Journal of Structural Biology 174 (2011) 352–359

Contents lists available at ScienceDirect

Journal of Structural Biology

journal homepage: www.elsevier.com/locate/yjsbi

Nucleolar structure across evolution: The transition between bi- and tricompartmentalized nucleoli lies within the class Reptilia

Françoise Lamaye [a], Sonia Galliot [b,c], Lorenzo Alibardi [d], Denis L.J. Lafontaine [b,c], Marc Thiry [a,*]

[a] Cellular Biology Unit, GIGA-Neurosciences, University of Liège, C.H.U. Sart Tilman, B36, 4000 Liège, Belgium
[b] Fonds de la Recherche Scientifique (FRS-FNRS), Institut de Biologie et de Médecine Moléculaire (IBMM), Université Libre de Bruxelles (ULB), Belgium
[c] Center for Microscopy and Molecular Imaging (CMMI), Académie Wallonie – Bruxelles, Charleroi-Gosselies, Belgium
[d] Dipartimento di Biologia evoluzionistica sperimentale, University of Bologna, Bologna, Italy

ARTICLE INFO

Article history:
Received 23 November 2010
Received in revised form 9 February 2011
Accepted 10 February 2011
Available online 16 February 2011

Keywords:
Nucleolus
Ribosome synthesis
rRNA
Reptiles
Cytochemistry
Immunocytochemistry
DNA

ABSTRACT

Two types of nucleolus can be distinguished among eukaryotic cells: a tricompartmentalized nucleolus in amniotes and a bicompartmentalized nucleolus in all the others. However, though the nucleolus' ultrastructure is well characterized in mammals and birds, it has been so far much less studied in reptiles.

In this work, we examined the ultrastructural organization of the nucleolus in various tissues from different reptilian species (three turtles, three lizards, two crocodiles, and three snakes). Using cytochemical and immunocytological methods, we showed that in reptiles both types of nucleolus are present: a bicompartmentalized nucleolus in turtles and a tricompartmentalized nucleolus in the other species examined in this study. Furthermore, in a given species, the same type of nucleolus is present in all the tissues, however, the importance and the repartition of those nucleolar components could vary from one tissue to another. We also reveal that, contrary to the mammalian nucleolus, the reptilian fibrillar centers contain small clumps of condensed chromatin and that their surrounding dense fibrillar component is thicker. Finally, we also report that Cajal bodies are detected in reptiles. Altogether, we believe that these results have profound evolutionarily implications since they indicate that the point of transition between bipartite and tripartite nucleoli lies at the emergence of the amniotes within the class Reptilia.

1. Introduction

The nucleolus is a distinct subnuclear domain present in all eukaryotic cells. Although the nucleolus is considered today as a multifunctional domain, its primary function is ribosome biogenesis (Olson, 2004; Boisvert et al., 2007; Sirri et al., 2007, 2008, Hernandez-Verdun et al., 2010). Transcription of ribosomal genes, maturation/processing of ribosomal RNAs and assembly of rRNAs with ribosomal proteins all occur within the nucleolus (Hadjiolov, 1985).

At the electron microscope level, the nucleolus appears mainly composed of three components: fibrillar centers (FCs), the dense fibrillar component (DFC) and the granular component (GC) (Thiry and Goessens, 1996). The FCs appear as round structures of variable size, with a very low electron opacity. They are partially surrounded by the densely contrasted DFC. The FCs and DFC are embedded in the GC that mainly consists of granules 15–20 nm in diameter. Using complementary approaches, a spatio-temporal map of ribosome formation in these three nucleolar components was obtained including the localization of rDNA, rRNAs, snoRNAs, as well as several proteins belonging to transcription and processing machineries and ribosomal proteins (Thiry et al., 2000). It was established that transcriptionally active ribosomal genes are located in the FCs and the intimately associated DFC (Cheutin et al., 2002; Derenzini et al., 2006). Therefore, the FCs and associated DFC can be considered a functional entity within the nucleolus producing rRNA molecules that accumulate in the GC where they undergo maturation and assemble into ribosome subunits. Early processing of the pre-rRNAs occurs in the DFC and later processing reactions in the GC.

Despite the above classical description of tripartite nucleolar organization in mammalian cells, many eukaryotic cells have only two morphologically distinct nucleolar components (Thiry and Goessens, 1996).

Previously, based on an extensive review of the literature, we speculated that the emergence of a third nucleolar compartments coincides with transition between anamniote and amniote vertebrates (Thiry and Lafontaine, 2005). At this transition lies the reptile group comprised of turtles, lizards, snakes and crocodiles. However, to this date, the fine structure of the nucleolus has been only reported in two lizard species (Hubert, 1975; Faure et al.,

* Corresponding author. Fax: +32 4 3665173.
E-mail address: mthiry@ulg.ac.be (M. Thiry).

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doi:10.1016/j.jsb.2011.02.003

1987). The nucleolar organization in other groups of living reptiles therefore remains completely unknown.

In the present study, we explored the fine structure of the nucleolus in different species of reptiles belonging to four groups of living reptiles. For this purpose, we applied the acetylation method on different reptilian tissues in order to distinguish clearly the different nucleolar components. We also used different cytochemical (AgNOR proteins) and immunocytological (DNA, fibrillarin, nucleolin) markers to investigate the structural organization of the reptilian nucleolus. Our results clearly indicate that turtles aside, all the living reptiles have a tripartite compartmentalization of the nucleolus. These findings are consistent with the view that during evolution, the tricompartmentalized nucleolus emerged within the class reptilia.

