Brazilian Journal of Medical and Biological Research (2004) 37: 997-1003
ISSN 0100-879X

Colocalization of coilin and nucleolar proteins in Cajal body-like structures of micronucleated PtK_2 cells

N.P. Silva[1], D.M. Christofolini[1], R.A. Mortara[2] and L.E.C. Andrade[1]

[1]Disciplina de Reumatologia, Departamento de Medicina, and [2]Departamento de Microbiologia, Imunologia e Parasitologia, Universidade Federal de São Paulo, Escola Paulista de Medicina, São Paulo, SP, Brasil

Abstract

Cajal bodies (CB) are ubiquitous nuclear structures involved in the biogenesis of small nuclear ribonucleoproteins and show narrow association with the nucleolus. To identify possible relationships between CB and the nucleolus, the localization of coilin, a marker of CB, and of a set of nucleolar proteins was investigated in cultured PtK_2 cells undergoing micronucleation. Nocodazol-induced micronucleated cells were examined by double indirect immunofluorescence with antibodies against coilin, fibrillarin, NOR-90/hUBF, RNA polymerase I, PM/Scl, and To/Th. Cells were imaged on a BioRad 1024-UV confocal system attached to a Zeiss Axiovert 100 microscope. Since PtK_2 cells possess only one nucleolus organizer region, micronucleated cells presented only one or two micronuclei containing nucleolus. By confocal microscopy we showed that in most micronuclei lacking a typical nucleolus a variable number of round structures were stained by antibodies against fibrillarin, NOR-90/hUBF protein, and coilin. These bodies were regarded as CB-like structures and were not stained by anti-PM/Scl and anti-To/Th antibodies. Anti-RNA polymerase I antibodies also reacted with CB-like structures in some micronuclei lacking nucleolus. The demonstration that a set of proteins involved in RNA/RNP biogenesis, namely coilin, fibrillarin, NOR-90/hUBF, and RNA polymerase I gather in CB-like structures present in nucleoli-devoid micronuclei may contribute to shed some light into the understanding of CB function.

Correspondence
N.P. Silva
Disciplina de Reumatologia
UNIFESP, EPM
Rua Botucatu, 740, 3º andar
04023-900 São Paulo, SP
Brasil
Fax: +55-11-5579-6665
E-mail: nps@reumato.epm.br

This work was supported by grants from FAPESP, CNPq and the Brazilian Society of Rheumatology (SBR). D.M. Christofolini was the recipient of a CNPq fellowship.

Publication supported by FAPESP.

Received June 2, 2003
Accepted March 4, 2004

Key words
- Cajal body
- Coiled body
- Micronuclei
- Cell nucleolus
- Nucleolus organizer region
- RNA polymerase I
- Nucleolar proteins

Introduction

Several structural and functional nuclear domains have been characterized over the last decades. The nucleolus, the most prominent nuclear domain, is involved in rRNA synthesis. In contrast, several other nuclear bodies have been poorly characterized in functional terms. One of the best known is the Cajal body (CB), first detected by light microscopy of sliver-stained nucleolar accessory bodies in 1903 by the Spanish cytologist Ramon y Cajal (1). Later, the same structure was characterized by electron microscopy as a round threaded non-membrane-limited structure measuring 0.2 to 1.0 μm in diameter and denominated coiled body (2-4). Today, the structure is called Cajal body

in honor of Ramon y Cajal (5).

Despite its ubiquitous presence in the nuclei of most animal and plant cells, its function has not been identified (6-10). In the early 90s, a novel protein enriched in the CB was recognized by human autoantibodies and designated p80-coilin (7). Anti-coilin antibodies were extremely valuable probes for studying the CB by immunofluorescence and immunoelectronic microscopic techniques. The renewed interest in this nuclear structure has stimulated studies of its behavior during cell division and cell proliferation (8-10). CBs are dynamic structures that disassemble during mitosis to reappear at late G1, after nucleolus reformation (8,9). They vary in number and size in different cell lines, being more prominent in fast growing cells (8,11-13).

Although the function of CB is not fully understood, several lines of evidence point to possible roles in the processing of small nuclear ribonucleoprotein (snRNP), histone genes processing and pre-rRNA (14-17). CBs are not involved in pre-mRNA transcription or splicing since they lack DNA, nascent pre-mRNA and some essential splicing factors such as SC-35 (16).

