Journal of Structural Biology **136,** 1–6 (2001)
doi:10.1006/jsbi.2001.4416, available online at http://www.idealibrary.com on IDEAL®

Further Ultrastructural Characterization of the Intranuclear Ring-Shaped Bodies of the Plant *Lacandonia schismatica*

Lourdes T. Agredano-Moreno,* Marco A. González-Jiménez,† M. de Lourdes Segura-Valdez,*,‡ Ernestina Ubaldo,* Clara H. Ramos,* Esteban Martínez,§ and Luis F. Jiménez-García*

**Departamento de Biología Celular, Facultad de Ciencias, UNAM, Circuito Exterior, Ciudad Universitaria, México, D.F., 04510, México; and †Instituto Nacional de Perinatología, ‡Instituto Nacional de Enfermedades Respiratorias, and §Instituto de Biología, UNAM, México, D.F., México*

Received March 1, 2001, and in revised form October 5, 2001

Ring-shaped bodies are found in the nucleus of *Lacandonia schismatica*, a rare plant with the sexual organs inverted. They are 0.5-μm-diameter structures that present an electron-dense external ring surrounding a central core. Ultrastructural studies indicate that these bodies contain RNA. The external ring is labeled with antibodies against small nuclear ribonucleoproteins, suggesting that they may be involved in pre-mRNA metabolism. In the present work we further characterized these intranuclear ring-shaped structures by serial-sectioning analysis. Moreover, we tested the presence of additional molecular elements related to pre-mRNA metabolism, such as SR proteins and poly(A)$^+$ RNA, using immunoelectron microscopy and ultrastructural *in situ* hybridization. Our results show that these nuclear bodies are spherical. They contain SR proteins involved in splicing and postsplicing events and little to no poly(A)$^+$ RNA. We also found similar nuclear bodies in other plant and animal species. Therefore, ring-shaped bodies in *L. schismatica* are spherical, highly compartmentalized nuclear structures that may be involved in pre-mRNA metabolism.

Key Words: **cell nucleus; *Lacandonia schismatica;* nuclear bodies; ring-shaped bodies.**

INTRODUCTION

Macromolecular domains involved in RNA metabolism are found within the mammalian cell nucleus. In plants, however, the reticulated and the chromocentric nuclear structures are described based upon heterochromatin arrangement. The nucleus of *Lacandonia schismatica* E. Martínez and C. H. Ramos (Lacandoniaceae:Triuridales), a rare plant with an inverted position of the sexual organs (Martínez and Ramos, 1989; Márquez-Guzmán *et al.*, 1989), is reticulated with a novel type of nuclear particle associated with it (Jiménez-García *et al.*, 1992; Agredano-Moreno *et al.*, 1994; Agredano-Moreno and Jiménez-García, 2000). In addition, the nucleus of this species also displays ring-shaped nuclear bodies.

The ring-shaped bodies of *L. schismatica* are 0.5–1 μm in diameter and they seem to be associated with the nucleolus, found free in the nucleoplasm, or found in close contact with some strands of compact chromatin (Jiménez-García *et al.*, 1992). The central core of these bodies may contain ribonucleoproteins (RNPs) (Jiménez-García *et al.*, 1992), as suggested by the preferential EDTA regressive staining of Bernhard (1969), while the external ring is enriched in snRNPs, as observed by immunoelectron microscopy studies (Agredano-Moreno *et al.*, 1994). These data suggest a role for these structures in pre-mRNA metabolism as assembly and/or storage sites of molecular elements involved in the splicing process (Agredano-Moreno *et al.*, 1994).

In order to further characterize and to compare the ring-shaped nuclear bodies found in *L. schismatica* to other known nuclear bodies, we have investigated their three-dimensional organization, their molecular composition, and their presence in other species by using standard transmission electron microscopy, immunoelectron microscopy for the splicing factor family of SR proteins, and ultrastructural *in situ* hybridization for RNA and poly(A)$^+$ RNA. We show that intranuclear ring-shaped bodies of *L. schismatica* are spherical structures that are enriched in SR proteins in the external ring. Similar structures are present in cells of other animal and plant species. Similarities to other nuclear bodies are discussed.