2. Materials and methods

2.1. Biological materials

Two turtles (*Trachemys scripta scripta*, Schoepff 1792 and *Pseudemys scripta elegans*, Wied-Neuwied 1839, Emydidae), a lizard (*Japalura splendida*, Barbour and Dunn 1919, Agamidae) and a snake (*Python regius*, Shaw 1802, Pythonidae) were purchased in authorized pet shops. The Nile crocodile (*Crocodilus niloticus*, Laurenti 1768, Crocodylidae) embryos (39 days) was provided by Samuel Martin, director of scientific research at the crocodile Farm (Pierrelatte, French). The different tissues and organs of these reptilian species used in the present study were summarized in Supplemental data Fig. S1. Fragments of small intestine and stomach from an adult Nile crocodile were provided by Dr. Mc Milinkovitch (Université libre de Bruxelles, Belgium). The epidermis of different other reptilian species were also examined: the turtle (*Emydura macquarri*, Gray 1831, Chelidae) (Alibardi and Thompson, 1999), the lizards (*Podarcis muralis*, Laurenti 1768, Lacertidae and *Anguis fragilis*, Linnaes 1758, Anguidae) (Alibardi et al., 2000), the snakes (*Liasius fuscus*, Peters 1873, Pythonidae and *Natrix natrix*, Linnaes 1758, Colubridae) (Alibardi and Thompson, 2003; Alibardi, 2005) and the crocodile (*Alligator mississippiensis*, Daudin 1801, Alligatoridae) (Alibardi and Thompson, 2001).

2.2. Electron microscopy

Small fragments of several tissues were fixed for 60 min at 4 °C in 1.6% glutaraldehyde in 0.1 M Sorensen's buffer (pH 7.4), acetylated as previously described (Wassef et al., 1979) or stained with AgNOR technique (Ploton et al., 1984). After washing in Sorensen's buffer, cells were dehydrated through graded ethanol solutions and then processed for embedding in either epon. Some fragments were fixed for 20–40 min at 4 °C in 4% formaldehyde in 0.1 M Sorensen's buffer (pH 7.4) and embedded in Lowicryl K4M using the technique of Roth et al. (1981).

Ultrathin sections were mounted on colloidin-coated grids, and stained with uranyl acetate and lead citrate before examination in a Jeol CX 100 transmission electron microscope at 60 kV.

2.3. Detection of DNA: in situ TdT reaction (Thiry, 1992)

Acetylated ultra-thin sections were incubated for 30 min at 37 °C on the surface of the following medium: 100 mM sodium cacodylate (pH 6.5), 10 mM beta-mercaptoethanol, 2 mM $MnCl_2$, 50 μg/ml bovine serum albumin (BSA) (sigma, USA), 20 μM 5-bromo-2-deoxyuridine (BUdR) triphosphate (Sigma, USA), 4 μM each of dCTP, dGTP, and dATP (Boehringer Mannhem, Germany) and 125 U/ml TdT (Boehringer; Mannheim, Germany). After three rinses in bi-distilled water, the different sections were incubated for 20 min in PBS (0.14 M NaCl, 6 mM Na_2HPO_4, 4 mM KH_2PO_4, 1% BSA, pH 7.2) containing normal goat serum (NGS) diluted 1:30. After rinses in PBS containing 0.2% BSA, pH 7.2, the next step of the treatment was a 4 h incubation at room temperature with mouse anti-BUdR antibodies (Roche, USA) diluted 1:50 in PBS pH 7.2 containing 0.2% BSA and 0.2% NGS. After four rinses in PBS containing 1% BSA, pH 7.2 and one in PBS containing 0.2% BSA, pH 8.2, sections were transferred to an incubation medium containing rabbit anti-mouse IgG coupled to colloidal gold (10-nm diameter, Amersham Life Science, Belgium) diluted 1:40 in PBS (with 0.2% BSA), pH 8.2, and incubated for 1 h at room temperature. Samples were rinsed with PBS containing 1% BSA, pH 8.2 four times, then four times with distilled water.

Several kinds of control experiments were carried out. First, TdT or labeled nucleotides were omitted from the TdT medium and in second control, the primary antibody was omitted.

2.4. Immunoelectron microscopy

Ultrathin sections of Lowicryl K4M-embedded cells were incubated for 30 min in PBS 0.1 M (0.14 M NaCl, 6 mM Na_2HPO_4, 4 mM KH_2PO_4, pH 7.2) containing normal goat serum (NGS) diluted 1:30 and 1% BSA, then rinsed with PBS 0.1 M, pH 7.2 containing 0.2% BSA. After a 3 h incubation with primary antibodies diluted in PBS 0.1 M containing 1:50 NGS and 0.2% BSA, the sections were washed with PBS 0.1 M, pH 7.2 containing 1% BSA and once in PBS 0.1 M, pH 8.2, containing 0.2% BSA and then incubated for 60 min with secondary antibody (goat anti-rabit) coupled to colloidal gold (10 nm in diameter) (Amersham Life Science) diluted 1:50 with PBS (pH 8.2) 0.1 M, containing 0.2% BSA. After washing with PBS (pH 8.2) 0.1 M, containing 1% BSA, the sections were rinsed in deionized water. Finally, the ultrathin sections were stained with uranyl acetate and lead citrate before examination in a Jeol CX 100 II transmission electron microscope at 60 kV.

For immunolabelling, several primary antibodies were used: anti-fibrillarin (rabit IgG) from *Xenopus laevis* and diluted at 1:100 (Dr. M. Caizergues-Ferrer (CNRS, Université Paul Sabatier, Toulouse) and anti-nucleolin (rabit IgG) from Carp (*Cyprinus carpio*) and dillued at 1:20 (Dr. M. Alvarez, Université d'Andres Bello, Santiago du Chili).

