Reproduction of the FC/DFC units in nucleoli

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ABSTRACT
The essential structural components of the nucleoli, Fibrillar Centers (FC) and Dense Fibrillar Components (DFC), together compose FC/DFC units, loci of rDNA transcription and early RNA processing. In the present study we followed cell cycle related changes of these units in 2 human sarcoma derived cell lines with stable expression of RFP-PCNA (the sliding clamp protein) and GFP-RPA43 (a subunit of RNA polymerase I, pol I) or GFP-fibrillarin. Correlative light and electron microscopy analysis showed that the pol I and fibrillarin positive nucleolar beads correspond to individual FC/DFC units. In vivo observations showed that at early S phase, when transcriptionally active ribosomal genes were replicated, the number of the units in each cell increased by 60–80%. During that period the units transiently lost pol I, but not fibrillarin. Then, until the end of interphase, number of the units did not change, and their duplication was completed only after the cell division, by mid G1 phase. This peculiar mode of reproduction suggests that a considerable subset of ribosomal genes remain transcriptionally silent from mid S phase to mitosis, but become again active in the postmitotic daughter cells.

KEYWORDS
cell cycle; FC/DFC units; nucleolus; rDNA; replication

Introduction
Nucleoli are formed around clusters of ribosomal genes, called Nucleolus Organizer Regions (NORs), which in mammalian cells are coding for 18S, 5.8S and 28S RNAs of the ribosomal particles. Each cell contains hundreds/thousands of gene repeats, but not all of them are transcribed. The transcriptionally active genes, which in cycling cells represent about a half of the entire rDNA (rDNA), are replicated in early S phase, and replication of the silent genes is postponed until late S phase.14,15

The essential structural components of the nucleoli, Fibrillar Centers (FC) and Dense Fibrillar Components (DFC), together compose FC/DFC units. All RNA polymerase I (pol I) dependent transcription seems to take place within these units, namely in DFC or at the border between DFC and FC. Early pre-rRNA processing mediated by the small-subunit processome, which includes fibrillarin, takes place in DFC. Structures exhibiting pol I transcription activity were visualized by light microscopy in the cells treated with DB (5,6-dichloro-1-β-D-ribofuranosylbenzimidazole), a specific inhibitor of RNA polymerase II. After that treatment, normally compact nucleoli unravel into necklace-like structures positive for rDNA transcription signal, pol I, its transcription factors, or components of early rRNA processing machinery. It is supposed that each bead in these necklaces corresponds to a single FC/DFC unit. Moreover, it is believed that the nucleolar beads correspond to individual transcriptionally active genes. The patterns of in situ hybridization staining for rDNA gene and spacer regions support this view.

Organization of FC/DFC units is still poorly understood. On hypotonically treated spread preparations, actively transcribed rDNA repeats appear as so-called Christmas trees, in which the “tree stem” represents a single DNA fibril, with the transcripts growing from it like the branches. But accommodation of the Christmas trees in the nucleolar compartments remains unclear up to this time. Data of electron microscopy and electron tomography suggest...
that active rRNA genes form coils surrounding FC.\textsuperscript{31} More recent studies using 3C assays,\textsuperscript{32,37-46} reviewed in ref.,\textsuperscript{47} show that the active genes, supposedly localized in the FC/DFC units, form loops. In each of these loops, promoter is joined to terminator through a number of proteins, among which transcription termination factor 1 (TTF-1) and protooncogene c-Myc seem to be particularly important;\textsuperscript{48} both are bound to non-transcribed spacer regions and regulate association of epigenetically activated rDNA genes to the nucleolar matrix.\textsuperscript{39} According to a core-helix model proposed by Denissov et al,\textsuperscript{32} the transcribing pol I complexes driven by actin revolve around the SL1 (selectivity factor 1) containing core, which is situated in FC and serves as an anchor for both promoter and terminator of the rDNA repeat; the nascent rRNAs exit radially into DFC.