An intriguing relationship between CB and nucleolus was observed in its first description in 1903, and was further strengthened by electron microscopy studies (18), and by the demonstration of several nucleolar antigens in CBs, such as snRNPs, fibrillarin, Nopp140, NAP57, and ribosomal protein S6 (16,19). Fibrillarin is a 34-kDa phosphoprotein found in association with uridine-rich small nucleolar RNAs which participates in rRNA processing (16). In contrast, the nucleolus organizer region (NOR)-90 protein, involved in the regulation of rRNA transcription, is not found in CBs. NOR-90 protein, the human upstream binding factor (hUBF), is a nucleolar transcription factor found in association with rRNA genes (20). The full length human p80-coilin was sequenced (21) and recently a self-association N-terminal domain and a cryptic nucleolar localization signal were identified within the protein (22). It was suggested that the self-association domain plays an important role in localizing coilin to CBs in HeLa cells and showed that coilin hyperphosphorylation reduces self-interaction (22). These observations led to the hypothesis that phosphorylation may control aspects of coilin function by altering its folding and subcellular localization.

In order to gain insight into the relationship between coilin and nucleolar proteins we chose to study their behavior in micronucleated cells. Micronucleation is a spontaneous phenomenon in some cell lines and can also be induced by treatment with microtubule inhibitors. In this process, in contrast to normal dividing cells, the genomic material is fractionated among several micronuclei but chromosome integrity is preserved (23). In PtK cells, a rat-kangaroo cell lineage, NOR is localized in the X chromosome. PtK_2 cells, the male lineage, have only one NOR-bearing chromosome per cell, and upon micronucleation only the micronuclei containing that chromosome will be able to form a normal nucleolus and produce rRNAs (23). Earlier studies have shown that NOR-devoid PtK_2 micronuclei present nucleolus-related fibrillar bodies or dots (24,25). These bodies were not recognized by anti-RNA polymerase I antibodies but reacted with antiserum against fibrillarin and with a monoclonal antibody against a 180-kDa protein also found in the nucleolus dense fibrillar component (DFC) (24). Benavente et al. (24) suggested that in the absence of rRNA gene, the proteins usually found in the DFC region assemble into spherical bodies not containing proteins normally found in the fibrillar center (FC) or granular component (GC). In contrast, another study conducted on the female lineage PtK_1 cells employing three different human autoimmune sera specific for FC, DFC, and GC antigens as characterized by electron microscopy and

immunoblotting, reported that each of these nucleolar antigens tested could be found in the dots (25). The behavior of coilin was not addressed in these papers.

In view of this body of indirect evidence linking the CB and the nucleolus, we decided to investigate the distribution of the CB marker protein coilin and a set of nucleolar autoantigens, fibrillarin, NOR-90/hUBF, PM/Scl, RNA polymerase I, and To/Th in PtK_2 cells submitted to micronucleation induced by the reversible microtubule inhibitor nocodazole (26).

It is worthwhile mentioning that several cellular proteins are specifically recognized by autoantibodies elicited in the course of autoimmune diseases, although the reason why these molecules became targets for the immune system is not clear. The initial recognition of one molecular species by autoantibodies may evolve to the recognition of other molecules that are related to the first one either spatially or functionally. All the molecular species addressed in this paper were shown to be autoantibody targets.

Material and Methods

Cell culture

PtK_2 cells obtained from the Culture Cell Laboratory of Dr. H. Armelin, University of São Paulo, were routinely grown in DMEM supplemented with 10% fetal calf serum at 37ºC in a humid 5% CO_2 atmosphere. Micronucleation was induced by culturing actively growing cells on circular coverslips for 48 h with 400 ng/ml nocodazole (Calbiochem, San Diego, CA, USA). Cells were fixed in 3% paraformaldehyde for 15 min and permeabilized in 0.2% Triton-X100 for 3 min before processing for double indirect immunofluorescence.