Two control experiments were carried out, in which either the primary antibodies were omitted, or the sections were incubated with antibody-free gold particles.

2.5. Quantitative evaluation

To evaluate the labelling density, the area of each compartment studied was first estimated morphometrically by the point-counting method (Weibel, 1969). After evaluating the areas (*S*a) occupied by the various compartments, we counted the number of gold particles (*N*i) over each compartment and calculated the labelling density ($Ns = Ni/Sa$).

3. Results

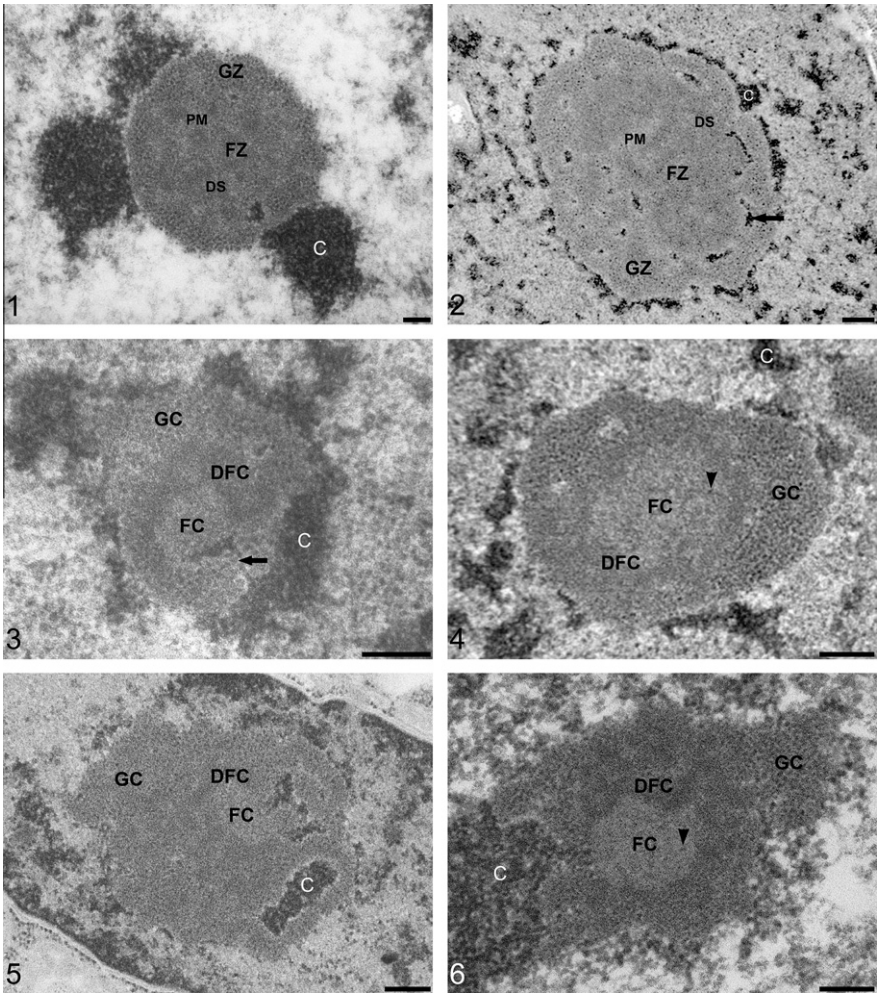
To determine the type of nucleolus present in four different groups of living reptiles, we examined the fine structure of nucleoli in different tissue fragments from several reptilian species: three turtles (*Trachemys scripta scripta*, *Pseudemys scripta elegans*, *Emydura macquarri*), three lizards (*Japalura splendida*, *Podarcis muralis*, *Anguis fragilis*), three snakes (*Python regius*, *Liasius fuscus*, *Natrix natrix*) and 2 crocodiles (*Crocodilus niloticus*, *Alligator mississippiensis*). To facilitate the discrimination between the various nucleolar components, we used the acetylation method known to enhance

the contrast of nuclear and nucleolar structures (Thiry et al., 1985, 1991).

