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High resolution detection of rRNA and rDNA in plant nucleoli with different activities by in situ hybridization

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In the present work we perform in situ hybridization with probes to different stretches of rDNA and electron microscopy of nucleoli with different activities, to gain insight into the ultrastructural organization of transcription and processing in the plant nucleolus. The main ultrastructural nucleolar components: fibrillar centers (FC), dense fibrillar component (DFC), and granular component (GC), are arranged in different ways depending on nucleolar activity. Heterogeneous FCs containing RNP fibrils and nucleolar perichromatin granules are frequently seen in nucleoli in the process of activation. DNA-RNA in situ hybridization with biotinylated probes spanning different sequences of the rDNA unit followed by immunogold detection of biotin, demonstrated the localization of the ribosomal transcripts in DFC, mainly in the zones around the FCs, in GC, and in the periphery of pale FC. The internal region of the heterogeneous FCs is labeled only in cells in the process of activation of transcription after dormancy. The distribution of the U3 probe indicates that the processing of the rRNA takes place in the DFC and inside the heterogeneous FCs, in which transcription occurs. DNA-DNA hybridization demonstrates the presence of rDNA in the compact and extended chromatin located in the interior and at the periphery of FCs and in nucleolar associated chromatin. Our results support the view that the plant nucleolus has a highly dynamic morphological and functional organization composed of a bipartite domain formed by FCs surrounded by DFC, which is associated with rRNA transcription and processing, and the GC representing a store of preribosomal particles. 2000 © Éditions scientifiques et médicales Elsevier SAS

plant cells / nucleolus / rRNA / rDNA / ultrastructure

1. INTRODUCTION

In eukaryotes the nucleolus shows similar well-defined structural components: the fibrillar centers (FCs), dense fibrillar component (DFC) and granular component (GC) (Jordan, 1984; Shaw and Jordan, 1995). The use of molecular probes to different nucleolar components in experiments of hybridization, immunolabeling and transcription in situ has contributed to localize the main biochemical processes to defined nucleolar compartments (Puvion-Dutilleul et al., 1991; Swarzacher and Wachtler, 1991; Shaw and Jordan, 1995; Brown and Shaw, 1998). Three different processes occur in the nu-

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cleolus: specific transcription of ribosomal genes by RNA polymerase I, processing of the primary transcripts by specific small nucleolar RNPs and assembly of the preribosomal particles (Hozak et al., 1994; Shaw and Jordan, 1995).

The nucleolus of active plant cells is made up of the three basic components: fibrillar centers (FCs), dense fibrillar component (DFC), and granular component (GC) (Martin et al., 1989; Shaw and Jordan, 1995; Brown and Shaw, 1998). Although ribosomal genes have been localized to FC and DFC, and rRNAs to DFC and GC by in situ hybridization (Leicht et al., 1992; Highett et al., 1993; Olmedilla et al., 1993), the exact functions of the FC and DFC are still far from understood.

The structure of the fibrillar centers changes with metabolic activity of the plant cell. The nucleolus of meristematic cells of dormant roots contains few large FCs, while in the nucleolus of active cells FCs are small and numerous (Medina et al., 1983; Acevedo et al., 1998). However, the morphological and functional changes of the FCs during the process of the activation of transcription were not studied. FCs present different structures in cells of various plants. Onion root meristematic cells in the G1 period of the interphase have low activity nucleoli with heterogeneous FCs and nucleolar perichromatin granules, which are postulated to contain unprocessed rRNAs (Moreno Díaz de la Espina and Risueño, 1982; Risueño et al., 1982), while similar cells of tomato and sugarcane present only clear FCs (Moreno et al., 1992; Acevedo et al., 1998).

The aim of this work was to investigate the organization of the transcription in nucleolar domains in plant nucleoli with different activities. With this purpose we localized the rRNA and rDNA by high-resolution in situ hybridization.

2. MATERIALS AND METHODS

2.1. Materials

Root meristematic cells from *Allium cepa* L. bulbs either unsoaked or grown in filtered tap water at room temperature were used.

2.2. Hybridization

Two different probes of human rDNA (B, C) obtained by *Eco*RI digestion and a probe obtained by *Bam*HI digestion (AD_{BB}) were kindly provided by Dr. Sylvester (Sylvester et al., 1986). Probe B is a 5.8 kb *Eco*RI fragment containing the ETS region and most of the 18S coding region (Erickson et al., 1981). Probe AD_{BB} is a 6.7 kb fragment containing part of the 28S coding region and a portion of the 5' intergenic spacer (IGS) (Erickson et al., 1981). Probe C is an 11.2 kb fragment of the 3' part of the IGS (Sylvester et al., 1986). Plasmids containing the probes were biotinylated by nick translation using the commercial bionick labeling system from Gibco BRL. Optimum probe sizes (100–500 bp) were monitored by agarose gel electrophoresis and the efficiency of the labeling by dot blot essay. The labeled probes were concentrated in a speed-vac (Savant) and resuspended in 10 µL of 50% formamide and 1 mL of 15 mg/mL tRNA. Then the probes were denatured in hybridization solution containing 20% dextran sulphate, 2% BSA and 4 × saline sodium citrate buffer (SSC) for 10 min at 75 °C.

The U3 probe contains a 41 base stretch from *Pisum sativum*, a very conserved sequence among numerous plants, which was a generous gift from Dr. P.J. Shaw (Beven et al., 1996).

2.3. Hybridization on filters

DNA from onion meristematic root cells was extracted with cetyltrimethylammonium bromide according to standard procedures (Ausubel, 1995). After digestion with *Bam*HI (Amersham), the DNA was loaded in a 0.6% agarose gel and separated by electrophoresis at 1V/cm for 24 h in Tris borate EDTA buffer. The DNA fragments were depurinated, denatured and transferred to nitrocellulose filters as described (Sambrook et al., 1989). Probes were labeled with digoxigenin by random primed (DIG DNA labeling and detection kit, Boehringer Mannheim). As a positive control of the heterologous probes, we used a probe from pea, pHA1, that contains the three rDNA genes and most of the non-transcribed spacer (Jorgensen et al., 1987).