MATERIALS AND METHODS

Transmission electron microscopy. Flowers, stems, and roots of *L. schismatica* were fixed for 1 h in 6% glutaraldehyde buffered

1047-8477/01 $35.00

in 0.16 M phosphate (pH 7.2). Postfixation was made in 1% osmium tetraoxide overnight. Samples were subsequently dehydrated in a graded series of ethanol and embedded in an epoxy resin (glycide ether 100, Merck). Thin sections 60–90 nm in thickness were contrasted with conventional uranyl acetate–lead citrate. Leaves of the tree *Gingko biloba* and flowers of the saprophytic plant *Voyria aphyla* (Gentianaceae: Gentianales) were processed for conventional electron microscopy in the same way. Tomato (*Lycopersicon esculentum*) root tips were processed for the phosphotungstic acid (PTA) preferential staining of chromatin (Vázquez-Nin *et al.,* 1973) by using PTA solution at pH 2.3. Rat liver and PtK2 cells were also processed as mentioned above. For serial-section analyses, thin sections were stained with lead citrate and uranyl acetate and ribbons were collected on slotted grids.

Immunoelectron microscopy. Immunoelectron microscopy was conducted as described (Agredano-Moreno and Jiménez-García, 2000). Briefly, fragments of flowers of *L. schismatica* were fixed with a mixture of 0.5% glutaraldehyde and 4% paraformaldehyde for 1 h at room temperature. Lowicryl K4M embedding was conducted as described (Roth, 1986), and 60- to 90-nm thin sections were collected on Formvar-coated gold grids. Grids were floated in Tris-buffered saline (TBS) containing 20 mM Tris (pH 7.6), 150 mM NaCl, 20 mM sodium azide, 1.0% Tween 20, 10% bovine serum albumin (BSA), and 10% normal goat serum for 60 min. Sections were then incubated with anti-SR monoclonal antibody 3C5 (Turner and Franchi, 1987; courtesy of Dr. D. Spector, CSHL), diluted 1:30 in TBS at 4°C for 16 h in a humidified chamber. Grids were washed for 15 min in TBS and then incubated with goat anti-mouse IgM secondary antibody coupled to 10-nm colloidal gold particles, diluted 1:10 in TBS for 1 h at room temperature. After incubation, the grids were rinsed for 15 min with TBS and for 10 min with water. Finally, samples were stained with uranyl acetate and lead citrate.

Genomic DNA extraction. Total DNA of *L. schismatica* was extracted according to standard protocols (Sambrook *et al.,* 1989), with modifications. Unless otherwise noted, all procedures were performed at 4°C. Briefly, plants of *L. schismatica* (1.32 g) were frozen in liquid nitrogen and ground to a fine powder in a mortar. Then, 10 ml of extraction buffer was added (100 mM Tris–HCl, pH 8, 50 mM EDTA, 10 mM β-mercaptoethanol, 1.3% SDS). The homogenate was incubated at 65°C for 10 min. Then 3.3 ml of potassium acetate was added, incubated for 30 min on ice, and then centrifuged for 45 min at 15 000 rpm. The supernatant was filtered into a tube containing 10 ml of isopropanol and incubated at −20°C for 1 h. DNA was collected and transferred to 350 μl of dilution buffer (50 mM Tris–HCl, pH 8, 10 mM EDTA). Two microliters of RNase (10 mg/ml) was added and incubated for 1 h at 37°C. A second precipitation of DNA was carried out by adding 37 μl of 3 M sodium acetate and 250 μl isopropanol for 2 h at −20°C. DNA was collected, rinsed in 80% ethanol, and resuspended in TE (10 mM Tris–HCl, pH 8, 1 mM EDTA). DNA was stored at 4°C. Total DNA was labeled by nick translation as described (López-Velázquez *et al.,* 1998) and then used as a probe.

