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Cell cycle redistribution of U3 snRNA and fibrillarin

Presence in the cytoplasmic nucleolus remnant and in the prenucleolar bodies at telophase

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SUMMARY

The distribution of the U3 small nuclear RNA during the cell cycle of the CHO cell line was studied by in situ hybridization using digoxigenin-labelled oligonucleotide probes. The location of the hybrids by immunofluorescence microscopy and at the ultrastructural level was correlated with the distribution of two nucleolar proteins, nucleolin and fibrillarin. The U3 snRNA molecules persist throughout mitosis in close association with the nucleolar remnant. U3 snRNA is present in the prenucleolar bodies (PNBs) and could participate in nucleologenesis in association with several nucleolar proteins such as nucleolin and fibrillarin. The interaction of U3 snRNP with the 5′ external spacer of pre-RNA newly synthesized by active NORs is proposed to be the promoting event of nucleologenesis.

Key words: U3 snRNA, fibrillarin, nucleolin, nucleologenesis, in situ hybridization, immunocytochemical localization, CHO cell

INTRODUCTION

In all eukaryotic cells, the nucleolus is devoted to the transcription of the ribosomal genes as well as to the processing and initial assembly of rRNA with ribosomal and non-ribosomal proteins (Hadjiolov, 1985). During interphase, ribosome biogenesis takes place in a defined nucleolar domain, which can be identified at the electron microscopic level. Cytochemical experiments have been carried out in order to localize the macromolecules involved in the different steps of ribosome biogenesis, in the different subcompartments of the nucleolus (Raska et al., 1990; Scheer and Benavente, 1990; Fischer et al., 1991, and references therein). Three major components are recognized, the fibrillar centres (FCs), the dense fibrillar component (DFC), and the granular component (GC). The fibrillar centres, which appear as low-contrast rounded structures, are surrounded by the dense fibrillar component formed by densely stained fibrils included in the granular component. Condensed chromatin is located at the periphery of the nucleolus and forms patches in the GC (Thiry et al., 1988), and contains rDNA as was demonstrated by in situ hybridization (Puvion-Dutilleul et al., 1991a,b).

In higher eucaryotic cells, the nucleolus disappears at the start of mitosis, and its subsequent re-formation is a dynamic and complex process. Nucleologenesis at telophase involves the precise distribution of material derived from the mother cell towards the active nucleolar organizer regions (NORs) of the daughter nuclei. At early telophase, two separate entities are present: (i) the NOR, which contains tandemly repeated rRNA genes, with tightly bound RNA polymerase I (Scheer and Rose, 1984) and topoisomerase I molecules (Guldner et al., 1986); and (ii) the prenucleolar bodies (PNBs), containing several nucleolar proteins (Jimenez-Garcia et al., 1989; Gas et al., 1985; Pfeifle et al., 1986; Schmidt-Zachman et al., 1987). The association of these two separate entities occurs only if the transcriptional activity of the rRNA genes is reinitiated (Benavente et al., 1987). Although the topological location of several antigens in these structures has already been precisely identified by immunocytochemistry, it is not yet clear whether small nuclear RNA components (snRNAs) are also associated; in particular that U3 snRNAs plays a role in pre-RNA processing (for review see Warner, 1990; Gerbi et al., 1990, and references therein), could be implicated in nucleologenesis.

The present study concerns the location during the cell cycle, of U3 snRNA and fibrillarin as components of snRNP particles, by in situ hybridization and immunocytochemistry, respectively. The results clearly show that U3 snRNA is present in the prenucleolar bodies and could participate in the re-formation of the nucleolus in association with different proteins such as nucleolin and fibrillarin.

MATERIALS AND METHODS

Cells and specimen preparation

Chinese hamster ovary cells (CHO cells) were obtained from Siminovitch (1976). For whole-cell observations, they were grown as monolayers on 18 mm × 18 mm coverslips. The coverslips were previously boiled in 0.1 M HCl for 20 minutes, rinsed in deionized water

and then autoclaved. Exponentially growing cells were fixed in 4% formaldehyde (Polysciences Inc.) in 0.1 M PBS, pH 7.2, for 10 minutes on ice, dehydrated in a graded ethanol series, permeabilized in 0.1% Triton X-100 in PBS buffer for 3 minutes at 4°C and treated with methanol at –20°C for 10 minutes.

For electron microscopy, the cells were fixed in situ with 1% glutaraldehyde (Ladd) in 0.1 M sodium cacodylate buffer, pH 7.2, during 15 minutes at 4°C. After washing, cells were treated with 0.5 mg/ml sodium borohydride, scrapped and dehydrated in ethanol and embedded in Lowicryl K4M (Chemische Werke Lowi, Waldkraiburg/FRG) at –30°C as recommended by the manufacturer. Polymerization was performed at –30°C under long wavelength UV light. Ultrathin sections were collected on 300 mesh nickel grids.

DNA probe and labelling

Two oligonucleotides complementary to the mouse U3B snRNA (Mazan and Bachellerie, 1988) were synthesized in an Applied Biosystems synthesizer: a 20mer oligonucleotide, complementary to nt 65-84: TCCTCGTGGTTTCGGGTGCT and a 23mer complementary to nt 101-123: AGAGCCGGCTTCACGCTCAGGAG. The two probes, called 'antisense probe' were labelled at the 3′ end by terminal transferase, with either digoxigenin-11-dUTP (DIG-dUTP: Boehringer-Mannhein) or [α-^{32}P]dCTP. In all experiments, the two antisense probes were mixed and used together in the same experiments. For control the specificity of hybrids, 20mer oligonucleotides corresponding to 65-84 and 23mer corresponding to 101-123 were synthesized: these probes has been called 'sense probes'.

Northern blot

Total cellular RNA isolated according to Le Meur et al. (1972) was separated in 5% acrylamide/urea gels and electrotransferred onto Amersham's Hybond-N membranes. Filters were prehybridized in the following mixture: 30% formamide, 5× SSC, 0.02% SDS, 0.1% sodium *N*-lauroylsarcosine, 5% blocking reagent (Boehringer-Mannheim) for 4 hours at 37°C (1× SSC: 0.15 M NaCl, 0.015 M sodium citrate). The hybridization mixture contained 0.1 ng/ml of each probe. The hybridization was performed overnight at 37°C. The filters were washed twice for 5 minutes in 2× SSC, 0.1% SDS at 37°C and in 0.2× SSC, 0.1% SDS at 22°C.

Detection of the hybrids was performed according to the standard manufacturer's procedure. Alkaline phosphatase polyclonal sheep anti-DIG-Fab conjugate was diluted 1:5000 in 150 mM NaCl, 100 mM Tris-HCl, pH 7.4, containing blocking solution. Incubations were at 22°C for 30 minutes, then washes were performed twice for 15 minutes, with the incubation buffer without blocking reagent. Alkaline phosphatase activity was detected using nitro blue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate solution (NBT and BICP reagents) at 22°C in the dark. Colour enhancement was stopped with Tris-EDTA buffer, pH 8.0.

In situ hybridization

For hybridization of cells grown on coverslips, 200 ml of hybridization mixture (30% formamide, 5× SSC, 10% dextran sulfate, 5% Denhardt's containing 1 ng/ml of each labelled probe) were laid on DEP-treated glass slides and coverslips were incubated in this medium overnight at 37°C, in a moist chamber. After in situ hybridization, the cells were washed with gentle agitation as follows: 5× SSC, 30% formamide, 2× 15 minutes at 37°C, 2× SSC, 2× 5 minutes at 37°C and 0.2× SSC, 3× 15 minutes at 37°C.

To detect hybridization probes, we used the TRITC or FITC/sheep anti-DIG Fab fragments or alkaline phosphatase/sheep anti-DIG Fab fragments (Boehringer-Mannheim) with a one-step procedure. When enzymatic marker was used, the activity was detected using a nitro blue tetrazolium salt and a 5-bromo-4-chloro-3-indolyl phosphate solution. After staining, the cells were mounted in Mowiol medium (Sigma Chemical Co.) containing 4′,6-diamino-2-phenylindole: 2HCl (DAPI) at a concentration of 0.25 mg/ml to localize DNA. In control experiment, increasing concentration of unlabelled probe was added to the labelled probe in order to saturate specific binding sites. In order to test the background of anti-DIG antibodies, another control experiment was performed in absence of labelled antisense oligoprobes; hybridization was carried out with hybridization buffer only.