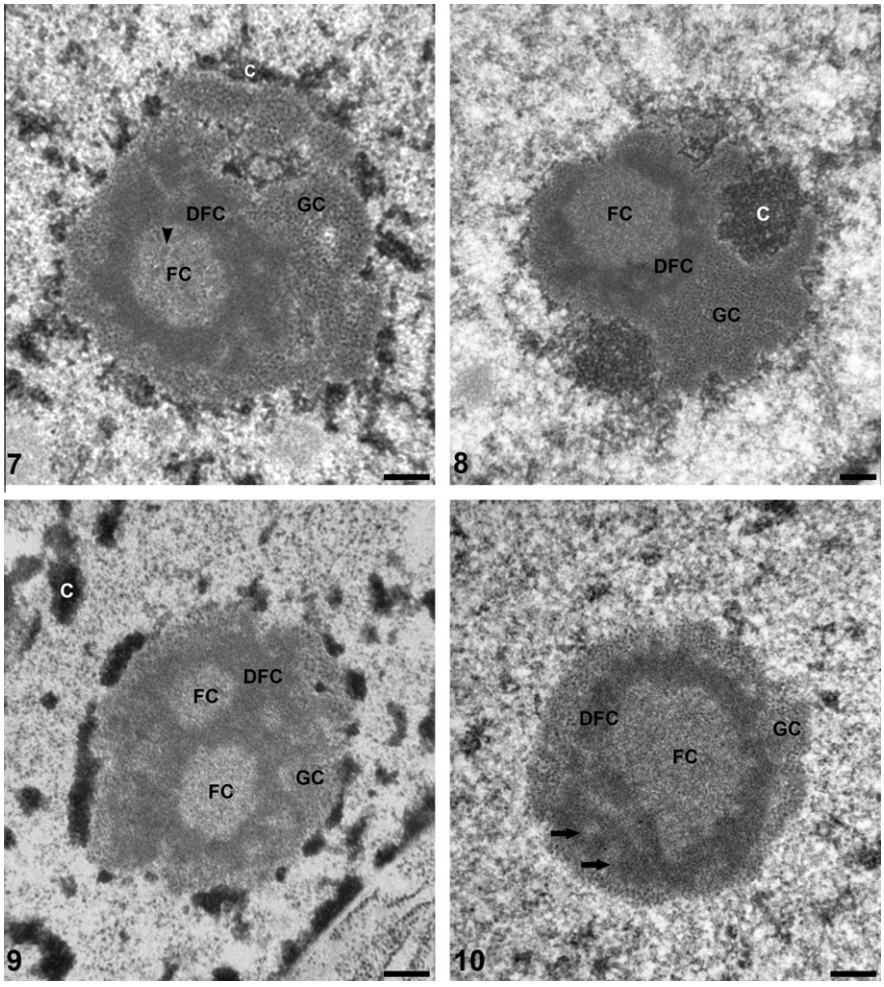
Under these experimental conditions, one or two compact nucleoli, whose size and shape varied from one tissue to another, were detected in reptilian cells (Figs. 1–10). In lizards (Figs. 3 and 7–10), snakes (Fig. 4) and crocodiles (Figs. 5 and 6), whatever the species and the tissues studied, we could easily detect the three fundamental nucleolar components (FC, DFC and GC), as classically described in the bird and mammalian cell nucleolus. As illustrated in the crocodile *Crocodilus niloticus*, nucleolar compartmentalization was also independent of the developmental stage (Figs. 5 and 6). By contrast, in turtles (Figs. 1 and 2, Supplemental data Figs. S2–S5), cell nucleoli were only formed by two main nucleolar compartments whatever the species and the tissues analysed, a fibrillar zone (FZ) containing dark strands (DS) and pale material (PM), as well as, a granular zone (GZ).

In the tricompartmentalized nucleoli of lizards, snakes and crocodiles, one, or sometimes several, FCs were obvious (e.g. see Fig. 9). They appeared as spherical areas of low electron density formed by fine fibrils. Careful examination of this nucleolar compartment revealed that some small clumps of condensed chromatin could be occasionally detected inside the FCs (Figs. 4, 6 and 7). The FCs were partially surrounded by a layer of densely-stained fibrous material, the DFC. This discontinuous rim around the FC could be very thick (Figs. 7–10) and often formed extensions, in the form of repetitive loops in the GC (see e.g. Fig. 10, arrows). Both fibrillar components (FC + DFC) were embedded in the GC that mainly consists of granules of about 15–20 nm in diameter. Besides the three fundamental nucleolar components, the tricompartmentalized nucleolus in reptiles was generally surrounded by a sheath of perinucleolar chromatin. On occasions, this condensed chromatin penetrated deeply into the nucleolar interior by way of spaces, nucleolar interstices, and reached the FCs forming a 'bridge' (Fig. 3, arrow). Nevertheless, in some tissues as in the Sertoli cells of testis from the lizard *Japalura splendida* (Fig. 10), nucleoli appeared to be almost completely devoid of intranucleolar and perinucleolar condensed chromatin.

The bicompartmentalized nucleoli of turtles (Figs. 1 and 2 and Supplemental data Figs. S2–S5) were composed of one or several irregular, compact, fibrillar structures, the fibrillar zone (FZ) and a region formed of granules, the granular zone (GZ). The FZ generally occupied the central part of the nucleolar body, the granules being preferentially found at the periphery. Careful examination of the FZ revealed that this nucleolar compartment was not



Figs.1–6. Bipartite (Figs. 1 and 2) and tripartite (Figs. 3–6) organization of nucleolus in different species of reptiles as revealed with the acetylation method. In turtles, *Pseudemys scripta elegans* (Fig. 1) and *Trachemys scripta scripta* (Fig. 2), the nucleolus is formed of two compartments: a granular zone (GZ) and a fibrillar zone (FZ). The latter is composed of dark strands (DS) and a pale material (PM). In the lizard, *Anguis fragilis* (Fig. 3), the snake, *Python regius* (Fig. 4), and the crocodile, *Crocodilus niloticus*, at embryonic (Fig. 5) and adult (Fig. 6) stage, the nucleolus consists of three main compartments: the fibrillar centre (FC), the dense fibrillar component (DFC) and the granular component (GC). In Fig. 2, the arrow points to an intranucleolar layer of chromatin. In Fig. 3, the arrow points to a 'chromatin bridge' that connects the perinucleolar sheath of chromatin with the interior of the nucleolus at the FC. In the Figs. 4 and 6, the arrowhead points to a small clumps of condensed chromatin situated inside the FC. C: condensed chromatin. Fig. 1 stomach, Fig. 2 trachea, Fig. 3 epidermis, Fig. 4 eye, Figs. 5 and 6 stomach. Bars represent 0.25 μm.



Figs.7–10. Tripartite organization of the nucleolus in different tissues from a lizard, *Japalura splendida*, as observed after the acetylation method. C: condensed chromatin; DFC: dense fibrillar component; FC: fibrillar centre; GC: granular component. In the Fig. 7, the arrowhead points to a small clumps of condensed chromatin situated inside the FC. In the Fig. 10, the arrows points extensions of DFC, in the form of repetitive loops, in the GC. Fig. 7 stomach, Fig. 8 liver Fig. 3 small intestine, Fig. 10 testicle. Bars represent 0.25 μm.

