Behaviour of nucleolar proteins in nuclei lacking ribosomal genes

A study by confocal laser scanning microscopy

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Summary

The behaviour of nucleolar proteins in cycling PtK1 cells and in micronuclei with or without NORs was investigated by immunofluorescence using antibodies from autoimmune sera and confocal laser scanning microscopy. These antibodies were shown by electron microscopy to recognize antigens confined to only one of the three basic nucleolar components: fibrillar centres (FC), dense fibrillar component (DFC) and granular component (GC). Serial optical sections allowed us to determine the three-dimensional organization of these components in the nucleolus of cycling cells. Furthermore, clear differences were found in the distribution of the various antigens in micronucleated cells. Three patterns could be observed: (1) the FC antigens were found mainly in the nucleoli, but also in varying amounts in the dots; (2) surprisingly, the DFC antigens were found to accumulate preferentially in the dots; (3) the GC-specific marker stained intensively the nucleoli as well as the dots. The results are interpreted with regard to possible mechanisms for targeting nucleolar proteins to the site of nucleolar formation.

Key words: nucleolar proteins, three-dimensional organization, micronuclei, confocal microscopy.

Introduction

The translocation of proteins from the cytoplasm into the nucleus is likely to occur in at least two steps: (1) the transport to the nucleus; and (2) the targeting to their specific sites. These two steps may be under the control of two different mechanisms. In the first step, the role of signal sequences, chaperone molecules and specific receptors in the pore complexes has been demonstrated for some proteins (Dingwall et al. 1988; Newmeyer and Forbes, 1988; Akey and Goldfarb, 1989; Hunt, 1989; Yamasaki et al. 1989). The second step is poorly understood (Roth and Gall, 1989). Proteins could reach their final position in the nucleus either directly or after forming preassembled complexes.

To gain insight into this latter mechanism, we chose to study the behaviour of nucleolar proteins in nuclei with and without nucleolar organizer regions (NORs). Our choice was dictated by the following considerations: (1) these proteins are assigned to a specific nuclear domain, the nucleolus, which is easy to identify by light microscopy; (2) the formation of nuclei lacking ribosomal genes can be experimentally induced by micronucleation (Ringertz and Savage, 1976); (3) antibodies specific for proteins confined to one subnuclear component that have been obtained from human autoantibody banks can be used as specific markers (Hernandez-Verdun et al. 1988; Masson et al. 1990).

From electron-microscopic studies it is known that the nucleoli, which provide structural support for rDNA transcription as well as for the maturation and storage of the preribosomal subunits, are subdivided into three morphologically distinct components: the fibrillar centres (FC), which correspond to the nucleolar organizer regions (NORs); the dense fibrillar components (DFC), where transcription takes place; and the granular component (GC), the site of maturation and storage of the ribosomal subunits (Goessens, 1984; Hadjiolov, 1985; Hernandez-Verdun, 1986; Scheer and Benavente, 1990). During active rDNA transcription these three basic nucleolar components are intermingled, rendering difficult the analysis of their three-dimensional arrangement. Electron-microscopic studies have yielded much information on the single nucleolar components at the ultrastructural level but do not permit an easy determination of their spatial distribution in the relatively large nucleolar volume. To overcome these difficulties we used confocal laser scanning microscopy (CLSM) to determine the relative positions of the three basic nucleolar components. For selective labelling we used antibodies shown by electron microscopy to recognize antigens confined to only one nucleolar component.

Each of these specific antibodies was also applied to micronucleated cells in order to investigate the distribution of the respective antigens in micronuclei that possess actively transcribed ribosomal genes and in those lacking these genes. Micronucleation can be induced experimentally when cells in culture are exposed to agents interfering with the mitotic spindle (Ringertz and Savage, 1976). We have shown that colchicine can induce the
formation of micronuclei containing single chromosomes in PtK cells (Frackowiak et al. 1986; Labidi et al. 1987a) and that these micronuclei are transcriptionally active (Labidi et al. 1987b). The ribosomal genes are segregated in these micronuclei containing the X-chromosome and the ribosomal RNAs could be found only in this kind of micronuclei (Labidi et al. 1990). However, in the micronuclei lacking ribosomal genes, the presence of nucleolar specific proteins has been demonstrated (Hernandez-Verdun et al. 1979; Benavente et al. 1988). In the present work, we describe the behaviour of certain nucleolar proteins in these different types of micronuclei.