For hybridization at the electron microscopy level, nickel grids bearing thin sections of glutaraldehyde-fixed and Lowicryl-embedded cells were floated on drops of reactive solutions placed on a parafilm sheet in a moist chamber. The grids were incubated in the hybridization solution (1 ng/ml of each DIG-labelled probes) for one or two hours at 37°C or 42°C. The most intense labelling of nucleoli was generally obtained with short hybridization times (1 hour) and low temperature (37°C). After washing in PBS buffer and saturation with 5% BSA in PBS, the grids were incubated with gold (10 nm)/sheep anti-DIG antibodies (Amersham) diluted 1:40 in PBS with BSA 1%, pH 8.2, at 37°C for 30 minutes. The grids were then washed successively in PBS with 1% BSA (2× 5 minutes) and distilled water (once for 2 minutes). The sections were next stained with 5% aqueous uranyl acetate for 10 minutes and rinsed in distilled water twice. They were observed under a 1200 EX JEOL electron microscope at an acceleration voltage of 80 kV.

Characterization and detection of antigens

Antibodies

The following antibodies were used: human autoimmune sera directed against fibrillarin (S4, kindly provided by Dr Reimer); monoclonal antibodies anti-fibrillarin (72B9, kindly provided by Dr Tan; Reimer et al., 1987); polyclonal rabbit antibodies raised against CHO nucleolin (Bugler et al., 1982); polyclonal rabbit antibodies against fibrillarin were obtained in our laboratory after immunization with *Escherichia coli*, *Xenopus laevis*-produced, protein (Lapeyre et al., 1990).

Western blot

Proteins were extracted from CHO cell nucleoli (Zalta et al., 1971). A 50 mg sample was separated by SDS-polyacrylamide gel electrophoresis according to Laemmli (1970). Western analysis was performed as previously described (Caizergues-Ferrer et al., 1991; Reuter et al., 1989) using human autoimmune S4 sera (1/500 dilution), *Xenopus laevis* fibrillarin antibodies (1/200 dilution) or rabbit anti-nucleolin antibodies (1/200 dilution).

Detection in whole cells

After fixation and washing, cells were incubated with primary antibodies against nucleolin (Bugler et al., 1982), diluted 1/50, or fibrillarin (72B9; Reimer et al., 1987) at 1/30, for 1 hour at 37°C, in a moist chamber. After washing in PBS with 1% BSA (3× 5 minutes each), they were incubated with FITC/goat anti-rabbit antibodies for detection of primary complex formed with anti-nucleolin detection or with Texas Red/goat anti-mouse antibodies for detection of anti-fibrillarin primary complex (Nordic Immunology, Tilburg, Netherlands), washed and finally mounted in Mowiol containing 0.25 mg/ml of DAPI.

Preparations were examined with a Leitz Ortholux II epifluorescence microscope equipped with a 100 W mercury arc lamp and appropriate filter sets for red, green and blue fluorescence. For photography, an NPL Fluotar ×100 objective lens, with a numerical aperture of 1,32 and a 400 ASA black and white film Kodak TMax 400 or a 400 ASA colour slide film FUJI were used.

Phase-contrast observation was with a Phaco NPL ×100/1.30 objective lens.

Confocal laser microscope

Cells were prepared as above, 0.5 mm optical sections were obtained using a Zeiss confocal laser scanning microscope equipped with a 63/1.4 NA oil immersion lens, and a HeNe laser (l=543 nm). Optical

sections spanning 2 or 3 nm were reconstructed using a Zeiss program package.

Detection on thin sections

Sections of glutaraldehyde-fixed and Lowicryl-embedded cells were treated for immunogold staining as previously described (Escande et al., 1985). The rabbit anti-nucleolin (1/50) and rabbit anti-*Xenopus laevis* fibrillarin antibodies (1/10) were used as primary antibodies. Goat/anti-rabbit antibody or Protein A complexed with 5 or 10 nm gold particles was used for detection of the primary complexes.

RESULTS

Specificity of probes and antibodies

In order to control the specificity of the antisense oligonucleotide probes used for in situ hybridization, northern blot experiments were performed. In the hybridization conditions determined for the in situ studies, the probes detected only a 217 nt RNA. Neither the abundant 5 S RNA nor 4 S RNA was detected (Fig. 1A).

The specificity of the antibodies used in indirect immunodetection was analysed by western blot analysis (Fig. 1B). The different sera used showed a specific reaction with a protein of a molecular mass of 34 kDa for fibrillarin (Fig. 1B, lane 2, *Xenopus laevis* fibrillarin antibodies; lanes 3-4, S4 human sera) and 100 kDa for nucleolin (Fig. 1B, lane 4). Utilization of the buffer described by Reuter et al. (1989), containing 100 mM $MgCl_2$, allows the specific detection under stringent conditions.

Comparative distribution of U3 snRNA and fibrillarin during interphase of CHO cells

On paraformaldehyde-fixed CHO cells, the in situ experiments were performed with digoxigenin-labelled antisense oligoprobes. The conditions of hybridization were determined in order to preserve the structure and allow the co-detection of antigens by immunocytochemistry. The DNA-RNA hybrids were visualized by indirect immunofluorescence using anti-digoxigenin antibodies conjugated with rhodamine (Figs 2A and 3C). Label was present in all nucleoli, small foci were also labelled in the nucleoplasm, but no label was found in the cytoplasm. DAPI staining allowed the localization of DNA in the same cells (Figs 2B and 3B). Fig. 2C shows the location of fibrillarin by immunofluorescence. As for U3 snRNA, all nucleoli were labelled and small foci were also labelled in the nucleoplasm. The number of these foci (2-3 per cell) and their small size suggests that they could correspond to coiled bodies (Raska et al., 1990) but analysis using additional specific antigens will be necessary to confirm their nature. Fig. 2D shows the location of nucleolin in the same conditions. All nucleoli were labelled while the coiled bodies were not (Gas et al., 1985). Control experiments demonstrate that addition of unlabelled probe to the hybridization medium induces a dose-dependent decrease and a disappearance of the labelling and argue in favour of the specificity of the signal (Fig. 3F). Furthermore, no hybridization signal was present when the sense probe was used (Fig. 3G,H).

For studies at the electron microscope level, we analyzed only undigested glutaraldehyde-fixed and Lowicryl K4M-embedded CHO cells. The nucleoli of the CHO cells were large and contained small FCs; the DFC was not only located at the FC periphery but also formed a reticulate network intermingled with the granular component (GC, Fig. 4). The roundish FCs appeared with moderate electron density compared to well-contrasted DFC (Fig. 4B). The control experiment to test non-specific background produced with anti-DIG antibodies shows no label (Fig. 4A). The gold particles, corresponding to U3 snRNA hybrids, were clearly most numerous over the nucleolus (Fig. 4B-C). In the cytoplasm and the nucleoplasm, only a few gold particles were observed with these probes; interchromatin granules in particular were never labelled (see Fig. 6A). The labelling was mostly present over, and at the periphery of, the DFC and at the nucleolar interstices present in the GC. The interior of the FCs was devoid of hybridization signal. A thin section of the same embedded cells was immunolabelled with anti-fibrillarin antibodies (Fig. 4D). Fibrillarin immunolabelling was detected in the DFC network whereas the FCs were devoid of labelling. A few gold particles were observed on the GC but were more frequently present on interstices containing condensed chromatin (Fig. 4D). The label was weak but reproducible and is thus clearly different from background labelling. Double labelling experiments showed

