and matrix cells of embryonic central nervous system have a biphasic distribution of their total nucleolar volume. This finding indicates that there are two populations of cells with two nucleoli, one with approximatively the same total nucleolar volume as mononucleolated cells and the other with almost double the volume. As synthetic activity and RNA content of the nucleoli are proportional to the total nucleolar volume, there are also two populations of cells with diverse steady equilibriums between the rate of synthesis of pre-rRNA and its migration to the cytoplasm.

The measurements of cytoplasmic basophilia indicate that cells with two nucleoli and larger nucleolar volume than mononucleolated ones have more ribosomes than those with one nucleolus and smaller nucleolar volume. All these results demonstrate not only different states in the regulation of pre-rRNA transcription in cells of the same tissue, in the same specimen, but also differences in the amount of the complete final product, the ribosome. The tissues with binucleolate cells with larger nucleolar volumes and higher uridine incorporation have cells committed to the mitotic cycle. On the contrary, neuroblasts and neurons, which are cells in indefinite G0, have no differences in nucleolar volume between cells with one and two nucleoli. The higher requirements of ribosomes of the former may be related to the mass increase necessary between mitoses. Although no quantitative estimation of the mitotic index was carried out in the present work, the number of cells in mitosis seems to be very much less than that of cells with two nucleoli and a large nucleolar volume.

There is no clear relation between ploidism or number of nucleolar organizing regions (NOR) on one side, and the rate of pre-rRNA synthesis on the other. In maize, a linear relation was found between the number of NOR and the RNA content of the nucleolus [*Lin*, 1955]. On the contrary, *Mohan and Ritossa* [1970] reported the same rate of synthesis of pre-rRNA in different clones of *Drosophila melanogaster* carring from haploid to tetraploid quantities of NOR. Subsequent works indicate that in this species the rate of pre-rRNA is more related to the number of sex chromosomes than to the quantity of NOR [*Biesdo and Richardson*, 1977; *Kubaneishvili* et al., 1983]. The present study shows that cells with the same ploidism, and in the same tissue of the same specimen, may have significantly different rates of pre-rRNA synthesis. These differences suggest that metabolic requirements may modulate the rate of transcription of the NOR in cells of the same genetic lineage. This modulation of synthesis is accompanied by an increased RNA content in the nucleolus and by an augmentation in the number of cytoplasmic ribosomes.

It is a common observation that very active cells have larger nuclei than inactive ones, and this difference is often related to the decondensation of compact chromatin [*Dupuy-Coin* et al., 1976]. However, a detailed study of the changes of the nuclear volume, decondensation of com-

pact chromatin and increase in the rate of RNA synthesis during neuroblast differentiation, casts some doubts on the existence of a simple relation between these parameters [*Vázquez Nin* et al., 1983]. The present results demonstrate no correlation between the rate of pre-rRNA and the nuclear volume in the cells studied. In spite of the diminution of the number of nucleoli during telophase and during period G1 of the interphase [*Anastassova-Kristeva*, 1971], there are evidences of genetic control of the number of nucleoli in cells in G0, such as mouse liver cells and lymphocytes [*Ivanyi*, 1971; *Flaherty* et al., 1972]. In other studies, the number of nucleoli was related to the transcriptional activity of the cell, such as in different types of erythroblasts [*Sasaki* et al., 1983]. However, the present results indicate that in some cell types there are cells carrying the same number of nucleoli but with different pre-rRNA transcriptional rates. The morphological parameter related to pre-rRNA transcriptional activity is the total nucleolar volume.

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