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The functional organization of the nucleolus in proliferating plant cells

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SUMMARY

The nucleolus is a prominent nuclear organelle which morphologically expresses all functional steps necessary for the synthesis of ribosomes, from transcription of rRNA genes to the assembly and maturation of preribosomal particles and their transport to the cytoplasm. Structurally, the nucleolus contains some basic components common to practically all cell types, namely fibrillar centers (FCs), the dense fibrillar component (DFC), and the granular component (GC); however, the organization and distribution of these components is highly variable, depending on cell identity and functional status. The different steps of ribosome biogenesis are not strictly correlated with the structural components of the nucleolus. Thus, FCs are most likely the anchoring sites for the accumulation of rDNA, and the sites where the assembly of transcription complexes takes place, but transcription of rRNA genes actually occurs at discrete points in the transition zone between FCs and the DFC. The DFC is a structurally homogeneous, but functionally heterogeneous component in which transcription and some early and advanced steps of pre-rRNA processing develop successively in a gradual fashion, from transition with FCs to transition with the GC. Finally, the GC is the site of the later steps of preribosomal processing, including the final assembly of ribosomal proteins for the export of mature particles to the cytoplasm.

The rate of ribosome biogenesis, as well as the structure of the nucleolus, are highly influenced by the proliferation status of the cell, and by factors regulating cell cycle progression. These factors are nucleolar proteins, such as nucleolin, which are targets of signal transduction mechanisms, being at the same time regulators of key steps in preribosome synthesis and processing. Thus, many features of the nucleolus, such as the structural organization of its components, the level and distribution of certain nucleolar proteins and, in general, the rate of ribosome biogenesis, show profound variations throughout cell cycle periods. Particularly interesting is the behavior of the nucleolus during mitosis, in which its structure is disorganized and its activity is stopped, even though the individual transcription and processing complexes are not disassembled, but carried from one cell generation to the next one in such a way that the daughter-cell nucleoli are built with materials coming from the parent-cell nucleolus. Transcription complexes remain assembled at the chromosomal nucleolar organizer in which the rRNA genes are clustered, and processing complexes are carried at the chromosome periphery, and then they are organized into discrete entities called prenucleolar bodies, whose fusion, together with the resumption of transcription and processing, originates the new nucleolus.

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RIBOSOME BIOGENESIS

The function of ribosomes in the translation of genetic information to synthesize proteins makes these small cytoplasmic ribonucleoprotein (RNP) particles play a key role in the cell's life. In eukaryotic cells, each ribosome is made up of four molecules of RNA (ribosomal RNAs; rRNAs) and around 80 protein species, which associate to form the two ribosomal subunits (60S and 40S).

The formation of the molecular components of the ribosome requires the expression of three sets of genes, whose transcription involves all three eukaryotic RNA polymerases (RNA pol): RNA pol I is specific for the commonly named ribosomal genes (or ribosomal DNA; rDNA), coding for three of the four rRNAs; RNA pol II transcribes the ribosomal protein genes; finally, RNA pol III transcribes the 5S rRNA genes. Consequently, the expression of these genes has to be synchronal and interdependent. Furthermore, the process of building and assembling preribosomal particles involves the activity of some other nonribosomal nucleolar proteins, whether enzymatic or not, and several species of small nucleolar RNAs (snoRNAs); all of these play regulatory roles and transiently associate with preribosomal particles, but they are not constituents of the mature ribosome.

The main steps of ribosome biogenesis in a eukaryotic cell are morphologically expressed as a dynamic and prominent structure of the interphase nucleus – the nucleolus – which is easily observed with the light microscope, and was discovered by Fontana in the last years of the 18th century. The nucleolar structure develops and disaggregates during the course of each cell cycle, apparently disappearing in mitosis. Observations by Heitz (1931) and McClintock (1934), that nucleoli originated after mitosis from secondary constrictions of certain chromosomes, led them to name these chromosomal loci "nucleolar organizers" or "nucleolar organizing regions" (NORs). During the decade of the 1960s, it was shown that NORs contain ribosomal genes, except 5S rRNA genes. Today, we know that the nucleolus is the result of the transcription of these genes, as well as of the processing of transcript RNA (pre-rRNA) up to the formation of ribosomal subunits, which are exported to the cytoplasm where they are finally assembled.

MOLECULAR ARCHITECTURE OF THE NUCLEOLUS

Structural components

Molecular components of the nucleolus are distributed in different ultrastructural domains, in which, sequentially, rRNA synthesis and processing, as well as the ribosome assembly, take place. The work of many different groups has led to the conclusion that there are a few constant (or almost constant) nucleolar subcomponents, namely fibrillar centers (FCs), the dense fibrillar component (DFC) and the granular component (GC), which are sometimes accompanied by other structures, such as vacuoles, interstices, etc. (Fig. 1) (Jordan, 1984). The relative distribution of these structural components is highly variable, depending on the cell type and the physiological state of the cell, so that it is impossible to define a "typical" structural model. Firstly, the differentiation of subcomponents may not be clear or sharp in many nucleolar types, but gradual transitions between adjacent components may be found. Secondly, the proportion and relative distribution of basic components show great differences; for instance, the major component of an active nucleolus from a plant meristematic cell is the DFC (see Fig. 1), whereas an active nucleolus from a mammalian cell culture contains up to 75% GC (Jordan and McGovern, 1981). Different nucleolar morphologies can even be found between nucleoli having similar proportions of subcomponents, such as the reticulate *versus* the compact model in animal cells, or segregated *versus* intermingled components in many cell types. Moreover, structural differences for a single component can be found; for instance, FCs from animal cells show a fibrous structure with a uniformly low electron density when observed under the electron microscope, whereas in plant cells two morphological types have been described for FCs, namely homogeneous, similar to those of animal cells, and heterogeneous, larger in size and showing dense inclusions of condensed chromatin in their interior, in addition to the fibrous content (Fig. 1) (Risueño *et al.*, 1982).