Indirect immunofluorescence

Rabbit anti-coilin antiserum R288 (7) (1:100), mouse monoclonal anti-fibrillarin antibody 72B9 (27) (1:30), and human antisera anti-NOR-90/hUBF (1:100), anti-To/Th (1:80), anti-RNA polymerase I (1:80), and anti-PM/Scl (1:100), kindly provided by Dr. E.M. Tan (Scripps Research Institute, La Jolla, CA, USA), were used as primary antibodies. Fluorescein-labeled sheep anti-rabbit IgG (1:100), Cy3-labeled sheep anti-mouse IgG (1:130), and Cy3-labeled goat anti-human IgG (1:140) antibodies were used as secondary antibodies (Sigma, St. Louis, MO, USA). Bisbenzimide H33342 fluorochrome tetrahydrochloride (Calbiochem) was used for chromatin staining. Coverslips were inverted for 1 h onto 50 µl R288, washed in 0.05% PBS/Tween 20, inverted for 1 h onto 50 µl of the counterstaining antibody and washed again. Subsequently, coverslips were inverted for 30 min onto each individual fluorescent conjugate. After washing, the coverslips were mounted with buffered glycerol on microscopy slides and sealed with nail varnish. Cells were imaged on a BioRad 1024-UV confocal system (BioRad, Hercules, CA, USA) attached to a Zeiss Axiovert 100 microscope, using a 40X N.A. 1.2 Plan-Apochromatic (DIC) water immersion objective. All images were collected by Kalman averaging at least 15 frames (512 x 512 pixels), using a maximum aperture (pinhole) of 2.0 mm. The confocal immunofluorescence microscopy images presented correspond to a single optical plane through the sample. Prints were generated by dye-sublimation with a Codonics NP1600 printer (28,29).

Results

PtK_2 interphase cells stained with anti-coilin antibodies showed either one or no visible CB. A fine speckled pattern was usually seen in the nucleoplasm. Interphase cells stained with anti-fibrillarin, anti-NOR-90/hUBF, anti-To/Th, anti-RNA polymerase I, or anti-PM/Scl antibodies showed the ex-

pected nucleolar pattern.

Nocodazole-treated PtK_2 cultures showed a high proportion of micronucleated cells, as assessed by chromatin staining. The nucleoli of micronucleated PtK_2 cells were consistently and brightly labeled with either anti-NOR-90/hUBF, anti-fibrillarin, or anti-RNA polymerase I antibodies. In contrast, micronuclei lacking a typical nucleolus were either not stained or displayed one to several round dots of different sizes brightly stained with anti-NOR-90/hUBF and anti-fibrillarin antibodies. By double indirect immunofluorescence, coilin was shown to colocalize