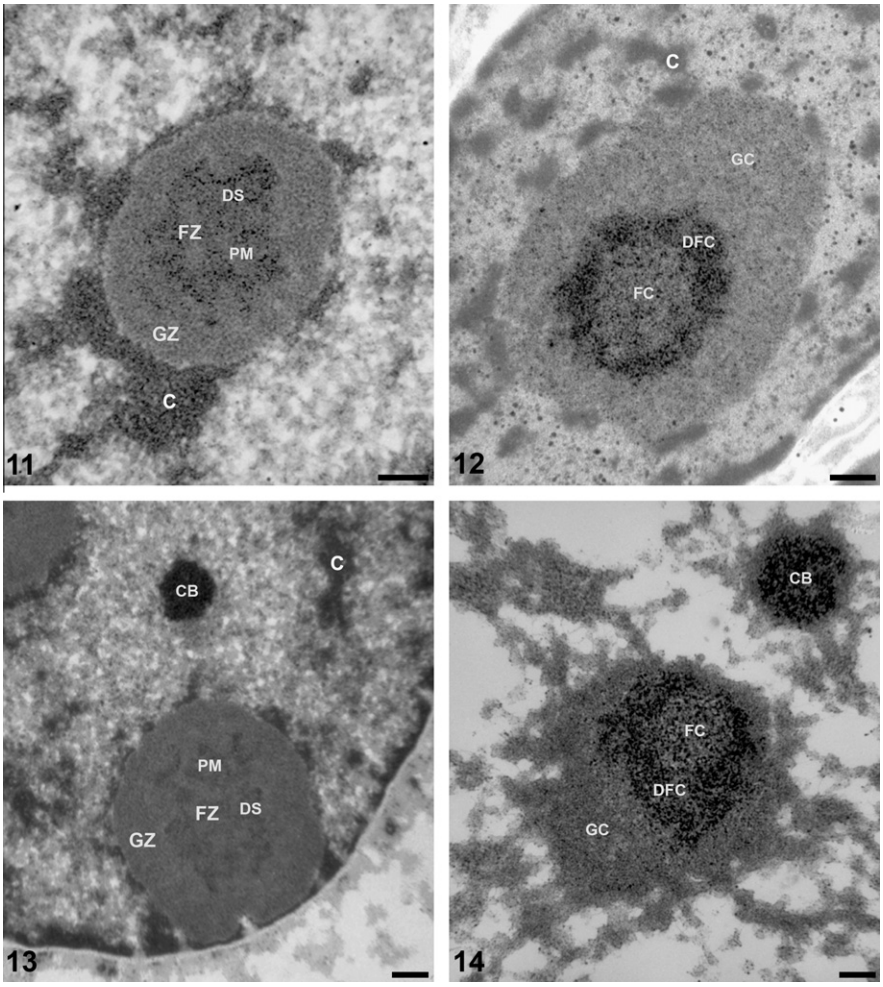
homogeneous; it contained several cordons of high electron density (DS) separated by pale material (PM) that did not form individual structure (Figs. 1 and 2). In addition to the two nucleolar components, clumps of condensed chromatin were generally found around and inside the nucleolar structure in turtles. The intranucleolar clumps of condensed chromatin were frequently observed at the transition between GZ and FZ (Fig. 2, arrowhead, Supplemental data Fig. S4) and, quite interestingly, defined somehow repetitive concentric rings (panel 2 and best seen in panels A and B, Supplemental). The layers of intranucleolar chromatin and of perinucleolar chromatin were also perfectly concentric, consistent with the idea that formation of the nucleolus results from the deposition of successive layers from the inside to the outside of the organelle.

To characterize better the two types of nucleolus in reptiles, we applied different cytochemical and immunocytological techniques on the tricompartmentalized nucleolus of the lizard *Japalura splendida* and on the bicompartmentalized one of the aquatic turtle *Trachemys scripta scripta*.

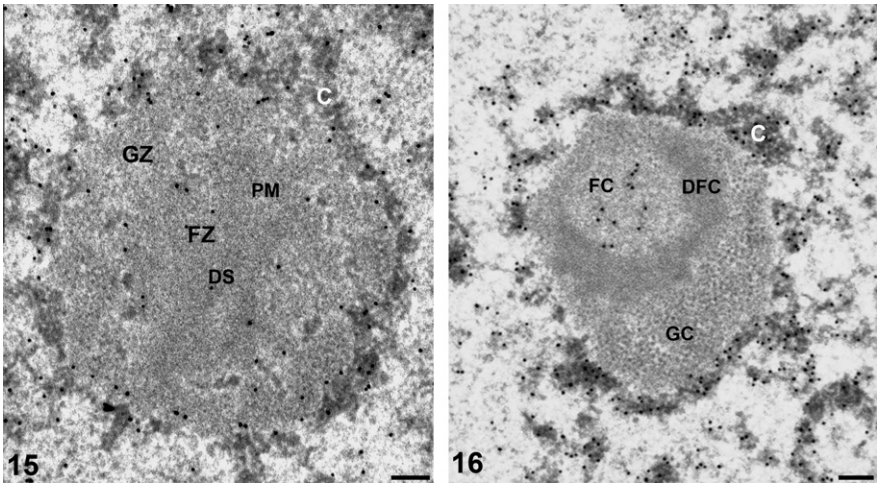
Firstly, we used the AgNOR staining which labels both the FC and the DFC in the tricompartmentalized nucleolus of mammalian cells. In the lizard nucleolus (Fig. 12), a silver-staining was observed in the DFC and, to a lesser extent in the FCs, as in the mammalian nucleolus. In the turtle nucleolus (Fig. 11), only the dense strands of the FZ were silver-stained.