In many cases, these morphological differences reflect functional alterations; for example, in plants, the existence of large nucleolar vacuoles containing preribosomal particles is the expression of a state of nucleolar hyperactivity (Moreno

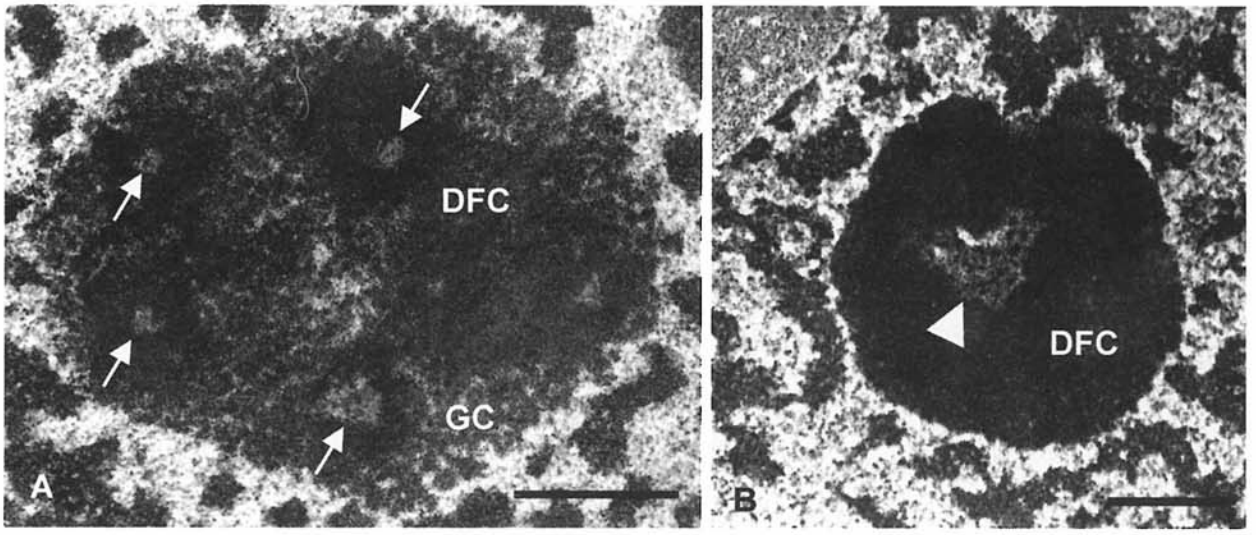

Fig. 1 - Structure of the nucleolus in onion root meristematic cells, observed under the electron microscope with conventional methods of sample preparation. **A**: Proliferating cell. In this active nucleolus, the bulk of the nucleolar body is occupied by the dense fibrillar component (DFC). Within this component, small lighter zones correspond to fibrillar centers (arrows) which are of the homogeneous type. The granular component (GC) surrounds the territories of the DFC. **B**: Quiescent cell. This inactive nucleolus is smaller, and is exclusively made up of DFC and a small number of heterogeneous fibrillar centers (rarely more than two per section) (arrowhead). This type of fibrillar center is characterized by a larger size and the presence of small inclusions of condensed chromatin in its interior. Bars indicate 1 μm.

Díaz de la Espina *et al.*, 1980). However, in many other cases, we do not know the functional cause that accounts for the great nucleolar plurimorphism appearing as the morphological expression of a cellular function that is common in all cases.

Correlation between structure and function of the nucleolus

Research efforts carried out in the past three decades attempting to locate in the nucleolar subcomponents the different steps of ribosome biogenesis have mostly been focused on answering two reciprocally interconnected questions: i) where are ribosomal genes located during interphase – in other words, what is the interphase counterpart of the mitotic NOR; and ii) where in the nucleolus is transcription located Since the nucleolus, as in no other case in the nucleus, is a prominent morphological marker of the expression of a particular set of genes (the ribosomal genes), the answers to these questions were considered, in principle, not to be too difficult, since the sites had to be confined within the territory of this organelle. However, the different macromolecules that play roles in ribosome biogenesis are highly compacted and packaged in the nucleolar body, which makes the *in situ* discrimination of single functional steps especially difficult. In fact, the search for answers to the above questions has resisted decades of intense effort from many research groups and has been the subject of hot debates.

With respect to the location of rRNA genes, the use of highly refined cytochemical techniques at the ultrastructural level, such as regressive EDTA staining capable of bleaching chromatin and enhancing the contrast of RNP-containing structures, the Feulgen-type osmium ammine staining for visualizing DNA, and the Ag-NOR method for staining proteins associated with the nucleolar organizer, among other techniques, allowed, in the mid-1980s, to reach the generally accepted conclusion that ribosomal genes were located in the nucleolar fibrillar components (FCs plus DFC). In the two subcomponents, chromatin was detected in an extended state (i.e., not structured as nucleosomes), with the sole exception of the inclusions characterizing heterogeneous FCs in plant nucleoli (Goessens and Lepoint, 1979; Fakan and Puvion, 1980; Risueño *et al.*, 1982; Derenzini *et al.*, 1982; Goessens, 1984).

Regarding the localization of transcription, a decisive step forward was the development of high-res-

olution ultrastructural autoradiographic techniques using short-pulse incorporation of a radioactively labeled RNA precursor (Granboulan and Granboulan, 1965; reviewed by Fakan and Puvion, 1980; Risueño and Medina, 1986). This technique, in association with cytochemical and ultrastructural studies, allowed the conclusion that the DFC was the site of transcription. Furthermore, ribosome biogenesis was shown to occur in all cellular models in a vectorial fashion, that is, from inside the nucleolus outward. While the former conclusion has been severely questioned by further work, the vectorial organization of ribosome biogenesis has repeatedly been confirmed in more recent studies (Jordan, 1987; Scheer and Benavente, 1990; Cerdido and Medina, 1995).

Already in these early studies, the functional counterpart of FCs was considered enigmatic, since DNA (rDNA), and some proteins related to rDNA transcriptional activity (AgNOR proteins) were found in them, but no direct marker of this activity could be shown (Goessens, 1984; Hadjiolov, 1985; Risueño and Medina, 1986). The debate was stimulated when immunocytochemical and *in situ* hybridization techniques for the detection of various molecular players of rDNA transcription became widely available. In fact, one of the first results obtained from this new methodological approach was that RNA pol I was reported to localize in FCs and not in the DFC (Scheer and Rose, 1984; Scheer and Raska, 1987). Shortly after, immunolocalization of nucleolar DNA and detection of rDNA by *in situ* hybridization produced the same results (Scheer *et al.*, 1987; Thiry and Thiry-Blaise, 1989). However, contrary results with the same techniques in other cell systems appeared almost immediately, showing the whole DFC as the exclusive site of localization of rDNA and transcription (Wachtler *et al.*, 1989). Our results with the onion cell model, using antibodies against the DNA and RNA polymerase, evaluated quantitatively, showed these macromolecules to be located in FCs and in a narrow rim of the DFC immediately surrounding them (Martín *et al.*, 1989; Martín and Medina, 1991).