In situ hybridization to detect total RNA. The ultrastructural *in situ* hybridization protocol was carried out basically as described (López-Velázquez *et al.,* 1998). Small fragments of floral buds of *L. schismatica* were fixed in 4% paraformaldehyde for 1 h at room temperature. Lowicryl thin sections (60–90 nm) were collected on Formvar-coated gold grids. One microliter of genomic DNA was labeled by nick translation in the presence of biotinylated dUTP. The reaction was carried out for 4 h at 16°C in order to obtain fragments of ~125 bp. Ten microliters of this nick-translated probe, 10 μl of formamide, and 1 μl of competitor *Escherichia coli* tRNA were denatured for 4 min at 90°C and immediately chilled on ice. For negative control experiments, no probe was added to the hybridization mixture. Hybridization buffer containing 4 μl of 50% dextran sulfate, 4 μl of 5% BSA, and 2 μl of 20× SSC was added. Grids were floated on 15 μl of this hybridization mixture and then incubated in a humid chamber for 18 h at 42°C. After being rinsed in 4× SSC at room temperature for 15 min, grids were incubated in streptavidin conjugated with 10-nm colloidal gold particles, diluted 1:20 in 4× SSC at 4°C overnight. Grids were rinsed with 4× SSC three times for 5 min each, rinsed with PBS three times for 5 min each, and rinsed with deionized water. Finally, sections were stained with uranyl acetate and lead citrate. Samples were examined at 80 kV with a Zeiss EM-10 transmission electron microscope.

In situ hybridization for poly(A)$^+$ RNA. *In situ* hybridization to poly(A)$^+$ RNA was performed as described (Agredano-Moreno and Jiménez-García, 2000). A biotin-labeled oligo(dT) 20-mer $(dT)_{20}$ (Boehringer) was used as a probe for *in situ* hybridization to poly(A)$^+$ RNA. Lowicryl sections were collected on Formvar-coated gold grids. Grids were floated in 10 μl of the hybridization mixture containing oligo$(dT)_{20}$, 2× SSC, 1 mg/ml of tRNA, 10% dextran sulfate, and 25% formamide. For negative control experiments, probe was not used in the hybridization mixture. Hybridization was performed at 42°C overnight. Grids were rinsed with 2× SSC three times for 5 min each, rinsed with PBS three times for 5 min each, and rinsed with deionized water. Uranyl acetate and lead citrate were used for contrast.

Quantitative analysis. To quantitatively evaluate the labeling for SR proteins, we estimated the number of gold particles using a 1-μm^2 grid. Values were represented as the number of gold grains per square micrometer.

RESULTS

Serial-Section Analysis of the Ring-Shaped Nuclear Bodies of L. schismatica

The serial-sectioning analysis of the ring-shaped bodies of *L. schismatica* revealed that they are spherical bodies that can be observed throughout five consecutive thin sections 70–90 nm in thickness, which results in bodies of about 0.5 μm in diameter. The shell is composed of strongly intermingled fibrils surrounding a less dense core of fibrils (Fig. 1). It is interesting to note that these bodies are associated with an environment enriched in *Lacandonia* granules.

Analysis of the Presence of SR Proteins

Ultrastructural immunolocalization using the monoclonal antibody 3C5 against SR proteins shows that the external ring that surrounds the central core is enriched in this family of nuclear proteins (Fig. 2; Table I) and, in some cases, thin fibers that radiate from them to the interchromatin space show intense labeling. Occasionally, the central core presents weak labeling.

