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Coiled bodies in the meristematic cells of the root of *Lupinus luteus* L.

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Abstract

The nature of nucleolar associate bodies in the meristematic cells of the root of *Lupinus luteus* L. was investigated using immunocytochemical methods, *in situ* hybridisation with light, confocal, and electron microscopy. The nuclear bodies of lupin proved to be structures containing fibrillarin and coilin, but devoid of rRNA and DNA, like animal coiled bodies (CBs). In lupin cells we have observed the occurrence of small nuclear ribonucleoprotein (snRNP) in the cytoplasm, in nucleoplasm, CBs and in nucleoli. This type of snRNP localisation pattern is in agreement with recently presented models of the small nuclear RNA cycle.

Additional key words: Ag-NOR proteins, immunogold, nucleolus, ribosomal RNA, Sm proteins, snRNA, splicing.

Introduction

The nucleus of eukaryotes is characterised by a high degree of differentiation into functional compartments thanks to which the synchronisation of basic metabolic processes such as replication, transcription or post-transcriptional modification of RNA is possible. The most thoroughly investigated compartment so far has been the nucleolus, widely thought to be responsible for the biogenesis of ribosomes (Hadjiolov 1985). Far less well-known, and more controversial, is the function of the compartments defined by the term nuclear bodies. In plant cells, nuclear bodies are characterised by considerable heterogeneity, and have been described as "karyosomes", "amorphous bodies" and "spherules" (Williams *et al.* 1983). Williams *et al.* (1983) attempted to classify nuclear bodies occurring in the meristematic cells of the root tips in plants. They reported that numerous (5 - 20 per nucleus) silver impregnated bodies, called "dense bodies" (DBs) occur in plant species with a reticulate nucleus and are dispersed in the nucleoplasm. Species of plants with areticulate nuclei have larger nuclear bodies, which are less numerous (2 - 3 per nucleus) and usually located in the direct vicinity of the nucleolus, and are thus named "nucleolus associated body" (NAB). The precise classification of nuclear bodies to each of the distinguished categories was considerably hindered by the heterogeneity of the ultrastructure of NABs and the occurrence of dense bodies connected to the nucleolus observed in some species. As a result of the introduction of immunocytochemical techniques and in situ hybridisation in recent years, new possibilities have been created for investigating the nature of nuclear bodies. In the cells of root meristems of *Pisum sativum* (Beven *et al.* 1995), spherical nuclear bodies lying near the nucleolus (NABs) were strongly labelled with the anti p80 coilin antibody, which testifies to the presence of coilin, a protein regarded as a marker of animal CBs. In plant nuclear bodies belonging to the NABs category, the presence of fibrillarin (Beven *et al.* 1996) and small nuclear RNA (Chamberland and Lafontaine 1993) was also revealed. Because plant NABs display similar immunocytochemical properties as animal CBs, the conviction that both kinds of nuclear bodies are homologous structures is expressed more and more frequently (Lafontaine and Chamberland 1995).

Although a great deal of information has been collected regarding the composition of CBs, their function remains an open question. The generally observed presence of small nuclear ribonucleoprotein

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Abbreviations: CBs - coiled bodies; DBs - dense bodies; NABs - nucleolus associated bodies; SFCs - splicing factor compartments; Sm - core spliceosomal proteins; TMG - tri-methyl guanosine.
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(snRNP) within CBs indicates that these structures are an important element in the cycle of small nuclear RNA. The model of the cycle of snRNA proposed by Mattaj and Englmer (1998) and Matera (1999) predicts that, within the nucleus, the primary transcripts of the UsnRNA genes acquire a cap at the 5' end; it is a methyl guanosine in position 7. The next stage of transformation after the export of snRNA particles to the cytoplasm is the binding of proteins belonging to the group defined by the term Sm. After the formation of a complex with Sm protein, hypermethylation of the cap takes place in the cytoplasm, yielding trimethyl guanosine (TMG), and then mature particles of snRNP are transported to the nucleus.

Recently the view has been presented that CBs may be the site of accumulation and recycling of snRNA (Bohmann *et al.* 1995). This belief is based on the results of biochemical as well as immunocytochemical investigations mainly in animal cells. The investigations on plants, so far not very numerous, have not made it possible to determine if a similar function may be ascribed to plant CBs.

The presence of fibrillarin and small nuclear RNA within animal CBs and their localisation near the nucleolus suggested that these structures might participate directly in the metabolism of ribosomal RNA. This type of function of animal CBs is precluded by the lack of rRNA within them, which was revealed thanks to the application of *in situ* hybridisation techniques (Carmo-Fonseca *et al.* 1993). It is not known whether the NABs of the root meristematic cells are the equivalent of animal CBs in this respect because no research has yet been attempted to determine whether NABs contain rRNA.