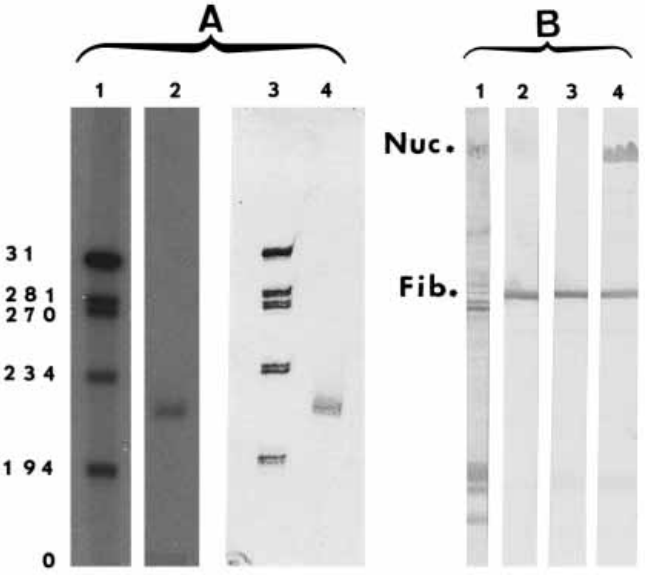

Fig. 1. (A) Northern blot analysis. Total RNA (5 μg) was separated by electrophoresis on a denaturing 7 M urea, 5% polyacrylamide gel, electrotransferred to nylon filter and hybridized with two 3′ labelled 20- and 23mer synthetic oligonucleotides complementary to U3 snRNA. Lane 1, [^{32}P]DNA marker; lane 2, hybridization with radiolabelled oligonucleotides; lane 3, digoxigenin-labelled size marker; lane 4, hybridization with digoxigenin-labelled oligonucleotides. (B) Western blot analysis. Immunoblot characterization of CHO cell nucleolus proteins separated on 12% SDS-PAGE. Lane 1, Coomassie Blue staining of the transferred nucleolar proteins. Nucleolin and fibrillarin are indicated. Lanes 2 and 3, characterization of fibrillarin with anti-*Xenopus laevis* fibrillarin (2) and anti-human fibrillarin S4 (3). Lane 4, characterization of fibrillarin and nucleolin with anti-CHO nucleolin and anti-*Xenopus laevis* fibrillarin. The bands were revealed by phosphatase-conjugated antibodies. Nucleotide numbers are indicated on the left.

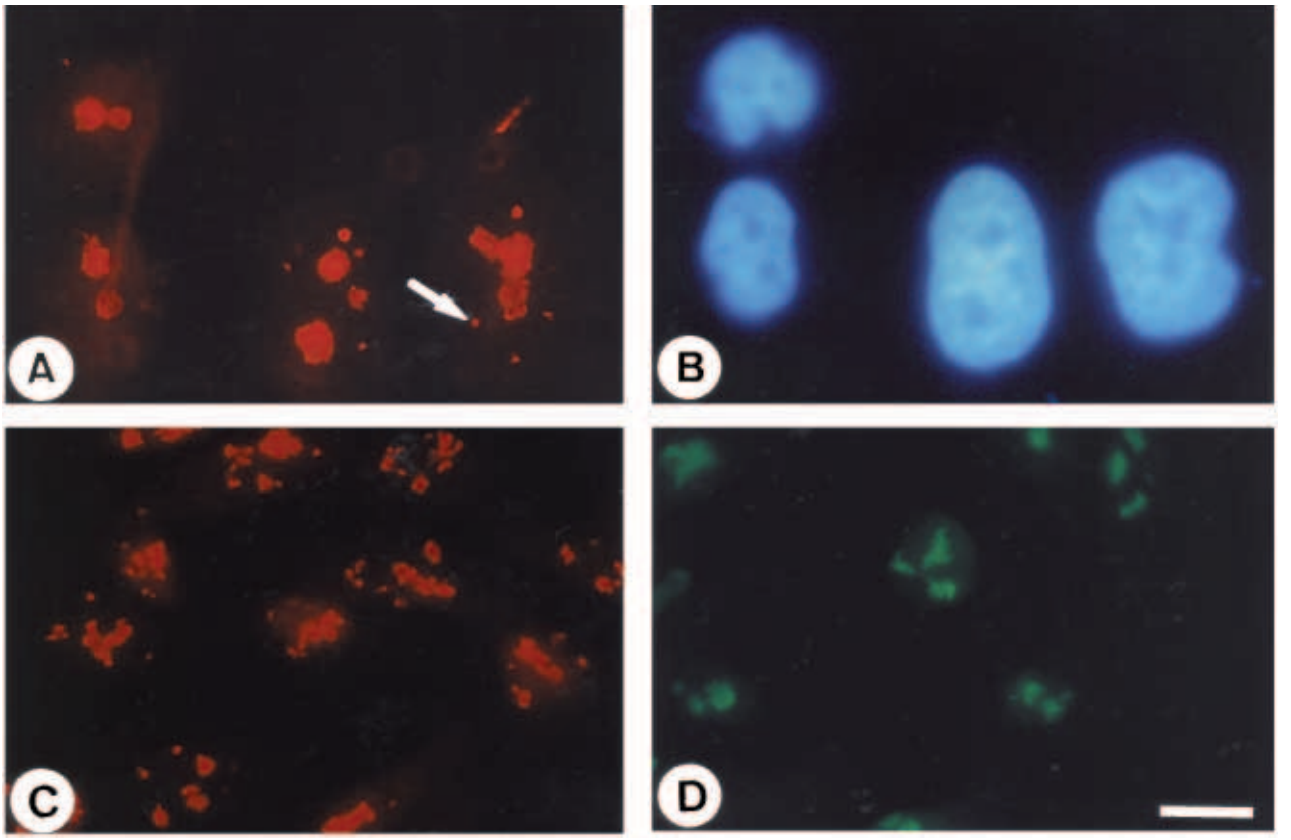

Fig. 2. Localization of U3 snRNA, fibrillarin and nucleolin during CHO cell interphase: (A) in situ hybridization for U3 snRNA localization. Hybrids were detected using TRITC/anti-DIG-Fab fragments. A bright fluorescence signal was present in some nucleolar components and in small foci (arrow). (B) The same cells were treated with DAPI dye to stain DNA. (C) Indirect immunofluorescence localization of fibrillarin using monoclonal antibodies 72B9 as first antibodies and Texas Red/goat anti-mouse antibodies as secondary antibodies. (D) Immunofluorescence localization using rabbit anti-nucleolin primary antibodies and FITC/goat anti-rabbit secondary antibodies. Bar, 20 μm.

that U3 snRNA and fibrillarin were associated in the DFC but also on the condensed chromatin in the GC (results not shown).

Distribution of U3 snRNA during the cell cycle

In situ hybridization experiments during the cell cycle were performed with digoxigenin-labelled antisense oligoprobes. The DNA-RNA hybrids were visualized using anti-DIG antibodies conjugated with alkaline phosphatase (Fig. 5A, C, E, G, I) or fluorescein (Fig. 5L-M) on whole cells. Localization of the DNA by DAPI staining in the same cells is shown in Fig. 5 (B, D, F, H, J, N). During prophase, U3 snRNA appears in the nucleolar remnant (A-B) but after nuclear breakdown it is detected as diffuse staining throughout the cytoplasm (C-D). When the chromosomes reach opposite poles, the cytoplasmic nucleolar remnants contain U3 snRNA. During late anaphase, we can observe enzymic deposits at the chromosome borders using alkaline phosphatase-labelled probes (G and I). During telophase, when chromosome decondensation occurs, the nuclear envelope is re-formed and small PNBs are weakly detectable within the daughter nuclei. With laser confocal scanning microscopy (Fig. 5L), we often observed that the daughter cells were not exactly phased at telophase, some containing numerous PNBs and others only a big spherical nucleolar remnant. The hybridization procedure on thin sections allowed the detection of label on the nucleolar remnant throughout mitosis. At telophase, the cytoplasmic nucleolar remnant containing U3 snRNA is observed in the proximity of the nucleus. When PNBs appeared in the nuclei, they were close to the nuclear envelope and gold labelled (Fig. 6A,B,C).

Distribution of nucleolar antigens during the cell cycle

Fig. 7 illustrates the distribution of fibrillarin during mitosis: B, E, H, K, N and Q correspond to DAPI-stained cells; C, F, I, L, O and R to immunofluorescence-treated cells. At prophase, like U3 snRNA, fibrillarin is distributed in a large and homogeneous nucleolar remnant centrally located in the nucleus, in close contact with the condensing chromosome (Fig. 7A-C). During metaphase (Fig. 7D-F), fibrillarin remains detectable at the chromosome periphery in a defined structure identified as nucleolar remnant by electron microscopy (results not shown). At anaphase, the labelling is present in close contact with the mass of the compacted chromosomes and one or several spots were immunostained at the chromosomal periphery and the chromosomal ends (Fig. 7G-I and J-L). During telophase, the nuclear envelope re-formed in close contact with the chromosomes, and numerous bright small foci were seen within the nuclei (Fig. 7M-O and P-R), which probably correspond to the PNBs observed by electron

microscopy (Fig. 6D). In many cells, round masses, the nucleolar remnants, appeared in the cytoplasm in addition to the typical nuclear staining (Fig. 7M-O and P-R).