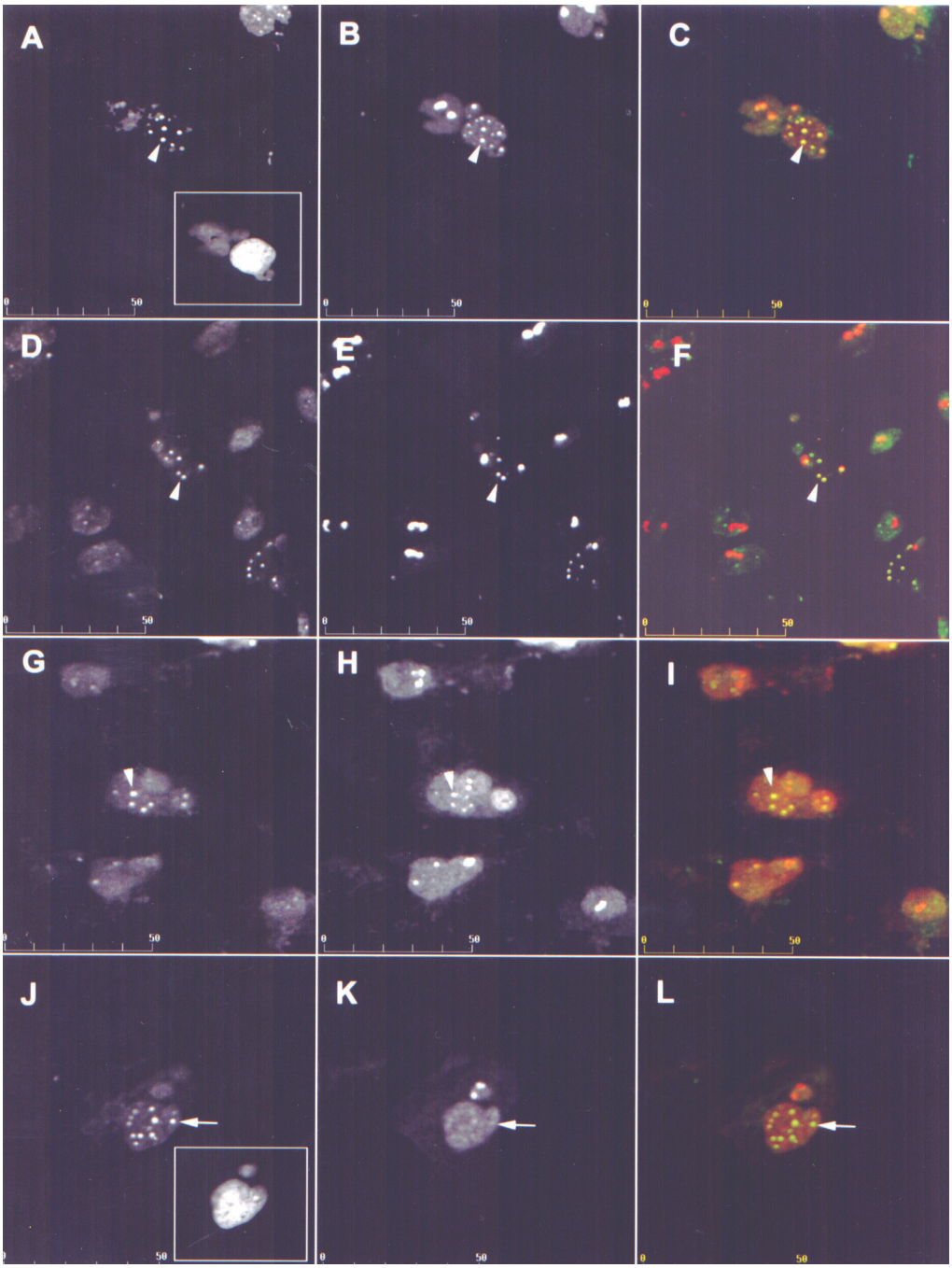

Figure 1. Coilin colocalizes with NOR-90, fibrillarin, and RNA polymerase I autoantigens in micronucleated PtK_2 round bodies. Confocal single plane optical sections were obtained through the nuclear region. *Panels C, F, I,* and *L* at the right column show merged images of the left and middle panels in each row, respectively. Arrowheads point at the colocalization (yellow/orange areas) of coilin (A) and NOR-90 (B), coilin (D) and fibrillarin (E), and coilin (G) and RNA polymerase I (H). Arrows point at nuclear bodies reacted with anti-coilin antibodies (J) but not with anti-PM/Scl antibodies (K). Round bodies are consistently absent in micronuclei displaying nucleoli. Insets in A and J show the bisbenzimide staining of the labeled nuclei. Magnification bars = 50 µm.

with both NOR-90 protein and fibrillarin at the round bodies in nucleolus-devoid micronuclei, as assessed by confocal microscopy (Figure 1, arrowheads in C and F, respectively). The round bodies were regarded as CB-like structures due to the simultaneous presence of coilin and fibrillarin.

Micronucleated PtK_2 cells stained with anti-RNA polymerase I antibody displayed the usual nucleolar pattern and it was also possible to detect stained bodies in some of the nucleolus-lacking micronuclei. Confocal microscopy observations showed that RNA polymerase I colocalizes with both coilin (Figure 1I, arrowhead) and fibrillarin in CB-like structures of nucleolus-devoid micronuclei.

Anti-To/Th (data not shown) and anti-PM/Scl antibodies only stained nucleoli and did not stain the CB-like structures formed in nucleolus-lacking micronuclei. Confocal microscopy showed no colocalization of the two autoantigens and coilin in nucleolus-devoid micronuclei (Figure 1L, arrow).