Analysis of the Presence of RNA and Poly(A)$^+$ RNA

When using genomic DNA as a probe to detect total RNA in the ring shaped-bodies of *L. schismatica,* we observed labeling in the external ring of these structures (Fig. 3a). To detect poly(A)$^+$ RNA,

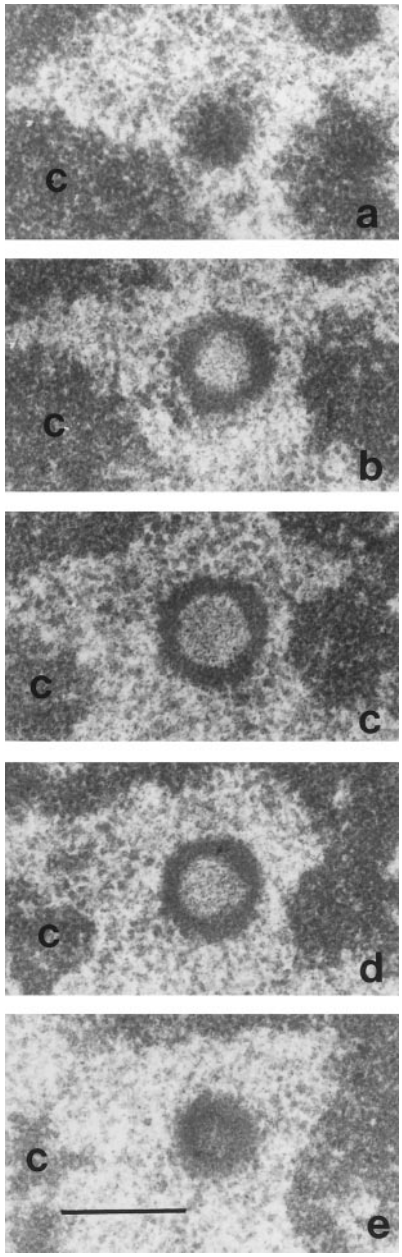

FIG. 1. Serial-section images of a ring-shaped nuclear body of *L. schismatica.* The complete structure is included in five consecutive thin sections and is embedded within a fibrogranular material, close to compact chromatin (c). Uranyl acetate–lead citrate. Bar is 0.5 μm.

we used a biotin-labeled oligo(dT) 20-mer $(dT)_{20}$ as a probe. Little to no poly$(A)^+$ RNA was detected in the ring-shaped structures (Fig. 3b). When some label is present, it is associated mainly with the external ring. In contrast, in the nucleoplasm a strong label is associated with fibers and *Lacandonia* granules as previously noted (Agredano-Moreno and Jiménez-García, 2000). No label was found when hybridization experiments were conducted in the absence of probe (Fig. 3c).

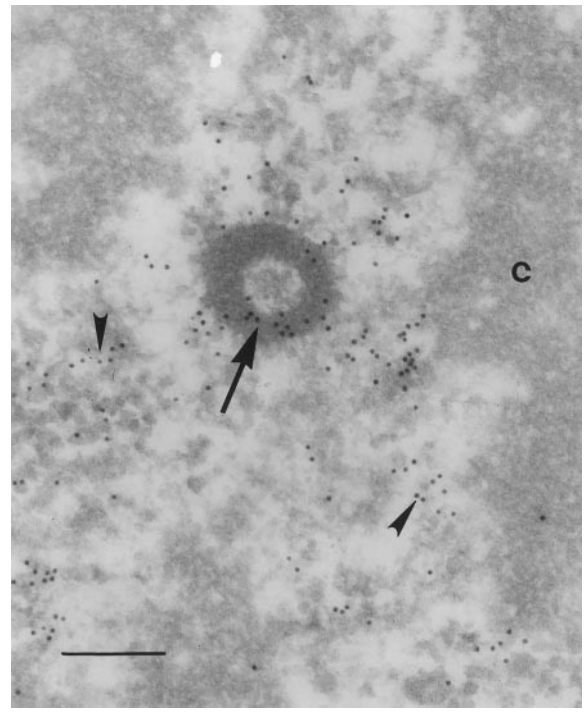

FIG. 2. Immunoelectron microscopy of SR proteins in a ring-shaped body of *L. schismatica.* Dense labeling is observed in the external ring of this structure (arrow) and in fibrils and granules in the surrounding nucleoplasm (arrowheads). c, chromatin. Bar is 200 nm.

