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ORIGINAL ARTICLE

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Nucleoli undergo structural and molecular modifications during hibernation

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Abstract The nucleolus is a very dynamic structure able rapidly to adapt its activity to the cellular metabolic state. An interesting physiological model characterized by drastic modifications of cellular metabolism is represented by hibernating animals. In the present study we investigated the hepatocyte nuclei of euthermic and hibernating edible dormice (*Glis glis*) with the aim of revealing, by means of ultrastructural and immunocytochemical analyses, possible modifications of nucleolar components during hibernation. Our observations demonstrate that, in deep hibernation, nucleoli undergo structural and molecular modifications: (a) they show numerous nucleoplasmic invaginations and clumps of dense fibrillar component extend from the nucleolar surface; (b) they are frequently in contact with coiled bodies and fibro-granular material, two nuclear bodies usually occurring in the nucleoplasm; (c) the dense fibrillar component contains significant amounts of small nuclear ribonucleoproteins, splicing factors usually distributed in the nucleoplasm. Taken together, these results suggest that during hibernation complex relationships are established between the nucleolus and nucleoplasm, probably related to functional activities peculiar to this physiological phase. However, since no evident nucleolar modification was found in early hibernating dormice, it seems likely that the particular structural and molecular arrangement of nucleoli establishes progressively during hibernation, becoming evident only in the deepest phase, and then disappears upon arousal.

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Introduction

In eukaryotic cells, the nucleolus is the site of ribosomal gene transcription and of rRNA processing and assembly with ribosomal proteins (reviews in Smetana and Busch 1974; Hadjiolov 1985). The various steps of this biochemical pathway take place in distinct nucleolar compartments. During interphase, three nucleolar components can generally be distinguished by electron microscopy: the fibrillar centers (FCs), the dense fibrillar component (DFC) and the granular component (GC) (for nucleolar nomenclature see Jordan 1984). The FCs, which appear as low-contrast round structures, are surrounded, either completely or in part, by an electron-dense rim consisting of tightly packed fibrils, the DFC, while the GC, made up of particles 15–20 nm in diameter, usually constitutes the main body of the nucleolus. Condensed chromatin is mostly located at the periphery of the nucleolus, forming a more or less continuous layer, but it also penetrates into the nucleolar body, appearing as isolated patches. Based on autoradiographic and biochemical studies, the primary steps of pre-ribosome formation have been assigned to the DFC, whereas the subsequent maturation stages occur in the GC (review in Hadjiolov 1985). On the other hand, the intranucleolar location of the transcriptionally active rRNA genes is still a matter of debate, some authors indicating the DFC (Fakan and Puvion 1980; Jimenez-Garcia et al. 1993), while others the FC or its border (Thiry and Goessens 1996).

The nucleolus is a very dynamic structure that can rapidly adapt its activity, and consequently its architecture, to the cellular metabolic state (for reviews see e.g. Busch and Smetana 1970; Hadjiolov 1985; Schwarzacher and Wachtler 1993; Shaw and Jordan 1995).

An interesting physiological model characterized by striking modifications of cellular metabolism is represented by hibernators. Hibernating animals undergo

drastic modifications of their activity during the seasonal cycle: in winter they are able to reduce their body temperature extremely to a few degrees above 0°C and their energy needs to a minimum level, while upon arousal the thermogenic processes restore in a very short time all normal metabolic and physiological functions (for reviews see e.g. Hoffman 1964; Lyman et al. 1982; Wang 1987; French 1988). In previous studies we have demonstrated that cell nuclei of hibernating dormice undergo striking changes in nucleoplasmic constituents (Zancanaro et al. 1993; Malatesta et al. 1994a, b, 1995, 1999; Tamburini et al. 1996). In the present study we investigated the hepatocyte nuclei of euthermic and hibernating edible dormice (*Glis glis*) with the aim of revealing, by means of ultrastructural and immunocytochemical analyses, possible modifications of nucleolar components occurring during hibernation.