Since organization of rDNA transcription centers within nucleoli still remains a subject of speculation, even less is known about re-organization of FC/DFC units in the course of cell cycle, particularly during and after replication. In the cycling cells, sufficient number of active ribosomal genes must be bequeathed upon the next generation. This could be accomplished by immediate restoration of the original chromatin structure on both helices arising in the wake of the replication fork. But in that case, the maternal cell would have excess of active ribosomal genes for a considerable part of interphase. Thus the simple symmetrical reproduction may prove to be unfavourable for cell homeostasis.

In the present study, correlative light and electron microscopy (CLEM) and specially produced cell lines allowed us to visualize FC/DFC units and precisely determine the corresponding stage of the cell cycle in vivo. Following the dynamics of the units in the cell cycle, we discovered a peculiar mode of their reproduction. Namely, the number of FC/DFC units increased in the course of S phase, but only by 60–80%. The duplication was completed in the daughter cells after mitosis.

Methods

Cell culture and cell lines

Human derived HeLa, HT-1080 (human fibrosarcoma), and primary LEP (human embryonic fibroblast, Sevapharm, Czech Republic) cells were cultivated at 37°C in Dulbecco modified Eagle's medium (DMEM, Sigma, #D5546) containing 10% fetal calf serum, 1% glutamine, 0.1% gentamicin, and 0.85g/l NaHCO\textsubscript{3} in standard incubators.

We produced 2 cell lines stably expressing: 1) GFP-RPA43 and RFP-PCNA (Smirnov et al, 2014); 2) GFP-fibrillarin and RFP-PCNA. The plasmid construct for RFP-PCNA was received from the Max Planck Institute for Molecular Cell Biology and Genetics, Dresden. GFP-RPA43 and GFP-fibrillarin vectors were received from Laboratory of Receptor Biology and Gene Expression Bethesda, MD.\textsuperscript{49} The constructs were transfected into HT-1080 cells using Fugene (Qiagen, #E2312), and G418 (GIBCO, #11811031) was used for selection of stable clones with 2-colored fluorescence.

Incorporation of DNA and RNA nucleotides

For labeling of replication and transcription sites, sub-confluent cells were incubated 5 min with 5-ethyl-2'-deoxyuridine (EdU) (Invitrogen, #C10337) at a final concentration of 10 μM and 5-fluorouridine (FU) (Sigma, #F5130) at a concentration of 100 μM. The cells were fixed in 2% formaldehyde freshly prepared from paraformaldehyde, permeabilized with Triton X-100, and processed for FU immunocytochemistry. The replication signal was visualized using EdU Alexa Fluor 647 Imaging Kit (Invitrogen #C10337). Additionally, we used incorporation of Cy3-dUTP and Cy5-dUTP, which were introduced into the cells by means of the scratch procedure.\textsuperscript{50}

Immunocytochemistry

Incorporated FU signal was visualized by a mouse monoclonal anti-BrdU antibody (Sigma, #B8434). Primary antibodies against human rRNA polymerase (pol I) and Upstream Binding Factor (UBF) were kindly provided by Dr. U. Scheer (Biocenter of the University of Wurzburg). We also used polyclonal (rabbit) anti-RPA43 (Thermo Scientific, #PIPA525184). For visualization of fibrillarin in nucleoli, we used antibodies against human fibrillarin or mouse monoclonal fibrillarin (clone 17C12), kindly donated by Kenneth M. Pollard (Scripps Research Institute, La Jolla, CA). Secondary anti-human, anti-rabbit, and anti-mouse antibodies were conjugated with Alexa 488 (Invitrogen, #H10120), Alexa 532 (Invitrogen, #A-11002), Cy3 (Jackson ImmunoResearch Laboratories, #109-165-088, #111-165-003,
Coverslips with the cells were then mounted in Mowiol.