The distribution of nucleolin during the cell cycle has already been described (Gas et al., 1985) but additional data concerning the behaviour of nucleolin during telophase are shown in Fig. 8. Electron microscopy of CHO mitotic cells shows, at late anaphase or early telophase, a cytoplasmic nucleolar remnant strongly labelled for nucleolin (Fig. 8A). Later, when chromosomes decondense at telophase, the labelling of this remnant decreases (Fig. 8B). In some thin sections (Fig. 8C), small fibrillar connections are observed

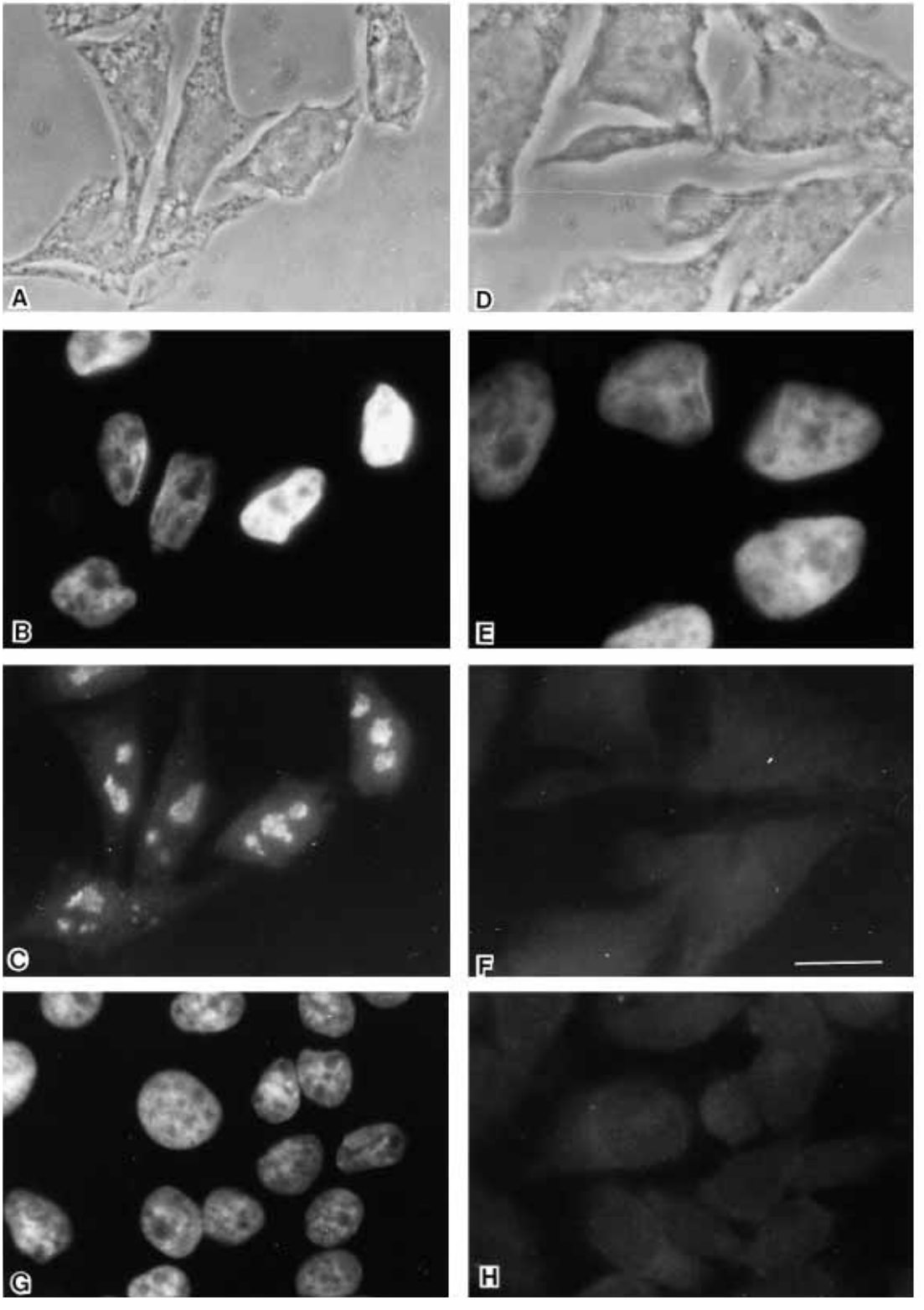

Fig. 3. Control experiments: (A) phase-contrast. (B) Same cells treated with DAPI to stain DNA. (C) Same cells treated for in situ hybridization with TRITC-DIG-labelled antisense oligoprobes. (D) CHO cells, phase-contrast. (E) Same cells treated with DAPI staining. (F) Same cells treated for in situ hybridization with a 100-fold higher concentration of unlabelled antisense oligoprobe. (G) CHO cells treated with DAPI stain. (H) Same cells treated for in situ hybridization with sense oligoprobes. Bars: (A-F) 20 μm; (G-H) 25 μm.