Filters were pre-hybridized for 3 h at 63 °C in 5 × SSC, 0.1% Sarkosyl, 0.02% SDS and 1% blocking reagent (from Boehringer Mannheim). Hybridization was carried out overnight under the same conditions by adding about 0.02 µg of heat-denatured probe to the pre-hybridization solution, blots were washed at room temperature for 10 min in 2 × SSC, 0.1% SDS and then for 30 min in 0.1 × SSC, 0.1% SDS at 63 °C. Finally, digoxigenin-labeled probes were detected with an anti-digoxigenin antibody conjugated to alkaline phosphatase and the colorimetric substrates NBT and X-phosphate.

2.4. In situ hybridization (ISH)

Roots were fixed in 4% paraformaldehyde (PFA) for 2 h at room temperature in 0.1 M phosphate saline buffer (PBS), pH 7.2, dehydrated in an ethanol series and embedded in LRW resin as described (Moreno Díaz de la Espina et al., 1992). Probe was denaturalized by heating to 70 °C for 10 min and quickly chilling on ice. A mixture containing 0.2 µg/mL of the probe, 5% BSA, and 1 mg/

mL tRNA from *E. coli*, 2 × SSC, and 50% dextran sulphate was employed. Nickel grids with the sections, were incubated overnight at 40 °C in a humidified chamber on the top of drops containing the probes at a concentration of 1 µg/µL in hybridization solution containing 50% formamide, 10% dextran sulphate, 2 × SSC, 1% BSA and 0.75 µg/mL tRNA. After incubation the grids were washed twice in 1 × SSC and then in PBS with 0.1% Tween (PBST). For DNA-DNA hybridization the B probe was employed. DNA was denaturated by placing the grids into a solution of 70% formamide/2 × SSC at 70 °C for 4 min. Then the grids were placed in ice cold 70% ethanol for 2 min, 50% ethanol and distilled water before air drying. Denaturation of the probe and incubation were carried out as described for DNA-RNA hybridization. For enzymatic digestions, the grids with the sections were floated on the enzymatic solutions in a moist chamber at 37 °C. As the RNase pre-treatment of sections was not effective on the nucleolus, double digestions were performed. Grids were first floated on 0.3% proteinase K in 10 mM Tris-HCl, pH 7.4, for 2 h and then on 2% RNase A in the same buffer for 2 h. As controls, hybridization using plasmids without the insert and hybridization using no probe were carried out.

The biotinylated probe was detected by immunocytochemistry with a gold conjugated anti-biotin antibody (British Biocell). After blocking in 0.1% PBST, containing 1% BSA and 0.1% gelatin for 15 min at room temperature, the grids were incubated in the humidified chamber with the antibody at 1:200 dilution in the blocking solution for 1 h at room temperature, washed twice in PBS and later in deionized water. Controls were performed omitting the second antibody in the incubation solution. After drying, grids were stained either conventionally with uranyl acetate and lead citrate or with the Bernhard's EDTA staining preferential for ribonucleoproteins (Bernhard, 1969), modified for plant cells by Risueño and Moreno Díaz de la Espina (1979).

The distribution of labeling was quantified by using 30 micrographs from randomly chosen nucleoli taken from three different hybridizations with each probe. The final magnification was × 65 000. Gold particle numerical densities (number of particles per square micron) in the different nucleolar domains and in the rest of the nucleus were calculated using a semiautomatic procedure in the computer with a digitizer tablet. When it was not possible to define the limits of the border region between FC and DFC, the particles in this interface were included half in the count of each component.

2.5. Electron microscopy

Roots were fixed in either 4% paraformaldehyde (PFA) in PBS, pH 7.2, or 2% glutaraldehyde in 0.025 M cacodylate buffer, pH 7, and embedded in LRW. EDTA staining (Bernhard, 1969) was performed as previously described (Risueño and Moreno Díaz de la Espina, 1979).

3. RESULTS

3.1. Ultrastructure

The onion nucleolus shows a compact organization in thin sections, with the three typical nucleolar components: FCs surrounded by an extensive DFC and a peripheral GC. Their morphological characteristics vary according to nucleolar activity (*figure 1*). Almost inactive nucleoli, as those of quiescent cells have an extensive compact DFC and a scarce peripheral GC. They show few and large heterogeneous FCs forming a track connected to and to some extent invaded by condensed nucleolus associated chromatin masses (NACs), (*figure 1A*), Risueño and Moreno Díaz de la Espina, 1979; Risueño et al., 1982; Medina et al., 1983). In nucleoli which are beginning to become active the intranucleolar track expands, and the sections show large heterogeneous FCs connected to the NAC. The FCs are looser and contain less condensed chromatin inclusions (*figures 1B, 4D, F*). After EDTA preferential staining, these large FCs present ribonucleoprotein fibrils in their interiors and also nucleolar perichromatin granules (nopgs), constituting the only case in which ribonucleoproteins are localized inside FCs in this system (*figure 4C*). Highly active nucleoli present a looser organization with numerous pale FCs surrounded by narrow zones of DFC and abundant GC around them (*figure 1C*).

Although the nucleolar organization is highly dynamic, the cytochemical and immunocytochemical characteristics of the nucleolar components are very constant. Nucleolar ribonucleoproteins are localized by EDTA staining in the DFC and GC of the active nucleoli (*figure 4A*) and also in the FCs of the reactivating nucleoli (*figure 4C*); while DNA is mostly localized in the heterogeneous FCs and the DFC surrounding the FCs by immunolabeling (not shown; see Martin et al., 1989).

3.2. In situ hybridization

To detect more specifically the nucleolar products, we performed DNA-RNA in situ hybridization and immunogold detection, with several heterologous probes which span different coding and non-coding regions of the ribosomal cistrons (*figure 2*). The probes of the human coding sequences for 18S and 28S rRNAs hybridize selectively with the restriction fragments of the onion rDNA as demonstrated by the Southern blots, while those corresponding to the IGS do not. The intensity of the hybridization with the human coding probes is lower than that obtained with the more closely relat-