**Light microscopy**

Confocal images were acquired using SP5 (Leica) confocal laser scanning microscope equipped with a 63×/1.4NA oil immersion objective. Live cell imaging was performed with a spinning disc confocal system based on Olympus IX81 microscope equipped with Olympus UPlanSApo 100×/1.4NA oil immersion objective, CSU-X spinning disc module (Yokogawa) and Ixon Ultra EMCCD camera (Andor). For live cell imaging cells were maintained in glass bottom Petri dishes (MatTek) at 37°C and 5% CO2 using a microscope incubator (Okolab).

**Correlative light and electron microscopy (CLEM)**

Cells were grown in glass bottom Petri dishes with grid (MatTek). DIC images and confocal z-stacks in the regions of interest were obtained on the spinning disk microscope. After that, cells were fixed in 2% glutaraldehyde (EMS, #16019) in Soerensen buffer pH 7.3, post-fixed in 2% OsO4(EMS, #19140), dehydrated in a series of ethanol solutions and embedded in epoxy embedding mixture (EMS, #14130). The blocks containing cells were separated from the coverslips after brief submerging in liquid nitrogen. The region of interest was localized by the α-numeric imprint on the surface of the block. Thin sections made on Leica Ultracut UCT ultramicrotome were mounted on formvar/carbon–coated nickel grids, contrasted with lead citrate and uranyl acetate, examined on the transmission electron microscope Morgagni (FEI) at 80kV. Comparing the ultrastructural images with optical sections of the z stacks, we looked for correspondence between GFP-positive nucleolar beads and FC/DFC units which appeared distinctly on the thin sections.

**Software and data analysis**

For measurement of FC/DFC units in 3D confocal images, we developed a MatLab based software. The program identifies each unit by creating a maximum intensity projection of the confocal stack and blurring the projection with a Gaussian filter (σ = 8–10 pixels), thresholding the blurred image with a value obtained by Otsu’s method for automatic threshold selection. After that the optical section whereupon the unit had maximum intensity was identified. The final result contains 3D coordinates of each unit, its size (FWHM), the value of χ², and integral intensities in the spheres with radii 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 pixels respectively. FC/DFC units were counted after deconvolution with Huygens software.

**Results**

**Nucleolar beads correspond to FC/DFC units**

To observe the cell cycle related dynamics of the FC/DFC units in vivo, we used the earlier produced cell line51 based upon human fibrosarcoma (HT1080) with stable expression of RFP-PCNA (the sliding clamp protein) and GFP-RPA43 (a subunit of pol I) (Fig. 1A). Control staining showed that RFP-PCNA in these cells well colocalizes with replication signal (incorporated Cy5-dUTP), and GFP-RPA43 colocalizes not only with anti-pol I antibody signal, but also with the transcription signal (incorporated FU), so that the nucleolar transcription foci correspond to the observed nucleolar beads.51 For another visualization of the nucleolar beads, we produced a cell line with stable expression of RFP-PCNA and GFP-fibrillarin (Fig. 1B). In these cells, RFP-PCNA colocalizes with incorporated Cy5-dUTP (not shown); GFP-fibrillarin colocalizes with anti-fibrillarin and anti-pol I antibody staining (Fig. 1B).

On the thin resin sections of these cells, FC/DFC units appeared as multiple, well defined, and mutually separated structures (Fig. 2). The well contrasted GFP-positive foci represented nucleolar beads (Supplement A). Employing CLEM, we established correspondence between the RPA43-positive nucleolar beads and FC/DFC units (Fig. 3A-F). This enabled us to study dynamics of the units on individual cells in vivo by observing RPA43 and fibrillarin signals on the beads.

CLEM analysis also allowed us to estimate the size of FC/DFC units at different stages of interphase. We measured area occupied by the middle sections of the units and calculated the diameter of the corresponding sphere. Thus we found that the average size of the units did not change significantly from middle G1 through G2 phase. In our estimates, mean value of the
diameter in the cells expressing RFP-PCNA and GFP-RPA43 was 250 ± 43 nm.

