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Original Article

Three-dimensional Electron Microscopy of Ribosomal Chromatin in Two Higher Plants: A Cytochemical, Immunocytochemical, and In Situ Hybridization Approach[1]

PATRICK M. MOTTE,[2] ROLAND LOPPES, MONIQUE MENAGER, and ROGER DELTOUR

Laboratoire de Morphologie Végétale (PMM,RD) et de Génétique Moléculaire (RL), Département de Botanique, Université de Liège, B4000 Liège, Belgium, and Laboratoire d'Histologie, Embryologie et Cytogénétique, Faculté de Médecine, 51100 Reims, France (MM).

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We report the 3-D arrangement of DNA within the nucleolar subcomponents from two evolutionary distant higher plants, *Zea mays* and *Sinapis alba*. These species are particularly convenient to study the spatial organization of plant intranucleolar DNA, since their nucleoli have been previously reconstructed in 3-D from serial ultra-thin sections. We used the osmium ammine-B complex (a specific DNA stain) on thick sections of Lowicryl-embedded root fragments. Immunocytochemical techniques using anti-DNA antibodies and rDNA/rDNA in situ hybridization were also applied on ultra-thin sections. We showed on tilted images that the OA-B stains DNA throughout the whole thickness of the section. In addition, very low quantities of cytoplasmic DNA were stained by this complex, which is now the best DNA stain used in electron microscopy. Within the nucleoli the DNA was localized in the fibrillar centers, where large clumps of dense chromatin were also visible. In the two plant species intranucleolar chromatin forms a complex network with strands partially linked to chromosomal nucleolar-organizing regions identified by in situ hybridization. This study describes for the first time the spatial arrangement of the intranucleolar chromatin in nucleoli of higher plants using high-resolution techniques. (*J Histochem Cytochem 39:1495-1506, 1991*)

KEY WORDS: Nucleolus; rDNA; Nucleolus-associated chromatin; Root cells; Anti-DNA antibody; Osmium ammine; *Zea mays*; *Sinapis alba*.

Introduction

The chromatin organization of the interphase nucleus has been investigated for many years. From recent data, we can argue that interphase chromosomes occur in well-defined nuclear domains (8,21,29,30,36,37). Concerning the nucleolar chromatin, it is well known that each metaphasic nucleolus organizer region (NOR), which contains the genes coding for the precursors of ribosomal RNA (rDNA), is a well-defined part of an NOR-bearing chromosome. The number of NOR-bearing chromosomes varies according to the species. At the end of telophase each NOR, or part of it, is responsible for the formation of one nucleolus (9,11,17,31). During interphase the nucleoli may fuse together. However, the exact localization of the nucleolar chromatin, i.e., of the rDNA molecule, within the active nucleolus is still unknown and is the subject of controversy (see Discussion). Its three-dimensional distribution is still totally unknown. By electron microscopy, five subcomponents can be distinguished in a transcriptionally active nucleolus: the fibrillar centers (FCs), the dense fibrillar component (DFC) which surrounds the FCs, the granular component (GC), the highly contrasted nucleolus-associated chromatin (NAC) observed at the periphery of the nucleolus, and small, less electron-opaque spaces, referred to as nucleolar vacuoles.

The unambiguous localization of rDNA in these subcomponents could be essential for the understanding of the functional organization of the nucleolus. In the present study we have used DNA-specific osmium ammine staining on thick sections, immunocytochemical technique, and in situ hybridization to localize rDNA and to study its spatial distribution in an active plant nucleolus at the electron microscopic level. Compared with animal material, the plant cell nucleolus exhibits specific advantages: (a) The NAC, restricted to heterochromatic knobs located at the nucleolar periphery, are parts of the interphasic NORs (32,36,37; and this report). On the contrary, in animal nucleoli, a peripheral ring of NAC of an unknown nature is always observed. The relationships between this peripheral chromatin and the intranucleolar rDNA are not well defined (43); (b) the DFC is large and constitutes the main structure of the plant active nucleolus; and (c) the active nucleoli of the two plant species here studied have been previously three-dimensionally reconstructed from serial ultra-thin sections (10,32).

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[2] Correspondence and present address: Dr. Patrick M. Motte, Max-Planck-Institut für Züchtungsforschung, 10 Carl-von-Linné Weg, D-5000 Köln 30, Germany.

The localization and spatial arrangement of ribosomal chromatin is discussed and compared with the recent data on rDNA distribution in both plant and animal nucleoli.

Materials and Methods

Germination Procedure

Batches of 30 seeds of *Zea mays* and *Sinapis alba* were germinated in petri dishes on cotton wool and filter saturated with distilled water. They were incubated in darkness in an Ehret-controlled temperature chamber at 16°C and 9°C, respectively. Germination began when the seeds were placed in contact with water. The maximum percentage of germinated seeds was always over 90%, and the rates of germination were very similar for both species. One hundred twenty hours after germination embryonic roots were about 3 cm in length.

Tissue Preparation

The distal millimeter of roots was excised from quiescent embryos and, 120 hr after sowing, rapidly immersed in a solution of 4% formaldehyde (freshly prepared from paraformaldehyde) in 0.1 M Sorensen buffer (pH 7.0) and maintained for 2 hr at room temperature. The root caps were excised and discarded. The root fragments were washed three times in 0.1 M Sorensen buffer at 4°C. The samples were then embedded in Lowicryl K4M at low temperature according to Carlemalm et al. (6) with longer infiltration periods. Semi-thin and ultra-thin sections were cut with a diamond knife in the cortex 1 mm from the root tips with a Sorval Porter Blum MT2 ultramicrotome. After the reactions described below, the sections were observed with a Siemens Elmiskop 101 or a Zeiss electron microscope EM109 at 80 kV.

Osmium Ammine-B (OA-B) Staining

OA-B was synthesized according to Olins et al. (33). Semi-thin sections about 0.5 µm thick realized in maize and *Sinapis alba* root fragments were mounted on 300-mesh gold grids without formvar support. Before the OA-B staining the sections were treated with 1 mg/ml pre-digested pronase (Boehringer; Mannheim, FRG) for 1 hr at 37°C in Tris-HCl 10 mM (pH 7.2). For the staining, the grids were first floated, sections facing the solution, on the surface of a 5 N HCl solution for 25 min at room temperature. The grids were then thoroughly washed with distilled water and floated on some drops of water until osmium ammine-B solution was ready. A 0.2% aqueous solution of OA-B was bubbled with SO_2 for 20 min. The staining of DNA was performed for 60 min at 37°C in a well-covered container, sections floating on the solution. The grids were thoroughly jet-washed with distilled water. We performed successively two ultra-thin and one semi-thin serial sections. The two ultra-thin sections were stained with uranyl and lead (Ur-Pb), while the thick section was stained with OA-B.

Some thick sections were observed with a Jeol 200CX at 200 kV. Sections were tilted with an eucentric goniometer stage from −18 to +18° by 6° steps. Stereo pairs of tilted images were mounted to be observed with a pocket stereoviewer according to Ploton et al. (35).