Besides the nucleolar labelling, the AgNOR staining also allowed identifying Cajal bodies in the cell nuclei of both species (Figs. 13 and 14). In most sections, one, sometimes two, Cajal bodies were observed in the nucleoplasm but they did not appear in direct contact with the nucleolus. Reptilian Cajal bodies contained coilin as revealed by immunogold labelling with antibodies anti-p80 (Supplemental data Fig. S6).

To pinpoint the precise localization of DNA within the reptilian nucleolus, we applied the immunogold labelling TdT method on acetylated material. In all reptilian tissues examined (Figs. 15 and 16), we observed an intense label over condensed chromatin blocks. Gold particles were also detected in the interchromatin spaces, preferentially at proximity of the clumps of condensed chromatin. In the lizard nucleolus (Fig. 16), besides the presence of an intense label over intranucleolar and perinucleolar condensed chromatin, DNA was clearly detected over the FCs. By contrast, the DFC and the GC appeared completely devoid of gold particles. In the bipartite nucleolus of turtles (Fig. 15), in addition to the condensed chromatin associated with the nucleolus, a labelling was present in FZ, preferentially over the cordons of high electron density. These observations were confirmed by quantification of the labelling density over the different cellular compartments in both different cell types (Figs. 17 and 18). Indeed, the labelling density was very high over the condensed chromatin, but it was still significant over the FC in the lizard nucleolus and over the dark



Figs.11–14. Localization of AgNOR proteins in the bicompartmentalized (Figs. 11 and 13) and tricompartmentalized (Figs. 12 and 14) nucleoli of reptiles. In the turtle, the dark strands (DS) of the fibrillar zone (FZ) are argyrophilic (Fig. 11). In the lizard, *Japalura splendida*, the dense fibrillar component (DFC) and to a lesser extent, the fibrillar centres are positive (Fig. 12). In both cell types, Cajal bodies (CB) are strongly labelled with silver. C: condensed chromatin; GC: granular component; GZ: granular zone; PM: pale material of the FZ. Figs. 11 and 12 small intestine; Fig. 13 and 14 liver. Bars represent 0.25 μm.



Figs.15 and 16. Localization of DNA in the bicompartmentalized and tricompartmentalized nucleoli of reptiles. An intense labelling is observed over the clumps of condensed chromatin (C) associated with the nucleolus. In the turtle, *Trachemys scripta scripta* (Fig. 15), gold particles are preferentially found over the dark strands (DS) of the fibrillar zone (FZ). In the lizard, *Japalura splendida*, the gold labelling is detected over the fibrillar centre (FC). In both cell types, Cajal bodies are also intensely stained. GC: granular component; GZ: granular zone; PM: pale material of the FZ. Fig. 15 testicle, Fig. 16 stomach. Bars represent 0.2 μm.

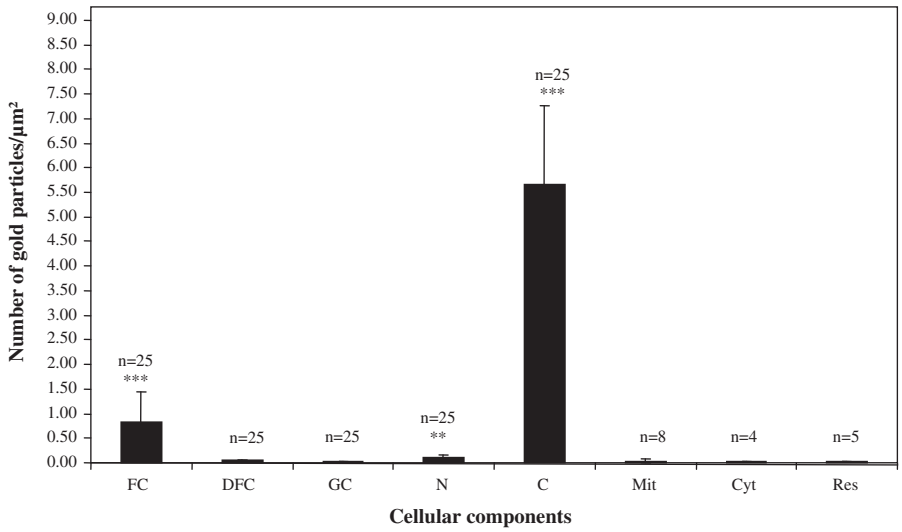


Fig.17. Gold particles densities (number of particles per square micrometer) over different cellular compartments in intestinal epithelium in the lizard, *Japalura splendida*, as revealed with the TdT method. C: condensed chromatin; Cyt: cytoplasm; DFC: dense fibrillar component; FC: fibrillar centre; GC: granular component; N: nucleoplam; R: resin. Student's *t*-test for cellular compartments vs. resin (***$P < 0.001$).

