NUCLEOLAR DEVELOPMENT IN THE INTERPHASE OF THE CELL CYCLE

A. SACRISTÁN-GÁRATE, M. H. NAVARRETE AND C. DE LA TORRE

Departamento de Citología, Instituto de Biología Celular (C.S.I.C.), Veldazquez, 144, Madrid-6, Spain

SUMMARY

Stereology of nucleoli at 3 different points of the cell cycle, in the middle of the G1, S and G2 of interphase, was accomplished in a naturally synchronous cell population rendered binucleate and thus 'labelled' and made identifiable by 1 h caffeine treatment in root meristems of Allium cepa L.

Consistent structural changes were found so that nucleolar parameters by themselves can locate a cell at any interphase period.

The growth of the nucleolus in the course of interphase takes place exclusively in its granular portion. The growth rate of this last component was found to be greater in the first half of interphase (5.4 μm² h⁻¹) than in the second (2.9 μm² h⁻¹), as calculated for a nucleus with only one fused nucleolus. The changes in volume of the nucleolar components during interphase do not parallel the gene dosage.

Nucleolar surface at each point seems to be the factor which determines the growth rate in the preceding interval, since these rates are inverted for fused and unfused nucleoli.

The nucleolar volume occupied by lacunae is minimal in the S-period and shows an enormous increase by the middle of G2.

We think our data may be the structural basis on which a model for nucleolar functioning in proliferating cells could be built.

INTRODUCTION

During the interphase of the cell cycle the molecular composition of a cell changes, implying changes in some of its functions (Baserga, 1968).

We considered that it would be of interest to study the structure of the nucleolus in 3 different functional states of the interphase chromatin, namely the G1, S and G2 periods, since the nucleolus is an intranuclear formation which results primarily from the activity of the structural genes contained in that portion of the chromatin – the nucleolar organizer – where the ribosomal cistrons are clustered.

In addition, the nucleolus itself seemed to be a very appropriate object for this sort of study since it is possible to discern at least 2 different structural components in it, fibrillar and granular, which have been positively correlated with 2 different functions: transcription of the ribosomal cistrons and maturation of the transcription product (La Cour & Crawley, 1965; Bernhard, 1971). Also, it is known that its 2 components are immediately redistributed when inhibition of transcription is induced (Bernhard, 1971), which shows what a responsive structure the nucleolus is.

In this respect, nucleolar morphology by itself indicates the phase of growth in Tetra-
A. Sácrıstán-Gdrate, M. H. Navarrete and C. de la Torre

hymena cultures (Satir & Dirksen, 1971). The fact that the nucleolus has a regulatory role in the expression of other nuclear genes (Harris, Sidebottom, Grace & Bramwell, 1969; Déak, Sidebottom & Harris, 1972) suggests that its functioning in the cell cycle must be strictly programmed.

Considering all this, we evaluated the changes in the structural composition of nucleoli of cells that were situated at 3 different interphase points, corresponding to the middle of the $G_1$, the $S$ and the $G_2$ periods. We used a naturally synchronous cell population labelled as binucleate by 1 h of caffeine treatment (Giménez-Martín, González-Fernández & López-Sáez, 1965) in which the $G_1$, $S$ and $G_2$ timing was precisely determined (González-Fernández, Giménez-Martín & de la Torre, 1971).

Important changes in the absolute rate of increase of the different nucleolar components during interphase were detected. We believe that the present report throws new light on nucleolar physiology in cycling cells, improving upon earlier attempts in which the heterogeneity of the synchronized population obscured the validity of observations for middle and late interphase (Erlandson & De Harven, 1971; Noel, Dewey, Abel & Thompson, 1971).

MATERIAL AND METHODS

The material used was root meristem of Allium cepa L. The onion bulbs were grown in the dark at a constant temperature of 25 ±0.5 °C in cylindrical receptacles in tap water which was renewed every 24 h and aerated continuously by bubbling air at 10–20 ml min−1. The bulbs were placed so that only their bases remained submerged in the water.

Labelling of a synchronous cell population

Caffeine is a drug which selectively inhibits the formation of the cell plate in those cells that are progressing through their telophase during treatment. Treatment with caffeine therefore 'labels' a synchronous population of cells by rendering them binucleate. The roots, still attached to the bulbs, were treated with 0.1 % caffeine in tap water for 1 h.