Electron Microscopic Immunocytochemistry

Yellow ultra-thin sections of embedded *Zea mays* fragments were mounted on formvar-coated gold grids. For labeling the grids were processed at room temperature as described below. The sections were incubated on 20 µl of PBS, pH 7.5, containing 0.5% BSA, 0.1% Tween 20 (PBST), and normal goat serum diluted 1:20, for 30 min. Then the grids were floated on 10 µl of PBS containing IgM mouse monoclonal DNA antibodies (30 µg/ml; Boehringer) and 1:50 normal goat serum, for 3 hr. The grids were washed with PBST twice for 10 min, then were incubated on 20 µl of PBST (pH 8.2) containing 1:75 goat anti-mouse IgM coupled to 5-nm gold particles

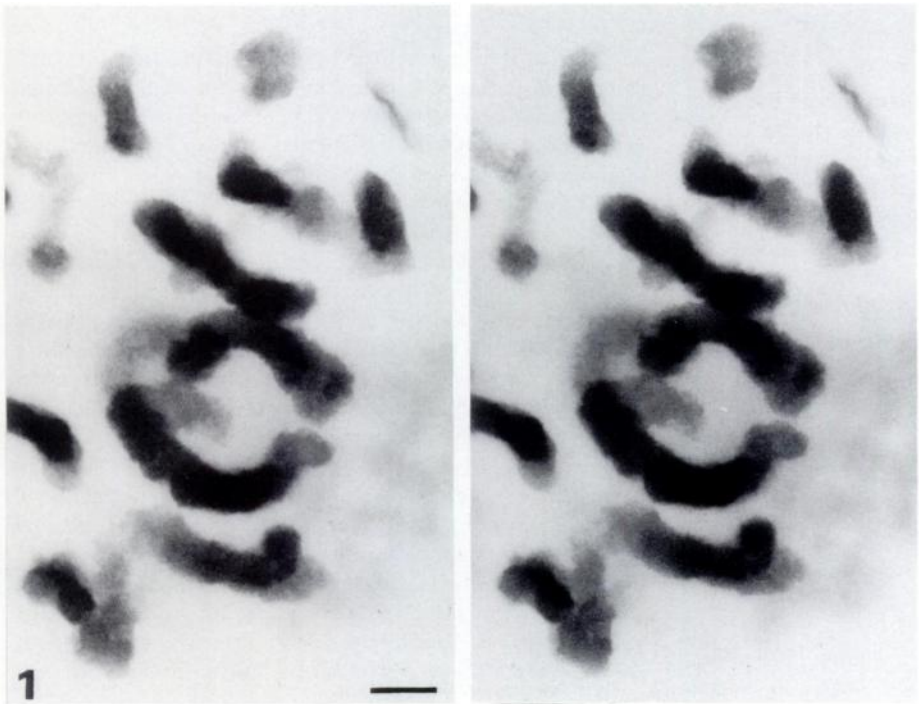


Figure 1. Stereo pair of *Zea mays* metaphase chromosomes stained with OA-B complex, observed at 200 kV. One-µm thick section. Long chromosome segments located at different level in the section are stained all along their length without interruptions. This demonstrates that the OA-B complex penetrates through the entire Lowicryl section. Note that when a chromosome is longitudinally sectioned the portion within the section is less thick and thus is less stained. Original magnification × 6800. Bar = 1 µm.

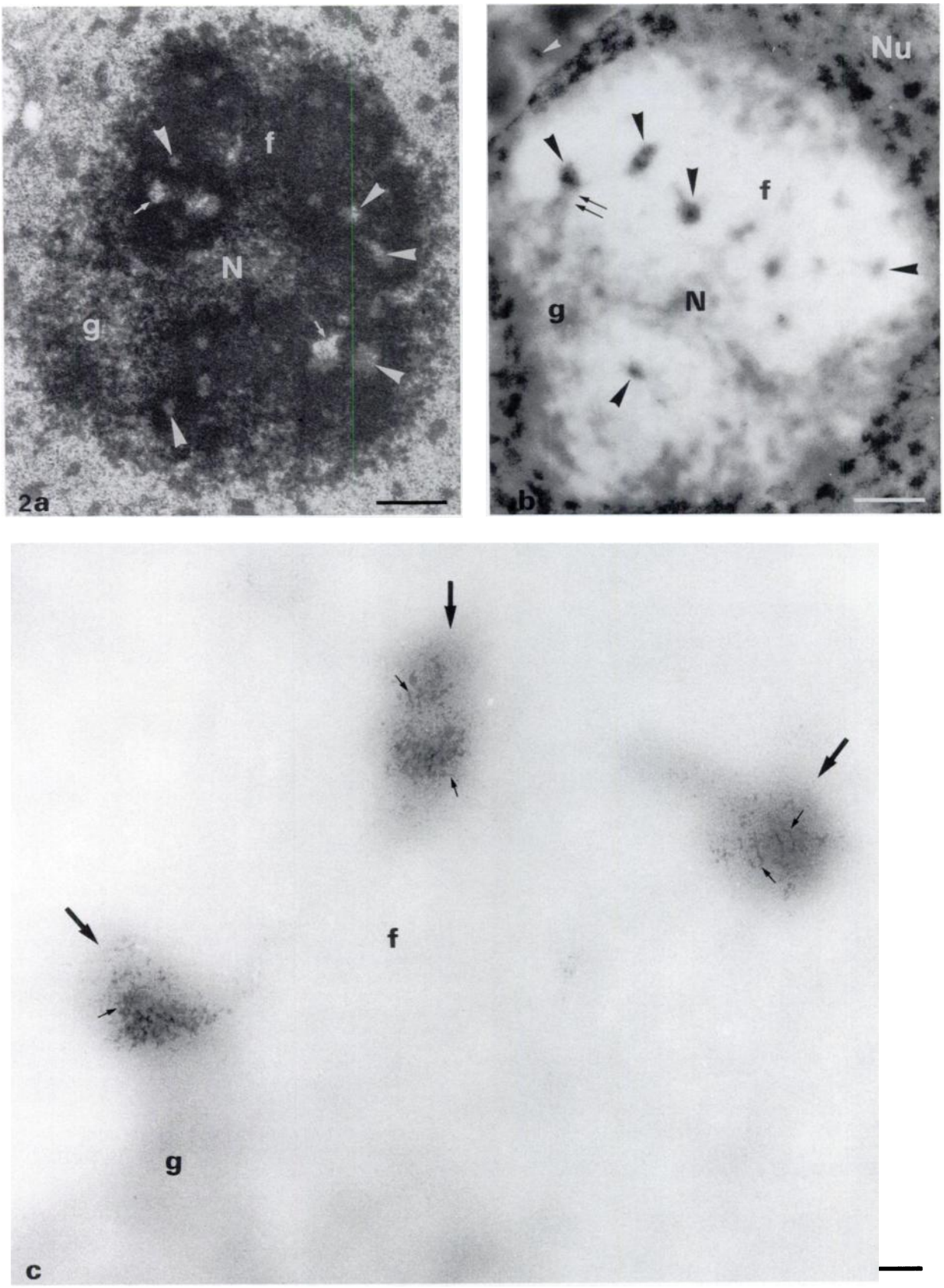


Figure 2. (**a**) Electron micrograph of a Lowicryl K4M-embedded nucleolus (N) of *Zea mays*, a monocotyledon. The subcomponents are visible and well discriminated: the DFC (f) is the main component surrounding a few FCs (arrowheads) often associated with small vacuoles or interstices (small arrows). The whole fibrillar component (DFC and FCs) which forms the nucleolonema is enclosed within the granular component (g). Uranyl and lead staining. (**b**) Semi-thin section of a nuclear portion of *Zea mays* stained with osmium ammine-B complex. The DNA-containing structures are well contrasted in the nucleoplasm (Nu). At low magnification, the nucleolar subcomponents are still easily visible owing to their remnant electron opacity after the pronase digestion. The DFC (f) appears like homogeneous bleached large zones and the GC (g) like interspersed clear gray and bleached zones. The FCs (arrowheads) appear like small contrasted areas embedded in the DFC and contain DNA stained by the OA-B complex. In some cases the granular component is directly in contact with a fibrillar center (double arrows). The cytoplasmic DNA is also visible (white arrowhead). (**c**) High magnification of the three upper left FCs (arrows) of **b**. Clumps of chromatin fibers (small arrows) are well visible inside small areas probably corresponding to FCs. DNA fibers are not observed within the surrounding DFC (f). g, granular component. Original magnifications: **a**,**b** × 6000; **c** × 20,000. Bars: **a**,**b** = 1 μm; **c** = 0.1 μm.