Discussion

There is major interest in understanding how the synthesis, processing and transport of macromolecules are coordinated within the cell. The study and characterization of subcellular organelles are of growing interest and provide clues to understanding the complexity of life processes and how cell malfunctioning may lead to disease. The CB is an intriguing subcellular organelle that appears to be related to the nucleolus and also to be involved in snRNP import. In order to obtain some information about CB we investigated the CB-specific protein coilin and nucleolar autoantigens in cells submitted to micronucleation, a process in which only those micronuclei containing NOR genes will be able to form a functional nucleolus. Our aim was to determine the distribution of the CB-specific protein coilin and selected nucleolar antigens inside NOR-lacking micronuclei.

The detection of NOR-90/hUBF in the round bodies of nucleolus-devoid micronuclei agrees with previous reports that proteins of the DFC nucleolar region such as fibrillarin gather in nuclear aggregates (dots) in micronuclei devoid of rRNA genes (24). Fibrillarin is found in the nucleolus at the interface between DFC and FC and is also detected in CB (16). In micronuclei devoid of rRNA genes we found it localized exclusively in the round bodies together with NOR-90 and coilin.

Although anti-RNA polymerase I antibodies could be shown to stain the bodies in nucleolus-lacking micronuclei, the staining was rather weak in comparison with either anti-NOR or anti-fibrillarin antibodies. In a previous study on micronucleated PtK_2 cells, Benavente et al. (24) could not detect RNA polymerase I in the round structures present in micronuclei devoid of NOR. In contrast, Hernandez-Verdun et al. (25), using antisera against nucleolar proteins localized in FC, DFC, and GC regions, reported that FC antigens could be detected in variable amounts in the dots of micronucleated PtK_1 cells. However, since the antisera used in the latter study were only characterized by electron microscopy and Western blotting and were not compared to known human autoantibodies, they might have been reacting with a set of autoantigens other than fibrillarin, NOR-90, and RNA polymerase I.

The occurrence of fibrillarin-bearing round bodies in micronuclei devoid of typical nucleoli in micronucleated cells has been reported by Benavente et al. (24). However, the simultaneous presence of coilin, NOR-90 protein, and RNA polymerase I in CB-like structures is a new and interesting observation. Neither PM/Scl nor To/Th antigens, both located in the nucleolar GC region, were detected in the nuclear dots as assessed with autoimmune sera. GC regions are regarded as the sites of ribosomal assembly and our results agree with previous findings

showing that GC antigens, such as ribosomal protein S1 and the nonribosomal protein NO38, do not segregate in the round bodies of nucleolus-devoid micronuclei (24). It is relevant to point out that a similar pattern of macromolecule association with p80-coilin in a different experimental model has been demonstrated by our group. In adenovirus-infected HeLa cells regular CBs disappear and p80-coilin colocalizes with NOR-90/hUBF, RNA polymerase I, and fibrillarin in large agglomerates. PM/Scl and To/Th proteins are not found in these agglomerates (30).

It has been recently shown that in *Xenopus laevis* oocytes coilin shuttles between nucleus and cytoplasm and might be involved in the transport and targeting of U7 snRNP to the *Xenopus* CB (15). All three RNA polymerases can be detected in *Xenopus* CBs and these structures have been suggested to be the site where RNA polymerase transcription and processing complexes are assembled (5). RNA polymerase I has not been detected in the CBs of a variety of cell lines under normal growth conditions (19). It is possible, however, that the CB-like structures in nucleolus-devoid micronuclei represent the aggregation of inactive proteins functionally related to ribosomal biogenesis that may not remain in the CB long enough to accumulate to detectable levels under normal growth conditions.