Materials and methods

Seven adult individuals of the edible dormouse *G. glis* were used in this study. Wild animals were trapped over a 2 year period in central Italy. This dormouse, like many other wild-living animals in Europe, is protected by law and only a limited number of individuals are available for the purpose of multiple investigations subject to permission from the local authorities. The animals were maintained in an external animal house and provided with food and bedding material; under such conditions they spontaneously began to hibernate in December and awoke in March. Four animals were sacrificed during hibernation (January, three after at least 6 days of continuous hibernation, one 36 h after a periodic arousal) and three during the euthermic period (July). Dormant animals were taken from the cage and immediately killed by cervical dislocation. Euthermic animals were anesthetised with ether and sacrificed as described above. Samples of liver were quickly removed and small fragments were fixed by immersion in 4% paraformaldehyde in 0.1 M Sörensen phosphate buffer at 4°C for 2 h. After washing in Sörensen buffer and in phosphate-buffered saline (PBS), free aldehydes were blocked in 0.5 M NH_4Cl in PBS at 4°C for 45 min. Following washing in PBS, the specimens were dehydrated through graded concentrations of ethanol and embedded in LRWhite resin. Ultrathin sections were placed on grids coated with a Formvar-carbon layer and then either used for morphological and morphometric analyses or processed for immunocytochemistry.

Several antibodies were employed: a mouse monoclonal anti-(Sm)-snRNP (small nuclear ribonucleoprotein) antibody (Lerner et al. 1981), a chicken antibody against the hnRNP (heterogeneous nuclear RNP) core protein (Jones et al. 1980), a rabbit polyclonal anti-fibrillarin antibody (Lapeyere et al. 1990), and a rabbit polyclonal anti-p80 coilin (Andrade et al. 1993) antibody. Sections placed on nickel grids were floated for 3 min on normal goat serum (NGS) diluted 1:100 in PBS and then incubated for 17 h at 4°C with the primary antibody diluted with PBS containing 0.1% bovine serum albumin (BSA, Fluka) and 0.05% Tween 20. After rinsing, sections were floated on NGS, and then reacted for 30 min at room temperature with the secondary gold-conjugated antibody (Aurion) diluted 1:3 in PBS. When antibodies were raised in chickens, a secondary rabbit anti-chicken IgG (EY Labs) diluted 1:100 in PBS, Tween, BSA was applied for 30 min at room temperature prior to the gold-conjugated marker. Following the last incubation, all sections were rinsed and air-dried. As controls, some grids were treated with the incubation mixture without the primary antibody, and then processed as described above. In the case of the chicken antibodies, some grids were also incubated in the absence of both the primary antibody and the rabbit anti-chicken IgG bridge probe.

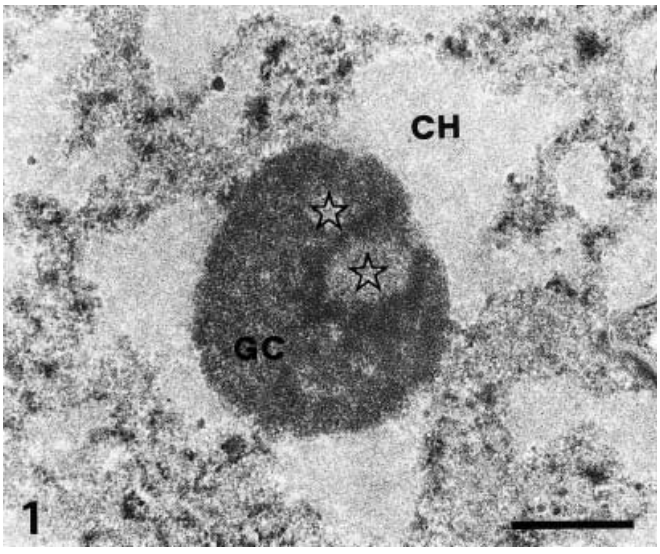


Fig. 1 Hepatocyte nucleus from a euthermic edible dormouse. The nucleolus shows a compact structure, with two fibrillar centers (*open stars*) surrounded by dense fibrillar component and abundant granular component (*GC*). Clumps of condensed chromatin (*CH*) surround the nucleolar body. *Bar* represents 0.5 µm

In order to identify clearly the nuclear structural constituents containing RNPs all sections were stained by the EDTA method (Bernhard 1969). The specimens were observed in a Zeiss EM 902 and a Philips CM12 electron microscope operating at 80 kV.