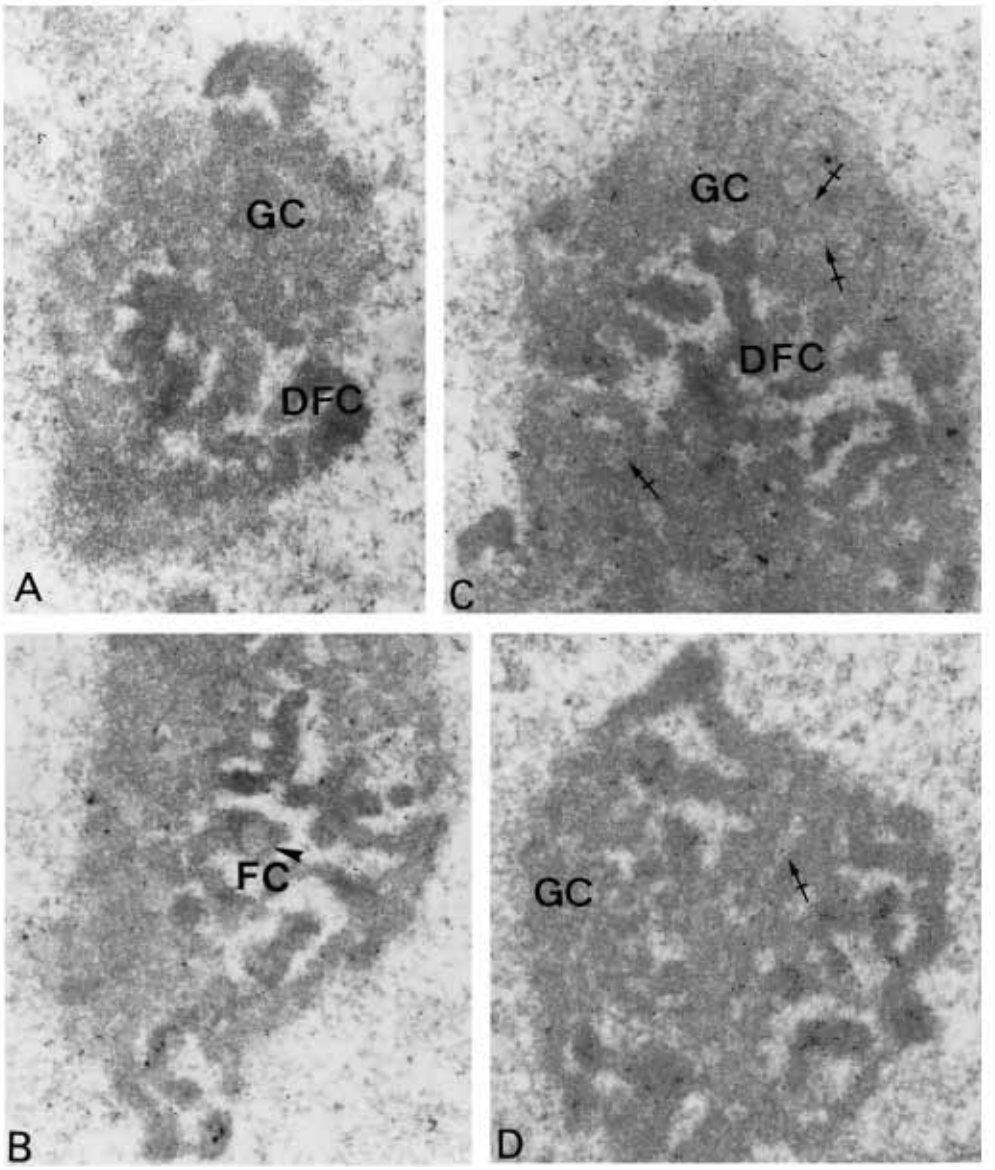

Fig. 4. Nucleoli of CHO cells after the in situ hybridization technique for detecting U3 snRNA (A-C) or after indirect immunogold staining for fibrillarin localization (D). (A) In situ hybridization in the absence of labelled antisense oligoprobe: no gold particles are seen over the nucleolus. (B-C) In situ hybridization in the presence of gold-labelled antisense oligoprobes. Numerous gold particles are seen over and at the margin of DFC and the GC but the FC (arrowhead in B) is gold-free. Label is also found over the intranucleolar invagination of condensed chromatin (arrows in C), in particular in the GC. (D) Immunogold staining for fibrillarin localization: gold particles are clearly seen over the DFC. The clumps of chromatin enclosed in the GC are also labelled (barred arrow). (A-D) ×24,000.

between the nucleolar remnant and the nuclear pore complexes, suggesting that nucleolin, probably with other molecules, is imported, via the nuclear pore complex from the nucleolar remnant, into the nucleus. These molecules could move into the PNBs, which are frequently visible in the nuclear envelope proximity.

DISCUSSION

In situ hybridization of digoxigenin-labelled probes allowed us to localize U3 snRNA during the cell cycle in CHO cells, without using antimitotic drugs.

During interphase, U3 snRNA is preferentially located in the nucleolus. The use of both post-embedding in situ hybridization with short oligonucleotide probes (the choice of oligonucleotides was made according to the predicted U3 snRNA secondary structure; Parker and Steitz, 1987) and immunogold procedures, allowed us to visualize directly the U3 snRNA and fibrillarin in the same substructure of the nucleolus. As judged from the distribution of gold particles, U3 snRNA is located (i) preferentially over and at the periphery of the DFC and (ii) over the GC; and a few particles decorate the compacted chromatin enclosed in the nucleolar interstices. In the DFC, the physical association between fibrillarin and U3 snRNA could correspond to an active snRNP that functions in the earliest cleavage step of the pre-rRNA within the 5′ ETS (Kass et al., 1990). This view is consistent with the study by Puvion-Dutilleul et al. (1991a), using a 5′ ETS probe to localize the substructures where these early events occur. A few gold particles were observed over the GC, both with U3 snRNA probes and with fibrillarin. The U3 snRNP present in the GC could be involved in other steps of rRNA processing taking place in the GC (Bachellerie et al., 1983; Tague and Gerbi, 1984). Like U3 snRNA, fibrillarin was preferentially detected on the DFC. It is, however, noticeable that the condensed chromatin, forming small foci within the nucleolar GC, also exhibited a label. These small foci correspond to condensed

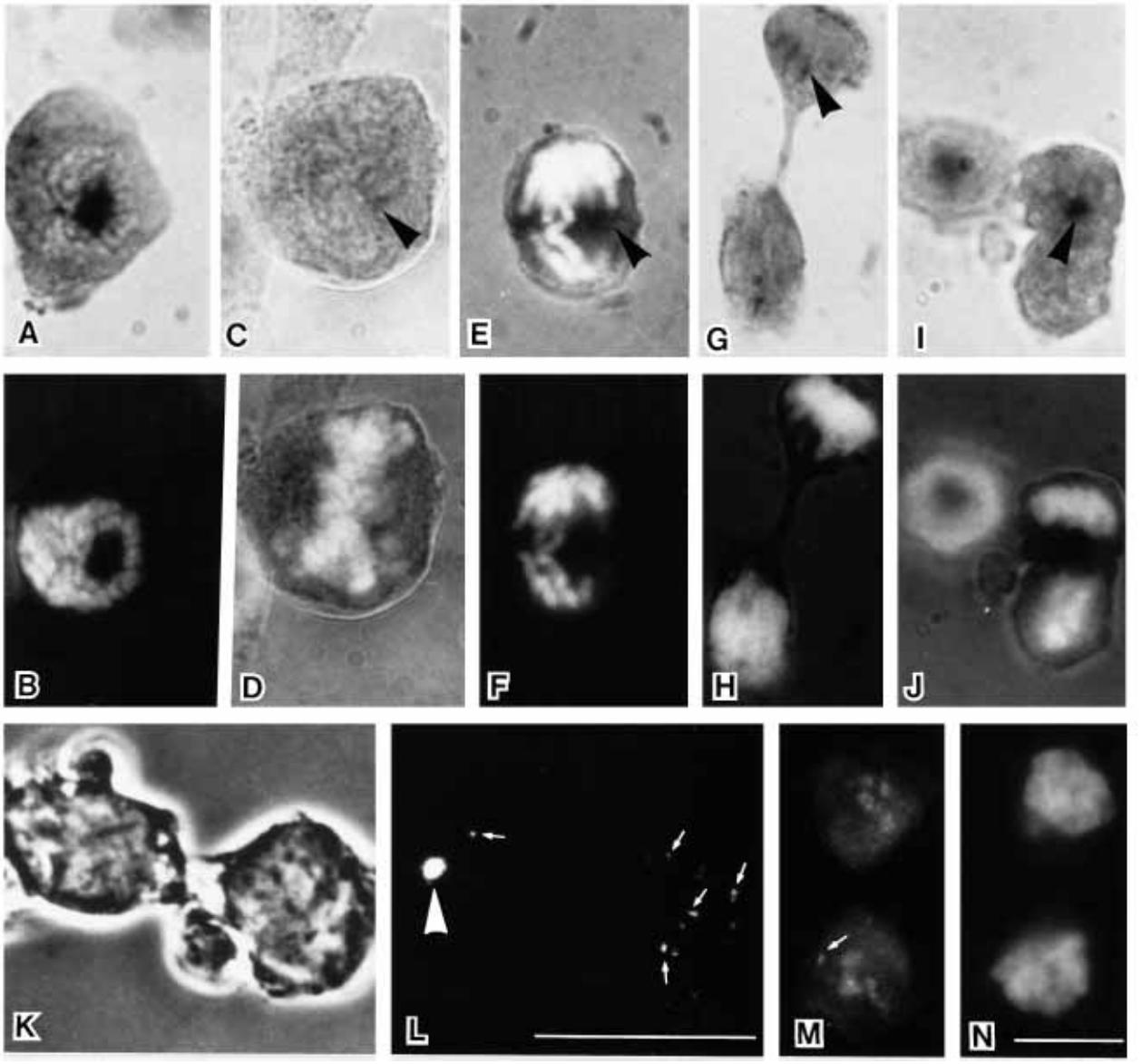

Fig. 5. Cellular distribution of U3 snRNA during mitosis. Shown are immunocytochemical localization of hybrids with alkaline phosphatase/anti-DIG Fab fragments (A,C,E,G,I) or with FITC/anti-DIG Fab fragments (L,M). To identify the different phases of mitosis, the CHO cells were stained before mounting with DAPI to visualize the DNA (B,D,E,F,H,J,N). (A-B) Prophase cell, (C-D) metaphase cell, (E-F) early anaphase, (G-H) and (I-J) late anaphase, (K-L) and (M-N), late telophase cells, respectively. At prophase, staining is localized to the nucleolar remnant. At metaphase, stain is distributed around chromosomes, while in anaphase, it is present in the interzone (arrowhead). At telophase (K-L), two distinct sites are immunolabelled: (K) phase-contrast; (L) same cells examined by confocal scanning laser microscopy. The view corresponds to reconstruction of four 0.5 μm optical sections from a single nucleus. Note the numerous small punctate nuclear structures characteristic of the appearance of PNBs (arrows) and a big spot (arrowhead), which could correspond to nucleolar remnant. Bar, 20 μm.

chromatin and the DNA present has been identified as rDNA (Puvion-Dutilleul et al., 1991a). We suggest that the condensed rDNA could correspond to transitory untranscribed genes. In fact, the nucleolus is a dynamic structure that can rapidly adapt ribosomal biogenesis to the cellular metabolic state. When 'silent' rDNA becomes actively transcribed, the early steps of the processing of nascent pre-rRNA are thought to be handled by chromatin pre-associated snRNPs.