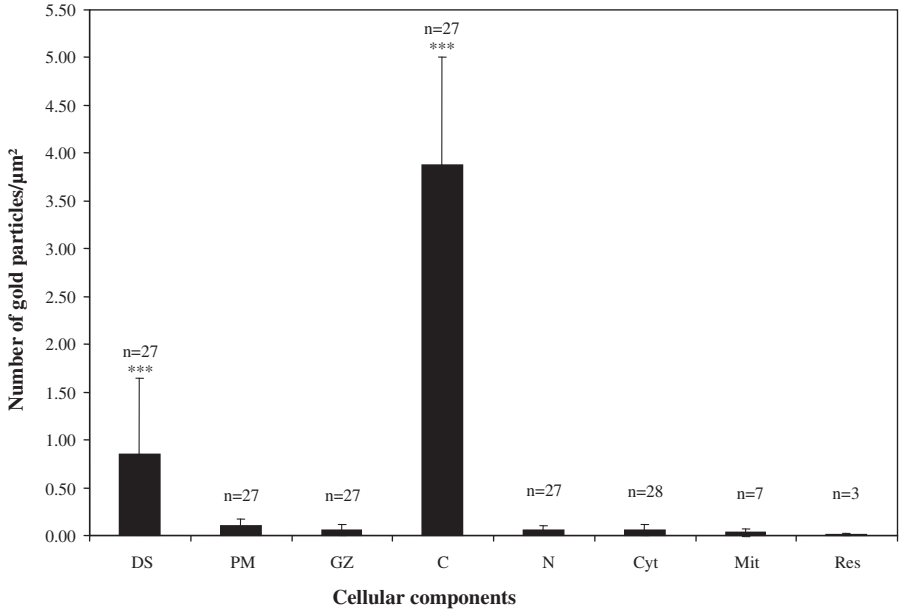


Fig.18. Gold particles densities (number of particles per square micrometer) over different cellular compartments in intestinal epithelium in the turtle,*Trachemys scripta scripta*, as revealed with the TdT method. C: condensed chromatin; Cyt: cytoplasm; DS: dark strand; GZ: granular zone; N: nucleoplam; PM: pale material; R: resin. Student's *t*-test for cellular compartments vs. resin (**$P < 0.005$, ***$P < 0.001$).

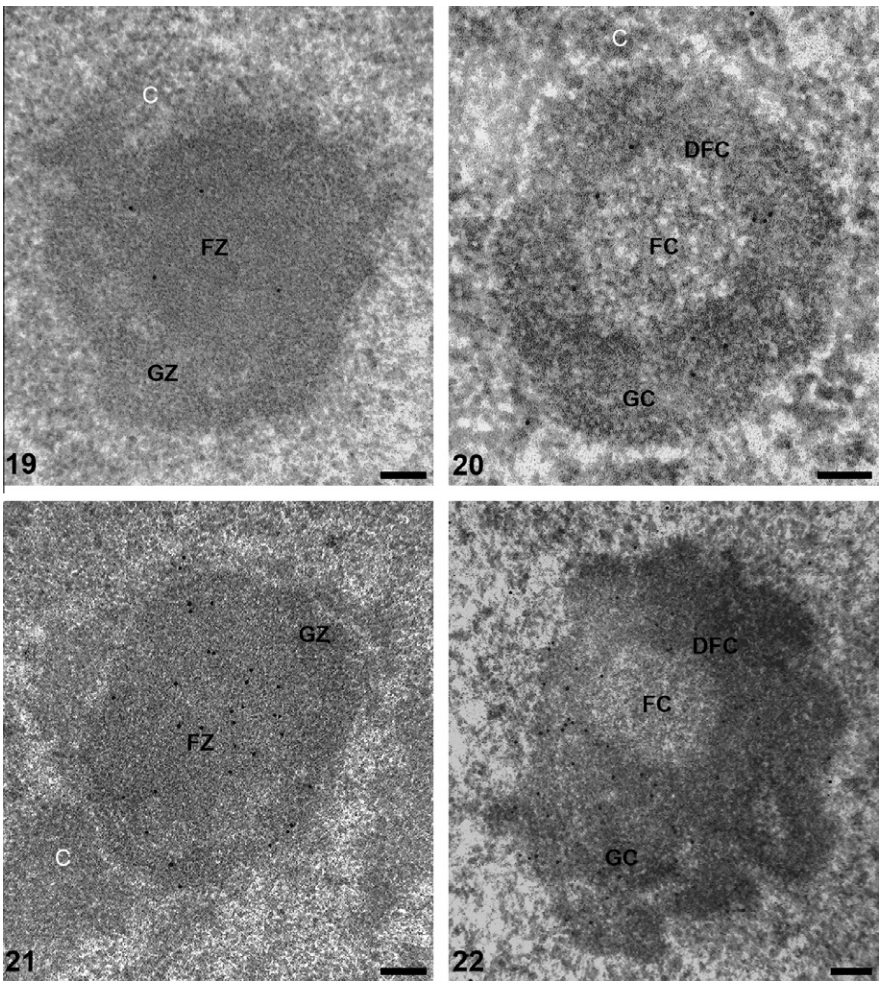
strands of FZ in the turtle nucleolus. By contrast, the labelling density was not significant in the DFC and the GC of lizard nucleoli and in GZ in turtle nucleoli.

Finally, the distribution of two abundant and well-conserved nucleolar proteins, fibrillarin and nucleolin, in both reptilian species was investigated using specific antibodies revealed by gold particles. The electron micrographs of lizard nucleoli showed that anti-fibrillarin antibodies preferentially labelled the DFC (Fig. 20), whereas anti-nucleolin antibodies strongly marked the GC and the DFC (Fig. 22). In both cases, no labelling was observed over the FCs. This distribution is consistent with the known localization of both proteins in mammals (Thiry et al., 2009).

In the cell nucleoli of the turtle, anti-fibrillarin antibodies labelled the FZ (Fig. 19) whereas anti-nucleolin antibodies strongly marked the GZ and the FZ (Fig. 21). However, we could not distinguish where were localized gold particles inside the FZ because the distinction of both components was difficult under the specific experimental conditions used in this experiment (see Section 2). The quantification of the distribution of the gold particles confirmed these observations (Supplemental data, Figs. S7 and S8).