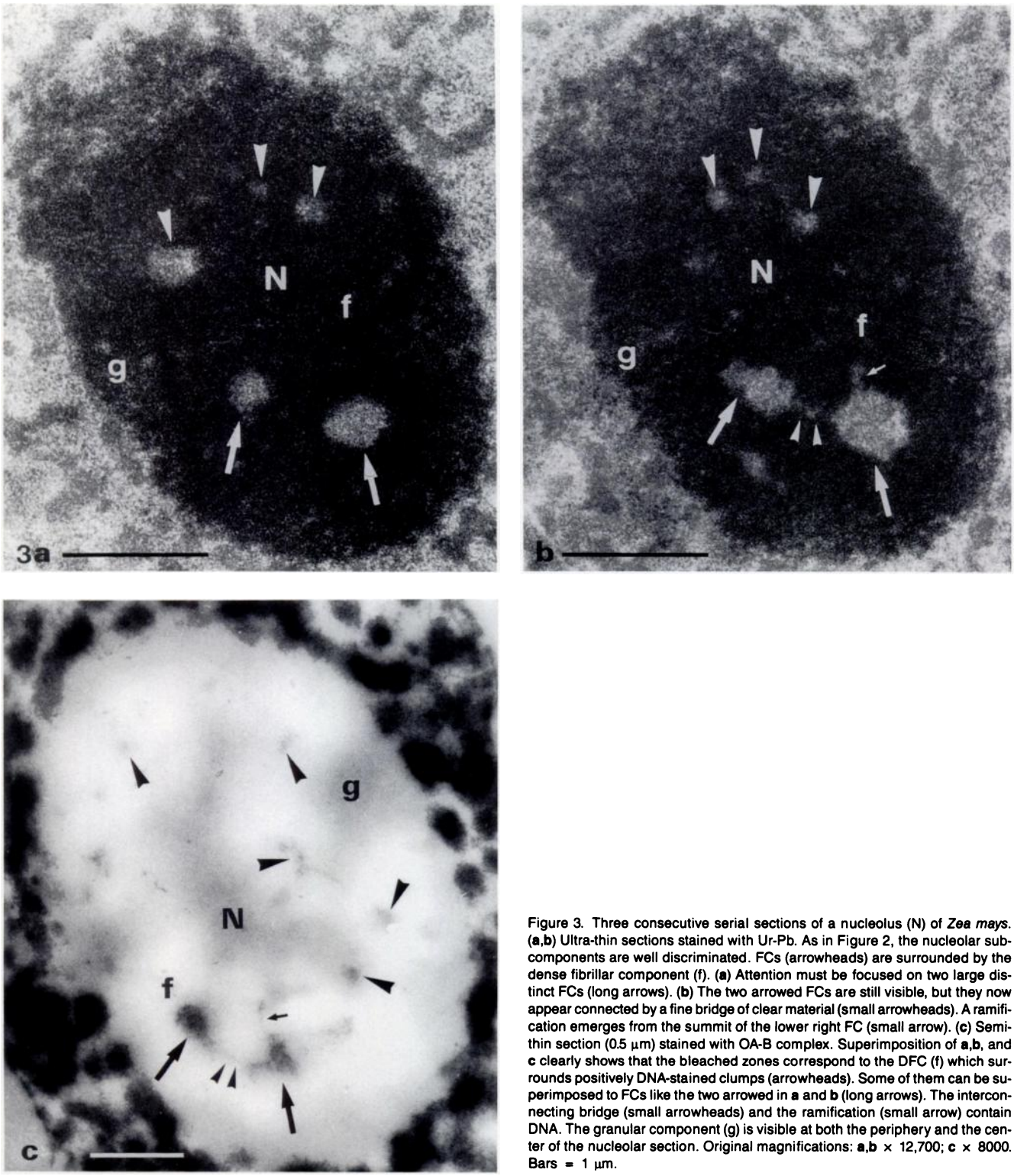


Figure 3. Three consecutive serial sections of a nucleolus (N) of *Zea mays*. (**a**,**b**) Ultra-thin sections stained with Ur-Pb. As in Figure 2, the nucleolar subcomponents are well discriminated. FCs (arrowheads) are surrounded by the dense fibrillar component (f). (**a**) Attention must be focused on two large distinct FCs (long arrows). (**b**) The two arrowed FCs are still visible, but they now appear connected by a fine bridge of clear material (small arrowheads). A ramification emerges from the summit of the lower right FC (small arrow). (**c**) Semithin section (0.5 μm) stained with OA-B complex. Superimposition of **a**,**b**, and **c** clearly shows that the bleached zones correspond to the DFC (f) which surrounds positively DNA-stained clumps (arrowheads). Some of them can be superimposed to FCs like the two arrowed in **a** and **b** (long arrows). The interconnecting bridge (small arrowheads) and the ramification (small arrow) contain DNA. The granular component (g) is visible at both the periphery and the center of the nucleolar section. Original magnifications: **a**,**b** × 12,700; **c** × 8000. Bars = 1 μm.

(Janssen Life Sciences; Beerse, Belgium) for 60 min. The grids were rinsed three times for 10 min in PBST and three times in distilled water. The sections were stained with uranyl acetate. Controls were performed by incubating sections with either distilled water, PBS, or normal mouse serum in place of the anti-DNA antibody. Ultra-thin sections labeled with the anti-DNA antibody were also stained by the OA-B as described above, without pronase digestion.

In Situ Hybridization Procedure

Preparation of the Biotinylated Probe. Plasmid pMrl (48) contains parts of the 25S and 18S rRNA coding regions and one complete spacer region of a maize rRNA transcription unit. The 0.75 KB Sma I fragment of the 25S rRNA coding sequence was subcloned into pUC9. The presence in the insert of Bgl II and Bam HI sites was verified. One microgram of the plas-

mid (pULG-1) was labeled with biotin-11-dUTP by nick translation using the BluGENE kit from Bethesda Research Laboratories (Gaithersburg, MD). The probe was purified by gel filtration on Sephadex G-50, precipitated by ethanol, then dissolved in 50 µl sterile water and stored at −20°C. Spots of 6 pg (in 3 µl) of the probe were easily detected on nitrocellulose filters using streptavidin–alkaline phosphatase conjugate, then nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate according to the recommendations of the supplier.