Under the experimental conditions used, the binucleate cells so obtained have a $G_1$ of 3.5 h, their $S$ period lasts until 9.7 h after caffeine treatment, and $G_2$ ends by 11.9 h after the caffeine treatment (Giménez-Martín et al. 1965; González-Fernández et al. 1971). Hence, roots were fixed at the times corresponding exactly to the middle of each of the 3 interphase periods.

Light microscopy

Preparation of specimens. For measurements on the cytoplasm and the nucleus, roots prepared for electron-microscope observation (see below) were used; 2-μm sections were obtained with an LKB Ultratome.

For measurements on the nucleolus, we used squashes of silver-impregnated meristems (Fernández-Gómez, Stockert, López-Sáez & Giménez-Martín, 1969). This technique is also an electron-microscope staining technique for preferential contrasting of nucleolar protein(s) (Rusueño, Fernández-Gómez & Giménez-Martín, 1973). The preparative scheme was as follows:

1. Fix roots in 1:1 mixture of 10 % formaldehyde and 1 % hydroquinone for 2 h.
2. Wash in distilled water, 10 min.
3. Place in 2 % silver nitrate solution at 65 °C overnight.
4. Wash in distilled water, 10 min.
5. Reduce in the mixture used in step (1) for 2 h.
6. Wash, 10 min.
Nucleolar development in interphase

(7) Place in sodium thiosulphate solution (the photographic fixer, 'Ilfofix') for 1 h.
(8) Wash in distilled water, 10 min, and squash.

We checked that nucleoli did not suffer any appreciable degree of deformation when squashed by comparing the maximum values obtained on sections of roots embedded in Epon with those obtained in squashes. This must be because of the high density of the nucleoli.

Measurement of areas. Sixty nuclei (corresponding to 30 binucleate cells) from each stage were studied in roots fixed at the times corresponding to the middle of \(G_1\), \(S\) and \(G_2\) of the binucleate cell cycle. A Zeiss photomicroscope with a micrometer in the ocular lens was used to measure the area occupied by whole cell, nucleus and nucleoli, under a 100 x objective and using a 2 x Optovar. The diameters were so recorded and the sectional areas estimated.

Estimation of cell, nuclear and nucleolar volumes. In the case of the whole cell, the areas recorded were multiplied by the minimal dimension in order to estimate the volume, as in the root the cell grows by elongation. As the values were obtained in binucleate cells, the total cell volume was divided by 2 in order to get the cell volume corresponding to each nucleus.

The nuclear and nucleolar volumes were estimated by considering either their spherical or ellipsoidal shape in each case.

Electron microscopy

Preparation of specimens. Roots were fixed for 2 h in 3 % glutaraldehyde in Millonig's buffer pH 7.0, and postfixed for 1 h in 2 % osmium tetroxide in the same buffer. The roots were dehydrated in an ethanol series and embedded in Epon 812. Sections 60 nm thick were cut with an LKB Ultratome and contrasted with uranyl acetate in ethanol and lead citrate solution.

Observations were carried out with an EM 300 Philips microscope.

Quantitative electron-microscope techniques. Ten different sections of binucleate cells (roughly corresponding to 30 nucleolar sections) were studied for each of the 3 periods recorded. Weibel (1969) considers that the recording of 6 electron micrographs is good enough for this sort of stereological estimation. The sections were randomly selected from 4 different grids, each from a different root.

Micrographs were taken at final magnifications up to x 30000. They were studied with a planimeter and the areas occupied by total nucleolus and its granular, fibrillar, lacunar and vacuolar areas were recorded. The percentages of nucleolar components, i.e. their volumetric densities, were so estimated. The means and standard deviations of these values were calculated for each of the 3 interphase periods, and finally related to the total nucleolar volume (in \(\mu m^3\)) as estimated under the light microscope.

Observations

Values of cell parameters

In Table 1 we have recorded the mean values and the standard deviations (s.d.) of the estimated volumes for whole cell, nucleus and nucleolus in each of the 3 interphase periods under study.

In the case of nucleoli, we considered 2 different situations independently, namely, nuclei where the 2 nucleoli were found as distinct entities and, on the other hand, those where the 2 nucleoli had fused into one larger nucleolar mass. As can be seen, in the case of the nucleus with 2 unfused nucleoli, the total nucleolar volume per nucleus is always smaller than in nuclei with one fused nucleolus.