Analysis of the Presence of Ring-Shaped Bodies in Other Species

We then analyzed the presence of ring-shaped structures in other animal and plant species by means of conventional electron microscopy. Our results show that similar nuclear structures are present in the primitive tree *G. biloba,* in the plant *V. aphylla,* and in animal cells such as rat hepatocytes and PtK2 cells (Figs. 4a, 4b, 4d, and 4e). They were also observed in tomato (*L. esculentum*) root tips (Fig. 4c). For tomato cells we performed preferential staining for chromatin using acidic PTA. As

TABLE I

Quantitative Analysis of the Labeling for SR Proteins in Nuclear Compartments

Nuclear compartment	Gold grains/μm^2
Chromatin	19 g/μm^2
Interchromatin region	325 g/μm^2
External ring	250 g/μm^2
Internal core of the ring	0 g/μm^2

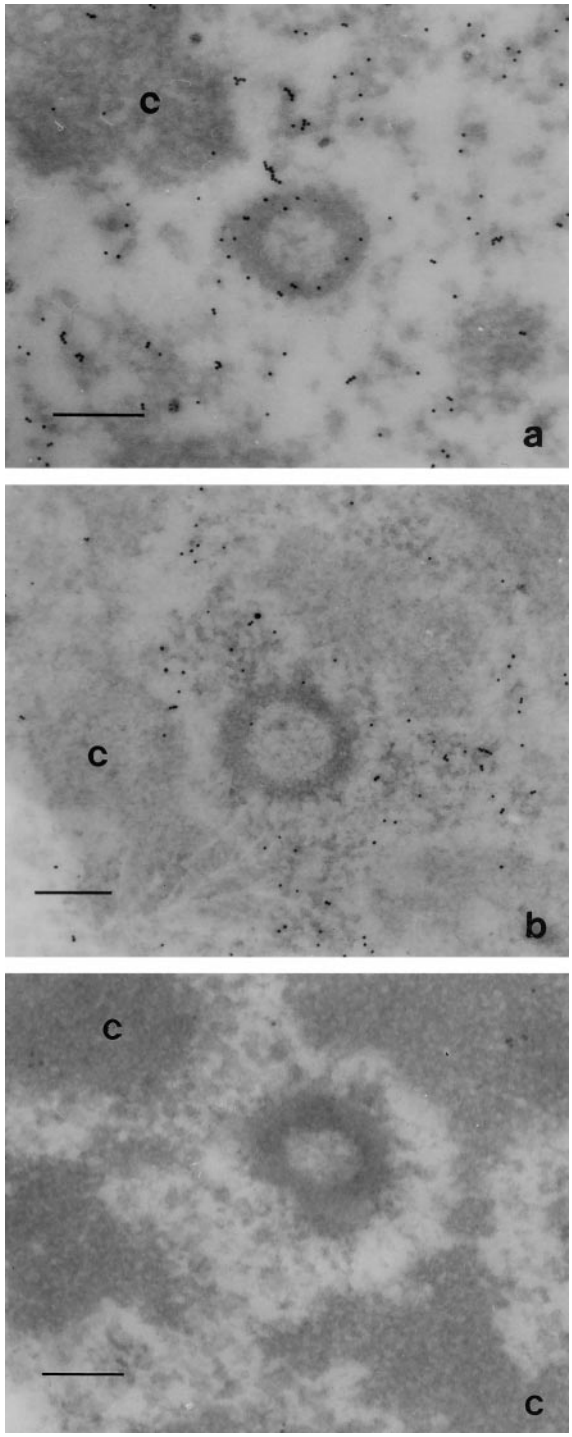

FIG. 3. Ultrastructural *in situ* hybridization to detect RNA in a ring-shaped nuclear body of *L. schismatica.* (a) Total RNA. The external dense ring and surrounding nucleoplasm are labeled. (b) Poly(A)$^+$ RNA. Some gold particles are associated with the external shell of the body while intense labeling is observed in the nucleoplasm. (c) Negative control. No probe was used. c, chromatin. Bars are 200 nm.