In Situ Hybridization (rDNA–rDNA). Yellow sections of *Zea mays* samples, collected on formvar-coated gold grids, were first submitted to enzymatic digestions performed at 37°C in a moist chamber. The sections were digested for 1 hr by 1 mg/ml pre-digested pronase in 10 mM Tris-HCl (pH 7.2), then treated for 1 hr by pre-boiled RNAse (1 mg/ml in 10 mM Tris-HCl, pH 7.2). The grids were then subjected to three 10-min rinses in distilled water and air-dried. Cellular DNA was denatured for 10 min at 80°C in 75% (v/v) deionized formamide in 2 × SSC (1 × SSC = 0.15 NaCl, 0.015 M sodium citrate, pH 7.0). The sections were then immediately dipped into ice-cold 0.1 × SSC, dehydrated in an ethanol series of 50%, 75%, and absolute ethanol, and air-dried for a few minutes. The probe diluted in the hybridization buffer (50% deionized formamide/10% dextran sulfate/1 × Denhardt's solution/1 mM EDTA/600 mM NaCl/500 µg.ml^{-1} herring sperm DNA/25 mM Tris-HCl, pH 7.2) was denatured for 4 min at 100°C. Hybridization buffer contained biotinylated probe at 0.8–1.0 µg/ml. The grids were floated overnight at 40°C in a moist chamber on 5 µl of the denatured hybridization solution. Grids were then floated three times for 10 min on drops of PBS, incubated successively in mouse anti-biotin antibody (Dako; Santa Barbara, CA) and goat anti-mouse IgG conjugated to gold particles 3–5 nm in diameter (Janssen), and finally stained with uranyl acetate before examination. Controls were performed by incubating sections on either hybridization buffer or hybridization buffer containing the biotinylated pUC9 plasmid.

Results

One hundred twenty hours after the onset of germination, the fully functional nucleolus was capable of both synthesizing and processing pre-rRNAs (50). It was composed of well-segregated granular and fibrillar components.

Osmium Ammine-B Staining

The osmium ammine complex is known to be a specific stain for DNA in electron microscopy (12,13). To increase the contrast of the chromatin fibers and to study three-dimensionally the intranucleolar

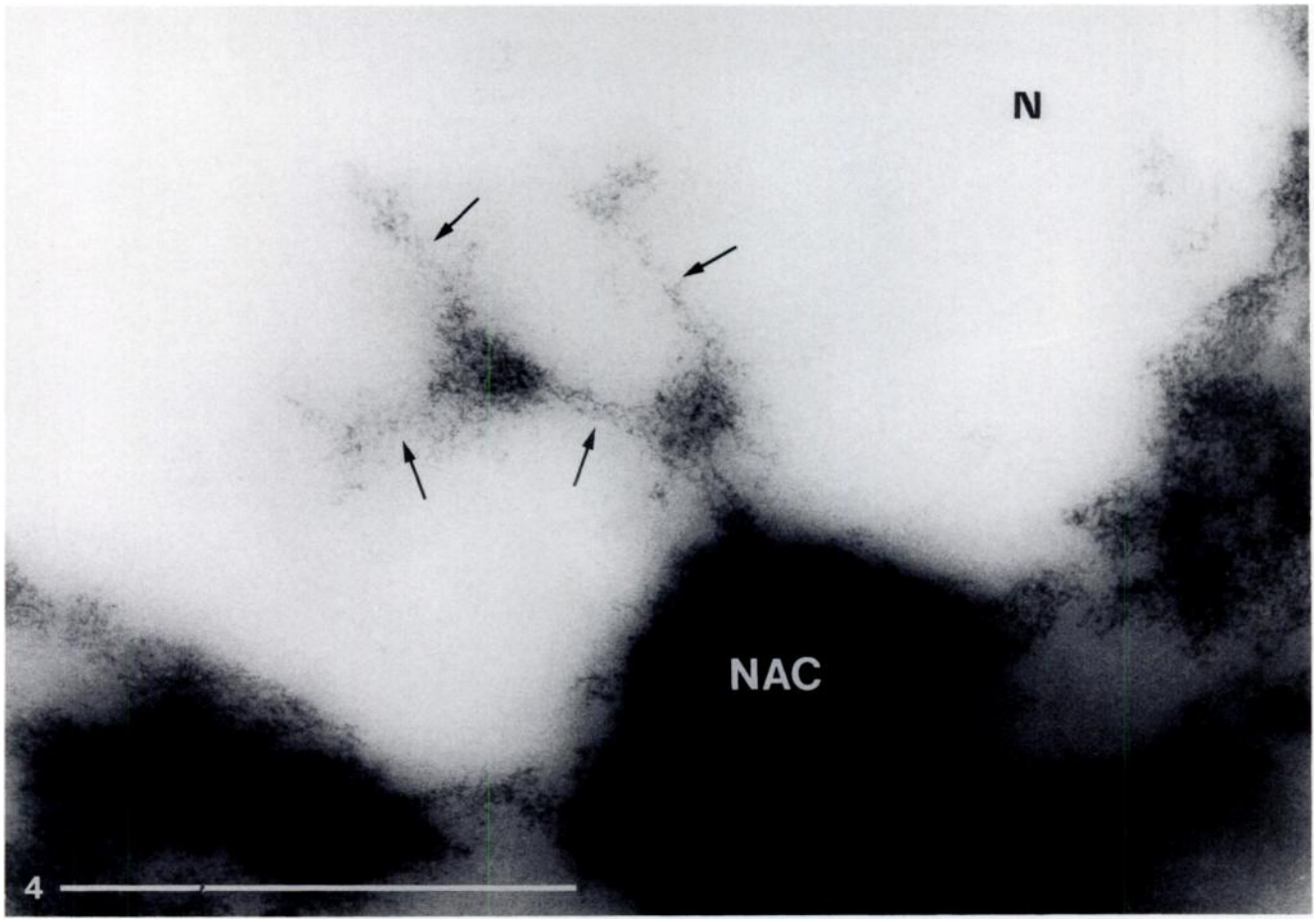
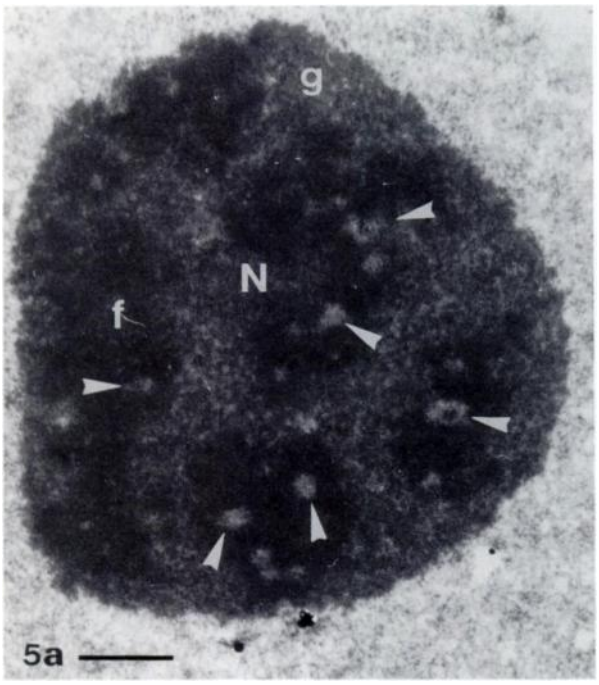