4. Discussion

In this work, in order to bring experimental support to the model that the transition between bi- and tripartite nucleoli occurred with the emergence of the amniotes, we have explored the fine ultrastructural organization of the nucleolus across the group of living reptiles. Our results demonstrate that in living reptiles, both types of nucleolar organization, bi- and tripartite, are present. We observed a bicompartmentalized nucleolus in all species of turtle



Figs.19–22. Immunoelectron microscopy of fibrillarin and nucleolin within the reptilian nucleolus. In the bicompartmentalized nucleolus of the turtle, *Trachemys scripta scripta*, fibrillarin (Fig. 19) is essentially located in the fibrillar zone (FZ) whereas nucleolin (Fig. 21) is found in the fibrillar zone and in the granular zone (GZ). In the tricompartmentalized nucleolus of the lizard, *Japalura splendida*, fibrillarin (Fig. 20) is detected in the dense fibrillar component (DFC) whereas nucleolin (Fig. 22) is observed in the granular component (GC) and the dense fibrillar component. C: condensed chromatin; FC: fibrillar centre. Figs. 17–20 small intestine. Bars represent 0.2 μm.

studied. By contrast, we found a tricompartmentalized nucleolus in lizards, crocodiles and snakes. This study is based on 2–4 species per reptilian group, and our findings are consistent regardless of the species, the tissues or the developmental stage analyzed.

In all reptiles, excepting for turtles, the nucleolus shows a tripartite organization as observed in mammalian and birds. Both fibrillar components of nucleoli (FC and DFC) contain AgNOR proteins. DNA is only found in FC. Fibrillarin is located in the DFC and nucleolin is found in the GC and the DFC. All our results confirm the tripartite organization of nucleoli in most reptiles. However, compared to the mammalian nucleolus, the tricompartmentalized nucleolus of reptiles presents several peculiarities. First, small clumps of condensed chromatin are sometimes detected inside the FC, an observation that could explain the homogeneous distribution of DNA in the reptilian FC. Inside the mammalian FC, DNA is always in a decondensed state and is preferentially localized in its cortex (Derenzini et al., 2006). Second, the layer of DFC in the reptilian nucleolus is particularly thick and frequently forms repetitive loops penetrating deeply into the surrounding GC.

In turtles, like in anamniote vertebrates and arthropoda (Thiry and Lafontaine, 2005), the nucleolus is organized in two major morphological areas: a centrally located fibrillar zone and a peripheral granular zone. We show further that the FZ is composed of a dark and a pale component, the DS and PM, respectively. However, DS and PM are distinctly different from the FC and the DFC of tricompartmentalized nucleoli as the electron dense material does not form a ring around the material of low electron density but forms dark strands embedded in clear areas. Moreover, only dark strands contain AgNOR proteins and DNA. A heterogeneous FZ has already been observed in the nucleolus of the insect cell line Sf9 (Thiry et al., 1991) although rarely reported in bicompartmentalized nucleoli. For example, in yeast, the FZ is exclusively formed by densely-contrasted cordons (Thiry and Lafontaine, 2005).

Together, these data are consistent with the earlier hypothesis that during evolution, the fibrillar part of nucleoli specialized into separate domains, leading to the formation of two morphologically and functionally distinct components: the FC and the DFC, respectively (Thiry and Lafontaine, 2005). They also suggest that the FC appears as specialized areas of tricompartmentalized nucleoli where rRNA genes are concentrated. The emergence in amniote vertebrates of a third nucleolar compartment, the FC, a repository of RNA polymerase I complexes ready to engage rRNA gene transcription, might impart regulatory functions to nucleolar processes (Thiry and Lafontaine, 2005).

Our results are further consistent with the idea that turtles are primitive reptiles. We show indeed that only turtles among the living reptiles present a bicompartmentalized nucleolus, a type of nucleoli characteristic of invertebrates and anamniote vertebrates (Thiry and Lafontaine, 2005). The exact phylogenetic position of

turtles is the most disputed aspect in the reconstruction of the land vertebrate tree of life. Traditionally, textbooks present turtles as primitive reptiles that are the only living survivors of the anapsid reptiles those that lack temporal opening in the skull (Willinston, 1917; Romer, 1966; Lee, 2001). Some of the morphological and most of the molecular phylogenetic evidence seriously challenge this classical view and place turtles as advanced diapsid reptiles in which the two temporal windows in the skull were secondarily closed (Rieppel and Reisz, 1999; Zardoya and Meyer, 2001; Hill, 2005; Iwabe et al., 2005). However, an expanded study of the relationships among the vertebrates and other chordates based on combined, nearly complete 28S and 18S rRNA genes supported the original form of the anapsid hypothesis, with turtles as the sister group of all the other amniotes (Mallatt and Winchell, 2007). Finally, a basal position of turtles to the living reptile clades was also clearly supported by timing of organogenesis data (Werneburg and Sánchez-Villagra, 2009). The morphological evidences presented here are in agreement with the earlier hypothesis that turtles are ancient.

Finally, the present study also evidences for the first time the presence of Cajal bodies in reptilian cell nuclei. Such structures have been reported in other vertebrate groups as well as in insects, plants and yeast (Jennane et al., 1999; Varheggen et al., 2000; Bogolyubov and Parfenov, 2008). Cajal bodies are enriched in snRNP and snoRNP and play a role in their maturation (Cioce and Lamond, 2005; Morris, 2008). The fact that these structures accumulate common components reveals the existence of evolutionarily conserved functions (Cioce and Lamond, 2005).

Acknowledgments

The authors wish to thank Drs. M. Caizergues-Ferrer and M. Alvarez for their generous gift of antibodies. They also acknowledge the skilful technical provided by F. Skivée and P. Piscicelli. This work received financial support from the "Fonds National de la Recherche Scientifique Médicale" (Grant No. 3.4551.10). F.L. is PhD grant holders of the FNRS.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jsb.2011.02.003.

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