This cascade of results was accompanied by a reinterpretation of the former autoradiographic data, considering that the duration of the tritiated uridine pulse, together with the utilization by the cell of the newly synthesized RNA containing the radioactive precursor, could allow the detection of the radioactive signal in sites corresponding to early pre-rRNA processing, in addition to the sites of synthesis. This re-interpretation produced strong controversy based on the limitations of the methods used (Medina, 1989; Jordan, 1991; Scheer *et al.*, 1993; Mosgöller *et al.*, 1996). For our part, we combined the interpretation of the new immunocytochemical and *in situ* hybridization data with the former autoradiographic results; this combination, together with a careful ultrastructural study of the morphological features of FCs and the surrounding DFC, allowed us to propose a definition of a new nucleolar structural subdomain, which we called "the transition area between FCs and the DFC" and which we suggested to be the site of nucleolar transcription (Martín *et al.*, 1989; Medina, 1989; Medina *et al.*, 1990; Martín and Medina, 1991).

The most recent progress in finding a definite solution to the problem of establishing unequivocally the site of nucleolar transcription has involved the use of newer methodologies, such as confocal microscopy, but has also taken advantage of the increasing availability of probes containing smaller rDNA fragments for *in situ* hybridization, allowing the mapping of pre-rRNA processing steps with higher precision (Lazdins *et al.*, 1997; Puvion-Dutilleul *et al.*, 1997). Furthermore, the development of a non-isotopic method for labeling transcription, based on the incorporation of Br-UTP (which is not only less hazardous, but also more precise than autoradiography) (Wansink *et al.*, 1993; Dundr and Raska, 1993), has been decisive in this objective.

Experiments carried out in our laboratory on isolated onion cell nuclei, consisting of the combination of the visualization of nucleolar transcription after Br-UTP incorporation with the immunodetection of fibrillarin, a component of the RNP complex involved in the early processing of pre-rRNA, under the confocal microscope, showed a focal arrangement of transcription sites, and the existence of detectable nucleolar domains in which only transcription, and not processing, could be localized; other domains in which the two processes overlapped were also detected. Complementary experiments of transcription *in situ* under the electron microscope revealed the transition area between FCs and the DFC to be the key domain for nucleolar transcription. (De Cárcer and Medina, 1999). The interpretation of

these results is that transcribing chromatin is organized as loops emerging from FCs, which come into (or give rise to) the DFC, being located in the transition area FC-DFC (Fig. 2).

Therefore, our previous proposal on the key role played by the transition area FC-DFC in nucleolar transcription was totally confirmed. Moreover, different studies by others were also in support of locating transcription to a restricted area comprising the outer periphery of FCs and the inner periphery of the DFC; that is, a nucleolar domain very similar to the transition area FC-DFC previously proposed by us (Testillano *et al.*, 1994; Hozák *et al.*, 1994; Shaw *et al.*, 1995; Shaw and Jordan, 1995; Melcák *et al.*, 1996; Mosgöller *et al.*, 1998). In fact, the term "transition zone" has actually been used in a prestigious recent review to indicate the commonly recognized site of nucle-

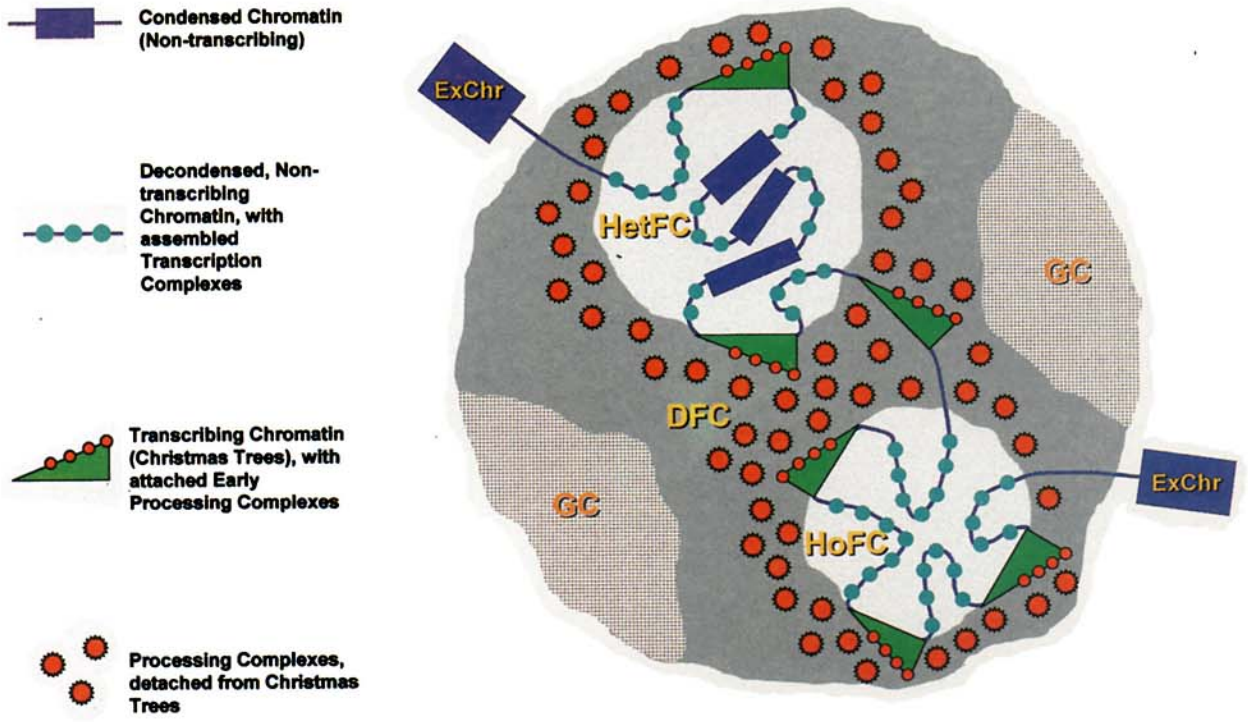

Fig. 2 - Schematic representation of the *in situ* localization, in the nucleolar structural components, of the main molecular entities involved in transcription of rRNA genes and in the early processing of pre-rRNA. Some pictograms, whose meaning is indicated at the left side of the picture, have been used to represent macromolecular assemblies of functional significance. They have been drawn in colors according to the following code: *Green*: Transcription; *Blue*: DNA/Chromatin; *Bluish-Green*: Assembled Inactive Transcription Complexes; *Red*: Early Processing. These pictograms have been placed on an idealized view of the nucleolar structure, which, for clarity's sake, has not been drawn to scale (for example, fibrillar centers are enlarged), and is shown to contain, at the same time, the two types of fibrillar centers (FCs) that appear in plant cells, namely heterogeneous (HetFC) and homogeneous (HoFC); this feature is rather infrequent.