The above-mentioned problems have led us to initiate research on the localisation of snRNA, rRNA and the fibrillarin and coilin proteins in meristematic cells of *Lupinus luteus* L. roots. This species is characterised by a non-reticulate type of nucleus containing nuclear bodies included in the NAB category by Williams *et al.* (1983). Nuclei of the non-reticulate type are a convenient research material as they have a few relatively large nuclear bodies and a low content of condensed chromatin localized next to the nuclear membrane which considerably facilitates performing observations.

Materials and methods

Plants: Seeds of *Lupinus luteus* L. were placed on cellucotton in Petri dishes moistened with distilled water at a temperature of 22 °C in the dark. After 48 h, when the roots of the seedlings had reached about 1.5 cm in length, root tips were collected and fixed.

Transmission Electron Microscopy (TEM): Samples were processed for electron microscopy by:
a) conventional techniques (paraformaldehyde - osmium fixation, lead citrate - uranyl acetate staining),
b) Ag-NOR staining (Wróbel and Stoynova 1992),
c) immunocytochemical and *in situ* hybridisation schedule (paraformaldehyde fixation and embedding in LR gold resin; Majewska-Sawka and Rodriguez-Garcia 1996).

Isolation of protoplasts: Root tips were fixed in 4 % paraformaldehyde solution in PBS buffer pH 7.2 and hydrolysed in a mixture of 1 % cellulase Onozuka R10 (*Serva,* Heidelberg, Germany) and 27 U cm^{-3} pectinase (*Sigma,* St Louis, USA) in 0.01 M citric buffer pH 4.8 for 4 h at 37 °C. The suspended protoplast matter was spread on gelatine-coated slides placed on dry ice and air-dried. Then the protoplasts were rinsed with 0.1 % Triton X100 solution in PBS buffer, pH 7.2, and air-dried.

Immunolabelling: The following primary antibodies were used: human anti coilin (gift from Dr. Ochs; diluted 1:200), rabbit anti coilin (gift from Dr. Lamond; diluted 1:50), human anti fibrillarin (*Sigma*; diluted 1:4), human anti fibrillarin (gift from Dr. Mol; diluted 1:70), human anti Sm core spliceosomal proteins (kindly provided by Dr. Plombo; diluted 1:800), mouse anti tri-methyl guanosine (TMG) on a 5' end of splicing U snRNA (*Oncogene Sci.*, Boston, USA; diluted 1:35), mouse IgM to single- and double-stranded DNA (*Roche,* Mannheim, Germany; 10 μg cm^{-3}) and mouse anti DIG (*BioCell,* Cardiff, UK; diluted 1:25).

In immunolabeling experiments ultrathin sections and protoplasts were blocked 15 min in 1 % BSA in PBS (for anti TMG snRNA additional 15 min of blocking with 5 % non-fat milk was applied), followed by incubation with primary antibodies in humid chamber in 4 °C overnight (diluted in 1 % BSA in PBS). Grids and slides were then washed in PBS and incubated in secondary antibodies in PBS containing 0.2 % BSA at 37 °C for 1h. The following secondary antibodies were used for tests in TEM (diluted 1:30): anti mouse IgM with 20 nm gold particles (*BioCell*), anti mouse IgG with 10, 15 and 20 nm gold particles (*BioCell*), anti rabbit 10 nm gold particles (*Sigma*), anti human 10 nm gold particles (*Sigma*) and 15 nm gold particles (*BioCell*). The following secondary antibodies were used for immunofluorescence tests (diluted 1:70): anti mouse IgG FITC (*Sigma*), anti human Cy3 (*Sigma*).

Ribosomal RNA detection: For *in situ* hybridisation, an inner 18S repeat element (1200 bp in length) of *Pisum sativum* (kindly provided by Dr. G. McFadden) was cloned into pBluescript KS plasmid. 18S rRNA sense and antisense probes labelled with digoxygenin were

synthesised using the DIG-RNA labelling kit (*Roche*). *In situ* hybridisation to ultrathin sections was carried out as described previously (Majewska-Sawka and Rodriguez -Garcia 1996).

DNA detection: Two techniques were used for in situ detection of structures containing DNA at the ultra-structural level: immunolabelling with monoclonal anti-DNA antibodies described by Lafontaine and Chamberland (1995) and the terminal deoxynucleotidyl transferase-immunogold technique TdT (Thiry 1992).

Control reactions: Control experiments indicating the specificity of the reactions were carried out by omitting the primary antibodies. Additional controls were also performed: for high resolution *in situ* hybridisation, a sense probe was used; for TdT reaction, incubation without Br-dUTP nucleotides was performed.

Ultrathin sections were observed with the electron microscopes *TEM Jeol 1200* (Japan) or *Tesla 550* (Czech Republic) and protoplasts were analysed using confocal microscopy (*CLSM, Leica TCS 4D*, Germany) equipped with an argon-krypton laser.