**Materials and methods**

**Cells and immunofluorescence labelling**

PtK cells, cells were cultured in Eagle's minimum essential medium (EMEM) with 0.85 g 1-1 NaHCO3 supplemented with 10% fetal calf serum and 2% glutamine. Micronucleation was induced by culturing actively growing PtK cells for 48 h in the presence of 1 µg ml-1 of colchicine. For immunofluorescence the cells were grown on coverslips, rinsed in phosphate-buffered saline (PBS) and fixed with 3% formaldehyde in PBS containing 1% Triton X-100, or in cold acetone for 4 min. Cells were incubated with sera at the following dilutions: 1/50 for V11, 1/100 for G04, 1/100 to 1/500 for A10. The antibodies were revealed by FITC-conjugated goat anti-human IgG at 1/200 dilution and the DNA was visualized with DAPI. The mounting medium was the anti-fading Citifluor LTD (City University, London).

Human TG and HeLa cells were grown in EMEM with 10% heat-inactivated fetal calf serum.

**The autoimmune sera**

The sera were screened from a human autoantibody bank. Out of the 306 sera containing nucleolar autoantibodies, as identified by indirect immunofluorescence, we selected those that did not contain additional autoantibodies against a variety of nuclear and cytoplasmic antigens. The presence of autoantibodies against nRNP, Sm, La/SS-B, Sc70, J01, PCNA and Ro/SS-A antigens was checked as already described. Among the selected sera, we chose those exhibiting the highest selectivity for one of the nuclear components. Finally, we worked with three different sera from patients with systemic autoimmune disease: V11, A10, G04. The antigens recognized by these sera were identified on immunoblots using the procedure already described by Masson et al. (1990).

**Electron microscopy**

The standard procedures for electron microscopy on PtK cells have been described previously (Gérard et al. 1989). For the immunolabelling, we used the two sensitive cytochemistry described for immuno-electron microscopy.

1. Cells were washed twice in PBS and fixed for 1 h at room temperature with either phosphate-buffered 4% paraformaldehyde or 2% paraformaldehyde containing 0.1% glutaraldehyde. After fixation, free aldehyde groups were blocked by incubation in 0.5 M NH4Cl for 2 h at room temperature. Fixed cells were rapidly plunged into liquid nitrogen after cryoprotection and then cryosectioned according to the method of Tokuyasu (1975).

2. Cell monolayers seeded on Millipore chambers (Millipor HA) were fixed in situ by quick-freezing using the Reichert-Jung Cryovacuum block, according to the method of Eacaghi (1981). The frozen monolayers were stored in liquid nitrogen and submitted for 3 days to cryosubstitution by acetone and then a mixture of 1.5% OsO4 and acetone before embedding in Epon. To permit labelling of antigenic sites on osmium-fixed samples, HeLa cell monolayer sections were pretreated with a saturated aqueous solution of sodium metaperiodate (Bendayan and Zollerger, 1983) for 20 min before incubation with antibodies.

The labelling with the different antibodies was performed by incubations with appropriately diluted sera followed by several washes and incubation with 10 nm protein A–gold particles or 10 nm anti-human IgG–gold particles. The procedures have been described in detail previously (Hernandez-Verdun et al. 1988; Masson et al. 1990). The control assays were carried out with a pool of normal human sera.

**Confocal laser scanning microscopy**

The laser scanning microscope (Carl Zeiss, Oberkochen, FRG) used in this study has been described previously (Robert-Nicoud et al. 1989). It is equipped with three laser excitation systems, confocal optics and a high-precision mechanical scanning stage (0.25 µm in the X/Y plane and 50 nm for the Z axis) operated by an MPC controller through an IEEE interface. The acquisition of data is through an 8-bit 512 X 512 frame buffer coupled by an IEEE interface to a central DEC Micro Vax II processing system. Image processing was carried out using TCL-image (Multihouse, TSI Amsterdam) an image analysis software developed at the Delft Centre for Image Processing. Confocal sections were obtained using an oil-immersion Plan-Apochromat 63/1.4 objective and zoom factors between 4 and 8. Excitation was at 488 nm; fluorescence emission was measured using a FT 510 diaphragm and a longpass filter (LP615). Series of up to 15 optical sections 0.5-1µm apart were used to generate stereoscopic reconstructions of the structures according to the method of Inoué and Inoué (1986) or Brakenhoff et al. (1986). Three-dimensional reconstructions were computed using Periconor 2001 (MS21 Guyancourt France) and displayed by the back-to-front colour-range method (Gérard et al. 1988).