The nucleolus is organized from a continuous segment of chromatin – the NOR chromatin that contains the rRNA genes – flanked by the extranucleolar chromatin (ExChr). Nucleolar chromatin preferentially gathers or anchors in FCs in a decondensed state (except for the small inclusions of condensed chromatin appearing in HetFCs, typical of low-active or inactive plant nucleoli) and is transcriptionally inactive, even though the components of the transcription complex can be found assembled within FCs. Transcription of rRNA genes occurs in loops of chromatin emerging from the interior of FCs to their periphery at discrete points, which are located at the transition area between FCs and the dense fibrillar component (DFC). The nucleolar chromatin that connects FCs to one another also contains transcription units. Early pre-rRNA processing occurs when the nascent pre-rRNA is still being synthesized, before transcription termination; therefore, transcription and processing markers are found to co-localize in transcription units. Then processing complexes detach from these transcription units and randomly distribute throughout the proximal zone of the DFC (the areas nearest FCs). Later steps of processing and maturation of ribosomal precursors occur in the distal zone of the DFC and in the granular component (GC) and are not represented in the figure.

olar transcription (Scheer and Hock, 1999). Nevertheless, the so-called "transcription foci", detected under the confocal microscope, are claimed not to be topologically related to FCs in some papers (Thompson *et al.*, 1997; Shaw *et al.*, 1998).

According to these and previous experiments, FCs are the sites in which the assembly of transcription complexes (polymerase, topoisomerase, transcription factors), and the activation of chromatin for transcription take place, although these complexes are actually inactive in transcription at the core of FCs (Fig. 2). However, FCs have recently been proposed to be mere reservoirs of rDNA and transcription-associated enzymes in the inactive state (Mosgöller *et al.*, 1998). These authors maintain that the different molecular elements are disassembled inside FCs, and their assembly only occurs at the periphery just before their activation. In our opinion, the attachment of proteins to DNA within FCs is supported by the evidence of a non-nucleosomal, fully extended structure of the DNA in FCs of both animal cells (Derenzini *et al.*, 1987) and plant cells, in which only heterogeneous FCs, typical of some inactive or low-active plant cells, contain at the same time both condensed and decondensed rDNA chromatin (Figs. 1B and 2) (Risueño and Medina, 1986; Motte *et al.*, 1991). Certainly, the cause of rDNA unfolding in FCs could be different from its association with the proteins of the transcription complex, but the structural and functional analogy of FCs to the mitotic nucleolar organizer, in which rDNA-protein association has been demonstrated (Weisenberger and Scheer, 1995; Suja *et al.*, 1997), favors the idea of the existence of assembled, but silent, transcription complexes within FCs. This is opposed to the hypothesis of a reservoir containing the disassembled pieces. Moreover, direct evidence of rDNA-UBF association during interphase, indicating potential but not current transcription, has recently been reported (Junéra *et al.*, 1997).

These concepts on FCs have received further support from a recent study on the organization of ribosomal transcription after DRB inhibition of RNA pol II transcription (Panse *et al.*, 1999). This drug is capable of disorganizing the structure of the nucleolus into necklace-like structures without inhibiting the transcriptional activity of RNA pol I, in such a way that each bead of the necklace might correspond to a single transcription unit (Weisenberger and Scheer, 1995). Each bead, in which rDNA, UBF, and transcriptional activity were detected, was shown to be composed of a small FC partially surrounded by the DFC. Furthermore, the individual beads were linked by the DFC. The authors suggest that FCs could correspond to anchoring sites for rDNA, in which the transcribed sequence would be located at the boundary between FC and the DFC; an interpretation totally in agreement with the above-described model for FCs. This model is also compatible with the interpretation of these structures as part of the nucleolar matrix, providing structural support for the organization of rDNA and transcription complexes (Hozák *et al.*, 1994; Moreno Díaz de la Espina, 1995).

Furthermore, since the rDNA is a continuous strand, flanked by extranucleolar chromatin, there is rDNA that extends through the DFC between FCs, and this rDNA is actively transcribing (Fig. 2). The FC-connecting DNA was previously detected by us using an anti-DNA antibody (Martín *et al.*, 1989), and its transcriptional activity was inferred from the presence of RNA polymerase in the same sites (Martín and Medina, 1991). Also, the data from Motte *et al.* (Motte *et al.*, 1991) using osmium ammine staining in plant cells confirmed the existence of thin fibers of extended DNA filaments connecting FCs to one another. Three-dimensional reconstruction from confocal optical sections of our transcription *in situ* experiment demonstrated the transcriptional activity of these DNA fibers (De Cárcer and Medina, 1999). The recent study on DRB-treated nucleoli, showing that the individual beads of the necklace-like structure are linked by the DFC (Panse *et al.*, 1999), is additional support for the existence of active DNA connecting FCs.

Regarding the localization of pre-rRNA processing, the first conclusive data came from autoradiographic experiments after tritiated uridine incorporation. Pulse and chase experiments, consisting of precursor incorporation followed by its later substitution by cold uridine for a certain time before fixation, showed the labeling to localize in the GC, indicating that the precursor, incorporated into the primary transcript, moved to this component in the course of its processing (Fakan and Puvion, 1980). Moreover, the GC was shown to be made up of preribosomal particles containing molecular intermediates of pre-rRNA processing, which were isolated from these particles (Royal and Simard, 1975).

Although there is now general agreement that the nucleolar GC is a site of pre-rRNA processing, new immunocytochemical and *in situ* hybridization studies have extended the localization of these steps of ribosome biogenesis by demonstrating that the processing of transcripts actually begins in the DFC, even with the pre-rRNA molecule still bound to template DNA, before transcription termination (Scheer and Benavente, 1990; Puvion-Dutilleul *et al.*, 1991; Mougey *et al.*, 1993; Shaw *et al.*, 1995). These findings lead to the conclusion that the DFC is actually the site of various steps in ribosome biogenesis, from transcription (in the boundaries of FCs) to advanced pre-rRNA processing, in the transition to the GC. Interestingly, in these different steps, various molecular elements play a part, which means that the DFC is actually a heterogeneous component, both from a molecular and a functional point of view. In fact, we have shown that fibrillarin and nucleolin localizations allow the definition of a proximal and a distal zone in the DFC, with respect to FCs (Fig. 2) (Martín *et al.*, 1992; Cerdido and Medina, 1995). More recently, the different intermediates of pre-rRNA processing detected by *in situ* hybridization were shown to occupy different domains of the DFC (Lazdins *et al.*, 1997).