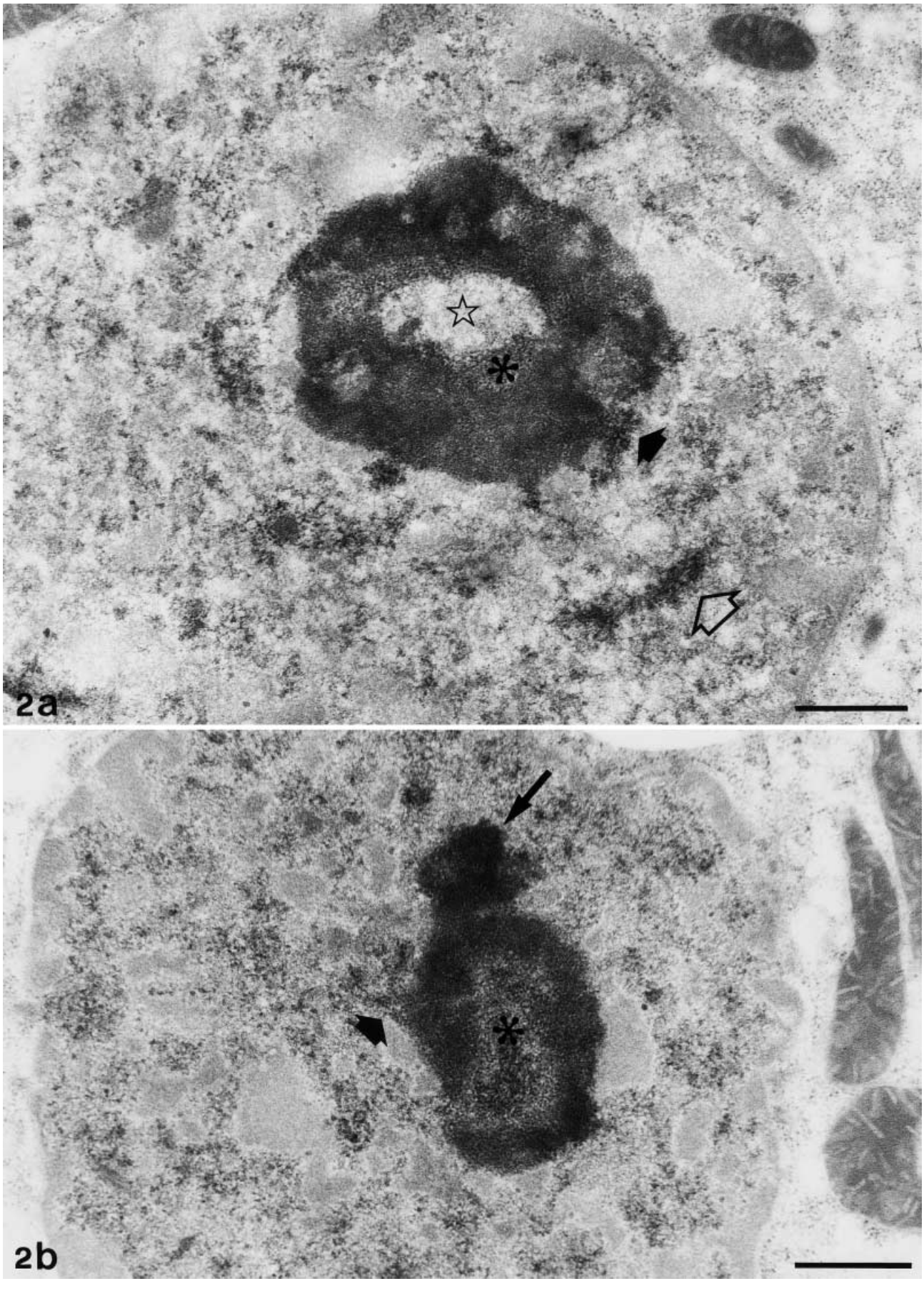
Morphometric analyses on nucleoli and on nucleolar structural components (FCs, DFC and GC) were carried out on sections prepared for ultrastructural morphology. Areas of nucleoli as well as of each nucleolar component were measured on 35 randomly selected micrographs (final magnification ×42,000) from euthermic and deeply hibernating dormice by using a computerized image analysis system (Image Pro-Plus for Windows 95). In hibernating animals nucleoplasmic invaginations were excluded from the nucleolar surface measurement. In each nucleolus the total FC, DFC and GC areas were calculated and expressed as the percentage of FC, DFC or GC area per nucleolus. The mean ± standard error of the mean (SE) values for all parameters were then calculated. Statistical analysis of the results was performed by the non-parametric Mann-Whitney U-test and the statistical significance was set at $P \leq 0.05$.