Results

Ultrastructure and cytochemistry of CBs: In cross-sections of the nucleus, 1 - 2 CBs are usually visible, while analysis using confocal microscopy reveals that there may be a maximum of 8 of them in the nucleus. The diameter of CBs varies from 0.2 - 0.7 μm. Lupin CBs are structures built of densely packed fibrills mixed with granules (Fig. 1*A*). Staining with $AgNO_3$ showed that CBs contain silver-absorbing nucleolar protein and are strongly impregnated like the fibrillar component of the nucleolus (Fig. 1*B*).

Immunogold localisation of DNA: The localisation of DNA in lupin root meristematic cells was achieved using the method with anti-DNA antibodies and the even more sensitive TdT technique. In both cases, labelling is restricted to the area of the nucleus. The highest density of traces occurs over the condensed chromatin, which builds the chromocentres. More particles of gold over the chromocentres were found by using the TdT method (Fig. 2*B*) than by using the anti-DNA antibodies (Fig. 2*A*). Very few traces were found over the nucleolus. CBs are totally devoid of labelling regardless of the method used.

Localisation of coilin and fibrillarin using the immunogold method: It was essential for the classification of nuclear bodies in lupin cells to test whether they contained coilin, a protein recognised as a CBs marker in mammalian cells (Bohman *et al.* 1995). The immunocytochemical reaction with the human anti-coilin antibody revealed the presence of coilin in lupin cells mainly within the nuclear bodies. A few particles occur near areas occupied by condensed chromatin and in the nucleolus (Fig. 3*A*). The CBs of lupin contain the nucleolar protein fibrillarin, which was proved by labelling using human anti-fibrillarin. Labelling was observed over the nucleolus but did not occur in the interchromatin spaces (Fig. 3*B*).

Ultrastructural localisation of rRNA, snRNA and snRNP: Using a digoxygenin-labelled complementary probe for 18 S rRNA we localised rRNA within the nuclei of lupin root meristematic cells. A very high concentration of traces occurs in the nucleolus, although their distribution is not uniform. The traces are grouped mainly in electron dense areas while the electron transparent areas are devoid of labelling. In the area outside the nucleolus, the traces are less numerous but more frequent in the area adjacent to the condensed fragments of chromatin. The lack of any traces is characteristic of CBs both in the immediate vicinity of the nucleolus and in the nucleoplasm (Fig. 3*C*). The ultrastructural localisation of the splicing system elements: Sm protein (Fig. 4*A*) and mature snRNA (Fig. 4*B*), showed them to be concentrated in CBs. Slight labelling occurs within the nucleoplasm (Fig. 4*A,B*).

Immunofluorescent localisation of Sm and TMG: Localisation of snRNP was investigated in the protoplasts of lupin root meristematic cells using antibodies for the epitopes of Sm protein and trimethyl guanosine cap. The strongest fluorescence indicating high concentrations of TMG was observed in CBs, while signals originating from the region of the nucleoplasm were considerably weaker (Fig. 5*A*). The TMG occurring in the CBs colocalised with the presence of fibrillarin. The colocalisation of signals of both antigens was also observed within the nucleolus (Fig. 5*B*). Confocal microscopy revealed the colocalisation of TMG and Sm protein also in the CBs. SnRNP were uniformly distributed in the nucleoplasm (Fig. 5*A,E*), or they occurred in the form of speckles (Fig. 5*C,D,F*). Sometimes, signals were also observed in the cytoplasm pointing at the presence of TMG colocalised with signals originating from the labelled anti-Sm antibodies (Fig. 5*C,D*). Both Sm protein and TMG snRNA occur within the nucleolus. They form characteristic spots in the central zone of the nucleolus (Fig. 5*E,F*).