This functional heterogeneity is in apparent contradiction with the uniform, homogeneous morphology of the DFC, in which the conventional methods of sample preparation have never produced reports on any internal structural differentiation, even though changes in size, distribution and organization of the DFC have been described in dependence on the cell type, or on the functional state of the cell (Risueño and Medina, 1986; Schwarzacher and Wachtler, 1993; Shaw and Jordan, 1995).

Furthermore, our study, simultaneously combining the detection of transcription and processing markers, has shown that, in addition to the radial functional differentiation of the DFC from the periphery of FCs outwards, a lateral differentiation of this component around FCs can be established (De Cárcer and Medina, 1999). As described above, our experiments demonstrate that transcription occurs in the transition area between FCs and the DFC; however, chromatin loops containing transcription units are not uniformly extended around the whole boundary of the FC, but their emergence is restricted to discrete zones. As a result, alternating zones of transcription and processing are seen in this area (Fig. 2). In fact, the structural organization of individual units of transcription and early processing is vectorial (Scheer and Benavente, 1990), so that transcription located at the border of FCs is followed by an outer zone of co-localization of transcription and early pre-rRNA processing, and, more outward yet, by a zone of exclusive localization of the processing machinery (Fig. 2) (De Cárcer and Medina, 1999). However, this only occurs at certain points of the periphery of FCs, and the distribution of preribosomal complexes already detached from transcription units throughout the DFC seems to be variable (as if they filled empty spaces not occupied by transcription complexes), including their localization in sites close to FCs (Fig. 2). Obviously, this distribution does not follow a vectorial pattern.

THE NUCLEOLUS, CELL PROLIFERATION AND THE CELL CYCLE

The cell mechanisms governing cell proliferation and, consequently, cell cycle progression affect the regulation of all the basic activities of the cell (gene expression, protein synthesis, energy pathways, signal transduction, etc.). In particular, there is abundant experimental evidence that the process of ribosome biogenesis is highly dependent on regulators of cell cycle progression. Thus, factors stimulating cell growth and division produce an increase in the rate of ribosome biogenesis; this has been demonstrated in animal cells (Schnapp *et al.*, 1990; Hannan and Rothblum, 1995), as well as in plant cells (Karagiannis and Pappelis, 1994; Gaudino and Pikaard, 1997). Moreover, this rate is regulated during the cell cycle, when it increases from G1 to G2, reaching a peak just before ribosome biogenesis is stopped during mitosis, accompanied by the disassembly of the nucleolus. This was shown directly in mammalian cells (Enger *et al.*, 1968), in *Physarum* (Hall and Turnock, 1976), and in yeast (Fraser and Nurse, 1979); indirect evidence showed that this may also be the case in plant cells (De la Torre and Giménez-Martín, 1982). More precisely, from nucleolar RNA types, pre-rRNA synthesis is associated with cell cycle periods, whereas snoRNA synthesis takes place homogeneously throughout the whole interphase, resulting in stable molecules

whose half-life extends for up to one cell cycle (Weinberg and Penman, 1969).

The molecular mechanisms by which regulators of cell proliferation and cell cycle progression affect the rate of ribosome biogenesis are not totally understood. Actually, ribosome biogenesis is a complex process that requires the concerted activity of many factors regulating the rate of transcription of rRNA genes and the co-ordination of many steps in pre-rRNA processing and preribosome assembly. There are indications that some of these factors, proteinaceous in nature, may act as final receptors of the signal transduction cascade that begins at the cell surface with the primary receptors of growth factors (Bouche *et al.*, 1994). In particular, a set of nucleolar nonribosomal proteins have been shown to play a key role in ribosome biogenesis and to be controlled, in turn, by factors regulating the cell cycle and proliferation (Olson, 1991). Interesting examples in plant cells are the increase in solubilized proteins, from soybean isolated nucleoli, produced by auxin treatment (Chen *et al.*, 1983), or the expression of *MA16,* a gene of maize coding for a nucleolar protein, which is developmentally and environmentally regulated (Albà *et al.*, 1994).

In the onion, we have described alterations in the levels of fibrillarin, a nucleolar protein known to participate in pre-rRNA processing, depending on cell cycle progression and on the differentiation state of the cell. Thus, parenchymatic (differentiated) root cells showed lower levels of fibrillarin than meristematic (undifferentiated) root cells when proteins separated from the same amount of nuclei from the two tissues were probed with an anti-fibrillarin antiserum in a Western blotting experiment. Throughout the cell cycle, the variation in the levels of fibrillarin was quantitatively measured *in situ*, using the morphological ultrastructural differences between G1 and G2 nucleoli as criteria for differentiating the two periods. Labeling of G2 nucleoli was shown to be more than double that of G1 nucleoli (Cerdido and Medina, 1995). Morphological differences between G1 and G2 nucleoli, as well as differences in the content of fibrillarin, were confirmed recently using isolated nuclei purified from hydroxiurea-synchronized onion root meristems, immunostained with anti-fibrillarin antiserum (Fig. 3). Furthermore, a flow cytometry study was performed on isolated nuclei analyzed for the interphase period by propidium iodide staining (specific for DNA), and simultaneously analyzed for fibrillarin content (indirectly reflecting fibrillarin expression) by immunofluorescent staining using anti-fibrillarin antiserum. During the G1 period, fibrillarin expression was low, and progressively increased at a high rate until the S period; later, in G2, this expression continued to increase, but at a lower rate, so that the maximum level was reached just before mitosis (Fig. 3).