We have also estimated the total nucleolar surface per nucleus in the case of both fused and unfused nucleoli (Table 1). Contrasting with the differences in nucleolar volumes found in nuclei where nucleoli were either fused or unfused, the surface area of the nucleolar mass seems to be a constant for each interphase point, as the differences are always smaller than 10 % of the values.
Table 1. Values of cell parameters, means ± S.D.

<table>
<thead>
<tr>
<th></th>
<th>Mid $G_1$</th>
<th>Mid $S$</th>
<th>Mid $G_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume per 1 whole nucleus, $\mu m^3$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell</td>
<td>5599.3 ± 1713.1</td>
<td>8528.2 ± 2260.2</td>
<td>9740.4 ± 2097.1</td>
</tr>
<tr>
<td>Nucleus</td>
<td>502.3 ± 120.5</td>
<td>714.2 ± 162.5</td>
<td>870.4 ± 175.6</td>
</tr>
<tr>
<td>2 unfused nucleoli</td>
<td>85.4 ± 15.6</td>
<td>66.7 ± 18.8</td>
<td>86.4 ± 27.2</td>
</tr>
<tr>
<td>1 fused nucleolus</td>
<td>71.2 ± 23.0</td>
<td>97.5 ± 22.5</td>
<td>111.9 ± 15.6</td>
</tr>
<tr>
<td>Nucleolar surface per nucleus, $\mu m^2$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 unfused nucleoli</td>
<td>83.2 ± 18.1</td>
<td>100.3 ± 18.5</td>
<td>119.1 ± 26.7</td>
</tr>
<tr>
<td>1 fused nucleolus</td>
<td>83.1 ± 17.9</td>
<td>102.4 ± 16.3</td>
<td>112.3 ± 8.8</td>
</tr>
</tbody>
</table>

Fig. 1. Absolute values for nucleolar volume (lower pair of lines) and surface (upper pair) in binucleate cells at mid $G_1$, $S$ and $G_2$. Volume of nucleoli is larger when they are fused in one unique nucleolar mass (F). Two paired, unfused nucleoli (U) is the normal initial situation in onion, where there is a pair of homologous chromosomes with nucleolar organizer. Nucleolar surface is similar at each point whether nucleoli were fused or unfused. Values were estimated on 30 binucleate cells per point (60 nuclei).

We have represented the absolute values of nucleolar volumes and surfaces in the case of nuclei with fused or unfused nucleoli in Fig. 1. The constancy of nucleolar surface for nuclei at each of the 3 interphase points is evident, while volumes are different, though volumes and surfaces grow in a similar parallel fashion in all cases.

Factors of increase for volume and surface

We calculated the rates of volume increase for cell, nucleus and nucleoli as well as for nucleolar surface between the 3 interphase stages considered (Table 2). They show the kinetics of growth rather more clearly than the absolute values in Table 1. Cell and nucleus show a similar growth rate from $G_1$ to $G_2$, though the kinetics of growth
Nucleolar development in interphase

are not identical in the 2 intervals that we have considered (from mid $G_1$ to mid $S$, and from mid $S$ to mid $G_2$).

The growth rates for nucleolar volume and surface in nuclei with fused or unfused nucleoli are seen to be different whichever interval is considered, though the total nucleolar surface per point is roughly constant (Fig. 1).

Table 2. Factors of volume and surface increase

<table>
<thead>
<tr>
<th></th>
<th>Mid $G_1$ to mid $S$</th>
<th>Mid $S$ to mid $G_2$</th>
<th>Mid $G_1$ to mid $G_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Volumes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell</td>
<td>1.52</td>
<td>1.14</td>
<td>1.74</td>
</tr>
<tr>
<td>Nucleus</td>
<td>1.42</td>
<td>1.22</td>
<td>1.73</td>
</tr>
<tr>
<td>2 unfused nucleoli</td>
<td>1.40</td>
<td>1.15</td>
<td>1.57</td>
</tr>
<tr>
<td>1 fused nucleolus</td>
<td>1.32</td>
<td>1.29</td>
<td>1.71</td>
</tr>
<tr>
<td><strong>Surfaces</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 unfused nucleoli</td>
<td>1.20</td>
<td>1.19</td>
<td>1.43</td>
</tr>
<tr>
<td>1 fused nucleolus</td>
<td>1.23</td>
<td>1.10</td>
<td>1.35</td>
</tr>
</tbody>
</table>