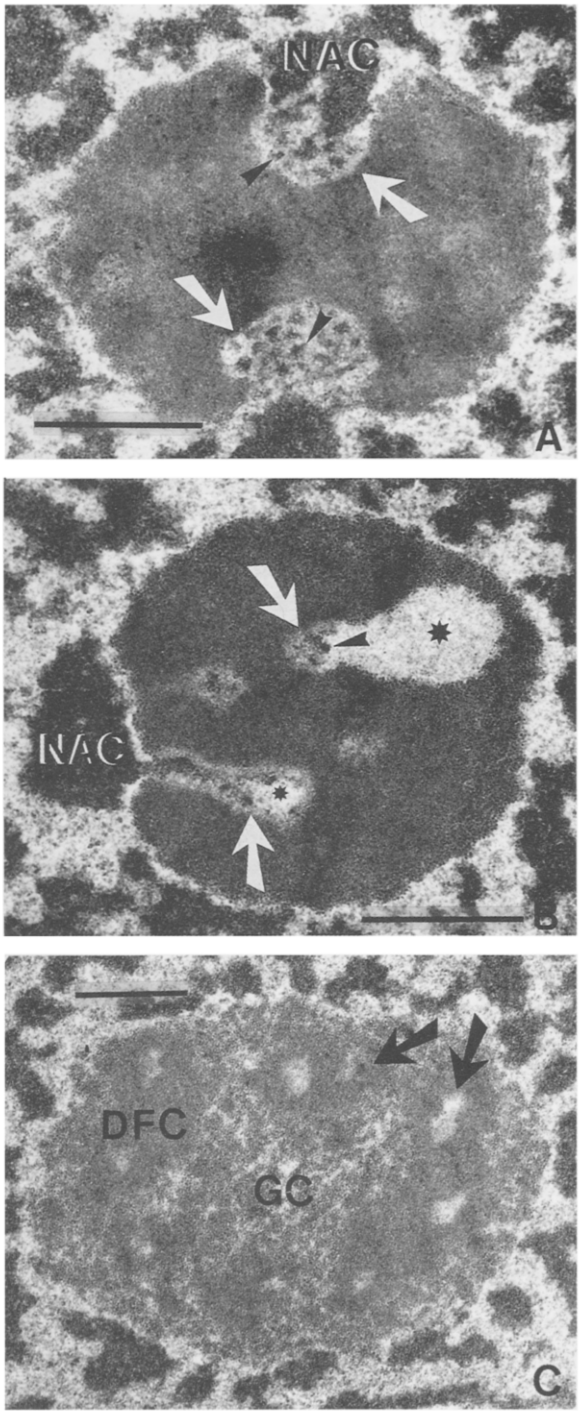


Figure 1. Evolution of the nucleolar organization in onion meristematic root cells from an inactive (**A**) to a very active (**C**) state. Glutaraldehyde-osmium fixation. Sections stained with uranyl acetate and lead citrate. **A.** Inactive nucleolus from a quiescent cell displaying two cross-sections of the large FC (large arrows) forming a track connected to the nucleolus associated chromatin (NAC), containing inclusions of condensed chromatin (arrowheads). × 22 000. **B.** Reacti-vating nucleolus after 6 h of soaking, similar to that shown in *figure 4C, D*. The large FC in close contact with the nucleolar cavity (*), is looser (large arrows) and displays less inclusions of condensed chromatin (arrowheads) than in quiescent cells. In both cases (**A, B**) the nucleolus has mostly a fibrillar organization with a very scarce peripheral granular component. × 21 000. **C.** Highly active nucleolus from a proliferative cell. It shows a loose organization with numerous and small pale FCs without chromatin inclusions (arrows), each surrounded by a dense fibrillar component (DFC) and immersed in abundant granular component (GC). × 14 000. Bars, 1 μm.

ed *Pisum* probe containing both sequences. (*figure 3*). These results demonstrate the cross-hybridization of the coding sequences between humans and plants, and the specificity of the hybridization analysis.

Enzymatic digestions were performed to test the specificity of the hybridization on sections. RNase digestion alone was not effective on the plastic sections. When double proteinase K and RNase pre-

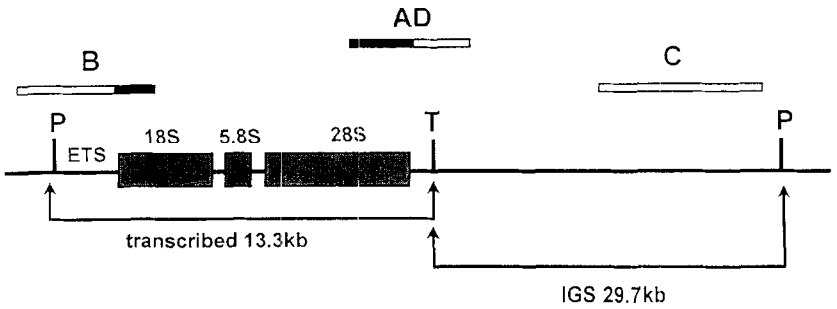


Figure 2. Localization of the probes along the general map of the human ribosomal DNA repeat unit, including transcribed and intergenic segments. The extent of each probe is delineated by an open bar. Probe regions of probable sequence conservation are filled in black. The lengths of the coding and non-coding regions are not represented at scale.

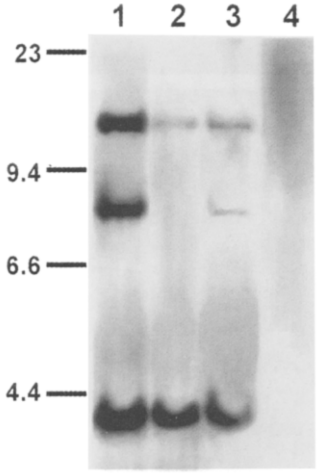


Figure 3. Southern blot analysis of the cross-hybridization of the human rDNA probes with the *Bam*HI restriction fragments of the onion rDNA. Lane 1, *Pisum* pHA1 probe; lane 2, probe AD; lane 3, probe B; lane 4, probe C. Markers, *Hin*dIII restriction fragments of lambda phage DNA. Probe B containing sequences of the 18S rDNA hybridizes with the three fragments of onion rDNA, while probe AD only hybridizes with two of them. Probe C containing a stretch of the IGS does not hybridize. The intensity of hybridization is higher with the more related probe of *Pisum*.

treatment was applied to sections the labeling of DNA/RNA hybridization is abolished, but the loss of ultrastructural organization produced in the nucleolus made it impossible to distinguish clearly the nucleolar domains (data not shown).

ISH with the specific probes for the coding regions (B and AD) showed labeling on the nucleolus and cytoplasmic ribosomes. As expected, the probe C specific for the IGS did not hybridize with the sections.