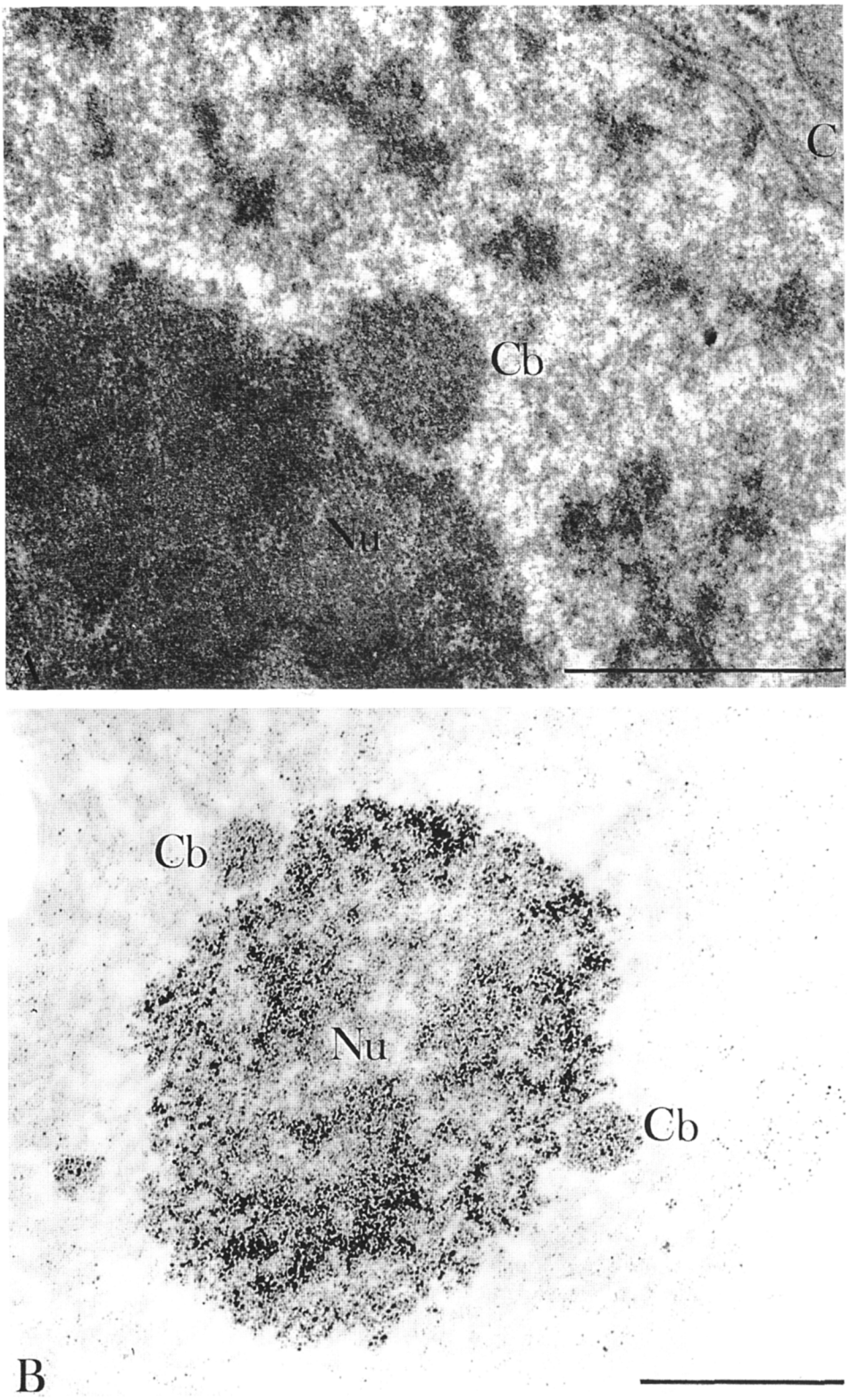

Fig. 1. *A* - Ultrastructure of the interphase nucleolus of lupin root cells. Oval nuclear body (CB) near the nucleus. Nuclear body built of a twisted bundle of fibrils with a diameter of 15 - 25 nm. Nu - nucleolus, C - cytoplasm. Physical contact of CB with the nucleolus (*bar* = 1 μm). *B* - Localisation of silver stained nucleolar proteins using Ag-NOR technique at the electron microscopy level. Nucleolar proteins are present in two nuclear bodies near the nucleolus (*bar* = 1 μm).