At present, the reason why coilin, fibrillarin, and NOR-90 gather at CB-like structures in nucleolus-lacking micronuclei is not understood. However, a hypothesis may be derived from the current body of knowledge. Both nucleolus and CB disassemble during mitosis and reassemble in G1. The metabolic requirements of the cell at G1 impose prompt reconstitution of functional nucleoli in order to provide the necessary protein synthesis. In the interphase nucleus, RNA polymerase I, NOR-90, and fibrillarin are found in nucleoli whereas coilin and fibrillarin are found in the CB. During mitosis coilin is phosphorylated at additional sites and CB disassembles (10). Coilin has been shown to carry a self-association N-terminal domain and a cryptic nucleolar localization signal (21,22). We may speculate that the hyperphosphorylated mitotic form of coilin is able to pick up dispersed NOR-90/hUBF, RNA polymerase I, and fibrillarin and direct them via the nucleolar localizing signal to the reforming nucleoli at early G1. This mechanism may contribute to a rapid reconstitution of the nucleoli in G1. Upon dephosphorylation and delivering macromolecules to the reforming nucleoli, coilin would activate the self-aggregation motif and gather to form the CB in the nucleoplasm. This sequence of events is in agreement with the previous observation that CB assembly only occurs after nucleolar reformation in G1. This mechanism would also be consistent with the present observations in micronucleated cells: due to the impossibility of delivering NOR-90/hUBF, RNA polymerase I, and fibrillarin to the nucleolus in nucleolus-devoid micronuclei, coilin would self-aggregate in CB-like structures containing these macromolecules.

Acknowledgments

We thank Dr. Eduard Chan (Shands Cancer Center and UF Autoimmune Disease Center) for thoughtful comments.

References

1. Ramon y Cajal S (1903). Un sencillo metodo de coloración seletiva del retículo protoplasmático y sus efectos en los diversos órganos nerviosos de vertebrados y invertebrados. *Trabajos de Laboratorio en Investigaciones Biológicas*, 2: 129-221.
2. Moneron A & Bernhard W (1969). Fine structural organization of the interphase nucleus in some mammalian cells. *Journal of Ultrastructure Research*, 27: 266-288.
3. Hardin JH, Spicer SS & Greene WB (1969). The paranucleolar structure, accessory body of Cajal, sex chromatin and related structures in nuclei of rat trigeminal neurons: a cytochemical and ultrastruc-