3.3. Localization of different types of rRNAs

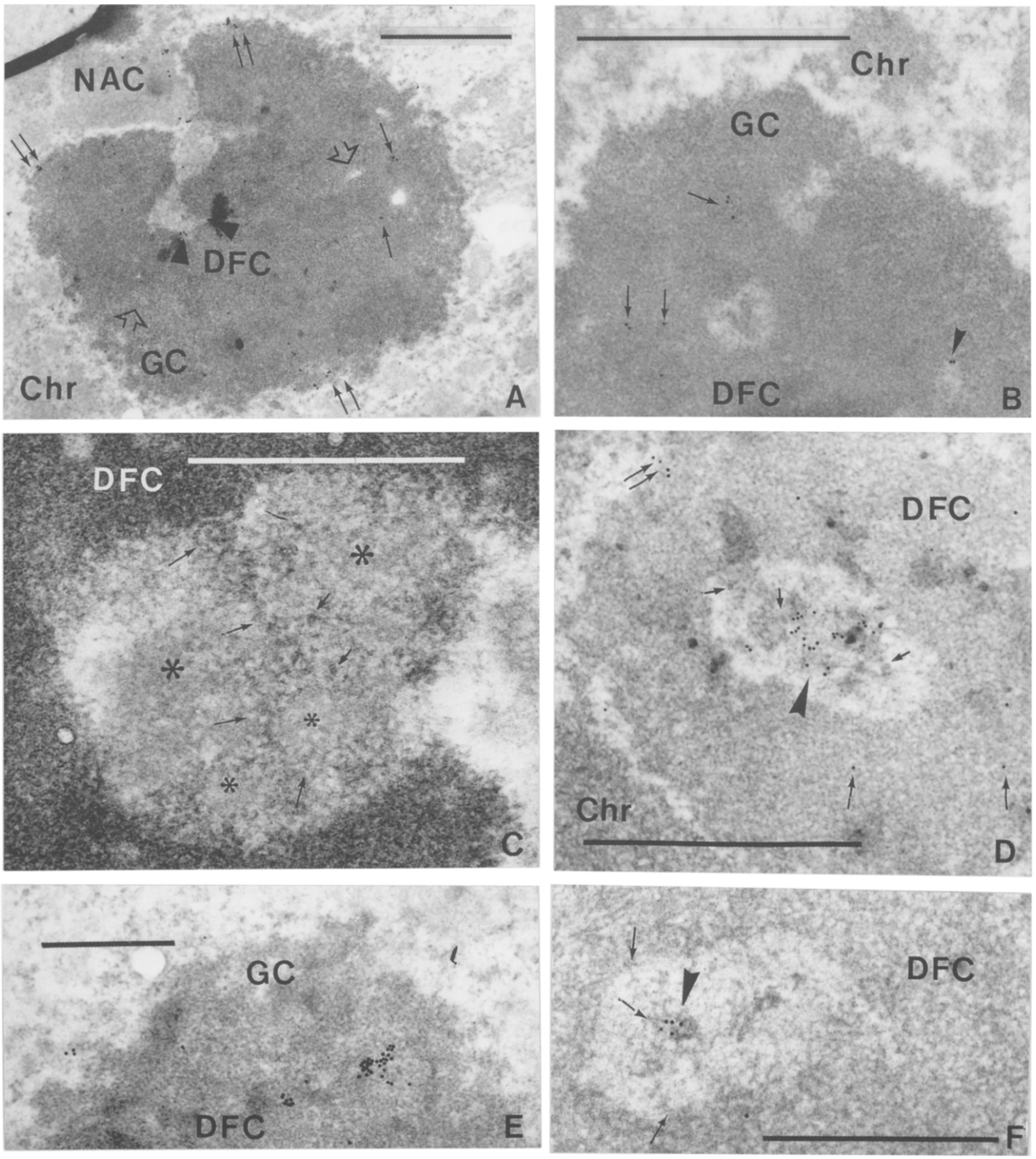
3.3.1. 18S rRNA sequences

To detect these sequences, probe B spanning the 5′ external transcribed spacer (ETS) and the 3′ part of the 18S rRNA coding sequence, was used (*figure 2*). After DNA-RNA hybridization with this probe on sections of active nucleoli from proliferating cells, both DFC and GC were labeled (*figure 4A, B*). The labeling on the DFC was not homogeneous, but showed a greater accumulation of gold particles in the zones of the DFC in close contact with the FCs. The clear FCs of active nucleoli show labeling in their peripheries, that is in the interface between them and the DFC, but never in their interiors (*figure 4B*).

Quiescent nucleoli, which are becoming active, have a large FC that contains ribonucleoprotein fibrils and nopgs, surrounding several pale round areas similar to individual FCs of highly active nucleoli, as evidenced by Bernhard's EDTA staining which is preferential for ribonucleoproteins (*figure 4C*). These large heterogeneous FCs are the only case of internal labeling of FCs with a probe to the 5′ region of the rRNA transcript (*figure 4D*), in contrast with the small clear FCs of active nucleoli which never were labeled except at their peripheries (*figure 4B*). There is also a high degree of labeling in close association to zones of nopgs in reactivating nucleoli, while the labeling on the compact DFC is scarce (*figure 4D*). We never observed labeling of ribonucleoprotein tracks in the nucleus indicative of pre-rRNA particle migration to the cytoplasm, either with this or the 28S probe (*figure 4A*).

3.3.2. 28S rRNA sequences

To detect the 25S rRNA we hybridized with probe AD spanning the 3′ portion of the 28S coding sequence and a portion of the non-conserved intergenic spacer (*figure 2*). The general pattern of distribution of the labeling was similar to that produced by probe B. Gold particles appeared distributed on the DFC, mainly in the zones around FCs, and more abundantly in the GC (*figure 4E*).



3.3.3. Intergenic spacer sequences

Probe C containing only sequences of the non-transcribed intergenic spacer did not hybridize to the sections (not shown).

3.3.4. U3 sequences

Gold particles are associated with the three main nucleolar constituents (FCs, DFC, and GC). The labeling is mainly concentrated in the DFC and the heterogeneous

FCs of the nucleoli in the process of activation, while that in the GC is very scarce (*figures 5, 6*).