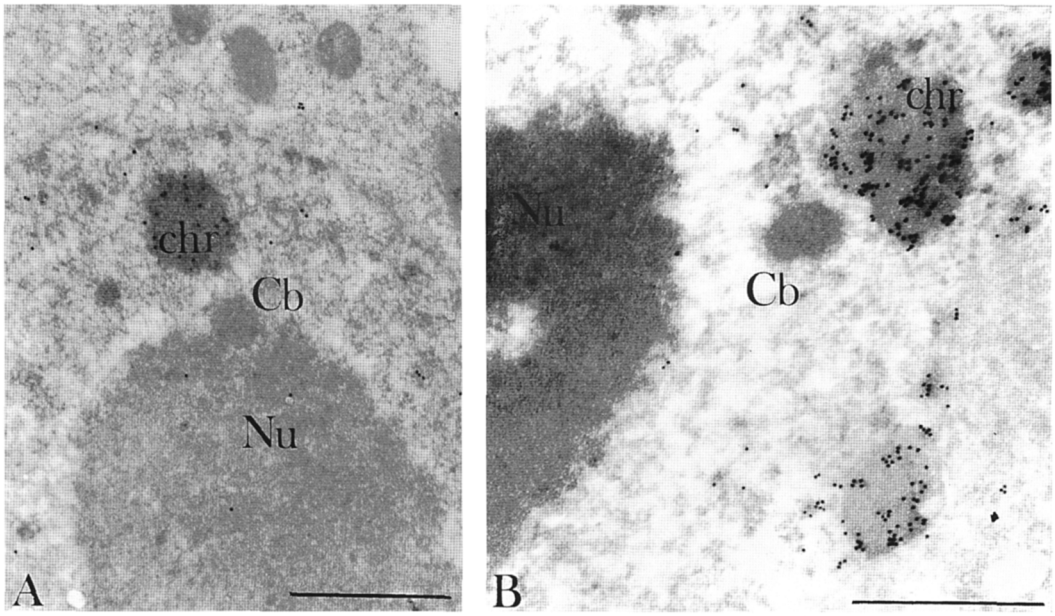

Fig. 2. Ultrastructural localisation of DNA. Neither immuno-localisation of DNA (*A*) nor the highly sensitive molecular TdT technique (*B*) demonstrated the presence of DNA within CBs. Strong and specific labelling can be observed within condensed chromatin (chr) (*bar* = 1 μm).

Discussion

The results obtained in this paper show that the nuclear bodies of lupin are, from the point of view of structural and cytochemical properties, the equivalent of coiled bodies in mammals. The basic structural elements forming the nuclear bodies of lupin are fibrills with a diameter of about 30 nm packed in a way similar to those in coiled bodies in animal cells (Bohmann *et al.* 1995). The presence of coilin in the nuclear bodies of lupin (NABs) represents a highly convincing argument for their classification into the same category as animal CBs.

The protein that colocalizes with coilin in animal CBs is fibrillarin (Carmo-Fonseca *et al.* 1993). Our results are consistent with these earlier observations since we found the presence of fibrillarin within both lupin CBs and nucleoli. This highly conservative protein together with U3 snoRNA, participates in the formation of ribosomal subunits (Baserga *et al.* 1991).

In our experiments using anti-DNA antibodies and TdT techniques, we observed labelling in lupin nuclei only over areas of chromatin, while the area of CBs was devoid of gold particles, which testifies to the absence of DNA in these structures. The lack of DNA in CBs, also noted in other species of plants and animals (Raška *et al.* 1991, Lafontaine and Chamberland 1995), excludes the possibility of transcription in the CBs, which is also indicated by the negative results of experiments using a pulse administration of Bromo-UTP (Raška 1996). Using immunogold *in situ* hybridization with a digoxygenin-labelled complementary probe for rRNA, no gold particles were found over areas of CBs in the nuclei of the meristematic cells of lupin. Our results are consistent with those of other researchers who did not demonstrate the presence of rRNA and also polymerase RNA I in plant CBs (Olmedilla *et al.* 1997, Smoliński and Górska-Brylass 1999a,b) or animal CBs (Raška *et al.* 1991, Carmo-Fonseca *et al.* 1993, Jimenez-Garcia *et al.* 1994) indicating that this type of nuclear bodies do not participate directly in the synthesis or accumulation of ribosomal RNA.

In lupin root meristematic cells the signals indicating the presence of TMG and Sm were detected both in the area of the nucleus and in the cytoplasm which is in agreement with the proposed models of the snRNA cycle (Mattaj and Englmeier 1998, Matera 1999). Cytoplasmic signals in lupin cells indicating the localisation of both TMG and Sm in the form of small concentrations are characterized by a relatively high intensity distinguishing them from the background. They colocalize with each other which indicates that they are not artifacts and suggest they may be the site of localisation of mature snRNP. The cytoplasmic location of snRNP was often noted in animal cells. Using immunocytochemical methods and *in situ* hybridisation in plants snRNP presence was not detected in the cytoplasm *Arabidopsis* (Boudonck *et al.* 1999), *Brassica napus* (Chamberland *et al.* 1999), *Zea mays* (Gulemetova *et al.* 1998) or a

