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

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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.,

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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