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Research articles

*Nucleologenesis*

# A class of nonribosomal nucleolar components is located in chromosome periphery and in nucleolus-derived foci during anaphase and telophase

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**Abstract**. The subcellular location of several nonribosomal nucleolar proteins was examined at various stages of mitosis in synchronized mammalian cell lines including HeLa, 3T3, COS-7 and HIV-1 Rev-expressing CMT3 cells. Nucleolar proteins B23, fibrillarin, nucleolin and p52 as well as U3 snoRNA were located partially in the peripheral regions of chromosomes from prometaphase to early telophase. However, these proteins were also found in large cytoplasmic particles, 1–2 μm in diameter, termed nucleolus-derived foci (NDF). The NDF reached maximum numbers (as many as 100 per cell) during mid- to late anaphase, after which their number declined to a few or none during late telophase. The decline in the number of NDF approximately coincided with the appearance of prenucleolar bodies and reforming nucleoli. The HIV-1 Rev protein and a mutant Rev protein defective in its nuclear export signal were also found in the NDF. The mutant Rev protein precisely followed the pattern of localization of the above nucleolar proteins, whereas the wild-type Rev did not enter nuclei until G1 phase. The nucleolar shuttling phosphoprotein Nopp140 did not follow the above pattern of localization during mitosis: it dispersed in the cytoplasm from prometaphase through early telophase and was not found in the NDF. Although the NDF and mitotic coiled bodies disappeared from the cytoplasm at approximately the same time during mitosis, protein B23 was not found in mitotic coiled bodies, nor was p80 coilin present in the NDF. These results suggest that a class of proteins involved in preribosomal RNA processing associate with chromosome periphery and with NDF as part of a system to conserve and deliver preexisting components to reforming nucleoli during mitosis.

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## Introduction

During mitosis the cell nucleus undergoes extensive structural reorganization. RNA transcription and processing cease, the nuclear skeleton depolymerizes, DNA detaches from the nuclear matrix, interphase chromatin condenses into chromosomes, the nuclear envelope breaks down and nuclear components disassemble. Although it is established that the nucleoli disintegrate during early prophase (Goessens 1984), the fate of various nucleolar components during the mitotic cycle is only beginning to be understood. The location of nucleolar components during the phases of mitosis appears to be highly variable, with each component following its own pathway back to the reforming nucleolus. However, a general pattern has begun to emerge suggesting that the nucleolar components fall into one of a few classes in their distribution during mitosis.

The first class of nucleolar proteins remains associated with the nucleolar organizer regions (NORs) throughout mitosis. These include RNA polymerase (pol) I (Scheer and Rose 1984; Haaf et al. 1988; Gilbert et al. 1995), transcription initiation factor UBF (Chan et al. 1991; Rendon et al. 1992; Roussel et al. 1993), the TATA-binding protein and TATA-binding protein-associated factor for RNA pol I (Jordan et al. 1996; Roussel et al. 1996). There is evidence that the RNA pol I transcription complex remains assembled on the rDNA promotor at the chromosomal NORs during mitosis (Roussel et al. 1996). DNA topoisomerase I (Guldner et al. 1986) and the NOR protein pp135 (Pfeifle et al. 1986) are also associated with the NORs during mitosis.

A second group of nucleolar proteins is located in the chromosome periphery from late prophase to early telophase. This group includes fibrillarin (Yasuda and Maul 1990; Azum-Gélade et al. 1994; Weisenberger and Scheer 1995), perichromonucleolin (Shi et al. 1987), proliferation-associated Ki-67 antigen (Verheijen et al. 1989; Starborg et al. 1996), protein B23/No38 (Ochs et al. 1983; Schmidt-Zachmann et al. 1987), nucleolin (Weisenberger and Scheer 1995), p52, p68 and p103

(Gautier et al. 1992), ribocharin (Hügle et al. 1985a), ribosomal protein S1 (Hügle et al. 1985b), protein No55 (Ochs et al. 1996), and HIV-1 Rev protein (Dundr et al. 1996). U14 snoRNA has also been localized in the perichromosomal region (Beven et al. 1996).

A third group of nucleolar proteins and RNAs are essentially uniformly distributed in the cytoplasm during prophase to telophase. Included in this group are 180 kDa nucleolar protein (Schmidt-Zachmann et al. 1984), ribosomal protein S6 (Jiménez-García et al. 1994) and phosphoprotein p130 (Pai et al. 1995). Several investigators have detected U3 snoRNA during prophase to anaphase distributed in the cytoplasm and in a nucleolar remnant but not associated with the condensed chromosomes (Azum-Gélade et al. 1994; Jiménez-García et al. 1994). However, other research groups have found U3 snoRNA enriched in the perichromosomal region (Gautier et al. 1994; Weisenberger and Scheer 1995; Beven et al. 1996). U8 snoRNA has been detected only in the cytoplasm during mitosis (Matera et al. 1994). Jiménez-García et al. (1994) and Weisenberger and Scheer (1995) have found pre-rRNA during prophase to anaphase in the cytoplasm, although Medina et al. (1995) and Beven et al. (1996) have localized pre-rRNA in the periphery of chromosomes.

Nucleolar reformation starts in early telophase when specific nucleolar proteins begin to associate with numerous prenucleolar bodies (PNBs) in newly formed daughter nuclei. When RNA pol I transcription is reinitiated at the chromosomal NORs in late telophase the PNBs are targeted to the NORs where they fuse and become the dense fibrillar components of the nucleolus (Scheer et al. 1993; Jiménez-García et al. 1994; Scheer and Weisenberger 1994).