Results

Electron microscopic examination of hepatocyte nuclei revealed that the nucleoli of deeply hibernating animals undergo structural modifications in comparison with those of euthermic individuals.

In euthermic dormice the nucleolus exhibited a rather compact structure, with some FCs surrounded by DFC

Fig. 2a, b Hepatocyte nuclei from deeply hibernating edible dormice. The nucleoli show nucleoplasmic invaginations (*open star*) in their interior and their shape appears irregular because of dense fibrillar component clumps extending from the nucleolar body (*arrow*). Fibro-granular material trails occur free in the nucleoplasm (*open thick arrow*) as well as in contact with the nucleolar surface (*thick arrows*) or enclosed in nucleoplasmic invaginations (*asterisks*). *Bars* represent 0.5 µm ▶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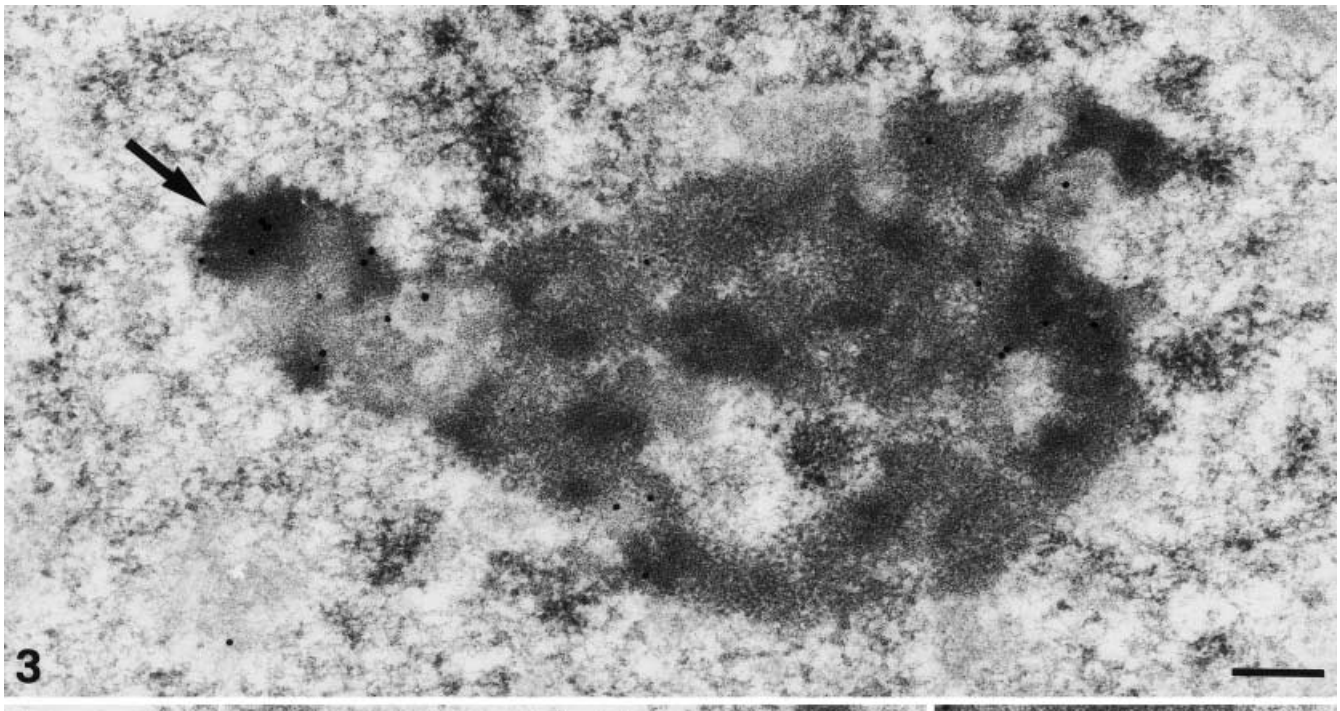
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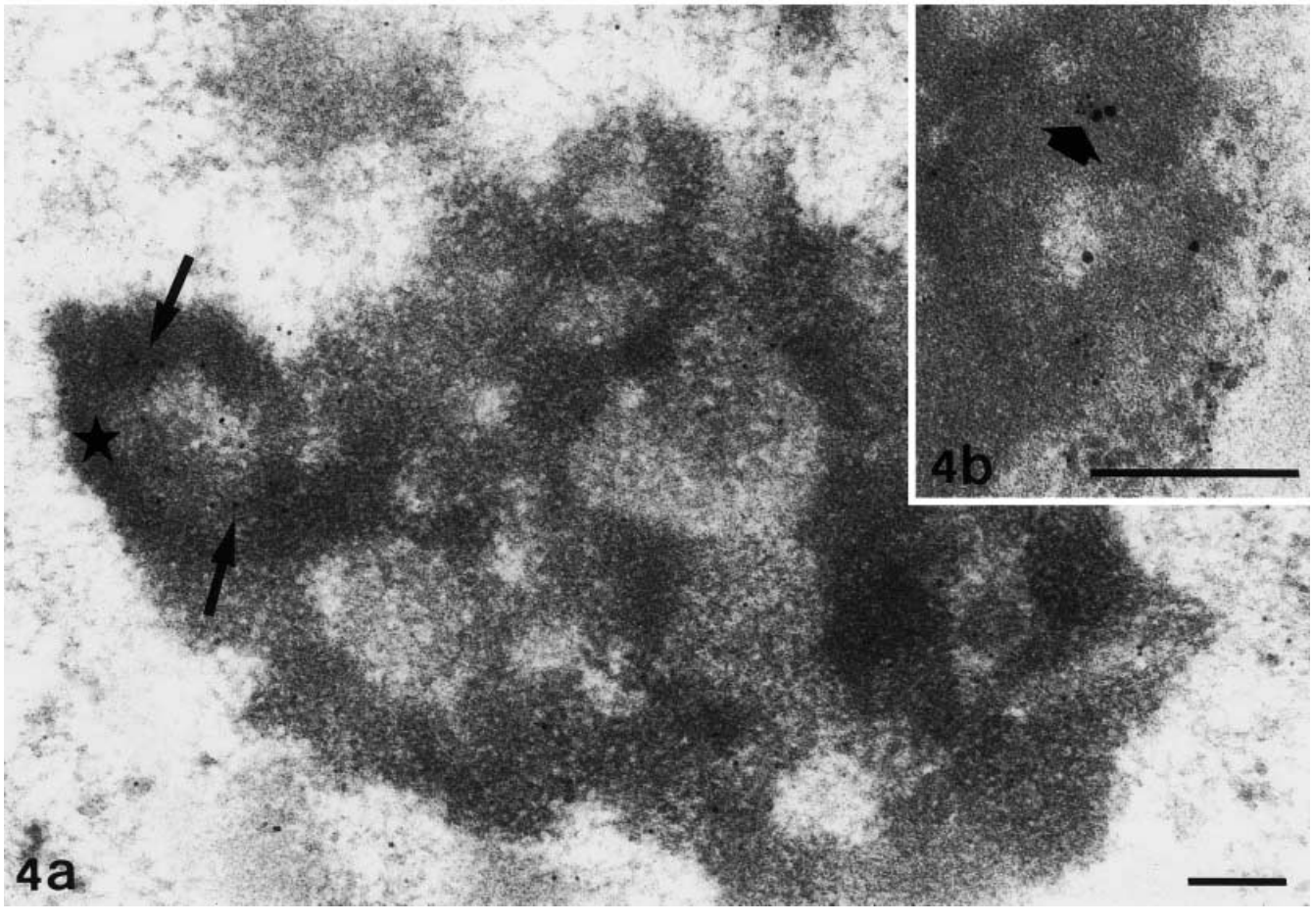
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Table 1 Quantitative characteristics of hepatocyte nucleoli from euthermic and deeply hibernating edible dormice. Values are means ± SE. (DFC, dense fibrillar component; FC, fibrillar centre; GC, granular component)