3.3.5. Localization of rDNA

After denaturation of sections which permit the detection of rDNA in addition to rRNA, and hybridization using B probe, the large heterogeneous FCs show labeling on their internal condensed chromatin inclusions and also in the fibers associated with their peripheries (*figure 4F*). The DFC and GC display sparsely distributed gold particles. The NAC displays the highest concentration of gold particles. The non-nucleolar chromatin is not labeled.

3.3.6. Quantitative analysis

Quantification of the gold particles on the different nucleolar domains showed that the distribution of labeling between the nucleolar compartments changes according to the metabolic activity of the cell. The highest labeling with coding probes B and AD occurs in the FCs of cells in the process of activation (*figure 7*). This compartment includes not only the labeling at the periphery of FCs where rRNA transcription occurs (Shaw et al., 1995), but also the labeling of the internal structures of the large heterogeneous FCs of activating nucleoli. Probe B containing the sequence of the ETS and part of the 18S, shows a higher labeling than probe AD in FC and DFC. This fact could be explained by a higher accumulation of nascent (non-completed) transcripts in this domain. On the other hand, probe AD containing a portion of the 28S rRNA and of the IGSs sequences, shows a higher density in the GC (*figure 7*). In fully active cells the numerical density of the labeling of probe B attains its highest level in DFC (*figure 8*). This is probably due to the decrease of the number and size of heterogeneous FC. The nucleoli of inactive cells present a lower density of labeling with probes B and AD, than more active nucleoli (compare *figure 9* with *figures 7* and *8*). The labeling obtained with probe B is more intense in FC while probe AD labels GC to higher extent (*figure 9*).

The U3 probe accumulates mainly in the DFC (*figure 10*). FCs also show a high density of gold particles due to their peripheral labeling, as well as to the localization of particles in the interior of FCs of nucleoli in the process of activation (*figure 10*). The probe C does not present numerical densities above background level in any nucleolar compartment (*figure 10*).

The numerical density of the labeling after DNA-DNA hybridization with B probe was estimated in FCs and in NACs. Both structures present a level of labeling significantly higher than the background noise. The numerical density of the gold particles in the NACs is more than twice that of the FCs (*figure 11*).

3.3.7. Controls

The very low level of labeling in the nucleoplasm after DNA-RNA hybridization using B, AD, C, or U3 probes, is similar to the background noise level. This observation shows that the probes associate with specific nucleolar RNA and not with hnRNA or snRNA present in the nucleoplasm. DNA-DNA hybridizations with probe B were carried out as positive controls. The NAC, which

◀ **Figure 4.** In situ hybridization with different probes to localize the nucleolar RNAs or rDNA in onion cells. **A.** Probe AD in a low active onion nucleolus. Bernhard's EDTA staining preferential for ribonucleoproteins. Gold particles localizing the 25S rRNA are more abundant in the granular component (GC) (double arrows) than in the dense fibrillar component (DFC), where gold particles are scattered (arrows). Labeling is also very abundant in the interphase between FCs and DFC (large arrowheads). The nucleolus associated chromatin patch (NAC), in continuity with the big FCs, is the only condensed extranucleolar chromatin patch displaying gold particles (small arrowheads). Extranucleolar ribonucleoproteins do not show labeling. Condensed chromatin (Chr). Small fibrillar centers (empty arrows). × 22 000. Bar, 1 μm. **B.** Probe B. Portion of an active nucleolus. The labeling is localized mainly in the DFC (arrows) and the peripheries of FCs (arrowheads). Clear FCs do not show labeling in their interiors, in contrast with the heterogeneous FCs (see **D**). Chromatin (Chr). × 45 000. Bar, 1 μm. **C.** EDTA staining of a large FC from a nucleolus in the process of activation. The nucleolar DFC is deeply contrasted. Within the large FC, pale roundish structures similar to the clear FCs of highly active nucleoli are discerned (asterisks). Surrounding them ribonucleoproteic fibrillar arrays similar to those shown in **D** radiate from the periphery of the clear areas and make contact with the periphery of the contrasted DFC (arrows). Contrasted nucleolar perichromatin granules, characteristic of this stage are also observed (small arrows). × 46 000. Bar, 1 μm. **D.** Probe B. Nucleolus in the process of activation after dormancy. The gold particles localizing the 18S rRNA appear mainly in the large heterogeneous FC (arrowhead), where nopgs accumulate at this stage (small arrows). A few gold particles are also spread over the DFC and the scarce peripheral GC (double arrows). Condensed chromatin (Chr). × 46 000. Bar, 1 μm. **E.** Higher magnification of the nucleolar granular component after hybridization with probe AD which detects a high signal for plant 25S rRNA in this component. × 44 000. Bar, 0.5 μm. **F.** Probe B, DNA-DNA hybridization. After denaturation of sections gold particles are observed on the condensed chromatin inclusions of the big FC of low active nucleoli (arrowhead). Fibrillar arrays are observed connecting the chromatin inclusions with the DFC (arrows). × 78 000. Bar, 0.5 μm.