for *L. schismatica* using anti-DNA antibodies (Agredano-Moreno *et al.*, 1994), we observed that these structures do not contain DNA (Fig. 4c).

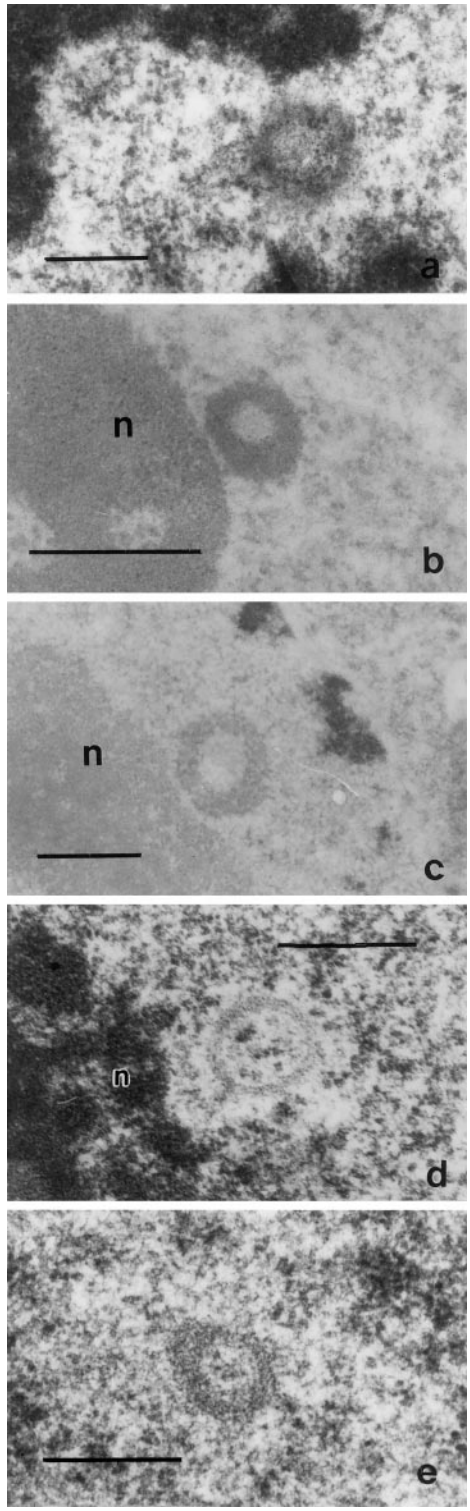

FIG. 4. Electron micrographs of ring-shaped bodies in other plant and animal cells. The tree *G. biloba* (a), the saprophyte *V. aphylla* (b), tomato (*L. esculentum*) (c), marsupial *P. tridactylus-apicalis* (PtK2) cells (d), and rat (*Rattus norvegicus*) hepatocytes (e). (a, b, d, e) Uranyl acetate–lead citrate; (c) PTA staining. n, nucleolus. Bars are 0.5 μm.