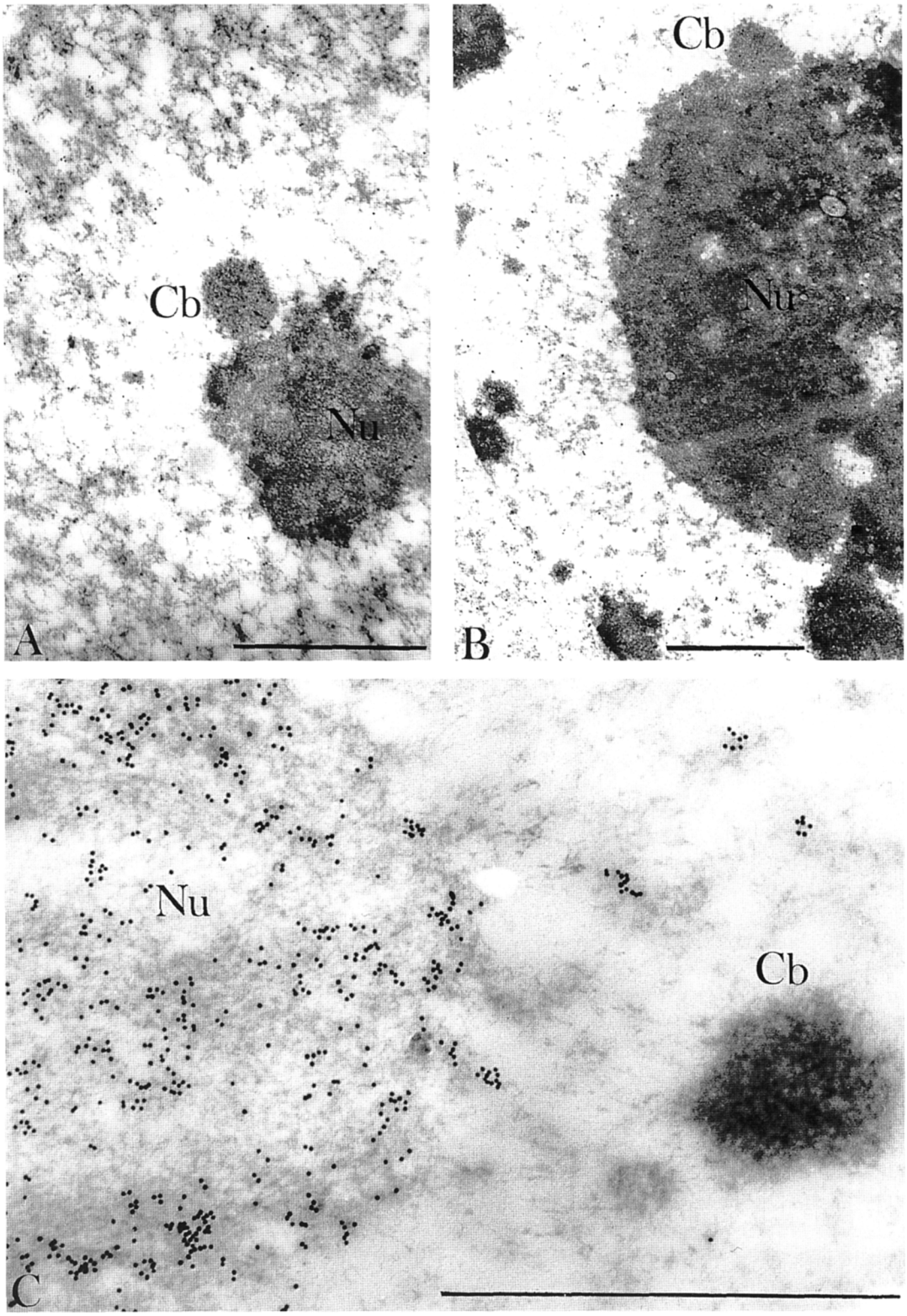

Fig. 3. Immunolocalisation of coilin (*A*), fibrillarin (*B*) and *in situ* hybridisation using 18S rRNA probe (*C*) (*bar* = 1 μm). *A* - Particles of colloidal gold indicating the localisation of coilin are mainly accumulated in the coiled body. Coilin also occurs in small quantities within the nucleolus and nucleoplasm. *B* - Fibrillarin, mainly accumulated within the nucleolus, is also present in the CB. A small quantity of this protein can be observed within the nucleoplasm and cytoplasm. *C* - CB in the nucleoplasm near the nucleolus do not indicate the presence of ribosomal RNA. Strong labelling is observed within the nucleolus.