	Nucleolar area (μm²)	FC area (μm²)	% FC	% DFC	% GC
Euthermia	1.20±0.07	0.05±0.01	8.51±0.93	24.74±1.61*	67.68±2.08
Deep hibernation	1.37±0.05	0.03±0.003	4.40±0.60	23.95±1.41*	72.22±1.55

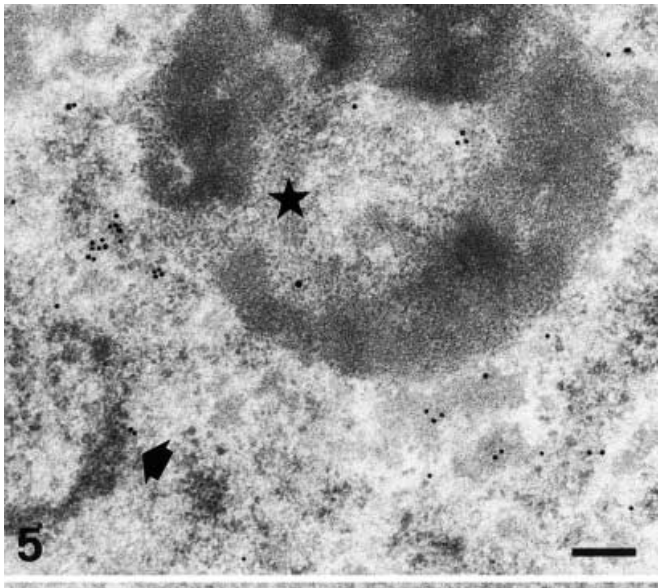
* Values identified by asterisks are not significantly different from one another

and abundant GC; condensed chromatin was mainly located at the periphery and only rare small patches occurred inside the nucleolar body, while nucleoplasmic invaginations were never observed (Fig. 1).

In the hibernating animal sacrificed 36 h after a periodic arousal the nucleoli were quite similar to those of euthermic individuals, apart from the presence of a few small nucleoplasmic invaginations (not shown).

In deeply hibernating dormice the nucleoli still showed clearly recognizable FCs, DFC and GC, but they appeared less compact than in euthermic individuals because of many nucleoplasmic invaginations, sometimes of remarkable size (Fig. 2a); moreover, they could also display irregular shapes due to dense fibrillar clumps extending from the nucleolar body (Fig. 2b). In addition, nucleoli of hibernating animals were frequently associated with two different types of nuclear body usually occurring in the nucleoplasm during deep hibernation: the coiled bodies (CBs) and the fibro-granular material (FGM). The CBs showed a typical substructure consisting in tangled electron-dense threads (Fig. 6); the FGM consisted in trails of a fibro-granular network meandering in the nucleoplasm, spreading out from the nucleolar surface or enclosed in nucleoplasmic invaginations (Figs. 2a, b, 5). The CBs were always observed in close association with the DFC, while the FGM did not seem to make preferential contact with any nucleolar structural component.

Immunocytochemical results revealed that the anti-fibrillarin antibody specifically labeled the nucleolar DFC in all animals; in deeply hibernating dormice the signal was also found in the dense fibrillar clumps extending from the nucleolar body (Fig. 3), thus demonstrating that they represent DFC regions. Moreover, the DFC of deeply hibernating dormice contained significant amounts of snRNPs (Fig. 4a, b), while in euthermic ani-