**Results**

**Electron-microscopic localization of the antigens**

From the sera containing antibodies with nucleolar specificity and showing no cross-reactivity with other nuclear or cytoplasmic proteins, we selected those found by immunofluorescence microscopy to label only part of the nucleoli. These sera were then applied to electron-microscopic (EM) preparations in order to determine if the antigens were confined to one of the three nucleolar components. We tested a number of electron-microscopic procedures and selected those with optimal antigen preservation for each of the different sera. Using these different procedures, we determined the nucleolar localization of seven sera. Three of these were found to detect antigens confined to only one of the basic nucleolar components and one stained two of them. The V11 serum was found to be specific for the fibrillar centres (FC); as shown in cryosections, gold particles were exclusively located on this region (Fig. 1A). The staining pattern obtained on cryosections, gold particles were exclusively located on this region (Fig. 1A). The staining pattern obtained on cryosections, gold particles were exclusively located on this region (Fig. 1A). The staining pattern obtained on cryosections, gold particles were exclusively located on this region (Fig. 1A). The staining pattern obtained on cryosections, gold particles were exclusively located on this region (Fig. 1A).

**Ultrastructure of PtK1 nucleoli and micronuclei dots**

PtK1 cells were chosen for this investigation for two reasons: (1) they possess a simple karyotype with only one
Fig. 1. Ultrastructural localization and characterization by immunoblotting of the antigens recognized by the three sera. (A) Immunolabelling of TG cell cryosection with V11 serum. Serum was applied at 1/10 dilution for 15 min and revealed using 10 nm protein A-gold particles. The gold particles are observed exclusively on the fibrillar centre (FC). ×45 000. (B) Immunolabelling of TG cell cryosection with A10 serum. Serum was applied at 1/50 dilution for 15 min followed by 10 nm protein A-gold particle labelling. The gold particles are distributed on the dense fibrillar component (DFC). ×48 000. (C) Immunolabelling of quick-frozen HeLa cell section with G04 serum. Serum was applied at 1/200 dilution for 2 h followed by 10 nm IgG-gold particle labelling. The gold particles are localized over the granular component (GC). ×48 000. (D) HeLa nuclear proteins blotted on nitrocellulose were incubated with the three sera. The specific bands were revealed by peroxidase-conjugated anti-human immunoglobulins. Lane a, V11 serum; lane b, A 10 serum; lane c, G04 serum.
Fig. 2. (A) Ultrastructural morphology of nucleoli in a cycling PtK<sub>1</sub> cell. Cells were fixed by glutaraldehyde and osmic acid and embedded in Epon; the sections were then contrasted by uranyl acetate and lead citrate. The two nucleoli exhibit a reticulated organization with a few FCs included in a network of DFC. ×25,000. (B) Ultrastructural morphology of nucleolus-like bodies (dots) in a micronucleated PtK<sub>1</sub> cell. Preparation procedure as above. The dots show a dense homogeneous fibrillar structure. ×25,000.

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Fig. 3. CLSM localization of the FC-specific marker. (A–B) Interphase PtK₁ cell. (A) Phase-contrast. (B) Superimposition of immunofluorescence (confocal optical section) and phase-contrast images. The labelling is seen in one nucleolus in 5 beaded structures. No labelling was observed in the other nucleolus at this focal plane, but was visible at other levels. ×2500. (C–D) Telophase PtK₁ cell. Stereoscopic reconstruction from 3 optical sections. Labelling is shown superimposed onto the phase-contrast image. Both newly formed nucleoli exhibit labelling in 3 or 4 regions. ×2800. (E–F) Micronucleated PtK₁ cell. (E) Phase-contrast. (F) Superimposition of immunofluorescence and phase-contrast images. The labelling is highest in the nucleoli (nu) and some dots are weakly labelled (arrows); others show no labelling (arrowheads). ×1800.
Fig. 4. CLSM localization of the DFC-specific marker. (A–B) Interphase PtK₁ cell. Stereoscopic reconstruction from 9 optical sections 0.5 μm apart. Labelling is shown superimposed on the phase-contrast image. Both nucleoli exhibit labelling as 6–7 beaded structures localized over the region with high contrast. ×2500. (C–D) Computer-assisted 3-D reconstruction from a series of 9 confocal immunofluorescence images showing the spatial organization of the labelled structures. Stereoviewing of these reconstructions shows a spiral distribution. ×4200. (E–F) Micronucleated PtK₁ cell. (E) Phase-contrast. (F) Superimposition of immunofluorescence (one confocal optical section) and phase-contrast images. The labelling is strongest over the dots and weaker in the nucleolar region (arrows). ×2100.
Fig. 5. CLSM localization of the GC-specific marker. (A–B) Interphase PtK₁ cell. (A) Phase-contrast. (B) Superimposition of immunofluorescence (one confocal optical section) and phase-contrast images. Strong labelling of the external regions of the nucleoli is evident. ×2300. (C–D) Micronucleated PtK₁ cell. Stereoscopic reconstruction from 10 optical sections 0.8 μm apart. Labelling is strong over both the nucleoli and dots. A weak nucleoplasmic labelling is seen mainly in micronuclei containing dots. ×1700. (E–F) Computer-assisted 3-D reconstruction from a series of 10 confocal immunofluorescence images showing the distribution of the labelling in the micronucleus. ×2200.
NOR per nucleolus, thus precluding the formation of complex structures, which could result from the association of several NORs in a nucleolus; (2) they are easily micronucleated to generate micronuclei with and without ribosomal genes. The PtK₁ nucleoli in cycling cells showed a typical reticulated structure, i.e., the fibrillar components formed a network (Fig. 2A). The GC is organized as aggregates located at the periphery of this network. In micronucleated cells, as already described (Hernandez-Verdun et al. 1979), some micronuclei possess a complete nucleolar structure, whereas others contain only fibrillar structures called dots, whose size varies between 0.1 and 0.6 μm (Fig. 2B). We have shown earlier that in each micronucleated cell only those micronuclei with a nucleolus are actively engaged in rDNA transcription (Labidi et al. 1990).