Table 3. Growth rate of nucleolar volume and surface

<table>
<thead>
<tr>
<th></th>
<th>Mid $G_1$ to mid $S$</th>
<th>Mid $S$ to mid $G_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Volume, $\mu m^3$ h$^{-1}$</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 fused nucleolus</td>
<td>3.3</td>
<td>3.5</td>
</tr>
<tr>
<td>2 unfused nucleoli</td>
<td>3.3</td>
<td>4.8</td>
</tr>
<tr>
<td><strong>Surface, $\mu m^3$ h$^{-1}$</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 fused nucleolus</td>
<td>3.9</td>
<td>2.4</td>
</tr>
<tr>
<td>2 unfused nucleoli</td>
<td>3.4</td>
<td>4.6</td>
</tr>
</tbody>
</table>

Growth rate of nucleolar volume and surface

As we knew the absolute increase in nucleolar volume and surface, as well as the time intervals between the 3 points under study (4-95 h between mid $G_1$ and mid $S$, and 4-20 h between mid $S$ and mid $G_2$) we were able to estimate the actual rate of growth per hour. The values are shown in Table 3. It can be observed that the rate of growth of nucleolar volume and surface is larger in the first interval considered (mid $G_1$ to mid $S$) for a fused nucleolus. Unfused nucleoli show their larger rate of growth in the second interval (from mid $S$ to mid $G_2$). It is striking that such differences in growth rate result in identical final nucleolar surfaces.

Distribution of nucleolar components

Under the electron microscope, the binucleate cells are also clearly distinguished (Figs. 3, 5). In Figs. 4 and 6, we can see nucleoli with their 4 different components: 8-nm fibrils, 15-nm diameter granules, and light spaces denominated vacuoles or lacunae according to their content (following Chouinard's terminology, 1970).

We quantified the percentages of fibrillar, granular, lacunar and vacuolar areas by
A. Sacristán-Gárate, M. H. Navarrete and C. de la Torre

doing planimetry on electron micrographs of nucleoli at each of the 3 interphase periods. This quantitation is valid since the distribution of the nucleolar components was found to be random (see Fig. 6). $G_2$ nucleoli sometimes presented a preferential location of granular moieties in the nucleolar periphery (see Fig. 4), so that exclusively

Table 4. Distribution of nucleolar components, expressed as percentages of total nucleolus, means ± S.D.

<table>
<thead>
<tr>
<th></th>
<th>Mid $G_1$</th>
<th>Mid $S$</th>
<th>Mid $G_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granules</td>
<td>$34.3 \pm 5.4$</td>
<td>$52.5 \pm 3.8$</td>
<td>$56.4 \pm 6.5$</td>
</tr>
<tr>
<td>Fibrils</td>
<td>$62.1 \pm 4.6$</td>
<td>$46.2 \pm 3.9$</td>
<td>$41.2 \pm 6.5$</td>
</tr>
<tr>
<td>Lacunae</td>
<td>$1.5 \pm 0.2$</td>
<td>$0.2 \pm 0.1$</td>
<td>$1.7 \pm 0.2$</td>
</tr>
<tr>
<td>Vacuoles</td>
<td>$2.1 \pm 0.3$</td>
<td>$1.1 \pm 0.2$</td>
<td>$0.8 \pm 0.1$</td>
</tr>
</tbody>
</table>

Table 5. Derived volumes of nucleolar components

<table>
<thead>
<tr>
<th>Estimated volumes of nucleolar components, calculated for 1 fused nucleolus ($\mu m^3$)</th>
<th>Mid $G_1$</th>
<th>Mid $S$</th>
<th>Mid $G_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrils</td>
<td>$44.2$</td>
<td>$45.0$</td>
<td>$46.1$</td>
</tr>
<tr>
<td>Granules</td>
<td>$24.4$</td>
<td>$51.2$</td>
<td>$63.1$</td>
</tr>
<tr>
<td>Lacunae</td>
<td>$1.1$</td>
<td>$0.2$</td>
<td>$1.9$</td>
</tr>
<tr>
<td>Vacuoles</td>
<td>$1.5$</td>
<td>$1.1$</td>
<td>$0.9$</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Derived growth factors</th>
<th>Mid $G_1$ to mid $S$</th>
<th>Mid $S$ to mid $G_2$</th>
<th>Mid $G_1$ to mid $G_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrils</td>
<td>$1.02$</td>
<td>$1.02$</td>
<td>$1.04$</td>
</tr>
<tr>
<td>Granules</td>
<td>$2.10$</td>
<td>$1.23$</td>
<td>$2.39$</td>
</tr>
<tr>
<td>Lacunae</td>
<td>$0.18$</td>
<td>$0.60$</td>
<td>$1.78$</td>
</tr>
<tr>
<td>Vacuoles</td>
<td>$0.72$</td>
<td>$0.83$</td>
<td>$0.60$</td>
</tr>
</tbody>
</table>