In our study, no label was observed on the FCs, indicating that U3 snRNA and fibrillarin were absent in this nucleolar compartment. Our results concerning the absence of fibrillarin from the FCs are in agreement with those obtained using a similar approach by Reimer et al. (1987), with a 72B9 antibody and an immunogold procedure. Moreover, Puvion-Dutilleul et al. (1991b) with the B-36 mAb-P2-G3 monoclonal antibody in mouse 3T3 and in HeLa cells, showed gold labelling on DFC but not on FCs. Only studies using immunoperoxidase electron microscopy (Lischwe et al., 1985; Ochs et al., 1985), demonstrated that both the DFC and the FCs exhibit a positive signal for fibrillarin. This discrepancy could be due to the different

protocols followed to fix and permeabilize the cells. In our studies, cells were not permeabilized but experiments were performed on thin sections following glutaraldehyde fixation and Lowicryl K4M embedding.

During mitosis, fibrillarin and U3 snRNA are present, first in the nucleolar remnant, then in the PNBs

In the present study, we have examined the location of the rRNA maturation machinery during mitosis and nucleologenesis. We showed (i) co-localization of U3 snRNA and fibrillarin throughout mitosis, (ii) the presence of these molecules in the persistent nucleolar remnant, (iii) and their presence in the PNBs of these snRNPs at the end of telophase. We propose that: (i) some parental nucleolar molecules remain associated in the nucleolar remnant; (ii) cytoplasmic entities are imported via the pore complex in order to form the PNBs in the nucleus;

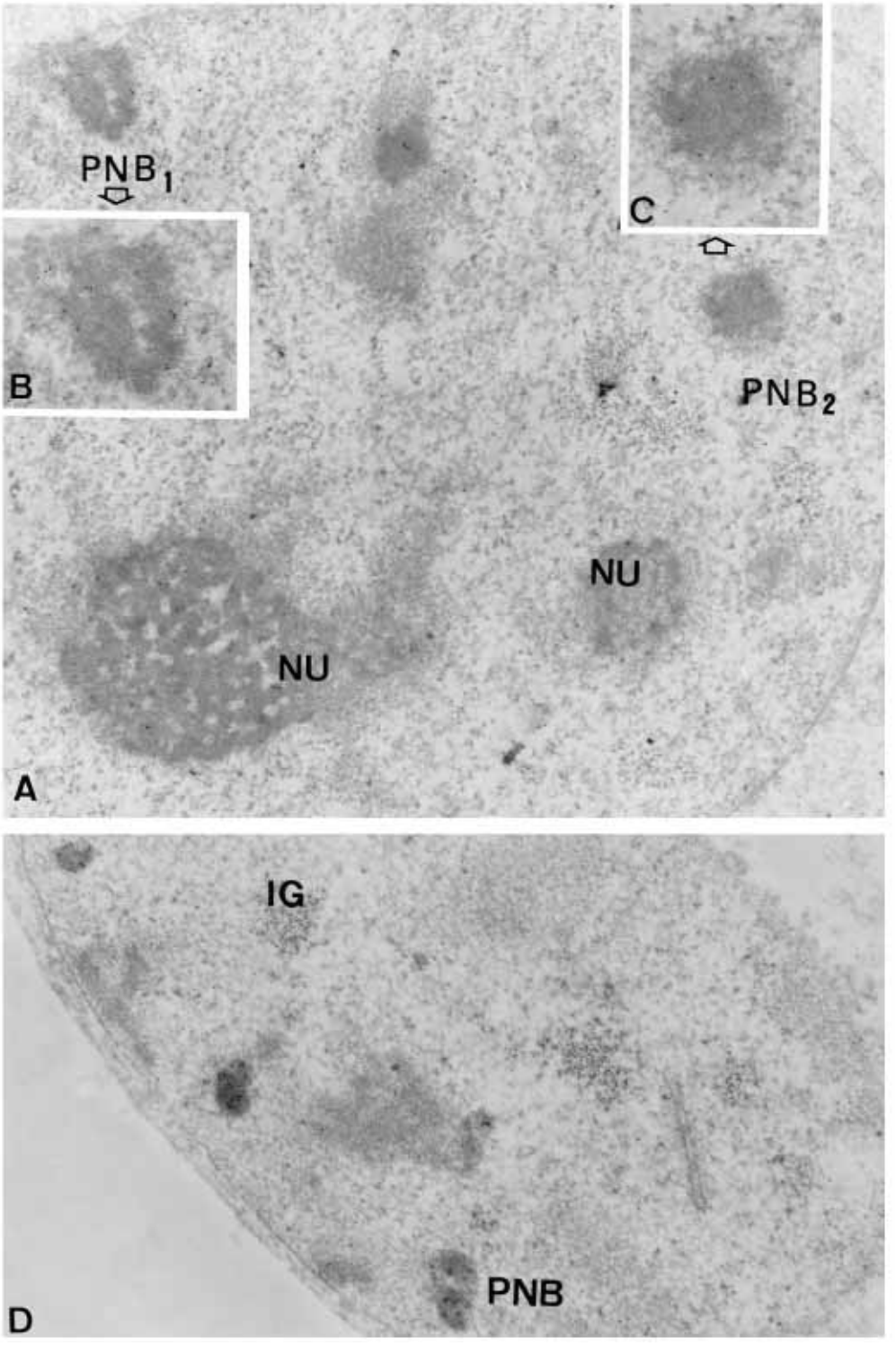

Fig. 6. (A) Ultrastructural localization of U3 snRNA after in situ hybridization on Lowicryl K4M-embedded telophase CHO cell. The PNBs (PNB_1 and $-_2$) and the nascent nucleoli (NU) are labelled. (B) and (C) show well-characterized enlarged PNB_1 and $-_2$ exhibiting the location of U3 snRNA over these structures. (D) Ultrastructural immunogold staining of fibrillarin in telophase cell: numerous PNBs are strongly labelled. IG, interchromatin granules. (A) ×18,000; (B-C) ×30,000; (D) ×18,000.