**FC/DFC units may lose GFP-RPA43 but not fibrillarin in early S phase**

The stage of cell cycle in our cell lines was determined by RFP-PCNA signal. Particularly, based upon the distinct morphological criteria,\(^\text{51,52}\) we divided S phase into 4 consecutive stages, S1–4 (Fig. 1A, B). Here S1 corresponds to early S phase, when dot-like replication foci are evenly distributed in the nucleoplasm. In S2, the foci preserve the granular aspect, but a fringe of coarse replication foci can be distinguished along the nuclear lamina. In S3, which represents mid S phase, there are conspicuous fringes along the nuclear lamina and around the nucleoli; in the nucleoplasm the foci are large and surrounded by vast spaces devoid of signal. At stage S4, i.e. in the very end of S phase, replication foci are large and sparse, distributed without any particular pattern, and altogether occupy a small part of the nuclear volume.

Short-term observations (up to 20 min with intervals 1 or 2 min) at different stages of interphase, showed that most FC/DFC units preserved a stable expression of pol I and fibrillarin during most part of G1, as well as in mid through late S phase, and G2. But at the stages S1 and S2 some units lost their GFP-RPA43 signal (Fig. 4A, arrows). This loss was probably transient; sometimes the units which have thus vanished seemed to re-appear within a few minutes (Fig. 4A, arrowheads). During the same stages of S phase, we also observed emerging of the new single dots with rapidly increasing intensity of the GFP signal, and splitting of the previously existing foci (Fig. 4B).

In the cell line expressing GFP-fibrillarin, we observed a similar multiplication of the units during S1 and S2 stages of S phase, but the fibrillarin signal never vanished (Fig. 4C).
Thus, FC/DFC units multiply in the course of early S phase; they also may lose pol I (most likely for a short period), but they do not lose fibrillarin.

Mobility of FC/DFC units

In the short-term observations with 1 min intervals between the succeeding confocal z-stacks, we found that nucleoli moved within the nuclei constantly and changed their form. In addition to that, FC/DFC units were also involved in a rather rapid motion (Supplement B). To estimate the speed of this motion, we selected one unit as a point of reference and measured every minute the relative displacement of other 10–20 closely situated units. The measurements were done on the 2D projections of z-stacks. The displacements [calculated as: \( d_i = (\Delta x_i^2 + \Delta y_i^2)^{1/2} \), where “i” relates to i-unit] were averaged and used as estimates of the relative speed of the units movement. The values obtained for 2D projections were multiplied by the factor 1.57 (\( \pi/2 \)) to get the corresponding 3D estimates. Thus defined, the relative speed of the FC/DFC units was about 0.5 \( \mu m/min \) at mid G1 and S4, but it was reduced to about 0.3 \( \mu m/min \) at S1 stage of S phase (Table 1).

Figure 2. FC/DFC units (arrows) in the nucleolus of a cell stably expressing GFP-RPA43. The units are surrounded by the nucleolar granular component (stars). NM – nuclear membrane. Scale bar: 1 \( \mu m \).

Figure 3. CLEM analysis of FC/DFC units. (A, B) Cells on glass-bottom dish supplied with grid; region of interest is framed. (C) The cell selected in (B); a confocal image showing expression of GFP-RPA43 (green) and RFP-PCNA (red). (D) An optical section of the same cell in green channel. (E, F) Ultrathin sections corresponding to the optical section in (D). The marked nucleolar beads in (D) correspond to FC/DFC units in (E) and (F) (framed numbers 1 through 7). Scale bars: 2 \( \mu m \)
Regular changes of FC/DFC units in the course of interphase

Counting the numbers of pol I and fibrillarin positive nucleolar beads in individual cells, we studied reproduction of the FC/DFC units during the cell cycle.