peripheral sections of nucleoli (i.e. minimal areas of intranuclear granular components) were not measured. These percentages ± S.D. are shown in Table 4. These percentual areas can be converted into percentual volumes, since the thickness of the sections used in the electron-microscope work can be considered a constant. Hence, they represent the volumetric density of each nucleolar component in the whole nucleolus (Weibel, 1969), expressed as a percentage.

The most striking fact is that the proportion of granules to fibrils in $G_2$ is the inverse of that found in $G_1$ nucleoli.

Volume of nucleolar components: their factors of increase

In order to estimate the real volume occupied by each of these nucleolar components in an average nucleolus at each interphase point, we referred these percentages to the nucleolar volume found in nuclei where there was only one fused nucleolus. The derived volumes (in $\mu m^3$) of these nucleolar components are recorded in Table 5.
The estimated values of fibrillar, granular and total nucleolar volumes are shown in Fig. 2. We can see how the volume of the fibrillar component remains rather constant, while granular moieties increase by a factor of 2.59 between mid G₁ and mid G₂. This is also apparent when comparing Figs. 4 and 6. G₁ nucleoli (Fig. 4) are mostly fibrillar, while an increase in granular moieties is evident in G₂ nucleoli (Fig. 6).

![Graph showing volume changes](image)

Fig. 2. Volumes of fibrillar (---) and granular (-----) components as well as total nucleolar volume (---) in binucleate cells with 1 large nucleolar mass per nucleus (fused nucleolus), in the 3 interphase periods studied.

<table>
<thead>
<tr>
<th></th>
<th>Mid G₁ to mid S</th>
<th>Mid S to mid G₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrillar part</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>Granular part</td>
<td>5.4</td>
<td>2.9</td>
</tr>
<tr>
<td>Lacunae + vacuoli</td>
<td>0.3</td>
<td>0.4</td>
</tr>
<tr>
<td>Total nucleolus</td>
<td>5.3</td>
<td>3.5</td>
</tr>
</tbody>
</table>

Lacunae and vacuoles are a minority fraction of the nuclear volume (less than 2%). Lacunae occupy a lower nucleolar portion at the middle of the S period, while there is a net increase (by a factor of 9.6) from mid S to mid G₂. The nucleolar volume occupied by vacuoles is larger at mid G₁ than in either of the other 2 interphase periods.

**Growth rate of nucleolar components**

As we knew the volume increase for every nucleolar component as well as the time in hours separating these 3 points, we were able to calculate their rates of growth.
shows that the rate of growth of the granular portion is higher in the first period of interphase (5·4 μm³ h⁻¹) than in the second (2·9 μm³ h⁻¹).

Lacunae and vacuoles as a whole decrease from mid G₁ to mid S as a negative rate of volume increase is found (−0·3 μm³ h⁻¹). This decrease in the volume of the light spaces of nucleoli explains why the volume increase of a whole nucleolus is not as great as might be expected when the volume increase of its granular portion is considered.

DISCUSSION

Caffeine is a drug which in our system selectively inhibits the formation of the cell plate in those cells that are progressing through telophase during treatment. Hence, caffeine can be considered as a cytological label for naturally synchronous cells and not a synchronizer. In this sense, it allows physiological studies in metabolically undisturbed cells to be accomplished, since these binucleate cells progress synchronously through their cycle in a manner similar to mononucleate cells. The parameters of their cycle are precisely determined under our experimental conditions (Giménez-Martín et al. 1965; González-Fernández et al. 1971).