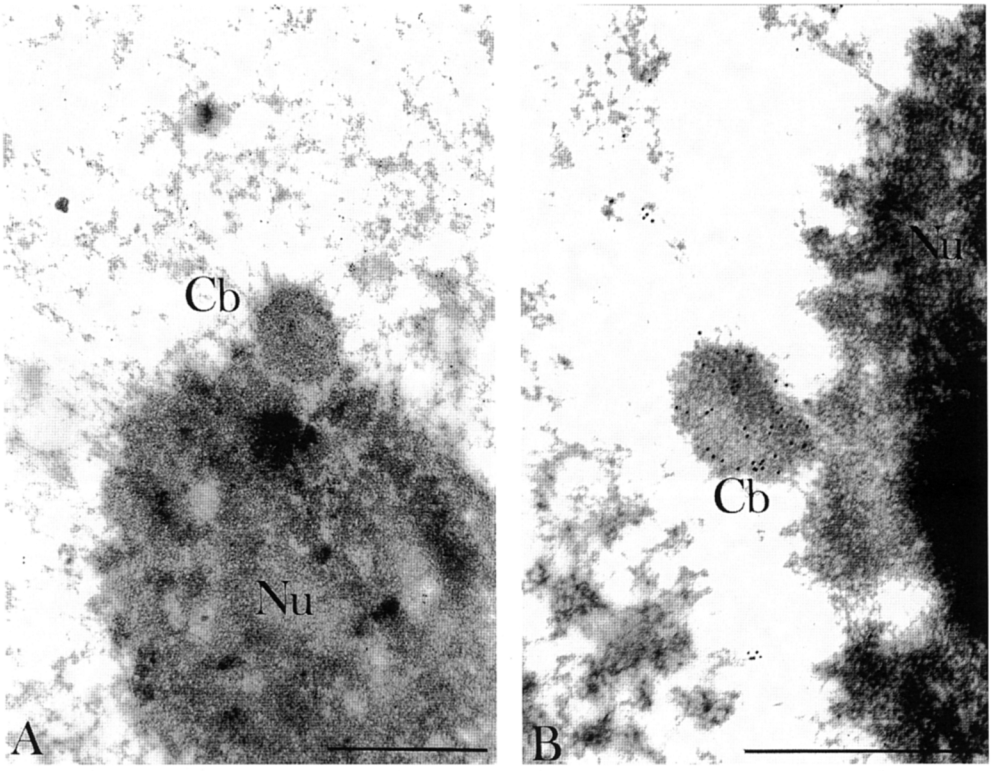

Fig. 4. Immunolocalisation of protein Sm (*A*) and TMG snRNA (*B*). SnRNP are accumulated selectively in CB, and also present within the nucleoplasm and in the nucleolus (*bar* = 1 μm).

low amount was detected *Pisum sativum* (Beven *et al.* 1995), *Daucus carrota* (Sanches-Pina *et al.* 1989). Such a differentiation of the results may be due to the low snRNP level in the cytoplasm or an insufficient sensitivity of the used methods. The immunocytochemical methods used by us do not allow us to state in what way the snRNP maturation process is spatially organized in the cytoplasm of lupin cells. In order to explain this problem it is necessary to undertake investigations using highly sensitive techniques at the electron microscope level.

The strongest signals indicating snRNP presence in lupin cells were found in CBs. The presence of snRNP in these nuclear subcompartments is commonly noted in animal (Carmo-Fonseca *et al.* 1993) and plant cells (Boudonck *et al.* 1999, Straatman and Schel 2001). In some lupin cells snRNP also occur in nucleoli which is in agreement with the observations of animal (Sleeman and Lamond 1999) and also plant cells (Boundock *et al.* 1999). In *Brassica napus* (Chamberland *et al.* 1999) nucleoli snRNP is localised in the fibrillar and granular area whereas in *Pisum sativum* snRNPs were registered in the central nucleolar vacuole (Beven *at al.* 1995). Our investigations in a confocal microscope, however, have shown snRNP in the center of lupin nucleoli but do not allow to localize it to a defined zone of the nucleolus.

The results of our investigations are in agreement with the results of Sleeman and Lamond (1999) who use time lapse microscopy and with the GFP method have found that snRNP after its reimportation from the cytoplasm into the nucleus initially accumulate in CBs and then in the nucleolus. Without performing additional experiments these authors could not state whether a direct snRNP transport exists between the CBs and the nucleolus. SnRNP transport from CBs to the nucleolar area seems highly likely as it could be linked to the utilization of nucleolar machinery capable of rRNA post-transcriptional modifications such as methylation and pseudouridylation (Bohman *et al.* 1995). SnRNA could also undergo this type of modification in the nucleolus which is necessary for its participation in splicing (Simpson and Filipowicz 1996). A morphological expression of this interaction seems to be the physical contact between CBs and the nucleolus characteristic for lupin cells and commonly observed in other plant (Simpson and Filipowicz 1996), and animal (Bohman *et al.* 1995) species.

Sleeman and Lamond (1999) assume that snRNP is transported from CBs or the nucleolus into the nucleoplasm and accumulates in the form of speckles. Speckles containing snRNP which we have also observed in lupin also occur in plants and animals (Huang and