After mitosis, the units appear from the unfolding transcriptionally competent nucleolar organizers (Fig. 5A). It seemed that each NOR was transformed into one nucleolar necklace. This supposition was confirmed in experiments with calyculin A, an inhibitor of protein phosphatases PP1 and PP2A, which causes more or less complete premature chromosome condensation, and reveals NORs as compact structures in interphase cells. Following the effects of calyculin A in living cells, we observed shrinking of the necklaces into individual NORs (Fig. 5B).
Within the early part of G1, assemblies of necklaces formed nucleoli. During that period, the pol I positive dots, which have gradually evolved from NORs and acquired the aspect of FC/DFC units, became smaller and more numerous. Additional 1h long observations in the period between mid G1 and S phase revealed no change in the number of units.

In the long-term observations which covered the entire cell cycle or its large parts, we found that the number of FC/DFC units increased by 60–80% during early S phase, namely, at S1 and S2 (Fig. 6A, B). The number did not change in the course of S3, from S3 to the end of G2, as well as for the most part of G1 phase. Thus the cells entered mitosis when duplication of the units had not been accomplished. In this respect, there was no difference between the cells expressing GFP-RPA43 and GFP-fibrillarin. Remarkably, in the daughter postmitotic cells, during middle and late G1 phase, total number of the FC/DFC units was higher than in the maternal cell at G2. Value of the increase varied between 10 and 20%, which seemed sufficient to compensate the incomplete duplication in early S phase (Fig. 6B).

Intensity of GFP-RPA43 signal on individual units did not change from middle G1 to the end of S phase, and distribution of the intensities had no tendency to asymmetry (Table 2).

**In HeLa and LEP cells, the nucleolar beads are also not completely duplicated in the course of S phase**

To verify our findings on another object, we used HeLa and primary LEP (human embryonic fibroblast, Sevapharm, Czech Republic) cells. HeLa cells were synchronized by mitotic shake. To obtain G1 and S phases, we incubated cells for 2 and 8 h (HeLa) or for 6 and 24 h (LEP) after the shake. Fixation was preceded with a short pulse of EdU (5 min) for precise identification of the stage of cell cycle. Then the cells were fixed with PFA solution and processed for

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**Table 1. Estimated speed of FC/DFC movements.**

| Cell cycle phase | Mean displacement (μm) | Displacement in 1 min (μm) |
|------------------|------------------------|---------------------------|
|                  | in 1 min | in 5 min | minimal | maximal |
| S1               |          |          |         |         |
| 2D               | 0.19 ± 0.01 | 0.20 ± 0.02 | 0.08 | 0.80 |
| 3D               | 0.33 ± 0.02 | 0.36 ± 0.02 | 0.00 | 1.05 |
| G1               |          |          |         |         |
| 2D               | 0.26 ± 0.02 | 0.29 ± 0.02 | 0.00 | 1.05 |
| 3D               | 0.45 ± 0.03 | 0.50 ± 0.03 | 0.00 | 1.05 |
| S4               |          |          |         |         |
| 2D               | 0.28 ± 0.01 | 0.32 ± 0.01 | 0.14 | 1.05 |
| 3D               | 0.48 ± 0.02 | 0.56 ± 0.02 | 0.14 | 1.05 |

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**Figure 5.** NORs and nucleolar necklaces in the cells expressing GFP-RPA43. Projections of confocal z-stacks. (A) After telophase, each mitotic NOR (arrow) gradually unfolds into a necklace (arrowheads). Nucleoli are formed from one or more necklaces. Emerging nuclei of the daughter cells are outlined in the first frame representing telophase. (B) Interphase cell treated with calyculin A for 1 h. A part of cell nucleus is shown. Nucleolar necklaces (arrowheads) gradually shrink into NORs (arrow). Scale bars: (A) 2 μm, (B) 3 μm
immunocytochemistry using antibodies against pol I or UBF (Fig. 6D,E). In HeLa cells, we found the following numbers of nucleolar beads: 251 ± 25 at G1, 280 ± 24 at S1 and 406 ± 42 at S4. Thus between G1 and late S phase the number increased on average by 62% (Fig. 6C). In LEP cells the numbers of the beads were: 76 ± 3 at G1, 114 ± 4 at S1, and 87 ± 5 at S3+S4. This corresponds to a 75% increase in the course of the S phase. The great dispersion of the mean value in this assay is due to the individual variability of the cells and lower contrast of the immuno-staining compared to the GFP signal in the transfected cell lines. Nevertheless, our data show that