In root meristems, the cell population has a clear-cut division cycle showing a cyclic pattern of replication, mitosis and synthesis of macromolecules, reflected in different cell compositions (Baserga, 1968), which affords good grounds for the use of the term ‘cell development’ in its proper sense. Now, since all development ultimately leads to morphogenesis, stereological studies covering the division cycle may be illuminating in the study of intracellular differentiation.

Consistent changes in nucleoli have been detected at 3 points of interphase separated by a fair distance, i.e. mid G₁, S and G₂ periods, so that the ratio between the fibrous and granular parts of the nucleolus can by itself be used to place a cell in any of the 3 interphase segments.

The standard deviation of the recorded measurements is, however, high. This is partially due to the natural variation between cell parameters in different roots, as well as to experimental errors in measurement. However, we believe this last source of error cancels out when similar parameters of cells in each of the 3 interphase periods are compared.

Nucleolar surface

The cell cycle provides us with a unique opportunity to test whether the larger volume of nucleoli when they are fused depends simply on fusion being an event that occurs late in the life of a cell and is merely a characteristic of mature nucleoli, or whether it has a functional meaning. As we see in Fig. 1, when fusion takes place, whatever the age of the cell may be, the resulting fused nucleolus always shows a larger volume than its unfused mates.

The nucleolar surface, surprisingly, remained constant, whether nucleoli had fused or not, for each of the interphase periods. Since nucleolar function in these synchronous cells must be identical at each interphase stage, whether nucleoli are fused or
Nucleolar development in interphase

unfused, it is valid to conclude that nucleolar surface may be a faithful indicator of nucleolar activity. The rationale for this is completely obscure, since no specific function can be ascribed to the size of the nucleolus/nucleoplasm interface.

Earlier reports in different materials showed this phenomenon, which Barr & Esper (1963) were the first to quantify. We can say that in the cell cycle nucleolar surface seems to be the factor that controls the volume growth rates for fused or unfused nucleoli. Those rates show a completely inverted pattern in both halves of interphase, though they lead to similar final nucleolar surfaces.

Constancy of the nucleolar fibrous components during interphase

In the course of interphase an interesting fact can be observed, namely that the amount of the fibrous component of the nucleolus is roughly constant (45 μm³), while growth of the nucleolus seems to take place exclusively in its granular portion. This essentially agrees with the results obtained by Noel et al. (1971), in hamster cells, though the decrease in granules and nucleolar volume reported by them in G₂ nucleoli seems to correspond better to the state of affairs in preprophasic nucleoli.

That nucleoli in G₁ are mostly fibrillar is consistent with other sorts of studies, for Hyde (1967) established that the cells in the quiescent centre of the root have nucleoli formed almost exclusively by 'pars fibrosa', and Clowes (1968) showed that this cell population was going through a very protracted G₁ period.

Given the 13,300 ribosomal cistrons per telophase nucleus of Allium cepa cells (Ingle & Sinclair, 1972), there will be 3.4 × 10⁻⁷ μm³ of fibrillar component per ribosomal cistron in G₁ nuclei or, expressed another way, about 300 ribosomal genes per μm³ of fibrillar component.

Our results suggest that the number of rDNA genes, however, does not at all determine the size of this fibrous part, for G₂ nucleoli have the same amount of it while they have twice as many ribosomal cistrons. The meaning of the constancy of this nucleolar component is obscure, as is the fact that nucleolar segregation (a lesion correlated with inhibition of nucleolar transcription) is unexpectedly characterized by a final loss of the granular portion of nucleoli, not of the fibrous moieties.

Growth of nucleolar granular moieties during interphase

The studies carried out by Bernhard’s group using high-resolution autoradiography (see Bernhard, 1971) showed that the fibrous part – which is probably where the 45 S RNA is located – is the precursor of the granular part, where the products from the maturing of the rRNA would presumably be.

We were able to estimate that the granular portion of the one large fused nucleolus grew at a rate of 5.4 μm³ h⁻¹ from mid G₁ to mid S, while in the interval between mid S and mid G₂ the growth rate was only 2.9 μm³ h⁻¹. If late replication of ribosomal cistrons were the case in Allium, as in some mammalian cell lines (Giacomoni & Finkel, 1972), the slowing down of the accumulation of granules in the second half of the interphase could be ascribed to physical incompatibility between replication and transcription, although the competition between these 2 gene functions might take place merely at the individual cistron level.
The estimated increase in granular portion (by a factor of 2.59) from $G_1$ to $G_2$ does not show the expected gene dosage, but instead exceeds it. If we bear in mind that the total nucleolar volume (i.e. the sum of the fibrous and granular parts) must be a function of the number of functional cistrons, the transcription rate, the maturation rate (methylation steps), and the rate of ribosomal RNA output (apart from any contribution of material coming from outside the nucleolus), it follows that any change in one of the factors may be masked if the other factors do not remain constant.