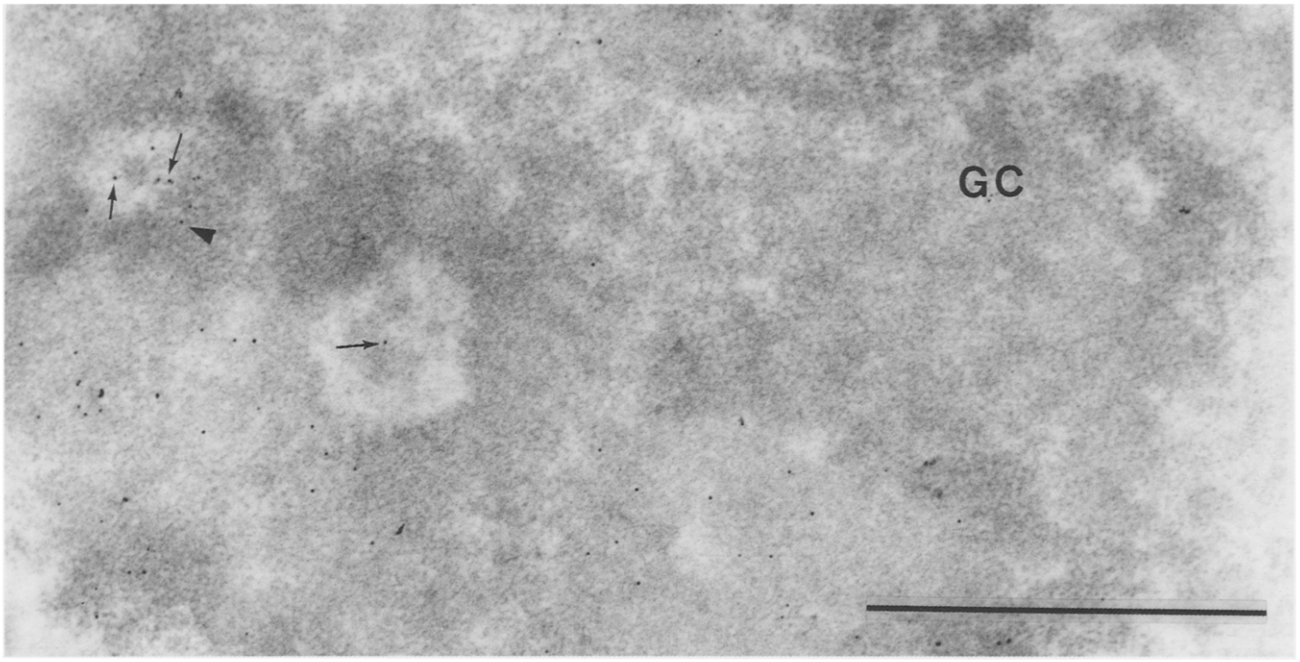


Figure 5. Localization of snoU3RNA in a low activity nucleolus of a root meristematic cell. In a heterogeneous FC the arrow points to labeled fibrils irradiating from a compact central body. The arrowhead indicates the gold particles associated with the DFC surrounding the FC. The granular component (GC) is almost devoid of labeling. × 52 500. Bar, 1 μm.

do not contain RNA, but is mainly constituted by compacted rDNA sequences (Panzera et al., 1996), depicted the highest level of labeling, almost the double of that of FCs (*figure 5*). Unspecific DNA corresponding to the plasmid without probe did not hybridize to the sections (not shown).

4. DISCUSSION

4.1. Ultrastructural organization of the plant nucleolus

The ultrastructural organization of the plant nucleolus is highly dynamic and consistent with nucleolar activity (Risueño and Medina, 1986; Shaw and Jordan, 1995). Meristematic onion cells (2n = 16) have about 7000 copies of rDNA grouped in five clusters (Panzera et al., 1996), although they develop a maximum of two nucleoli per nucleus. Stereological studies in synchronous onion meristematic cells have established a correlation between cell cycle position and nucleolar organization (Sacristán-Gárate et al., 1974). Quiescent meristematic onion cells have inactive nucleoli with a compact DFC and large FCs with condensed chromatin inclusions (Risueño and Moreno Díaz de la Espina, 1979; Medina et al., 1983). The present results of the DNA-DNA hybridization experiments demonstrate that these small masses of condensed chromatin located inside the FCs contain rDNA genes. During activation the large FC fragments into smaller ones, the chromatin inclusions decondense, eventually resulting in the highly active meristematic nucleolus showing numerous small FCs with little or no condensed chromatin inclusions, each surrounded by a layer of DFC (Risueño et al., 1982; Medina et al., 1983). Experimental inactivation of nucleoli by drugs leads to the reverse of these changes (Risueño and Medina, 1986).

4.2. In situ hybridization

Hybridization on filters demonstrates the cross-reactivity of the sequences of the coding regions of the rDNA genes between humans and onions, but not of those spanning the intergenic spacers and hence the specificity of the ISH analysis. The low densities of labeling, compared with other ISH experiments in which homologous or related probes were used (Puvion-Dutilleul et al., 1992; Olmedilla et al., 1993) are probably due to the lower complementarity of the rDNA sequences between the two species, as suggested by the hybridiza-

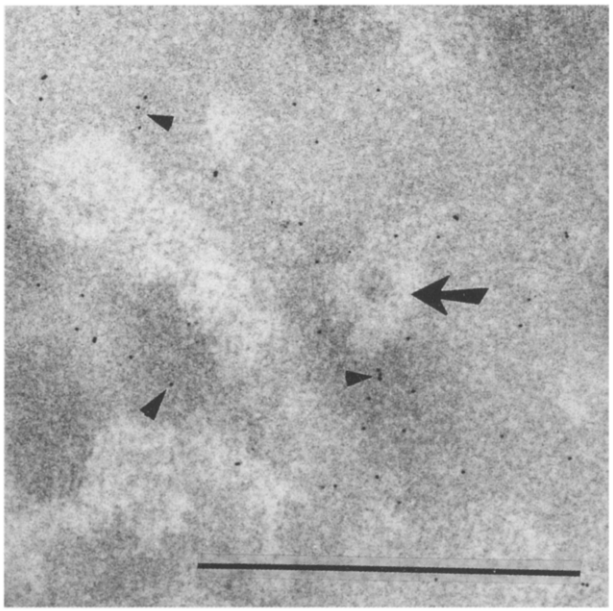


Figure 6. Localization of snoU3RNA in a nucleolus in the process of activation. The gold particles are localized in the fibrillar component (arrowheads). The interior of the FC is devoid of labeling (arrow). × 50 000. Bar, 1 µm.

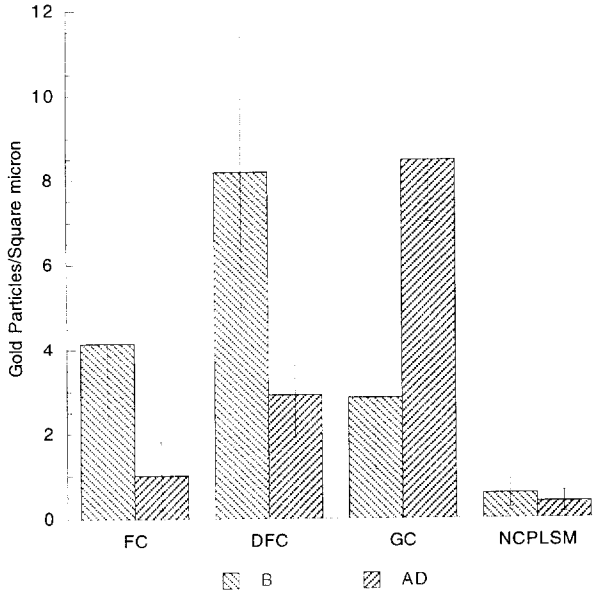


Figure 8. Distribution of the labeling obtained with probes B and AD in nucleoli of fully active cells.