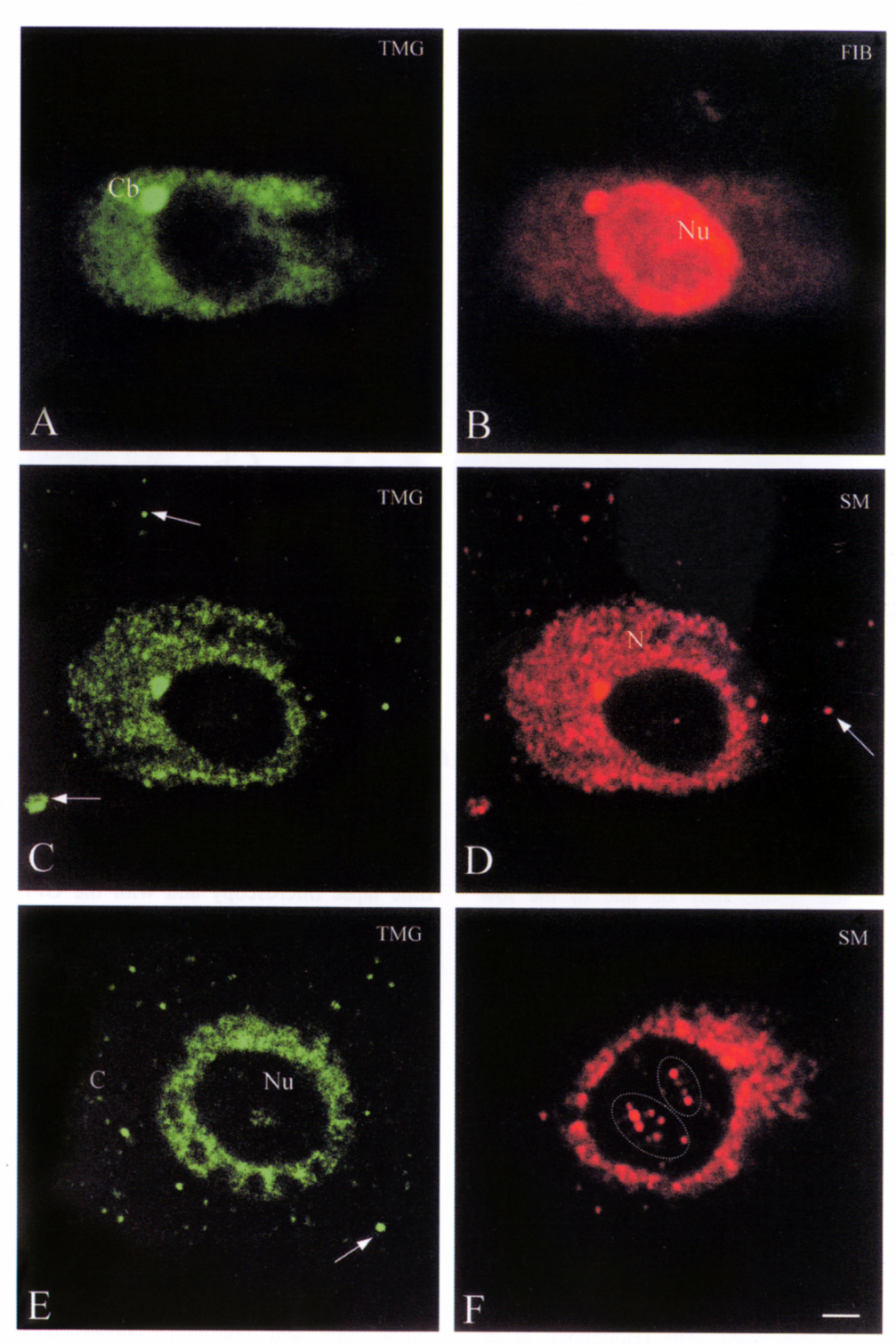

Fig. 5. Immunofluorescent "labelling pattern" in isolated protoplasts of lupin root cells: CLMS technique (green fluorscence FITC, red fluorescence Cy3) (*bar* = 1 μm). Double labelling of TMG snRNA (*A*) and fibrillarin (*B*). Colocalisation of both occurs in CB. Accumulation of snRNA (*C*) as well as splicing protein Sm (*D*) can be observed within CB. Within the nucleus they are distributed in a dispersed form. Punctual localisation of splicing factors can also be observed within the cytoplasm (*arrows*). Nucleolar and cytoplasmic localisation of TMG snRNA (*E*) and proteins Sm (*F*). Both splicing protein Sm and TMG snRNA occur within nucleoli. A characteristic pattern was observed in the form of concentrations in the central part of nucleoli (*F* - *marked areas*). Concentration of TMG are also present within the cytoplasm (*E* - *arrow*).

Spector 1996, Simpson and Filipowicz 1996). Investigations of the latest years devoted to this type of speckles indicated that they form a structurally and functionally complex nuclear compartment and the term splicing factor compartments (SFCs) has been proposed to define them (Spector 1993). Nuclear SFC compartments have been shown to contain among others: mRNA splicing factors, snRNA, transcription factors, 3'processing factors and ribosomal proteins. Electron microscope investigations have shown that the structural elements of SFCs are interchromatin granules and perichromatin fibrils. The role of these compartments is not clear and is the subject of numerous speculations (Misteli 2000).

In the light of our results we believe that lupin nuclear bodies because of their structural organization, chemistry and functions are the counterparts of animal CBs. The occurrence of this type of nuclear bodies both in animals and in plants indicates that CBs are a morphological expression of the same universal process conserved in the evolutionary process.

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