Figure 4. Semi-thin section of a maize nucleolus (N) stained with OA-B complex. One peripheral knob of the nucleolus-associated chromatin (NAC) is visible, from which a continuous and multibranched DNA-containing structure intrudes into the nucleolus. The ramifications are shown by arrows. Original magnification × 22,000. Bar = 1 µm.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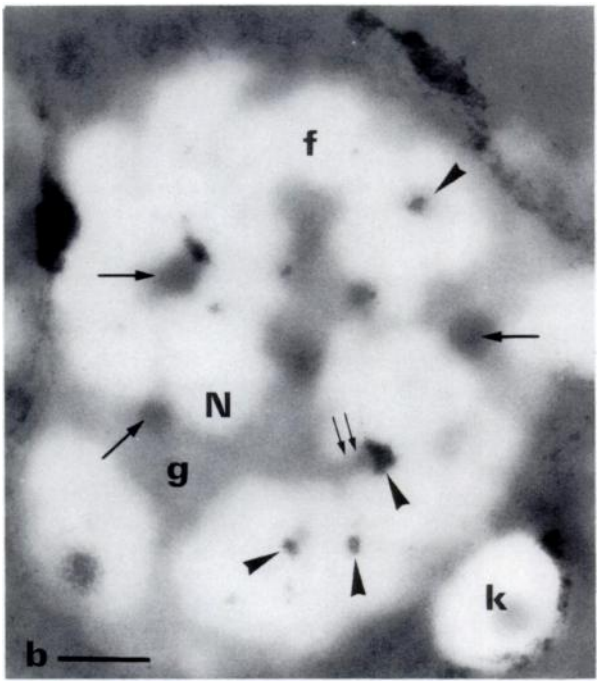
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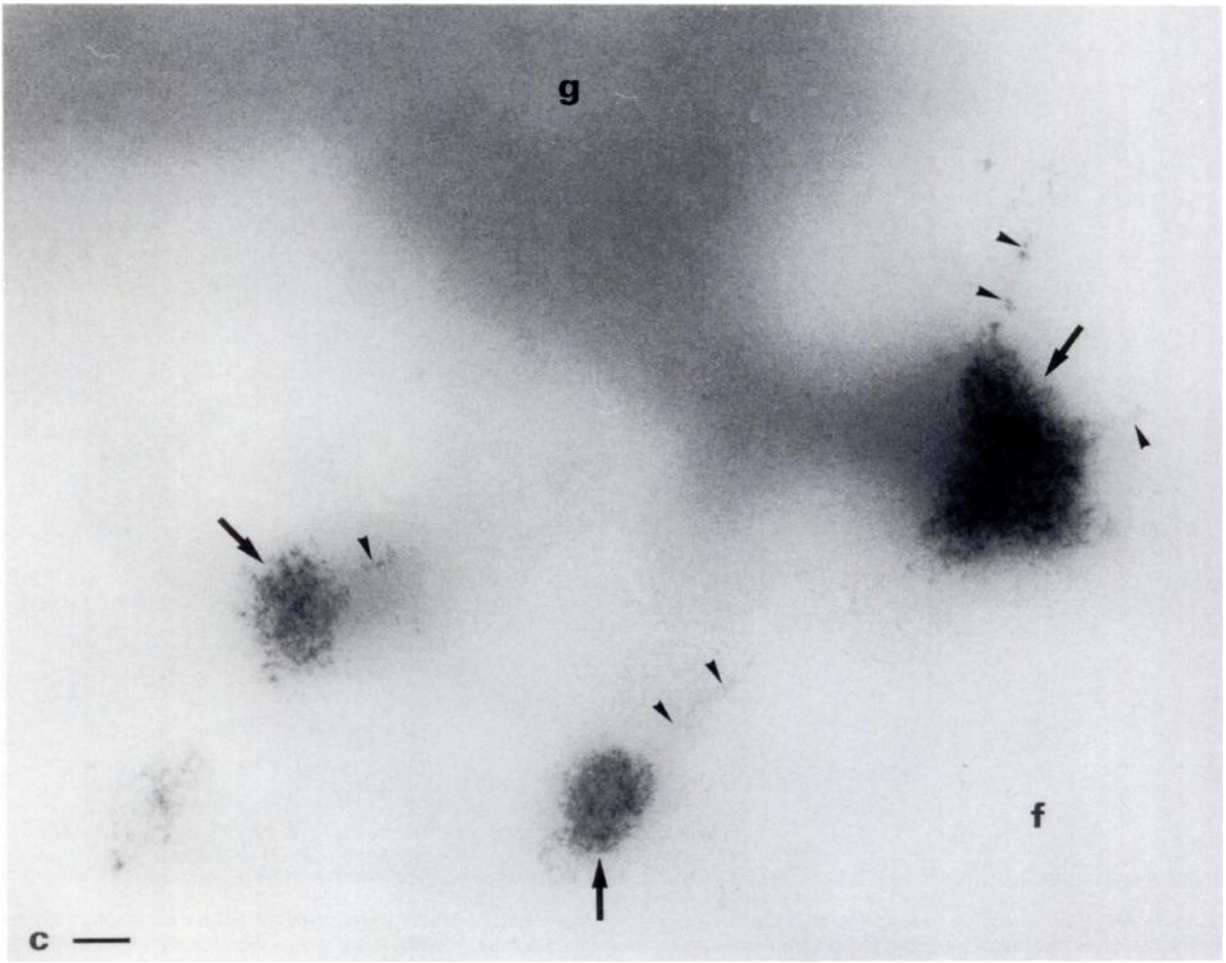
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DNA, the OA-B reaction was applied on semi-thin sections of Lowicryl K4M-embedded root fragments. In this polar embedding resin, the OA-B complex stained the DNA-containing structures throughout all the semi-thin section thickness, as seen very well on the tilts of the metaphasic chromosomes (Figure 1). It must be noted that in Lowicryl K4M sections the OA reaction is specific for the nucleic acids (12). However, the pre-treatment with hydrochloric acid removes all the ribonucleic acids from the section. Different times of HCl hydrolysis (from 10–35 min) were realized without modification of the specificity and quality of the staining (data not shown). When a DNAse digestion was performed, the contrast of DNA-containing structures disappeared (unpublished results). We performed a pronase digestion before OA-B staining to strongly decrease the electron opacity of the nucleolar section under the EM, which was mainly due to proteins. In our material and working conditions very good staining of chromatin fibers was obtained in *Zea mays* (a monocotyledon) as well as in *Sinapis alba* (a dicotyledon) (Figures 2–6). After the OA staining on thick sections the DNA was heavily stained in both the cytoplasmic organelles (mitochondria and plastids) and the nucleus (Figures 2 and 5). In the nucleoplasm, irregular clumps of chromatin, sometimes in contact with the nuclear envelope, were densely stained. From them, fine non-nucleosomal DNA filaments about 3 nm thick emerged and irradiated within interchromatin zones (data not shown). A large roundish space corresponding to the nucleolar body showed bleached areas surrounding some entities of higher electron opacity containing contrasted DNA filaments. The bleached areas correspond to the dense fibrillar component, which is known to contain a large amount of proteins (25,28). The small areas surrounded by the DFC correspond to the FCs. The granular component appeared like interspersed clear gray and bleached zones. To unambiguously identify the nucleolar subcomponents, we performed superimposition of successive ultra-thin sections stained with Ur-Pb and a thick section stained with the OA-B complex. Comparison of Figures 3a, 3b, and 3c clearly showed that the entities within the DFC were FCs containing chromatin fibers. Moreover, the DNA localization was sometimes restricted to particular regions of the FCs. The DFC and the GC did not contain any detectable DNA. At higher magnification on ultra-thin sections stained with OA-B, 10–13-nm thick granules considered as nucleosomes were seen in the FCs (Motte et al., manuscript in preparation). On some sections we could see very well the continuity and the ramifications of the intranucleolar chromatin which is located in a complex network (Figures 4–6). The internal NAC appeared to be ramified as soon as it intruded within the nucleolus (Figure 4). The ramifications were often visible at the level of large masses of dense chromatin probably corresponding to the core of the heterogeneous FCs (Figure 6). In some rare sections, loops of internal NAC were observed (data not shown).