Perhaps the best known of the nucleolar proteins related to cell proliferation and cell cycle events is nucleolin, which is the major protein of the nucleolus of actively proliferating cells (Tuteja and Tuteja, 1998; Ginisty *et al.*, 1999). Nucleolin is a multifunctional protein capable of controlling the structure of nucleolar chromatin, depending on its phosphorylation by cdc2 kinase, and of regulating the rate of preribosome production in response to its phosphorylation by casein kinase II (CKII), which is activated, in turn, by growth factors (Belenguer *et al.*, 1990; Olson, 1991; Bouche *et al.*, 1994). Furthermore, it has recently been demonstrated that nucleolin is critically involved in the first processing step of pre-rRNA. The interaction of nucleolin with the pre-rRNA substrate has been shown, as well as its role in the recruitment of factors, such as U3 snoRNP, to the cleavage site (Ginisty *et al.*, 1998). As mentioned above, nucleolin is one of the most abundant proteins in proliferating cells, whereas its expression is greatly reduced in quiescent cells. In fact, the cytological procedure for the silver staining of the nucleolar organizer (AgNOR staining), a well-known method for staining proliferating cells widely used in many laboratories and hospitals as a diagnostic method for malignant tumors, is based on the cytochemical detection of nucleolin (Lischwe *et al.*, 1979; Hozák *et al.*, 1992). By using this method, it has been shown that nucleolin levels are minimal in non-proliferating cells and in the G1 stage of proliferating cells, increase through interphase and reach maximum values in G2 (Sirri *et al.*, 1997). During cell division, nucleolin, together with other nucleolar proteins (fibrillarin, B23) and pre-rRNA, are found in the nucleolar materials that associate with the chromosome periphery in order to be carried to the daughter cells, and in prenucleolar bodies (see the next section). Moreover, the two kinases capable of phosphorylating nucleolin, namely cdc2 kinase and casein kinase II, are known to play cru-

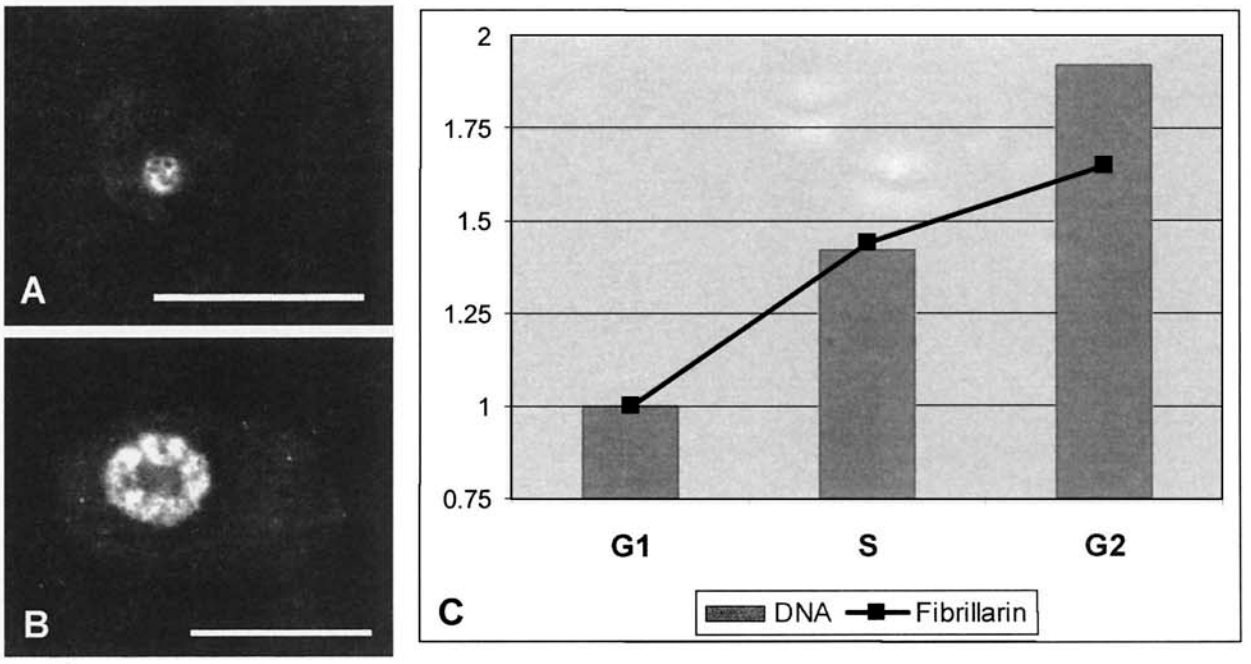

Fig. 3 - Differential expression of the nucleolar protein fibrillarin throughout the interphase periods of the cell cycle, estimated by the accumulation of the protein in the cell nucleus. A and B: Immunofluorescent localization of the protein in isolated nuclei from onion root meristematic cells synchronized by treatment with 0.75 mM hydroxiurea at 25°C, for 14 hours. A: Nucleus in G1, taken from a sample grown at 25°C for 10 hours after treatment. B: Nucleus in G2, taken from a sample grown at 25°C for 6 hours after treatment. Comparison of the two images shows great differences in the size and structure of the nucleolus, as well as in the amount and distribution of the protein between the two periods. C: Quantitative measurement, by flow cytometry, of the relative amount of fibrillarin in the nucleus throughout the interphase periods. The highest accumulation of the protein occurs in G2, but the rate of increase is higher from G1 to S than from S to G2.

cial roles in the regulation and co-ordination of cellular events involved in the cell cycle and in proliferation. It is supposed that nucleolin phosphorylation by these two kinases is the bridge connecting the cell cycle and proliferation events to regulation of ribosome biogenesis (Olson, 1991; Tuteja and Tuteja, 1998; Ginisty *et al.*, 1999). Yeast homologues of nucleolin have been described (Lee *et al.*, 1991; Gulli *et al.*, 1995).

We have recently characterized two nucleolar proteins of plant cells which we have called NopA64 and NopA100, which show interesting analogies with mammalian nucleolin (De Cárcer *et al.*, 1997). In particular, we have demonstrated that they show differential expression throughout the cell cycle, and that they play a part in nucleolar disassembly and reassembly during mitosis. These characteristics, as well as their phosphorylation features in relation to casein kinase and cdc2 kinase, make them suitable markers for studying the influence of mechanisms controlling the cell cycle and proliferation events in plants on ribosome biogenesis.

The nucleolus during mitosis

During cell division, the nucleolus is disassembled in such a way that it is not morphologically detectable. During prophase, the nucleolar constituents are either disaggregated between condensed chromatin masses or are dispersed in the cytoplasm. In metaphase, the only apparent nucleolar remnant is the nucleolar organizing region (NOR), a chromosome segment containing the rRNA genes. However, a portion of the nucleolar components are anchored at the chromosome periphery, forming a sheath-like structure, in order to be distributed rather equally between the two daughter cells; this localization is maintained during anaphase. Later, from early telophase, nucleolar reorganization begins to reconstitute the daughter cell nucleoli. The first sign of this reorganization is the formation of prenucleolar

bodies from the nucleolar materials carried by the chromosomes at their periphery. Prenucleolar bodies are recruited at the NOR and their fusion, together with *de novo* synthesis of pre-rRNA, gives rise to the new nucleolus (Moreno Díaz de la Espina *et al.*, 1976; De la Torre and Giménez-Martín, 1982; Ochs *et al.*, 1983; Hernandez-Verdun and Gautier, 1994; Azum-Gélade *et al.*, 1994).