CLSM localization
A precise localization of the antigens was obtained by superimposition of the phase-contrast and fluorescence images acquired using the same laser line. Furthermore, three-dimensional reconstructions were generated from serial optical sections by using different procedures (see Materials and methods).

FC-specific marker
The staining pattern obtained with the FC-specific marker (V11) exhibited three to five fluorescent spots per nucleolus in interphase cells (Fig. 3A–B). During mitosis, the labelling was found to be associated with two chromosomes. At telophase the newly formed nucleoli also displayed a distribution of these antigens in three to five sites per nucleolus, which could be seen on three consecutive optical planes 0.5 μm apart (Fig. 3C–D).

In micronucleated cells the FC marker exhibited a complex distribution. In each cell examined, the nucleoli were strongly labelled and the nucleoplasm was negative. Three types of dot-containing micronuclei were found: those with only positive dots, those with only negative dots and those containing both positive and negative dots. The latter case was the most frequent (Fig. 3E–F).

DFC-specific marker
With the DFC-specific marker (A10), the staining was confined to from one to ten discrete sites located in the dense structures of the nucleoli as observed by phase-contrast (Fig. 4A–B,C–D). The spatial arrangement of these fluorescent spots differed from one nucleolus to another. In cells where the nucleolonema was clearly visible by phase-contrast, it could be seen generally to form circular or semi-circular figures (Fig. 4C–D). The number of spots per nucleolus and the distance between them did show some variation; on the other hand, the size of the spots remained quite constant from one nucleolus to another. At prophase, the number of labelled sites decreased and they were more closely packed than in the interphase nucleoli.

In micronucleated cells, we found spot labelling in the nucleoli with the A10 serum but, surprisingly, a stronger labelling in all the dots. In some micronuclei the nucleoplasm contained dispersed antigens (Fig. 4E–F). Observations of spontaneously formed micronuclei showed that the increased antigen concentration was not the result of colchicine treatment, but rather the consequence of micronucleation.

Discussion
The nucleolus is a very complex structure with multiple components; it is known to be the organelle of ribosome biogenesis (Nigg, 1988). However, despite the fact that the morphology of the nucleolus has been extensively studied, we are still lacking precise answers to such fundamental questions as the process of nucleolus formation and the role played by ribosomal genes in the assembly of the nucleolus (Karpen et al. 1988). In the present study, we have been interested in determining if the behaviour of non-ribosomal nucleolar proteins depends upon the presence and state of activity of the ribosomal genes. One hypothesis is that the presence of ribosomal sequences is required for assembly of nucleolar constituents; alternatively, the proteins may have intrinsic self-assembly properties allowing formation of prepackaged ‘modules’, which would later accumulate at the site of rRNA synthesis. In addition, signal sequences could mediate the targeting of the protein to the nucleoli as was recently shown for viral proteins (Nosaka et al. 1989; Kubota et al. 1989). We took advantage of the possibility of inducing the formation of micronuclei that do or do not contain ribosomal genes and of using specific markers for antigens associated with different subnucleolar compartments for investigating these questions.