tural study. *Anatomical Record*, 164: 403-432.
4. Lafarga M, Hervás JP, Santa-Cruz MC, Villegas J & Crespo D (1983). The "accessory body" of Cajal in the neuronal nucleus. A light and electron microscopic approach. *Anatomical Embryology*, 166: 19-30.
5. Gall JG, Bellini M, Wu Z & Murphy C (1999). Assembly of the nuclear transcription and processing machinery: Cajal bodies (coiled bodies) and transcriptosomes. *Molecular Biology of the Cell*, 10: 4385-4402.
6. Moreno Diaz de la Espina S, Sanchez-Pina MA & Risueño MC (1982). Localization of acid phosphatasic activity, phosphate ions and inorganic cations in plant nuclear coiled bodies. *Cell Biology International* (*Reports*), 6: 601-607.
7. Andrade LEC, Chan EKL, Raska I, Peebles CL, Roos G & Tan EM (1991). Human autoantibody to a novel protein of the nuclear coiled body: immunological characterization and cDNA cloning of p80-coilin. *Journal of Experimental Medicine*, 173: 1407-1419.
8. Andrade LEC, Tan EM & Chan EKL (1993). Immunocytochemical analysis of the coiled body in the cell cycle and during cell proliferation. *Proceedings of the National Academy of Sciences, USA*, 90: 1947-1951.
9. Carmo-Fonseca M, Ferreira J & Lamond AI (1993). Assembly of snRNP-containing coiled bodies is regulated in interphase and mitosis - evidence that the coiled body is a kinetic nuclear structure. *Journal of Cell Biology*, 120: 841-852.
10. Carmo-Fonseca M, Bohmann K, Carvalho MT & Lamond AI (1994). P80-coilin. In: van Venrooij WJ & Maini RN (Editors), *Manual of Biological Markers of Disease*. Kluwer Academic Publishers, Dordrecht, The Netherlands.
11. Ochs RL, Stein Jr TW & Tan EM (1994). Coiled bodies in the nucleolus of breast cancer cells. *Journal of Cell Science*, 107: 385-399.
12. Ochs RL, Stein Jr TW, Andrade LE, Gallo D, Chan EK, Tan EM & Brasch K (1995). Formation of nuclear bodies in hepatocytes of estrogen-treated roosters. *Molecular Biology of the Cell*, 6: 345-356.
13. Malatesta M, Zancanaro C, Martin TE, Chan EKL, Amalric F, Lührmann R, Vogel P & Fakan S (1994). Cytochemical and immunocytochemical characterization of nuclear bodies during hibernation. *European Journal of Cell Biology*, 65: 82-93.
14. Bellini M & Gall JG (1998). Coilin can form a complex with the U7 small nuclear ribonucleoprotein. *Molecular Biology of the Cell*, 9: 2987-3001.
15. Bellini M & Gall JG (1999). Coilin shuttles between the nucleus and cytoplasm in *Xenopus* oocytes. *Molecular Biology of the Cell*, 10: 3425-3434.
16. Lamond AI & Earnshaw WC (1998). Structure and function in the nucleus. *Science*, 280: 547-553.
17. Ogg SC & Lamond AI (2002). Cajal bodies and coilin - moving towards function. *Journal of Cell Biology*, 159: 17-21.
18. Raska I, Ochs RL, Andrade LE, Chan EK, Burlingame R, Peebles C, Gruol D & Tan EM (1990). Association between the nucleolus and the coiled body. *Journal of Structural Biology*, 104: 120-127.
19. Bohmann K, Ferreira J, Santama N, Weis K & Lamond AI (1995). Molecular analysis of the coiled body. *Journal of Cell Science*, 19 (Suppl): 107-113.
20. Imai H, Fritzler MJ, Neri R, Bombardieri S, Tan EM & Chan EK (1994). Immunocytochemical characterization of human NOR-90 (upstream binding factor) and associated antigens reactive with autoimmune sera. Two MR forms of NOR-90/hUBF autoantigens. *Molecular Biology Reports*, 19: 115-124.
21. Chan EK, Takano S, Andrade LEC, Hamel JC & Matera AG (1994). Structure, expression and chromosomal localization of human p80-coilin gene. *Nucleic Acids Research*, 22: 4462-4469.
22. Hebert MD & Matera AG (2000). Self-association of coilin reveals a common theme in nuclear body localization. *Molecular Biology of the Cell*, 11: 4159-4171.
23. Labidi B, Broders F, Meyer JL & Hernandez-Verdun D (1990). Distribution of rDNA and 28S, 18S and 5S rRNA in micronuclei containing a single chromosome. *Biochemistry and Cell Biology*, 68: 957-964.
24. Benavente R, Schmidt-Zachmann MS, Hügle-Dörr B, Reimer G, Rose KM & Scheer U (1988). Identification and definition of nucleolus-related fibrillar bodies in micronucleated cells. *Experimental Cell Research*, 178: 518-523.
25. Hernandez-Verdun D, Robert-Nicoud M, Geraud G & Masson C (1991). Behaviour of nucleolar proteins in nuclei lacking ribosomal genes. A study by confocal laser scanning microscopy. *Journal of Cell Science*, 98: 99-105.
26. Kung AL, Sherwood SW & Schimke RT (1990). Cell line-specific differences in the control of cell cycle progression in the absence of mitosis. *Proceedings of the National Academy of Sciences, USA*, 87: 9553-9557.
27. Reimer G, Pollard KM, Penning CA, Ochs RL, Lischwe MA, Bush H & Tan EM (1987). Monoclonal autoantibody from a (New Zealand black x New Zealand white) F1 mouse and some human scleroderma sera target an Mr 34,000 nucleolar protein of the U3 RNP particle. *Arthritis and Rheumatology*, 30: 793-800.
28. Barros HC, Verbisck NV, da Silva S, Araguth MF & Mortara RA (1997). Distribution of epitopes of *Trypanosoma cruzi* amastigotes during the intracellular life cycle within mammalian cells. *Journal of Eukaryotic Microbiology*, 46: 332-344.
29. Procópio DO, da Silva S, Cunningham CC & Mortara RA (1998). *Trypanosoma cruzi*: effect of protein kinase inhibitors and cytoskeletal protein organization and expression on host cell invasion by amastigotes and metacyclic trypomastigotes. *Experimental Parasitology*, 90: 1-13.
30. Rodrigues SH, Silva NP, Delicio LR, Granato C & Andrade LEC (1996). The behavior of the coiled body in cells infected with adenovirus *in vitro*. *Molecular Biology Reports*, 23: 183-189.