We have schematically summarized the behavior of the different nucleolar components during mitosis in Fig. 4. This scheme assumes that the nucleolus essentially consists of an ordered aggregation of three types of complexes of nucleic acids and proteins, namely transcription complexes (rDNA and proteins), processing complexes or processomes (pre-rRNA and proteins) and preribosomal particles (rRNA and proteins). Paying particular attention to the first two types, available data demonstrate the existence of a link between them in the interphase nucleolus (Scheer and Benavente, 1990).

The onset of mitosis and the subsequent disorganization of the nucleolus involves inhibition of transcription and processing and, consequently, the inactivation of the two types of complexes and their separation. Interestingly, the major components of the proteinaceous complex capable of transcribing rRNA genes remain stably associated with these genes throughout mitosis in the NOR (Fig. 4). The components detected up till now include RNA pol I (Scheer and Rose, 1984), topoisomerase I (Rose *et al.*, 1988), and the transcription factors UBF and SL1 (Roussel *et al.*, 1996).This indicates that the molecular mechanism repressing rRNA synthesis during mitosis is a regulatory mechanism. Recent studies have demonstrated that the reversible phosphorylation of the transcription factor SL1 by cdc2 kinase is responsible for the loss of activity of this factor. Mitotic phosphorylation impairs the capability of SL1 to interact with UBF, this interaction being a pre-requisite for pre-initiation complex formation (Heix *et al.*, 1998). Moreover, UBF itself is also inactivated by phosphorylation (Klein and Grummt, 1999). The reversibility of these phosphorylation processes causes resumption of rRNA gene transcription after mitosis. But whereas SL1 activity is rapidly regained on entry into G1, UBF is reactivated later in G1, concomitant with the onset of RNA pol I transcription (Klein and Grummt, 1999).

The particular behavior during mitosis of the components of the transcription complex led us to hypothesize whether the components of the processing complex could behave in a similar way, i.e., to remain assembled during their mitotic inactivation. In order to test this hypothesis, we traced, in onion root cells, the mitotic course of some key components of this complex, namely rRNA itself and the nucleolar proteins fibrillarin and nucleolin (Medina *et al.*, 1995). The rRNA was detected by two methods, one direct and one indirect. The direct method was ultrastructural *in situ* hybridization with a rDNA probe capable of hybridizing not only mature rRNAs, but also the intermediate forms of pre-rRNA processing. The indirect method consisted of tracing the course of the RNA synthesized in the preceding G2 (according to classical data, this is mostly rRNA; see above), which was revealed by autoradiography on synchronous cells labeled in G2 by tritiated uridine. Immunofluorescence and electron microscope immunocytochemistry were used to detect fibrillarin and nucleolin. Additional observations indicated that the pattern observed for fibrillarin and nucleolin, detected with heterologous antibodies, was the same as that observed with homologous anti-NopA64 and anti-NopA100 antibodies, specifically detecting these two nucleolin-like onion nucleolar proteins (unpublished observations). In all cases, following nucleolar dispersion in prophase, the signals were detected in the chromosome periphery (perichromosomal sheath) during metaphase and anaphase, in irregular fibrillar masses located between chromosomes in ana-telophase, in prenucleolar bodies during telophase, and in the newly formed nucleoli after nucleologenesis. Moreover, as expected, ribosomes appeared labeled after *in situ* hybridization, but a dispersed cytoplasmic labeling was observed with all markers from metaphase till late anaphase (Fig. 4).

In a recent study, Dundr and Olson (Dundr and Olson, 1998) have confirmed these results by showing that the perichromosomal regions, as well as the so-called cytoplasmic nucleolus-derived foci that appear between early anaphase and late telophase, contain pre-rRNA sequences from the 5' ETS core, 18S, ITS1 and 28S segments, but do not contain the short-lived 5' ETS leader segment upstream from the primary processing site in 47S pre-rRNA. This indicates that high molecular weight processing intermediates are preserved during mitosis, in addition to other components of the processing machinery.