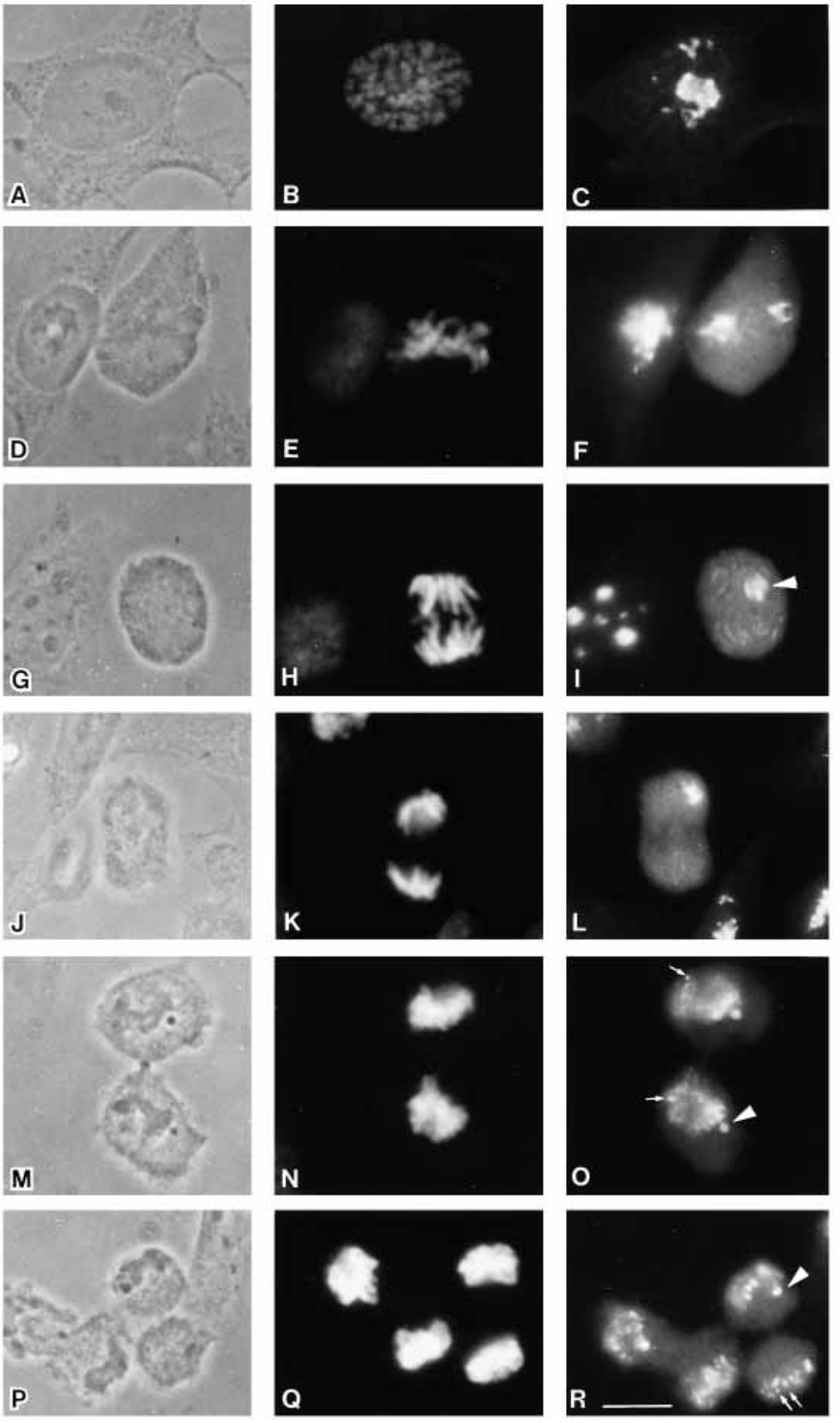

Fig. 7. Intracellular localization of fibrillarin in CHO cells throughout the cell cycle determined by indirect immunofluorescence using 72B9 antibodies as primary antibodies and with mouse anti-human Texas Red antibodies as secondary antibodies (C,F,I,L,O and R). Each vertical column represents the cell cycle pattern of cells as determined by phase-contrast, DAPI stain and by incubation with 72B9 antibodies, left to right, respectively (A-C), prophase; (D-F) metaphase; (G-I) early anaphase; (J-L) late anaphase; (M-R) telophase cells. Fibrillarin is in close contact with the chromosomal ends during metaphase. During anaphase, fibrillarin forms a coat over the chromosomes and remains partly in a large spot, which could correspond to the cytoplasmic nucleolar remnant (arrowhead in I). In telophase, several spots are seen in the re-forming nuclei (small arrows) and big fluorescent remnants (arrowheads in O,R) are observed in the cytoplasm of daughter cells. At this stage of nucleologenesis, these fluorescent structures are characteristic of the appearance of PNBs and nucleolar remnants, respectively. Bar, 20 μm.

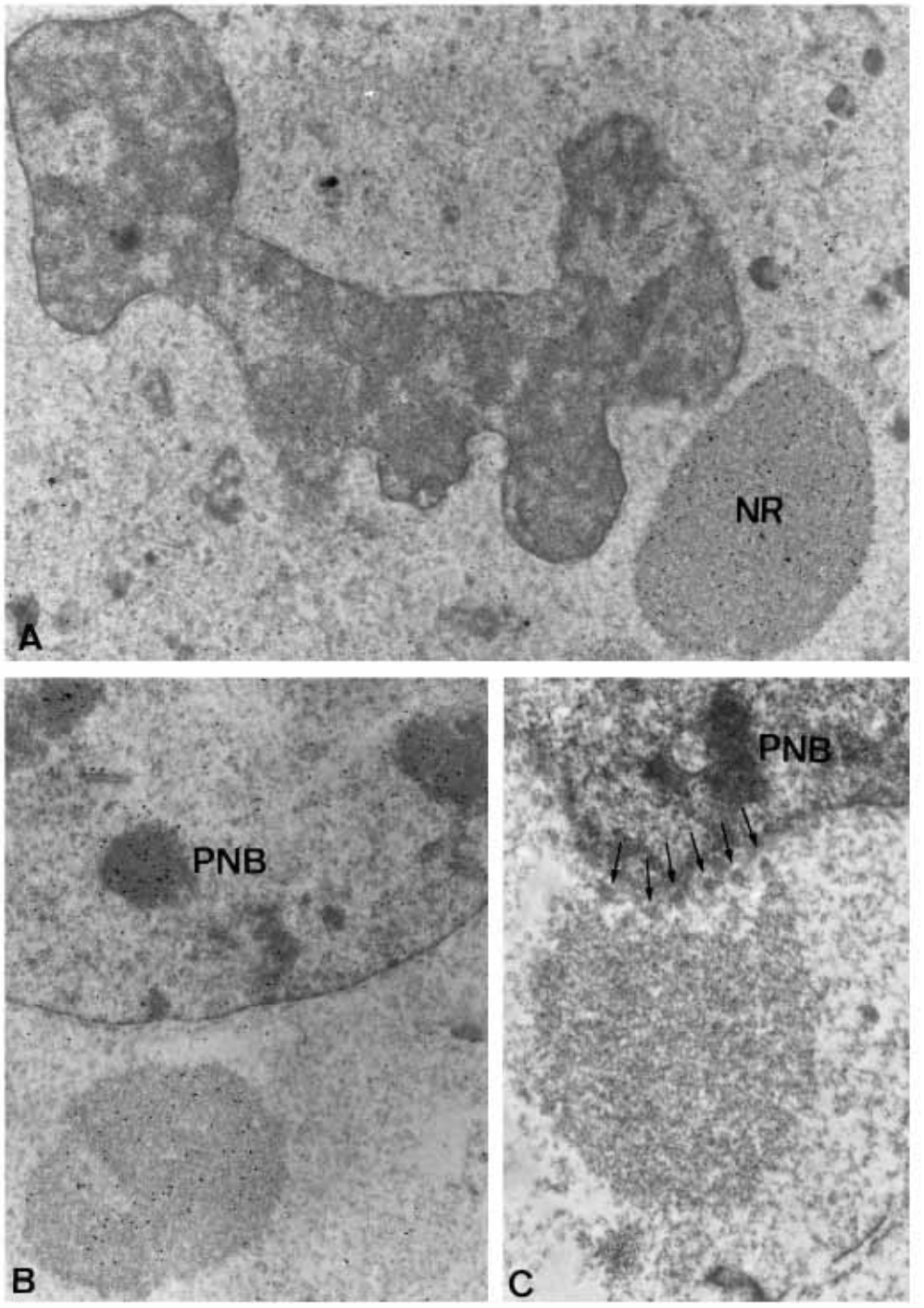

Fig. 8. Ultrastructural indirect immunogold detection of nucleolin during the late steps of mitosis in the CHO cell: (A) very early telophase: the re-forming nucleus contains condensed chromatin, and a large nucleolar remnant (NR), which is strongly labelled with anti-nucleolin antibodies, is observed in the cytoplasm. (B) Late telophase: numerous gold particles are clearly seen over the well characterized PNBs. In the cytoplasm, the nucleolar remnant presents fewer gold particles at this stage. (C) Late telophase: the nucleolar remnant is located in close proximity to the nuclear envelope, suggesting possible import of molecules from the nucleolar remnant into the nucleus (PNBs) via the nuclear pores (arrows). (A) ×20,000; (B) ×30,000; (C) ×28,000.

and (iii) association between the PNBs and the NORs is promoted by interaction of the ribonucleoprotein particle (U3 snoRNP) and the 5′ external spacer of the newly synthesized pre-rRNA.