Table 2. Intensity of GFP-RPA43 signal on individual FC/DFC units.

| Cell cycle phase | Average | Asymmetry of distribution |
|-----------------|---------|---------------------------|
| G1              | 0.56 ± 0.50 | −0.03 ± 0.50 |
| S1              | 0.62 ± 0.80 | 0.08 ± 0.55    |
| S4              | 0.59 ± 0.50 | −0.02 ± 0.40    |

Figure 6. Dynamics of the number of FC/DFC units in the course of cell cycle. (A) Counts of the GFP-RPA43 positive units in individual cells. (B) Averaged data for both GFP-RPA43 and GFP-fibrillarin positive units. The values are presented as proportions to the numbers at G1 phase. In (A) and (B) the number of units increases twice: between G1 and mid S phase by cca 70% in the daughter postmitotic cells at G1 by cca 15%. (C,D,E) Nucleolar beads stained with anti pol I antibody in synchronized HeLa and LEP cells. (C) Counts of the beads show incomplete duplication (increase by 62% in HeLa and by 75% in LEP cells) between G1 and late S (stages S3 and S4). (D,E): pol I (green) and incorporated EdU (red) in a HeLa (D) and LEP (E) cell. The replication signals correspond to S1 stage. Scale bar: 2 μm.
FC/DFC units are “underduplicated” in HeLa and diploid LEP cells as well as in the transfected cell lines.

**Common activators of rDNA transcription had no significant effect on reproduction of FC/DFC units**

Incomplete duplication of the FC/DFC units during S phase, followed by what seemed to be a compensatory increase in the daughter cells, suggested that ribosomal genes in a number of the units may remain transcriptionally silent from S3 to the end of G2 phase, but get activated in the cells of next generation. We attempted to reveal the nature of this silencing by studying effects of some activators of rDNA transcription.

Observing the individual cells in the period between G1 and S4 in the presence of 0.5 μM TSA or 1 μM AzdC, we found in all cases 60–80% increase in the number of FC/DFC units, just as without the treatment (Table 3A).

In another group of experiments we used roscovitine, an inhibitor of Cdc2-cyclin B kinase, which causes specific dephosphorylation of the basic transcription factor SL1 and can stimulate transcription in metaphase NORs.55,56 We treated cells at S3 stage with 150 μM roscovitine, and observed no increase in the number of pol I positive units (Table 3B).

These results indicate that the hypothetical silencing was resistant to TSA and AzdC dependent changes in the chromatin structure and did not depend on the phosphorylation of SL1.

**Discussion**

FC/DFC units of nucleoli play key role in cell metabolism being centers of ribosomal transcription and early rRNA processing. In the present work, we focused on the dynamics of these units in the course of the cell cycle. Using CLEM and specially produced cell lines, we showed that population of the units expressing both pol I and fibrillarin undergoes specific changes during 2 periods of interphase: early G1 and early S.

At early G1, when nucleoli are re-assembled after mitosis, NORs unfold into nucleolar necklaces with gradually multiplying beads so that each NOR produces one necklace (Fig. 5) This process is concluded by mid G1. Then, the number of the beads, or FC/DFC units, does not change until the end of G1.