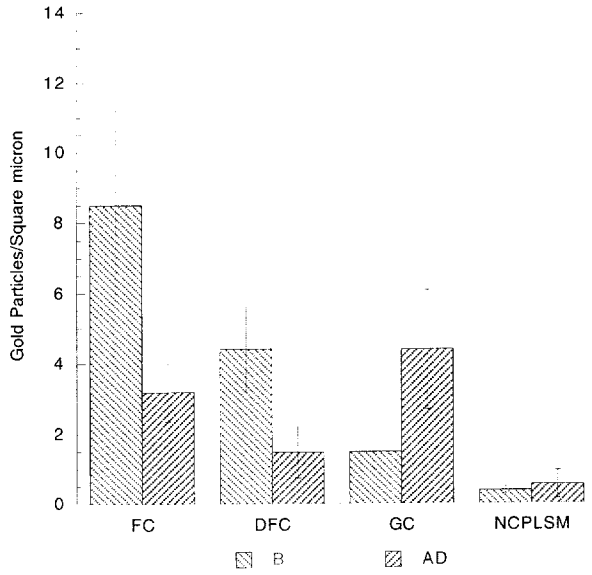


Figure 7. Distribution of the numerical density of rRNA labeling with probes B and AD, in the different nucleolar domains of cells in the process of activation. FC, fibrillar centers, frequently heterogeneous ones; DFC, dense fibrillar component; GC, granular component; NCLPSM, nucleoplasm.

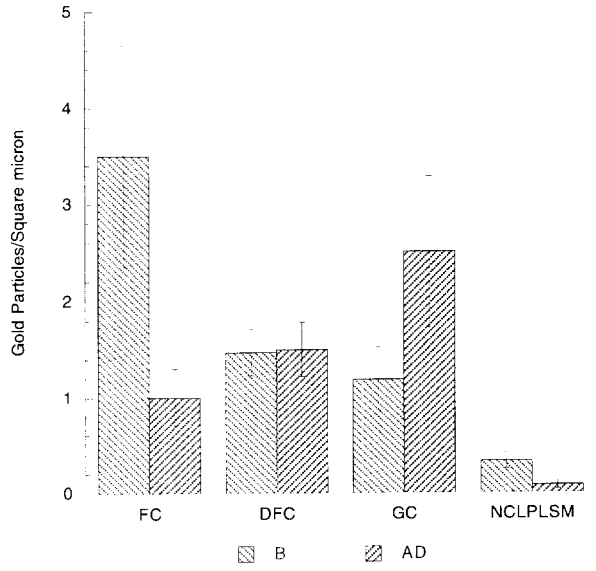


Figure 9. Distribution of the labeling obtained with probes B and AD in nucleoli of inactive cells.

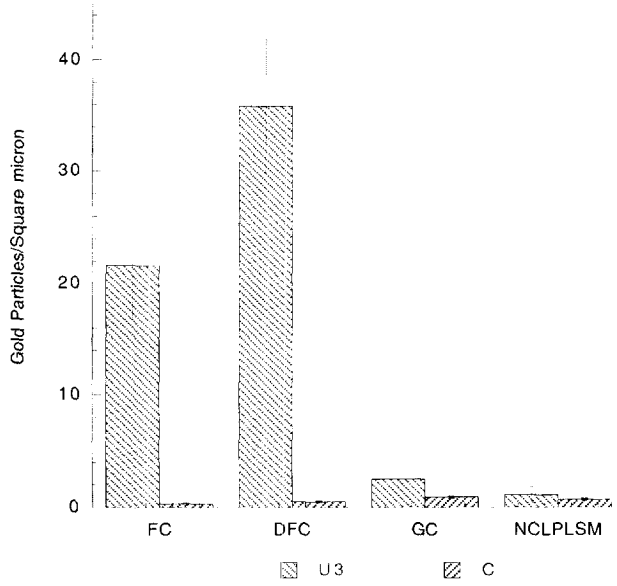


Figure 10. Distribution of labeling with U3 and C probes.

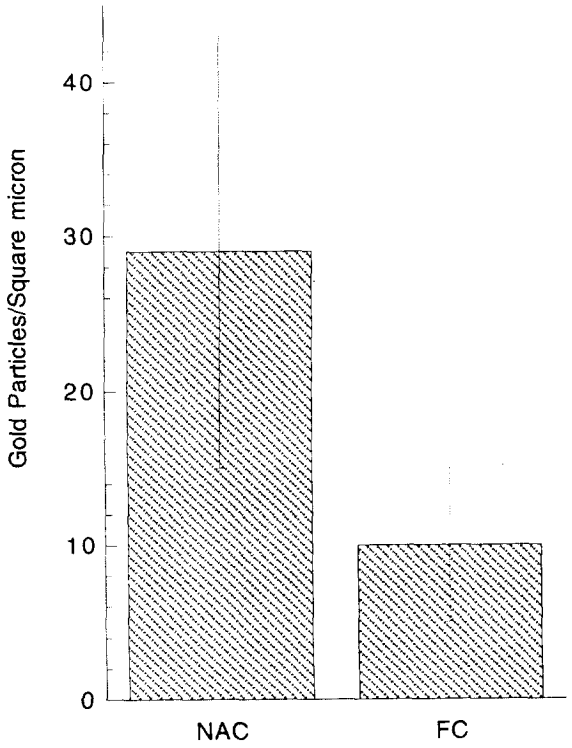


Figure 11. Distribution of the numerical density of rDNA labeling using B probe after denaturation of the DNA of the sections. The small clumps of compact chromatin located inside the FCs of nucleoli of cells in the process of activation after dormancy are significantly labeled. Background level is 0.8 ± 0.5 S.D.

tion signals obtained in Southern blots with human and pea probes.

ISH of biotinylated rDNA probes on thin LRW sections provides a precise localization of ribosomal gene products at the EM, better than that obtained by confocal microscopy (Leicht et al., 1992; Highett et al., 1993; Shaw et al., 1995; Beven et al., 1996).