Immunocytochemical Localization of DNA

We localized the DNA by means of a mouse monoclonal antibody directed against single- and double-stranded DNA. Immunolabeling was performed on ultra-thin Lowicryl sections. After the immunoreactions gold particles were essentially observed over the dense chromatin in the nucleus (Figure 7) and over the clumps of dense NAC located at the nucleolar periphery (not shown). Large nucleoplasmic areas were devoid of labeling. Over the nucleolus, gold particles were present over electron-clear areas corresponding to FCs. On some sections the labeling was observed at the border of the FCs (Figure 7a). To compare the respective efficiency of the OA-B complex and the anti-DNA antibody, we stained ultra-thin Lowicryl sections by both methods. We observed that the gold particles were strictly located over structures stained with the osmium ammine-B complex, but the density of gold particles over the dense chromatin was weak (Figure 7b). This is due to the reaction of the anti-DNA antibody with only the DNA molecule at the surface level of the section, whereas the OA-B stains DNA included in the entire section thickness. Thus, the use of the anti-DNA antibody is insufficient to reveal the continuity of the intranucleolar chromatin.

In Situ Hybridization

We performed in situ hybridization on ultra-thin Lowicryl sections of quiescent maize embryos because the quiescent state offers certain advantages: the NAC appears like two knobs adjacent to the periphery of the nucleolus, is very compact, and permits high signal/background labeling. Indeed, after the in situ hybridization we saw very well the gold particles concentrated in the region corresponding to the NAC (Figure 8). Therefore, these knobs correspond to the NORs which, as previously shown, stretch out and run inside the nucleolus during early germination.

Discussion

Localization of DNA in the Interphase Nucleolus

Previous ultrastructural and cytochemical studies have suggested the presence of DNA in the fibrillar component of plant and animal nucleoli (1,17,28). More recently, by means of the OA reaction

Figure 5. (a) Electron micrograph of a nucleolus (N) of *Sinapis alba*, a dicotyledon. The dense fibrillar component (f) appears as large electron-opaque areas surrounding many small FCs (arrowheads). The granular component (g), which is more compact than in maize, envelopes the entire fibrillar component. (b) Semi-thin section of a nucleolus (N) of *Sinapis alba* stained with OA-B complex. As in maize, one can clearly recognize the strongly bleached DFC (f) surrounding small FCs (arrowheads) which react positively with OA-B. The granular component (g) appears like a homogeneous clear gray zone. Note that, first, the GC is frequently observed in close contact with FCs (double arrows), and second, nucleolar vacuoles (long thin arrows) are present in both the DFC and GC. They appear as areas slightly more electron opaque than the GC. This is due to the intrinsic opacity of the acrylic resin, which is more abundant in this nucleolar subcomponent. In the DFC the vacuoles are always associated with the DNA internal to FCs. k is probably a karyosome. (c) High magnification of the three bottom right FCs (arrows) of b showing the DNA clumps located in the FCs. The DFC seems to be devoid of DNA. The contact between the GC (g) and the DNA internal to FCs is particularly well visible. Small threads of DNA (small arrowheads) originate from the large clumps. They are probably ramifications or interconnections between chromatin clumps, as more clearly observed in Figure 6. Original magnifications: a × 4700; b × 6000; c × 20,000. Bars: a,b = 1 μm; c = 0.1 μm.

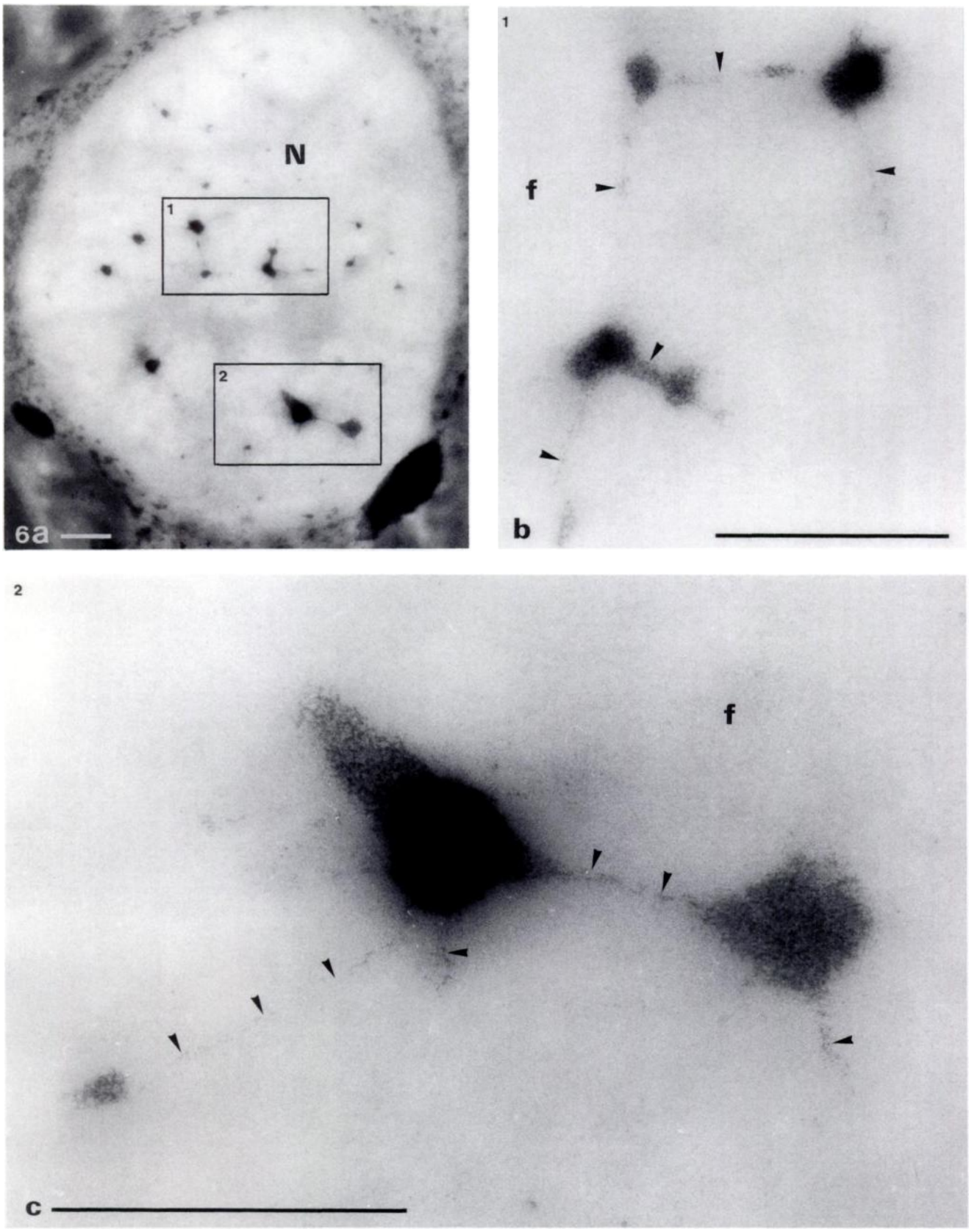

Figure 6. Semi-thin section of nucleolus (N) of *Sinapis alba* stained with OA-B complex. (**a**) The FCs form a complex network, the interconnections of which are small threads of DNA. (**b,c**) High magnifications of the two nucleolar portions 1 and 2. The progressive stretching out of the compact DNA (arrows) is clearly apparent (arrowheads). Some ramifications are so thin that they are probably constituted of one or a few rDNA molecules. Original magnifications: **a** × 4000; **b,c** × 20,000. Bars = 1 μm.