DISCUSSION

The Ring-Shaped Bodies Are Spherical Bodies

In the present work we performed serial-sectioning analysis of the ring-shaped bodies of *L. schismatica* in order to investigate whether they are spherical or cylindrical structures. Our results clearly show that these structures are spherical bodies that comprise an external ring of strongly intermingled fibers surrounding a central core of fibrils and granules. Although the structure is about 0.5 μm in diameter for a medial section, they should be considered spheres. Therefore, since the volume for a sphere is defined as $V = \frac{4}{3}\pi r^3$, this structure would be about 0.07 μm^3, with a maximum diameter of 0.5 μm. A similar structure was previously described in animal cells using a serial-sectioning approach. In fact, Ochs *et al.* (1995) found that ring-shaped nuclear bodies of roosters induced by stimulation with estrogens were also spherical bodies of about the same size. It is tempting to speculate that both structures may belong to a class of nuclear bodies with a similar function in plant and animal cells.

The Ring-Shaped Bodies of the Plant L. schismatica Contain SR Proteins

The functional role of the ring-shaped bodies in the nucleus of *L. schismatica* is unknown. The presence of SR proteins, a family of proteins involved in splicing and postsplicing events (Manley and Tacke, 1996), found in this study and the presence of snRNAs (Agredano-Moreno *et al.*, 1994) suggest that they may play a role in the processing of pre-mRNA such as in the assembly, transport, and/or storage of the molecular elements involved in the splicing process.

It is interesting to note that almost all molecular elements identified to date in the ring-shaped bodies of *L. schismatica* are located in the external ring. The central core of these structures contains ribonucleoproteins (Jiménez-García *et al.*, 1992; Agredano-Moreno *et al.*, 1994), although the EDTA regressive staining (Bernhard, 1969) is not specific but preferential for RNPs. Little to no SR protein is present, however.

The Ring-Shaped Bodies Contain Little to No Poly(A)$^+$ RNA

While RNA was detected in ring-shaped structures of *L. schismatica* by *in situ* hybridization using genomic DNA as a probe, little to no poly(A)$^+$ RNA was associated with the external ring when analyzed by using a specific probe. These results, however, do not rule out the possibility that the ring-shaped structures may contain factors involved in the 3′ end processing of pre-mRNA. In a previous work, the EDTA regressive method for RNP stained the central core. However, this method also may stain only structures containing proteins as suggested by the absence of label for RNA as observed by the absence of phosphorus (Hendzel *et al.*, 1999).

Ring-Shaped Bodies Are Present in Other Plant and Animal Cells

To date, intranuclear ring-shaped bodies have been observed in normal animal and plant cells (Jiménez-García *et al.*, 1992; Grande *et al.*, 1996), associated with viral infections (Puvion-Dutilleul and Puvion, 1991), under pathological conditions (Popoff and Stewart, 1968; Dyck *et al.*, 1994), and in tissues stimulated by hormones (Brasch *et al.*, 1989; Ochs *et al.*, 1995). A random search of structures of this kind in other plant and animal cells indicated that they may be present in a more widespread distribution than previously thought. Although the general structure is conserved in the different species studied, there are differences in the size, in the compaction grade of the outside ring, and in the fine structure of both the external ring and the central core. The presence of these structures in diverse plants such as the saprophytic plant *V. aphylla* and in the dicotyledon *L. esculentum* supports the notion that they may be present in many eukaryotes. It is noteworthy that even the primitive plant *G. biloba* displays such structures. Additionally, we have observed ring-shaped bodies in marsupial animal cells, such as the cell line PtK2, and in rat hepatocytes, which may have a different composition since the ultrastructure seems to display some differences, such as in the compactness and width.

Based on our ultrastructural data, including serial sectioning, immunoelectron microscopy, and ultrastructural *in situ* hybridization, we conclude that ring-shaped bodies in *L. schismatica* are spherical structures that contain RNA and SR proteins. The presence of ring-shaped structures in other plant and animal species suggests that they may belong to a class of a more general nuclear bodies that predate the origin of plants and animals. However, whether they are homologous structures or not remains to be elucidated.

We thank Dr. D. Spector (CSHL) for providing 3C5 antibody. This work was supported by CONACyT 28002N, México.

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