The background labeling in our conditions was minimal, not only in the controls with unspecific DNA, but also in the hybridization with the IGS probes. That demonstrates that the unspecific binding of the probes to DNA-binding proteins is minimal without protease pre-treatment. We eliminated this hydrolysis to preserve the fine ultrastructure of the nucleoli permitting accurate quantification of the gold particles in relation to the different compartments. The extent of non-specific binding of the anti-biotin antibodies to the tissue was checked by incubation with the antibody without probe and was found to be negative. Labeling on the nucleoplasm and condensed chromatin masses with the specific probes was also negative.

The hybridization signals within the nucleolar domains show differences depending on the probe used. Probe B, containing the 5′ ETS region and a part of the 18S rRNA, mainly hybridize with heterogeneous FCs, and with the peripheries of pale FCs. This relatively high labeling reflects an abundance of very early transcripts in these regions. The high density of labeling of the same regions using U3 probe indicates that the processing of newly synthesized transcripts is taking place at the periphery of FCs and in DFC. Coincidences of the distribution of U3 probe and early stages of pre-rRNA transcription were also found in the nucleolus of mouse cells (Puvion-Dutilleul et al., 1997).

Probe AD containing portions of the 28 S rRNA and IGS sequences, hybridizes with GC to a larger extent than probe B, in cells with different metabolic activities. These data seem to indicate an underrepresentation of 18S rRNA in relation to 25S RNA sequences in the GC. This may be due to the higher rate of transport of 18S rRNA in relation to 28S rRNA or could reflect the characteristics of probe B, which only contains about 30% of coding sequences available to hybridize with the processed precursors of the GC, while the percentage is much higher for probe AD.

The clear FCs of active nucleoli never contain rRNA products in their interiors in spite of the accumulation on their peripheries. The EDTA staining procedure preferential for RNA demonstrated the presence of

RNA containing fibrils and nucleolar perichromatin granules, which have been postulated to contain unprocessed rRNAs (Moreno Díaz de la Espina and Risueño,1982; Risueño et al., 1982) inside the large heterogeneous FCs of early reactivating cells. The high labeling observed in this type FCs is associated with newly found RNP structures, and with RNA-containing peripheral fibrils. These results agree with previous reports of RNA-RNA ISH suggesting the presence of rRNA on plant FCs (Olmedilla et al., 1993).

Our data are consistent with the idea that the pre rRNAs are highly organized in the nucleolus. The early transcripts are localized inside the heterogeneous FCs of nucleoli in the process of activation, in the peripheries of clear FCs and in the inner region of DFC. The bulk of the DFC contains elongating transcripts and intermediate stages of processing, as suggested by the results of the ISH with U3 probe, while the GC would be enriched in processed rRNA. Similar results have been obtained by ISH in plants (Olmedilla et al., 1993; Shaw et al., 1995; Beven et al., 1996) and mammalian systems (Puvion-Dutilleul et al., 1991, 1992); and also by autoradiography after tritiated uridine incorporation (Olmedilla et al., 1993). In contrast, BrUr incorporation in permeabilized onion nucleoli localize the main synthesis of rRNA in domains of the DFC different from the peripheries of FCs. The lack of quantification of the labeling, the difficulties of identifying small FCs in extended nucleoli from protoplast under the experimental conditions used, and also the long pulses of BrUr incorporation, which would detect the processing sites in addition to transcription sites, would probably account for the discrepancies with our results (Melcák et al., 1996). The latter is also applicable to previous autoradiographic studies in the same system (Risueño et al., 1982; see Martin et al., 1989 for discussion). Recently studies using BrUTP incorporation localized by immunofluorescence and immunoelectron microscopic procedures, as well as ISH with a probe to ETS, suggest that rRNA transcription takes place in small foci, each corresponding to the product of a single gene (Thompson et al., 1997).

The data of specific localization of rRNA by ISH, together with previous data of nucleolar ultrastructure (Risueño and Moreno Díaz de la Espina, 1979; Moreno Díaz de la Espina et al., 1980; Risueño et al., 1982; Medina et al., 1983), immunolocalization of DNA (Martin et al., 1989) and nucleolar proteins (Martin et al., 1992; Cerdido and Medina, 1995), reveal: 1) the existence of a fine and constant organization of the nucleolar functions in plants, in which, each structural domain would support specific nucleolar functions; 2) that FCs constitute core structures of the nucleolar activity; and 3) the presence of RNA-containing fibrils and nucleolar perichromatin granules inside the large heterogeneous FCs in very early reactivating nucleolus.

These data are consistent with those previously reported in plant cells (De la Torre and Giménez Martín, 1982; Shaw et al., 1995; Beven et al., 1996). Present results confirm and extend our previous ideas for nucleolar functioning in these systems (Martin et al., 1989; Medina et al., 1990). The morphological and functional association between the FC and DFC is very strong in the nucleolus and suggest a role for FCs as central structures for nucleolar transcriptional activity. A lot of evidence supports the idea that the assemblies formed by a FC and the surrounding DFC are basic structures for rRNA synthesis and processing. First, the tight association between FCs and DFC is constant in almost all kinds of plant nucleoli independently of their morphology (Risueño and Moreno Díaz de la Espina, 1979; Medina et al., 1983). Second, the application of hypotonic buffers or 5,6-dichloro-1B-D-ribofuranosylbenzimidazole (DRB) treatment to plant cells, produces structures containing FCs associated to a layer of DFC organized in necklaces (unpublished results), which are able to sustain rRNA synthesis in absence of an organized nucleolar structure (Scheer and Benavente, 1990; García Blanco et al., 1995). Third, the immunolabeling results of previous works (Moreno Díaz de la Espina, 1995), showed that the transcription and processing machinery, as well as the nucleolar DNA loops are anchored in the peripheries of FCs forming bipartite domains composed of a FC and the inner part of the DFC. And last but not least, the experimental results of short term Br-UTP incorporation (Hozák et al., 1994), as well as our results of in situ hybridization have demonstrated that the boundaries between FCs and DFC are the sites of rRNA transcription and processing. Taken all data together, it is tempting to hypothesize that the nucleolus has a morphological and functional organization composed of: 1) bipartite domains formed by FCs surrounded by DFC, which are associated with rRNA transcription and processing; and 2) the GC representing a store of preribosomal particles. The organization of these subdomains would be highly dynamic and vary according to nucleolar activity, cell type, and species (Risueño and Moreno Díaz de la Espina, 1979; Moreno Díaz de la Espina et al., 1980; Medina et al., 1983).

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