Nuclei with active ribosomal genes
In cycling PtK₁ cells, the two NORs are active and two reticulated nucleoli are formed. By laser scanning microscopy, we mapped the three-dimensional distribution of the proteins in the nucleolar volume. The GC marker was found to be confined to the nucleolar periphery, whereas the FC and DFC markers are centrally located in discrete sites. The finding that the structures labelled by the latter two markers appear to be intermingled has interesting bearing on the internal architecture of the nucleolus in these cells. In PtK₁ cells, there is only one NOR per nucleolus, but the FC marker was distributed in several discrete sites (generally 3–5). It has been shown in the phytohaemagglutinin (PHA)-stimulated human lymphocytes that the number of FCs is also higher than the number of NORs and can approach the number of ribosomal genes after 48 h in culture (Hozak et al. 1989; Ochs and Smetana, 1989). We propose that during interphase FCs alternate with substructures of another component in one functional NOR. It is tempting to
consider that this pattern reflects the alternation of transcribed and non-transcribed rDNA. It has already been shown by EM that DFC regions are intercalated between the FCs (Hozak et al. 1986; Thiry and Goessens, 1986; Ploton et al. 1987a). This hypothesis is well corroborated by the three-dimensional localization we observed for the antigens confined in the DFC and by the finding that the number of signals with the DFC marker is generally higher than for the FC marker. These results are in agreement with data from three-dimensional reconstruction of EM serial sections of pancreatic rat nucleoli (Géraud et al. 1990). Another interesting finding is the distribution of the DFC marker in sites arranged in spiral order.

Nuclei without ribosomal genes

In the micronucleated PtK cells, it has been demonstrated that some micronuclei contain an active nucleolus whereas others only possess several fibrillar dots (Phillips and Phillips, 1979; Géraud et al. 1989). These dots react with anti-nucleolar antibodies and are stained by NOR-silver staining (Hernandez-Verdun et al. 1979; Benavente et al. 1988b); therefore, they correspond to nucleolar constituents in nuclei lacking ribosomal genes (Labidi et al. 1990). In the present study, we obtained three new findings on the composition of these nucleolar dots.

First, we found that each nucleolar antigen tested can be present in the dots. These results are in contrast to those of a previous study in which only antibodies directed against the DFC were found to accumulate in the dots (Benavente et al. 1988a). This could be due to the fact that different antibodies were used and/or that confocal laser scanning microscopy allows better visualization of such small structures.

Second, there was a great variability in the amount of these nucleolar antigens in the dots. The FC antigen was found to be present in a smaller amount in dots than in the nucleoli, the GC-specific antigens were equally concentrated in both, and the DFC antigen was present at a higher concentration in dots. The distribution of the DFC antigens appears to be inconsistent with the type of activity that takes place in this nucleolar compartment. However, it is not the only example of accumulation of DFC proteins in non-transcribing structures, since Ag-NOR proteins have also been found in the dots (Hernandez-Verdun et al. 1979). These observations indicate that the material accumulated in dots does not only represent nucleolar proteins coating the chromosomes during mitosis and trapped in micronuclei at the time of nuclear envelope formation. The presence of these nucleolar proteins, including those specific for the FC, in all the micronuclei indicates that they were addressed to nuclei independently of the presence of ribosomal genes.

Third, the dots, while containing antigens specific for all three nucleolar components, have ultrastructural features similar to those of the DFC. Therefore, they could be considered as subnucleolar modules in which the proteins are not assembled in the same way as in a functional nucleolus. Prepackaged nucleolar modules with similar structures are known to occur during the first step of nucleologenesis at the end of mitosis. These so-called prenucleolar bodies (PNBs), contain nucleolin (C23), the B23 proteins and Ag-NOR proteins (Ochs et al. 1985; Ploton et al. 1985b). Except for the finding that both dots and these PNBs contain Ag-NOR proteins, it is not known to what extent the compositions of these structures are identical. In any case, both structures seemed to behave similarly in the presence of active NORs. They are targeted to these latter sites and reorganize themselves to form functional nucleoli. Furthermore, the mere presence of NORs is not sufficient for this targeting to occur: active ribosomal RNA synthesis is required (Benavente et al. 1987; Karpen et al. 1988).

In conclusion, the present data indicate that the nucleolar proteins, in the presence of specific genes, are distributed in accordance with their role in ribosome biogenesis in functional nucleolar structures; alternatively, in the absence of ribosomal genes and rRNA they can aggregate in prepackaged independent modules. As a consequence of the aggregation, a single signal sequence in only one of the proteins would be sufficient to mediate the targeting of these preformed modules to their final position.

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