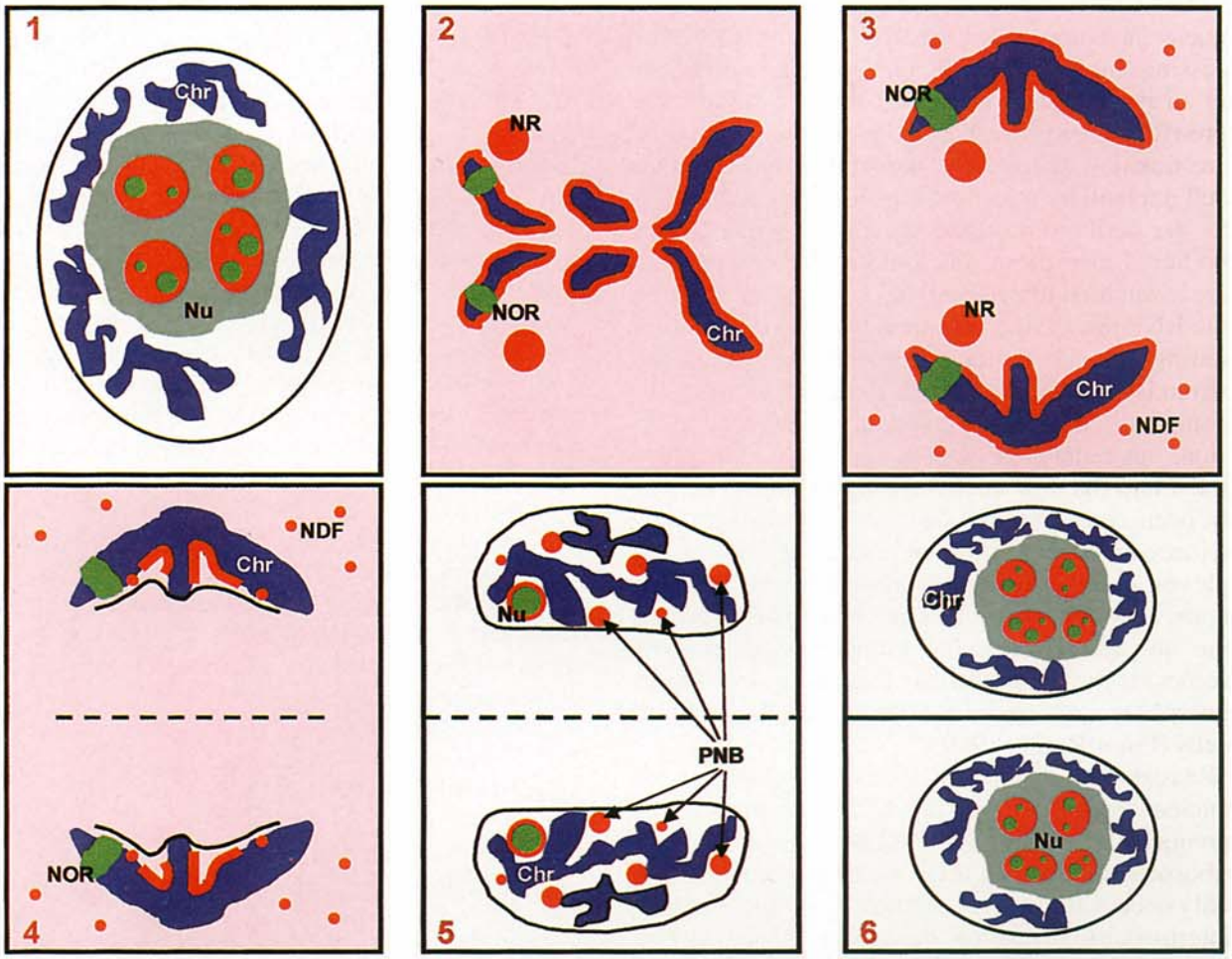

Fig. 4 - Schematic representation of the localization of the nucleolar components during mitosis and their transport from the parent cell to the daughter cells. Molecular components of functional significance have been represented with the following color code: *Green*: Transcription complexes (rDNA, RNA pol I, topoisomerase I, UBF, SL-1,…); *Red*: Processing complexes (pre-rRNA, fibrillarin, nucleolin, U3 snoRNA,…); *Blue*: Cromatin/Chromosomes.

1: Interphase of the parent cell. Active transcription complexes and processing complexes are organized in the nucleolus (Nu). Chr: Chromatin.

2: Metaphase. The nucleolus is disorganized and both transcription and processing complexes are inactivated, but the molecular constituents of the two types of complexes remain assembled. Transcription complexes are gathered in the chromosomal nucleolar organizer region (NOR). Processing complexes are found at the periphery of chromosomes (Chr) and dispersed in the cytoplasm (reddish color). In some cell types, their presence has also been reported in nucleolar remnants (NR).

3: Anaphase. The localization of both transcription and processing complexes is similar to that found at metaphase. Some cytoplasmic bodies called nucleolus-derived foci (NDF) are reported to contain processing complexes in certain cellular types.

4. Ana-Telophase. Chromosomes (Chr) begin to decondense, and fragments of the nuclear envelope appear, as well as the phragmoplast, which will originate the cell wall separating the two daughter cells. These are the first signs of nuclear reorganization that includes nucleologenesis. The nucleolar material located at the chromosome periphery begins to be reorganized as round bodies. Nucleolar remnants are disaggregated and their components are imported into the organizing nuclei, but nucleolus-derived foci (NDF) are still visible, as well as the nucleolar materials dispersed in the cytoplasm.

5. Telophase. The structure of chromosomes (Chr) resembles more and more interphase chromatin. In each of the two daughter cell nuclei, the reorganizing nucleolus (Nu) contains the rDNA (the nucleolar organizer, NOR), transcription complexes, and also components of the processing complexes. Transcription and processing are reactivated. All the rest of the nucleolar processing complexes appear organized in small round bodies, prenucleolar bodies (PNB). The materials of these PNBs are recruited at the reorganizing nucleoli, or NOR, in order to originate the active nucleoli of the two daughter cells.

6. Interphase in the two daughter cells. The organization of the nucleus and nucleolus is resumed in each cell, as it was in the parent cell.

The results taken together demonstrate that nucleolar components involved in pre-rRNA processing, including rRNA itself, which is probably in an incompletely processed form (coming from pre-rRNA newly synthesized in the preceding G2), are transferred from the parental to the daughter cell nucleoli by means of transient structures, such as the perichromosomal sheath and prenucleolar bodies. Since these macromolecular components are assembled in the interphase nucleolus, forming the RNP processing complex, their co-localization during mitosis in the same transient structures strongly suggests that at least a subset of these complexes do not disaggregate during cell division, but remain assembled and become incorporated into the new nucleolus. This idea has recently been confirmed by the discovery that the proteinaceous composition of nucleolar RNP complexes (containing nonribosomal nucleolar proteins, as well as ribosomal proteins and rRNA, i.e., the so-called processing complexes or processomes) is the same whether they are isolated from interphase cell nuclear extracts or from mitotic cells (Piñol-Roma, 1999).

Reassembly of the nucleolus at telophase, or nucleologenesis, consists of the recruitment of prenucleolar bodies at the NOR and restarting of ribosome biogenesis (Fig. 4). This restarting not only occurs at the level of transcription, but also at intermediate levels of processing of pre-rRNA molecules which were transcribed in the previous interphase (Medina *et al.*, 1995; Dundr and Olson, 1998). The mechanism of nucleolar reassembly strongly suggests that there is physical migration of prenucleolar bodies throughout the reorganizing nucleus towards the NOR, in which all of them would coalesce. Surprisingly, however, the first results obtained in living cells with video-fluorescence microscopy, using cells transfected with constructs expressing green fluorescent protein fused with proteins found in nucleolus-derived foci, showed rather "immobile" prenucleolar bodies, gradually dissolving at the same time as the new nucleolus was growing. It appears that the new nucleolus is, in fact, formed from the materials coming from prenucleolar bodies in a process that does not involve the coalescence of the intact bodies, but their previous disaggregation. These results, including video projection, were presented by Dr. M.O.J. Olson in collaboration with M. Dundr and T. Misteli at the 16th Wilhelm Bernhard Workshop on the Cell Nucleus, held in Prague in August, 1999.

On the other hand, non-nucleolar proteins have also been localized in the perichromosomal sheath (the so-called "chromosomal passenger proteins"). This clearly indicates that the formation of this structure is probably a general mechanism for the distribution of different cellular components between daughter cells (Earnshaw and Bernat, 1991; Hernandez-Verdun and Gautier, 1994), and its function is not limited to carrying nucleolar components. More hypothetical are other functions that have been proposed for this structure, such as a role in protecting chromosomes or a way of cross-linking them by keeping them tightly packaged (Shi *et al.*, 1987; Yasuda and Maul, 1990; Gautier *et al.*, 1992). These interpretations are all compatible with the role of the perichromosomal sheath in the accumulation and transport of nucleolar processing complexes by linking successive cell generations in the essential process of ribosome biogenesis.

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