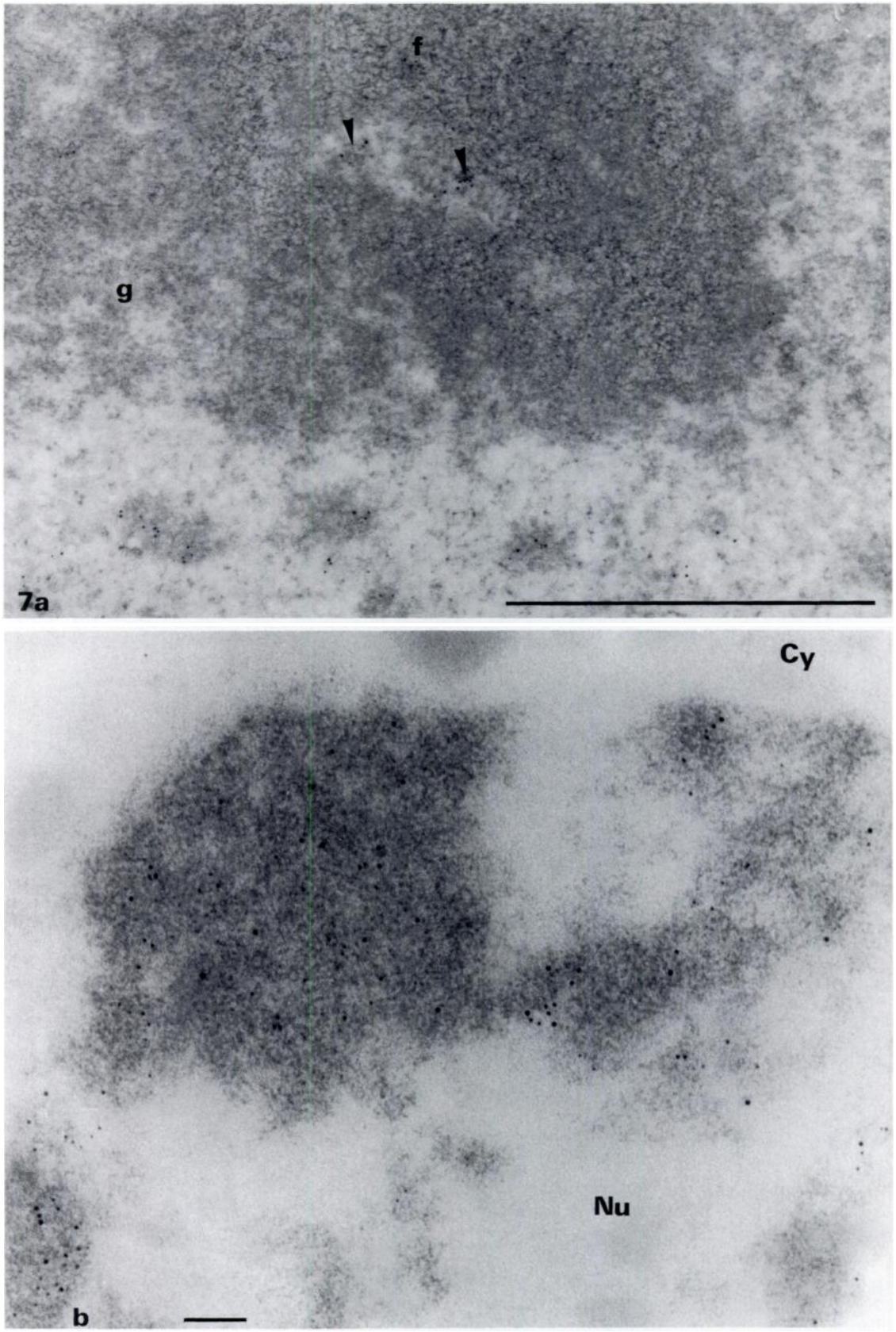

Figure 7. (a) Immunocytochemical localization of DNA on Lowicryl K4M sections of *Zea mays* using a monoclonal anti-DNA antibody. Uranyl staining. (a) View of an FC surrounded by a thick layer of DFC (f). Labeling is present in the FC (arrowheads). The DFC and the granular component (g) are devoid of gold particles. The fibrillar texture of the DFC is well visible on this micrograph. (b) Portion of a nucleus of *Zea mays* labeled with anti-DNA antibody and stained with the OA-B complex. Note that the HCl hydrolysis does not remove the gold particles. If there is a good superimposition of the detection methods, the density of the gold particles is not sufficient to detect all the DNA and to delineate precisely the DNA-containing regions. In contrast, the latter can be strictly mapped by the OA-B complex. Original magnification × 40,000. Bars: a = 1 μm; b = 0.1 μm.