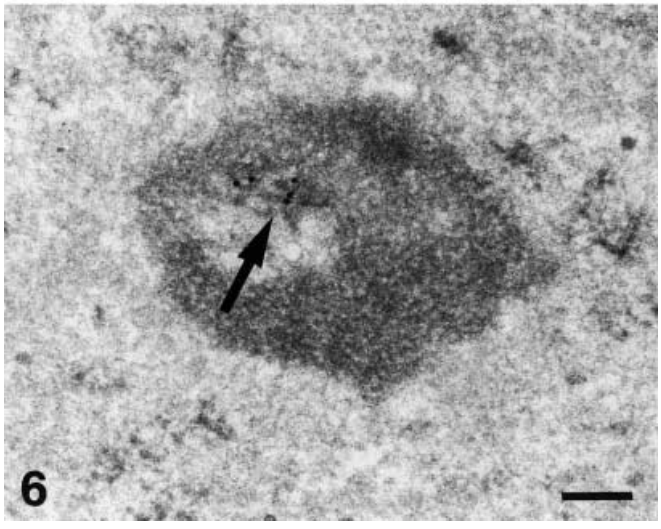


Fig. 5 Hepatocyte nucleus from a deeply hibernating edible dormouse. Immunolabeling with anti-hnRNP (heterogeneous ribonucleoprotein) antibody. The nucleolus appears devoid of gold grains while the fibro-granular material inside the nucleoplasmic invagination *(star)* as well as the fibro-granular trail in the nucleoplasm (*thick arrow*) are specifically labeled. *Bar* represents 0.2 μm

Fig. 6 Hepatocyte nucleus from a deeply hibernating edible dormouse. Immunolabeling with anti-coilin antibody. The coiled body associated with the nucleolus is specifically labeled *(arrow)*, whereas no gold grains are present in the nucleolar body. *Bar* represents 0.2 μm

◀ **Fig. 3** Hepatocyte nucleus from a deeply hibernating edible dormouse. Immunolabeling with anti-fibrillarin antibody. The probe specifically labels the dense fibrillar component inside the nucleolus as well as the dense fibrillar component clump extending from the nucleolar surface *(arrow)*. *Bar* represents 0.2 μm

Fig. 4a, b Hepatocyte nuclei from deeply hibernating edible dormice. **a** Immunolabeling with anti-snRNP (small nuclear ribonucleoprotein) antibody. The signal is evident in the nucleolar body (*arrows*), especially in the dense fibrillar component clump extending from the nucleolar surface (*star*). **b** Double immunolabeling with anti-fibrillarin (15 nm) and anti-snRNP (6 nm) antibodies. The probes colocalize in the dense fibrillar component (*thick arrow*). *Bars* represent 0.2 μm

mals as well as in the dormouse sacrificed 36 h after a periodic arousal the anti-snRNP antibody gave only a negligible nucleolar signal. On the other hand, no hnRNPs were found in the nucleolus in any physiological state studied (Fig. 5). Anti-coilin labeling was observed only in CBs (Fig. 6). Control grids displayed negligible levels of labeling (not shown).

Morphometric results (Table 1) revealed that the nucleolar area significantly increased in deep hibernation, while FC size as well as the percentage of nucleolar surface occupied by FCs strongly decreased. The percentage of nucleolar surface occupied by DFC remained unchanged in euthermic and deeply hibernating dormice, whereas that occupied by GC was higher in deep hibernation.

Discussion

Our observations carried out on hepatocytes of euthermic and hibernating edible dormice (*G. glis*) demonstrate that, in deep hibernation, nucleoli undergo evident structural modifications, showing numerous nucleoplasmic invaginations and DFC clumps extending from the nucleolar surface. Moreover, nucleoli are frequently in contact with CBs and FGM, two types of nuclear body usually occurring in the nucleoplasm during this physiological phase. Finally, the DFC contains significant amounts of snRNPs.

Taken together, our results suggest that during hibernation complex relationships are established between the nucleolus and the nucleoplasm. In fact, the numerous nucleoplasmic invaginations and the DFC clumps extending from the nucleolar surface, leading to an increase in the nucleolus/nucleoplasm interface, might be needed to facilitate the functional relationships between the two nuclear compartments.

In addition, the frequent association of nucleoli with nuclear bodies usually occurring in the nucleoplasm and containing nucleoplasmic factors strongly suggests possible functional interactions between nucleolus and nucleoplasm. These nuclear bodies – CBs and FGM – and their contacts with the nucleolus have been previously described in nuclei of deeply hibernating edible and hazel dormice (Malatesta et al. 1994a, 1999). In particular, the CBs, which have also been reported in cell types belonging to non-hibernating species, contain both nucleoplasmic and nucleolar factors, involved, respectively, in pre-mRNA and pre-rRNA functions. This suggests an ambiguous role for these structural constituents in RNP storage and/or assembly of pre-splicing complexes (for a recent review see Lamond and Earnshaw 1998), perhaps as “shuttling” organelles involved in a migration cycle touching different nuclear domains (Malatesta et al. 1994b). However, in spite of the frequent contacts between CBs and nucleoli occurring in cells of deeply hibernating edible dormice, no coilin – a protein highly enriched in CBs (Andrade et al. 1991; Raska et al. 1991) – has been detected inside the nucleolar body. Therefore, the nucleolar modifications related to the hibernating state are quite different from those observed in metabolically altered cells, whose nucleoli have been demonstrated to accumulate coilin (Carmo-Fonseca et al. 1992; Matera and Ward 1993; Haaf and Ward 1996; Lyon et al. 1997).