In mammalian cells, the distribution of fibrillarin during cell cycle has already been examined and conflicting results have been obtained (Jimenez-Garcia et al., 1989; Yasuda and Maul, 1990). Once again, we can explain the observed discrepancies by the use of different protocols. In fact, it could be proposed that the use of hypotonic media disperses the residual nucleolus and, in this case, the antigens were recovered forming a coat over the chromosomes (Leser et al., 1989; Spector and Smith, 1986). This could also be the case for several nucleolar antigens called perichromonucleolin (PCN), found on the surface of condensed chromosomes (Luji et al., 1987; Gautier et al., 1992a,b). Furthermore, CHO cells, which have prominent nucleoli during interphase, form large and easily detectable nucleolar remnants during mitosis. In other cell lines with no nucleolar remnant, several antigens and RNA, retained in the CHO nucleolar remnant, can disperse around the chromosomes. During mitosis, until anaphase, we have shown that fibrillarin, like nucleolin (Gas et al., 1985), is sequestered in

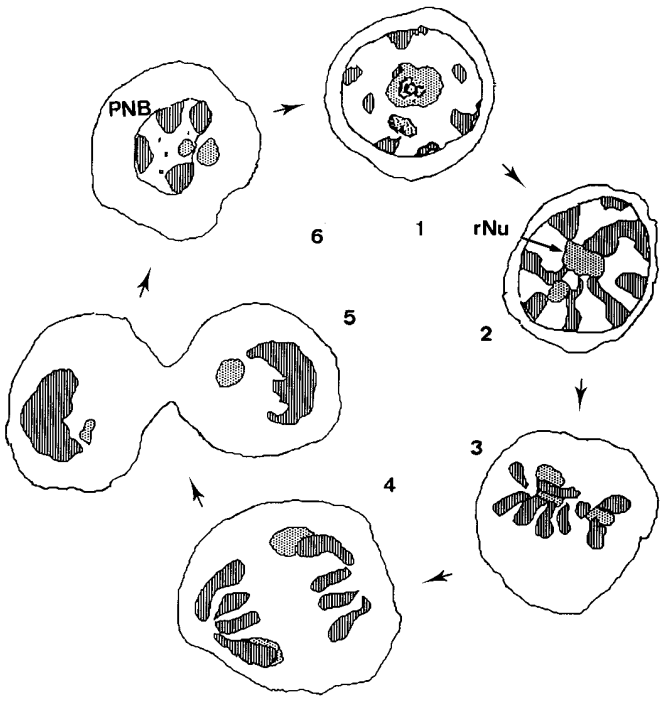

Fig. 9. Schematic drawing of the nucleolar cycle in CHO cells. (1) Interphase cell with active nucleolus. (2) At the prophase, chromatin (hatched) condensation occurs and the inactive nucleolus (dotted) forms the nucleolar remnant (rNu). (3) During metaphase, the nucleolar remnant is observed in close contact with the chromosome arms. (4) At anaphase, the nucleolar remnants progress to the opposite poles drawn by the chromosome arms. (5) At early telophase, the nuclear envelope re-forms in contact with the condensed chromosomes. The remnant nucleolus remains in the cytoplasm. (6) During late telophase, the chromatin decondenses, PNBs appear and molecular import from the nucleolar remnant into the daughter nuclei may occur.

the remnant nucleolus. A fraction of U3 snRNA is found dispersed in the cell but some is also present in this structure and we have shown that the amount increases during the progression of mitosis. We do not know if this accumulation corresponds to the parental molecules or to the newly transcribed ones that accumulated in this cytoplasmic nucleolar remnant. It has also been shown that some steps in the maturation of the U snRNAs of the splicing machinery occur in the cytoplasm and that re-entry into the nucleus is dependent on the addition of proteins (the Sm proteins) and on the trimethyl Cap structure (Hamm et al., 1990; Lamond, 1990). It has also been shown that the integrity of the 13 first nucleotides at the 3′ end of U3 snRNA are necessary for its nuclear targeting (Baserga et al., 1992); but how fibrillarin is directly involved in this process is still a puzzling question. A study of *Xenopus* development (Caizergues-Ferrer et al., 1991) suggests that fibrillarin is not involved in the nuclear targeting of U3 snRNA; furthermore, genetic analysis of yeast demonstrated that in fibrillarin mutants (*nop1*-) the U3 snRNA location is not affected (Tollervey and Hurt, 1990).

What is the function of the nucleolar remnant?

At the beginning of prophase, the typical nucleolar organization disappears. Only remnants of the nucleoli with moderate electron density are observed in several mammal cell lines after glutaraldehyde fixation (Brinkley, 1965). 'Persistent nucleoli' were either free in the cytoplasm or associated with the chromosomal arms (Noël et al., 1971). We suggest that these remnant structures correspond to metabolic nucleolar matrix derived from interphase nucleoli, resulting from the arrest of ribosomal transcription and chromosome condensation. This matrix retains several nucleolar antigens such as fibrillarin and nucleolin, and corresponds to a site for the segregation, at mitosis, of several nucleolar molecules. The nucleolar remnant directly attaches to the chromosomes, moves with them and becomes cytoplasmic when the nuclear envelope re-forms (Fig. 9). The initial separation of nucleus and cytoplasm by the formation of the nuclear envelope appears to involve an exclusion of nuclear material, which is later reimported by pore-mediated selective uptake. These nucleolar components could correspond to some of the proteins and snRNAs involved in nucleologenesis or to storage materials. This pathway for segregation of nucleolar material at mitosis is new with regard to the three different locations of nucleolar material that are frequently observed: during mitosis, the nucleolar proteins are (i) associated with NORs, (ii) dispersed within the cytoplasm, and (iii) coat the chromosomes (for review, Sommerville, 1986). We have previously shown that nucleolin, an AgNOR protein, is not only associated with NORs but also an important pool remains in the nucleolar remnant (Gas et al., 1985). In the present study, it appears clearly that several other nucleolar molecules, fibrillarin and U3 snRNA, remain transitorily sequestered in the nucleolar remnant.

Several lines of evidence suggest that post-mitotic reorganization of daughter nucleoli requires nucleolar components provided by the mother cell, which are, in part imported via the nuclear pores and in part introduced into the daughter nuclei when the nuclear envelope re-forms. The microinjection of antibodies against p68, a pore-complex glycoprotein, into mitotic cells under conditions known to inhibit the transport function of the pores prevented post-mitotic daughter cells from reaching interphase (Benavente et al., 1989). Interestingly, granular aggregates (morphologically identical to the nucleolar remnant of CHO) are often observed close to the nuclear envelope (Fig. 3a and c, of Benavente, 1991).

The molecular mechanisms for the considerable ultrastructural changes taking place at the end of telophase and which permit nucleologenesis, remain largely unexplained (Bell et al., 1992). At mitosis, several nucleolar proteins such as nucleolin and B23 (NO38), are phosphorylated by a cdc2 kinase (Belenguer et al., 1990; Peter et al., 1990) but the physiological consequences of these phosphorylation events have not been determined. It is attractive to postulate a relationship between the state of phosphorylation of these proteins and the mitotic disassembly of nucleoli and/or the sequestration of some of these molecules in the cytoplasm.

The way in which the PNBs fuse with the NORs is still puzzling. It has been clearly demonstrated that this step is dependent on RNA Pol I activity (Benavente et al., 1987). In the *Xenopus* O-Nu mutant, in the absence of rDNA genes, only pseudo-nucleoli that could correspond to PNBs are observed (Hay and Gurdon, 1967). For the first time, we have shown that U3 snRNA is present in the PNBs. We can postulate that other nucleolar snRNAs, which also interact with fibrillarin (Tyc and Steitz, 1990), are present in this structure and can

promote nucleolus formation via base-pairing interactions with the pre-rRNA. For U3 snRNA, the first interaction can occur in the 5′ ETS, at a site previously identified by cross-linking experiments. Furthermore, it has been shown (Ghisolfi, personal communication) that nucleolin is able to interact with the pre-rRNA in the vicinity of the U3 snRNA interaction site. We cannot exclude the possibility that some protein-protein interactions take place between the protein present in the NORs and that present in the PNBs. Both nucleolin and fibrillarin have a glycine+arginine-rich domain (GAR domain), possibly involved in protein-protein interactions. We postulate therefore that RNA-RNA interactions could be stabilized by such protein-protein interactions.

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