A model of nucleolar functioning has yet to be proposed to allow correlation of these morphological changes with other known biochemical events taking place in nucleoli. For instance, it is known that the rate of 18s RNA methylation in a mammalian cell line is roughly doubled by the end of interphase (Enger, Tobey & Saponara, 1968). Even if it were the same in our cells, we could hardly predict what the structural changes would be.

On the other hand, experimental induction of nucleolar enlargement, characterized by an enormous increase in granular moieties, has been positively ascribed to a rise in nucleolar RNA synthesis (Steele & Busch, 1966), paralleled by a decrease in cytoplasmic rRNA (Kleinfeld, 1966). It is reasonable that these findings could be extrapolated to explain our results concerning the increase in granular portions during interphase.

That the nucleolar volume throughout the cell cycle is not a function of the number of nucleolar genes is in accordance with other findings; for instance, Maher & Fox (1973) reported similar lack of correlation among 4 different species of Vicia. We feel that both kinds of results strongly suggest differential involvement of the ribosomal cistrons in transcription, perhaps by the so-called nucleolar organizer competition, which has received recent support from experiments such as those of Krider & Plaut (1972) with Drosophila, where again nucleolar $[^3H]$uridine incorporation was not a function of the number of rDNA genes either.

It seems that in the $G_2$ of meristematic cells either there is transcription of part of the total rDNA genes if all of them are functional, or the transcription rate must be much lower.

Xenopus individuals that are heterozygous for the Oxford mutation provide a good opportunity to study the relationship between number of ribosomal cistrons, nucleolar volume and rRNA production. Barr & Esper (1963) showed that those individuals with half the amount of rDNA copies had nucleoli as large as wild-type individuals, and Brown & Gurdon (1964) reported that both produced similar amounts of rRNA. Redundancy seemed to occur in wild individuals or heterozygous individuals were capable of compensating for the gene losses. Furthermore, nucleolar volume and/or nucleolar surface may reflect the rate of rRNA production.

**Lacunae and vacuoles in nucleoli**

As we see in Table 5, the nucleolar volume occupied by lacunae is minimal in the $S$ period while it rises 9.6 times from $S$ to $G_2$. Chouinard (1970) showed these lacunar spaces to be permanent components, where most of the `pars chromosoma' of inter-
Nucleolar development in interphase

phase nucleoli is found in a central core (see Fig. 4). It is tempting to correlate this lacunar diminution with decondensation of chromatin and its replication during the S period.

Vacuolar areas are rather more frequent in G1 than in the other 2 interphase periods. They decrease slightly during interphase.

Finally, as each interphase period is characterized by structurally different nucleoli, we should like to stress the fact that nucleolar characteristics by themselves can situate a cycling cell in interphase, and we think our data may be the structural basis on which a model for nucleolar functioning in proliferating cells could be built.

This work has been partially supported by the III Plan de Desarrollo, Spain.

One of us (A.S.G.) did this work during tenure of a fellowship given by the Spanish Ministry of Education and Science.

We wish to express our gratitude to Mr E. Blanco, Mrs M. C. Partearroyo and A. Partearroyo as well as to Miss M. L. Martínez for their excellent technical assistance.

REFERENCES


(Received 19 February 1974)

Fig. 3. Binucleate cell at the time corresponding to mid $G_1$. $\times 9380$.

Fig. 4. Nucleolus of a binucleate cell at mid $G_1$. Fibrillar (f) and granular (g) portions are indicated, as well as a lacuna (l) with its dense core and some vacuoles (v). $\times 22700$. 
Nucleolar development in interphase
Fig. 5. Binucleate cell at time corresponding to mid $G_2$. $\times 5700$.

Fig. 6. Nucleolus from a binucleate cell in mid $G_2$. The granular component (g) is seen around fibrillar areas. Lighter electron density than in Fig. 4 is noticed, as well as an increase in the granular portion. f, fibrils; v, vacuole. $\times 22700$. 