Finally, the colocalization in the DFC of snRNPs and fibrillarin, factors involved in early processing phases of pre-mRNA and pre-rRNA, respectively (Kass et al. 1990; Fakan 1994), suggests an involvement of this nucleolar compartment in both nucleolar and nucleoplasmic functions. Based on the detection of poly(A) RNA as well as of nucleoplasmic transcripts and processing factors in the nucleolus (Bond and Wold 1993; Kadowaki et al. 1994; Krause et al. 1994; Schneiter et al. 1995; Tani et al. 1995; Lyon et al. 1997), it has been suggested that, in addition to ribosome production, the nucleolus could also be involved in mRNA transport and/or degradation. If trafficking of snRNPs to the nucleolus usually occurs, maybe for snRNA maturation (Bohmann et al. 1995a, b), it is possible that during hibernation such a step becomes more evident because of the slowing down of the metabolic processes. SnRNPs have been found to accumulate specifically in the DFC, but we do not know whether this nucleolar compartment represents only a storage or also a functional site for these nucleoplasmic factors; in any case the DFC seems to play a key role in nuclear metabolism during hibernation. Previous reports have also demonstrated that physiologically inactive nucleoli contain significant amounts of nucleoplasmic RNPs (Biggiogera et al. 1994; Kopecny et al. 1996a, b). However, nucleoli of hibernating animals cannot be considered as completely inactive: a remarkable reduction in cellular metabolism obviously occurs, but some DNA, RNA and protein synthesis still continues at a low rate even in deep hibernation (Derij and Shtark 1985; Bocharova et al. 1992). Accordingly, nucleoli of hibernating animals maintain a general morphological appearance similar to that of euthermic animals, with clearly recognizable structural components and rare small patches of intranucleolar condensed chromatin. Such nucleoli appear quite different from physiologically inactive nucleoli (Biggiogera et al. 1994; Kopecny et al. 1996a, b) as well as from nucleoli experimentally altered by inhibitory drugs that interfere with certain steps of protein synthesis (segregated, degranulated or fragmented nucleoli) (for reviews see e.g. Busch and Smetana 1970; Bernhard 1971; Simard et al. 1974; Hadjiolov 1985). Moreover, morphometric analyses demonstrate that the modifications typical of nucleolar inactivation – nucleolar condensation, change to fewer larger FCs and decrease in amount of DFC (see e.g. Jordan and McGovern 1981; Lafarga et al. 1991; Schwarzacher and Wachtler 1993) – do not occur in hibernating animals. On the contrary, during hibernation nucleolar surface area increases and FC size decreases, a behavior characteristic of metabolically active cells, thus confirming once again the peculiarity of metabolism in hibernators.

Structural alterations of nucleoli similar to those observed in hepatocytes have been found in all tissues of

deeply hibernating edible dormice examined so far (Malatesta et al. 1999); however, the drastic modifications described in the present study have been observed in hepatocytes only, whereas in the other tissues minor structural changes occur. It is possible that in hepatocytes important nucleolar modifications are needed in order to allow the continuation of significant nucleolar activities during deep hibernation (as suggested by our morphometric data), since the liver maintains significant metabolic rates even in this physiological phase (Lyman et al. 1982; Wang 1987). An alternative hypothesis is that this nucleolar organization is such as to permit a most efficient reactivation upon arousal from hibernation. In fact, in the edible dormouse sacrified 36 h after a periodic arousal no evident nucleolar modification was observed, suggesting that the specific structural and molecular arrangement is established progressively during hibernation, becoming evident only in the deepest phase, and disappearing upon arousal. Tissues of hazel dormice also contain cells with irregularly shaped nucleoli (Malatesta et al. 1994a, 1995), indicating that the nucleolar modifications do not represent a species-specific characteristic, but features that can be more generally related to the phenomenon of hibernation.

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