Further significant changes take place during early S phase, when transcriptionally active ribosomal genes are replicated.14,15 FC/DFC units occasionally lost their RPA43 signal, but not their fibrillarin signal (Fig. 4B, C). This lead us to think that such disappearance of pol I, not observed at other stages, is directly connected to replication of the ribosomal genes. According to our earlier hypothesis,51 transcription is suspended in the FC/DFC units involved in rDNA replicating, which provides a basis for efficient separation of replication and transcription machineries in nucleoli. Our present data agree with this hypothesis. Remarkably, fibrillarin, which is associated with processed rRNA, but not with rDNA,28 is retained by the units throughout the S phase.

The loss of pol I must be of a short duration, for at the same period we observe an increase in the total number of pol I and fibrillarin positive FC/DFC units (Fig. 6). This increase may be detected directly (Fig. 4B, C) or by counting the units before and after early S phase (Fig. 6A, B). During the rest of S, as well as G2 and most of G1 phase, the number of FC/DFC units do not change.

In this work we assume that all DNA, including the ribosomal genes, is duplicated between G1 and G2 phases. But when we followed progress of individual cells through the cell cycle, we found that in the course of S phase the number of FC/DFC units increased only by about 70% (Fig. 6A, B); the

| Table 3. AzdC, TSA (A) and roscovitine (B) had no significant effect on reproduction of FC/DFC units. |  |
| --- | --- | --- | --- |
| **A. Relative increase in the number of units ranges between 60 and 80%.** | **B. The number of units at S3 does not change in presence of roscovitine** |  |
| **G1** | **TSA** | **AzdC** | **Control (0 min)** | **90 min** | **180 min** |
| 1 | 1.68 | 1.74 | 390 | 384 | 390 |
| 1 | 1.59 | 1.70 | 354 | 355 | 352 |
| 1 | 1.84 | 1.64 | 316 | 314 | 318 |
| 1 | 1.65 | 1.64 | 459 | 451 | 451 |
duplication was never completed until the end of interphase. But after mitosis, an additional multiplication of the units took place, so that each daughter cell by mid G1 acquired as many units as the maternal cell had at the same stage. Data obtained on HeLa and diploid LEP cells after synchronization agree with these findings (Fig. 6C, D).

Since there are good reasons to believe that FC/DFC units correspond, more or less precisely, to individual transcriptionally active repeats of rDNA, our data suggest that about 30% of active ribosomal genes remain silent from the middle S till the end of G2 phase, and get re-activated in the cells of next generation, at early G1 phase (Fig. 7). This may be important for maintaining optimal level of rDNA transcription. Production of ribosomes is regulated at different levels; it seems that one of the mechanisms prevents excessive production of the rDNA transcription centers after replication. Interestingly, neither intensity of pol I signal on the units, nor their average size measured on electron microscopic preparations, did change after replication of the active rDNA (Table 3). This suggests that transcription activity per unit remains at a stable level for the most part of interphase.

The hypothetical silencing of some ribosomal genes after replication appears to be so efficient, it could not be prevented by AzdC and TSA, nor reversed by roscovitine (Table 3). Likewise, the permanently inactive ribosomal genes, which are usually present in mammalian cells, did not respond to the treatment. Alternatively, duplication of the active genes may be complete, but not always followed by duplication of the FC/DFC units. In that case, some of the units (cca 30% of the entire pool in the studied cells), would accommodate more than one active gene until the end of interphase. But distribution of the signal intensity showed no tendency to asymmetry, neither before nor after early S phase. This suggests that an original number (probably just one) of active genes per unit is restored shortly after replication.

Our findings also indicate that restoration of rDNA activity after replication is not always symmetrical, i.e., one of the daughter helices may become silent. This hypothesis complements our data on asymmetrical NORs which regularly appear on certain r-chromosomes and cause a mitotic asymmetry.

Thus reproduction of FC/DFC units in nucleoli follows a peculiar pattern, which includes an incomplete duplication during early S phase. Our data suggest that a considerable subset of ribosomal genes remain transcriptionally silent from late S phase to mitosis, but become again active in the postmitotic daughter cells.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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