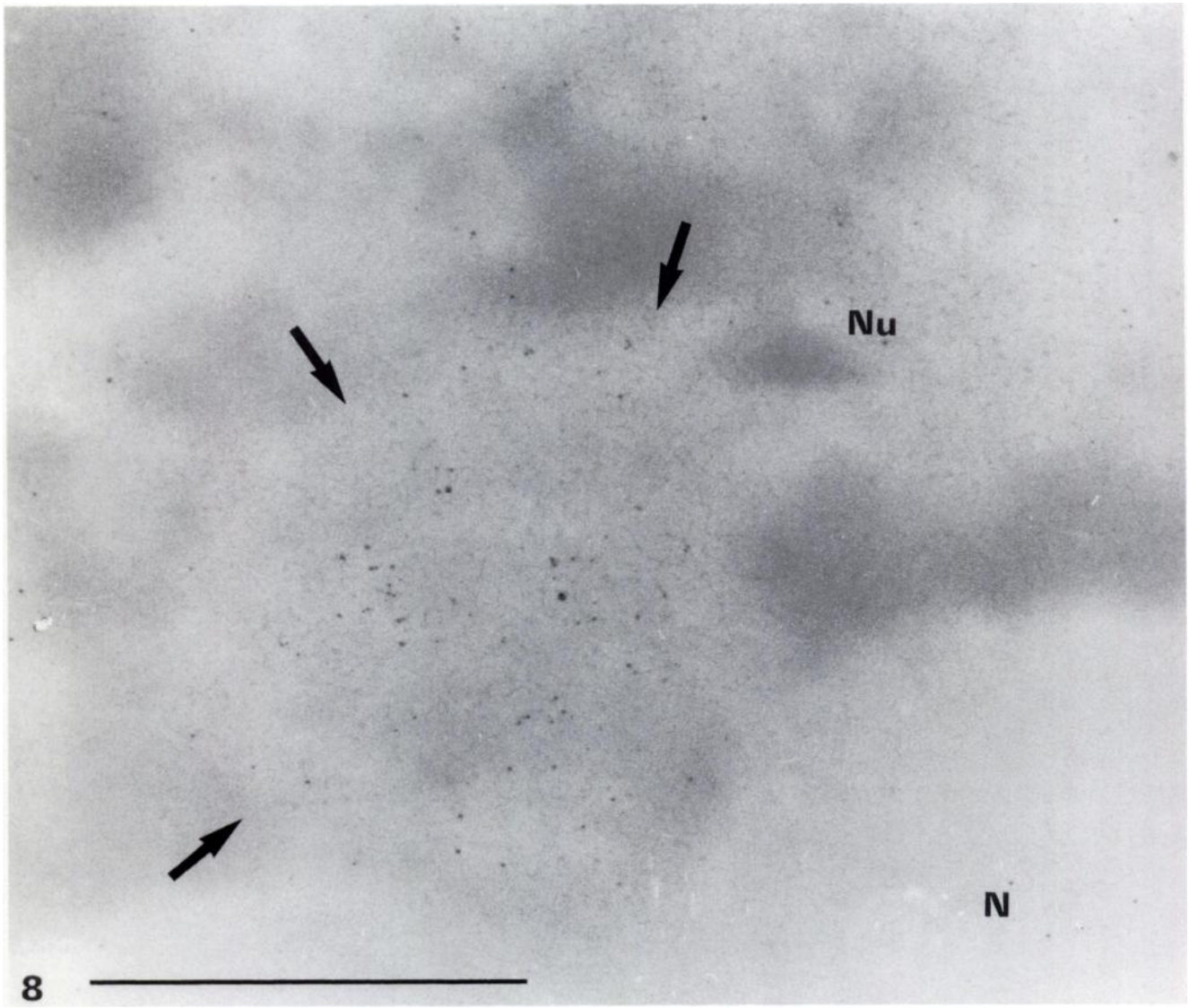

Figure 8. In situ hybridization on protease–RNAse-digested Lowicryl section of a quiescent maize nucleolus (N). The gold particles are restricted to the peripheral knob of the nucleolus-associated chromatin (arrows) in close association with fibers of DNA. The two knobs observed at the periphery of the maize nucleolus correspond to the two nucleolus-organizer regions present in this species. Original magnification × 20,000. Bar = 1 μm.

on animal tissues, Derenzini et al. (13) have established that DNA is present within the FCs and probably in the DFC, and is constituted of extended non-nucleosomal 3-nm thick filaments. However, after the OA reaction the identification of the FCs and tight DFC is not reliable in animal nucleoli. On the basis of qualitative (9,17) and quantitative (15) high-resolution autoradiographic studies, it has generally been admitted that the DFC corresponds to the transcriptionally active part of the interphase NOR. However, autoradiography can be criticized because a displacement of labeled primary rRNA transcripts could rapidly occur and/or the labeling could also result from 5S rRNA synthesized elsewhere in the nucleus and rapidly transferred into the DFC (44). In this connection, some recent biochemical studies have disclosed that the intergene spacer is transcribed in various animal cells (*Xenopus*, *Drosophila*, mammalian species) (3,7,14,20,22,45). The spacer transcripts are highly unstable in vivo and a rapid processing and degradation of them is likely to occur (20,45). Therefore, one can not state with certainty that DFC contains DNA. In addition, some recent immunocytochemical studies have led to challenge of this view. Scheer et al. (41,42) have detected the anti-RNA polymerase I antibodies exclusively in the FCs of various cell lines. A similar nucleolar localization was also found for anti-topoisomerase I and anti-DNA antibodies (38,40,46). Benavente et al. (4,5), using a different approach, support the idea that DFC is a genuine entity not constituted by the superimposition of transcriptional units of rDNA and primary transcripts. The rDNA localization with in situ

hybridization techniques in active animal nucleoli provides controversial results (47,51). Taken together, these results cast doubts on the localization of the primary sites of transcription in the DFC.

In the present study, we detect DNA mainly in the FCs of interphase plant nucleoli. The penetration of the OA complex throughout the section thickness shows any very weak quantity of DNA and thus allows study of the 3-D organization of DNA on thick sections. On semi-thin sections digested by pronase and stained by OA, the nucleolar subcomponents are well discriminated and recognizable by their electron opacity. The nucleolar subcomponents appear like a negative micrograph of an ultra-thin section stained with uranyl and lead. This was confirmed by the superimposition of successive ultra-thin sections and OA-stained thick section. It is beyond doubt that DNA chromatin fibers are located in FCs. A potential problem in the interpretation of our results is the possibility that the processes of the OA staining would specifically destroy the DNA fibers contained in the DFC. Since the OA complex stains very small DNA amounts in the FCs, in the nucleoplasm, and in the cytoplasmic organelles, it follows that if DNA is present in the DFC it should be structurally different and more flimsy than the other DNA fibers. Possibly this could occur following the pronase digestion. However, because we know of no data reporting such observations, this process appears to us highly improbable. Therefore, our results support the studies suggesting that rDNA is localized only in FCs in higher plant (36,37) as well as in animal cells (2,38,40–42,46).

Spatial Organization of the Intranucleolar Chromatin

Early cytological studies on the nucleolus have already described, in light microscopy, an intranucleolar filamentous structure generally called the "nucleolonema" (see 11 for references). These filamentous structures are detected in animal cells after drug treatment (16,18,26,39) or after removal of arginine from the culture medium (19), and in plant cells after treatment of isolated nuclei with a detergent (23,24). The nucleolonema may also appear as a natural structure in the epidermal cells of *Calpodes* in relation to the content of hemolymph ecdysteroid (27). In situ hybridization has shown that in human glioma cells, ribosomal genes form intranucleolar strands 0.2 μm thick (29).

By means of 3-D reconstructions of nucleoli from serial ultra-thin sections in the two species studied here, we have previously shown that the nucleolonema is ramified and appears as an arborescent structure mainly composed of fibrillar material, the axis of which appeared in transverse or oblique sections as homogeneous and heterogeneous FCs (10,32). However, we had no information on the localization of DNA and ribosomal genes in this filamentous structure. In the present study, we have shown a defined 3-D organization of the intranucleolar chromatin which forms a very complex filamentous structure within an active higher plant cell nucleolus. This structure is continuous and ramified. In 3-D reconstructions it was sometimes difficult to follow the continuity of the FCs. Indeed, we see very clearly on thick sections stained with the OA complex that the axis of the nucleolonema (containing DNA) can be so thin at some places (Figures 6 and 7) that it cannot be visible on ultra-thin sections stained with Ur-Pb. This led to the conclusion that the FCs are discrete entities. The body of the above data confirm here that each nucleolonema is fixed by its base to an interphase NOR-bearing chromosome from which it is a lateral extension.

The nucleolonemata probably contain the ribosomal genes since, during early germination, each of them comes from the stretching out and the intrusion within the nucleolus of the two peripheral knobs in which in situ hybridization has disclosed the presence of all the rDNA. These peripheral knobs correspond to the NORs. The two NORs adjacent to the nucleolar periphery observed in quiescent maize cells are still partially visible in the transcriptionally active nucleolus. They probably correspond to transcriptionally inactive rDNA of the heterochromatic segment of the interphase NOR not involved in the formation of the nucleolus (32,34,36,37). To our knowledge, this is the first time that in electron microscopy a very ordered spatial distribution of intranucleolar chromatin is described in situ in a particular nucleolar subcomponent, the FCs. As previously shown (9), the FCs of maize and *Sinapis* nucleoli are small entities (between 0.22–0.58 μm in diameter), although the transcription units of these two species measure respectively 1.7 and 1.9 μm. Moreover, the transcriptionally active ribosomal genes in one nucleolus could number several hundred (34). This means that the rDNA should be highly compacted in the FCs. In animal cells, Tröster et al. (49) have observed that these genes are compacted by a factor of 4.

Since we have observed arborescent nucleolonemata which sometimes present loops in two evolutionary distant plant embryos, we can propose that the nucleolonematal organization is general for higher plants.

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