In recent studies (Dundr et al. 1996), we observed that several nonribosomal nucleolar proteins accumulate in numerous large cytoplasmic particles termed nucleolus-derived foci (NDF) during anaphase and telophase. These foci appear to be an intermediate location of nucleolar components between chromosome periphery and the PNBs in newly formed nuclei. In these current studies the NDF are further characterized and shown to contain U3 snoRNA and No55 protein. In contrast, another nucleolar nonribosomal protein, Nopp140, and its associated protein, NAP57, were not associated with the NDF or with the PNBs in anaphase and telophase. These results confirm that different nucleolar proteins arrive in reformed nucleoli by different routes.

## Materials and methods

*Cells.* The monkey CMT3 cells (Gerard and Gluzman 1985) and COS-7 cells, human HeLa and mouse 3T3 cells were grown on poly-L-lysine coated glass coverslips in Dulbecco's modified Eagle's medium (DMEM) (GIBCO) supplemented with 10% fetal calf serum (GIBCO) at 37°C in 5% $CO_2$ atmosphere. The A5.9 line of CMT3 cells constitutively expressing the HIV-1 Rev protein and 2AB7 line expressing the *trans*-dominant negative HIV-1 Rev protein with a deletion of two amino acids (aa 78 and 79) in the nuclear export signal of Rev were described previously (Dundr et al. 1996). These two cell lines were cotransfected with pHyg plasmid that confers hygromycin resistance and they were cultured in hygromycin B containing DMEM (200 μg/ml). The cell lines were synchronized at the G1/S transition by a double thymidine block with 2.5 mM thymidine (Bootsma et al. 1964) and then released to proceed through mitosis.

*Immunofluorescence.* Cells grown on coverslips were washed in PBS and fixed with 3% paraformaldehyde for 20 min at room temperature. After washing in PBS, the cells were permeabilized with 0.2% Triton X-100 in PBS on ice for 5 min and then washed extensively with 1% BSA in PBS. Immunofluorescence was performed as previously described (Dundr et al. 1996). Fibrillarin was detected with human autoimmune serum S4 and nucleolar protein No55 with human autoimmune serum (kindly provided Dr. R.L. Ochs). Nopp140 was labeled with rabbit polyclonal antibody (RF11). Protein B23 was detected using anti-B23 monoclonal antibody (mAb) (kindly provided by Dr. P.K. Chan). Nucleolin was labeled using a polyclonal antibody against the N-terminal end of nucleolin. A 52–53 kDa nucleolar ribonucleoprotein was visualized with G04 autoimmune serum (kindly provided by Dr. D. Hernandez-Verdun). The Rev protein was detected using anti-Rev mAb (Repligen) and rabbit polyclonal antibody (American Biotechnologies). Coilin was detected using rabbit polyclonal antibody (R288) raised against a β-galactosidase fusion protein containing the C-terminal region of p80 coilin (kindly provided by Dr. E.K.L. Chan).

*Hybridization probes.* The human U3 snoRNA gene cloned in *Sma*I/*Pst*I sites of the Bluescript vector was kindly provided by Dr. R. Reddy. The hybridization anti-sense or sense probes (residues –6 to +50 relative to transcription start site) were produced by in vitro transcription with biotin-16-UTP (Boehringer Mannheim) using T7 RNA polymerase after linearizing with *Sma*I or T3 RNA polymerase after linearizing with *Pst*I, respectively. The sense probe was used as a control for the in situ hybridization.

*In situ hybridization.* Cells grown on coverslips were washed with PBS and fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. The cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min on ice, then washed with PBS and finally in 2×SSC. The hybridization mixture was prepared as described by Jiménez-García et al. (1994). Briefly, 100 ng of probe and 20 μg of tRNA were dried under vacuum. Ten microliters of deionized formamide was added and the mixture was denatured for 10 min at 70°C. The probe was immediately chilled on ice, and the hybridization mixture was made up to final concentrations of 2×SSC, 1% BSA, and 10% dextran sulfate. Onto each coverslip was placed 20 μl of hybridization mixture and incubated in a moist chamber for 16–18 h at 42°C. The coverslips were rinsed with 2×SSC/50% formamide at 37°C, 2×SSC and 1×SSC at room temperature for 30 min each. The cells were incubated with avidin-DCS-conjugated with Texas red (Vector) (2 μg/μl) in 4×SSC/0.25% BSA for 1 h, and then rinsed in 4×SSC, 4×SSC/0.1% Triton X-100, 4×SSC and PBS. Coverslips were mounted in Mowiol (Calbiochem) containing 1 mg/ml *p*-phenylenediamine. When in situ hybridization of U3 snoRNA was followed by immunoflourescence, after the rinsing of cells in PBS, the coverslips were incubated with anti-B23 mAb for 50 min at room temperature. After the incubation the coverslips were rinsed in PBS and incubated with sheep anti-mouse-fluorescein labeled secondary antibody (Amersham) for 45 min. The cells were washed several times with PBS, briefly with ethanol, air dried and mounted in Mowiol (Calbiochem) containing 1 mg/ml *p*-phenylenediamine.

*Fluorescence microscopy.* The samples were examined using a laser scanning confocal microscope (Noran) with either a Nikon 60×/1.4 or 100×/1.4 N.A. objective. The samples were subjected to an excitation wavelength of 488 nm (fluorescein) or 529 nm (Texas red) from an argon-ion laser. For double labeling, the con-

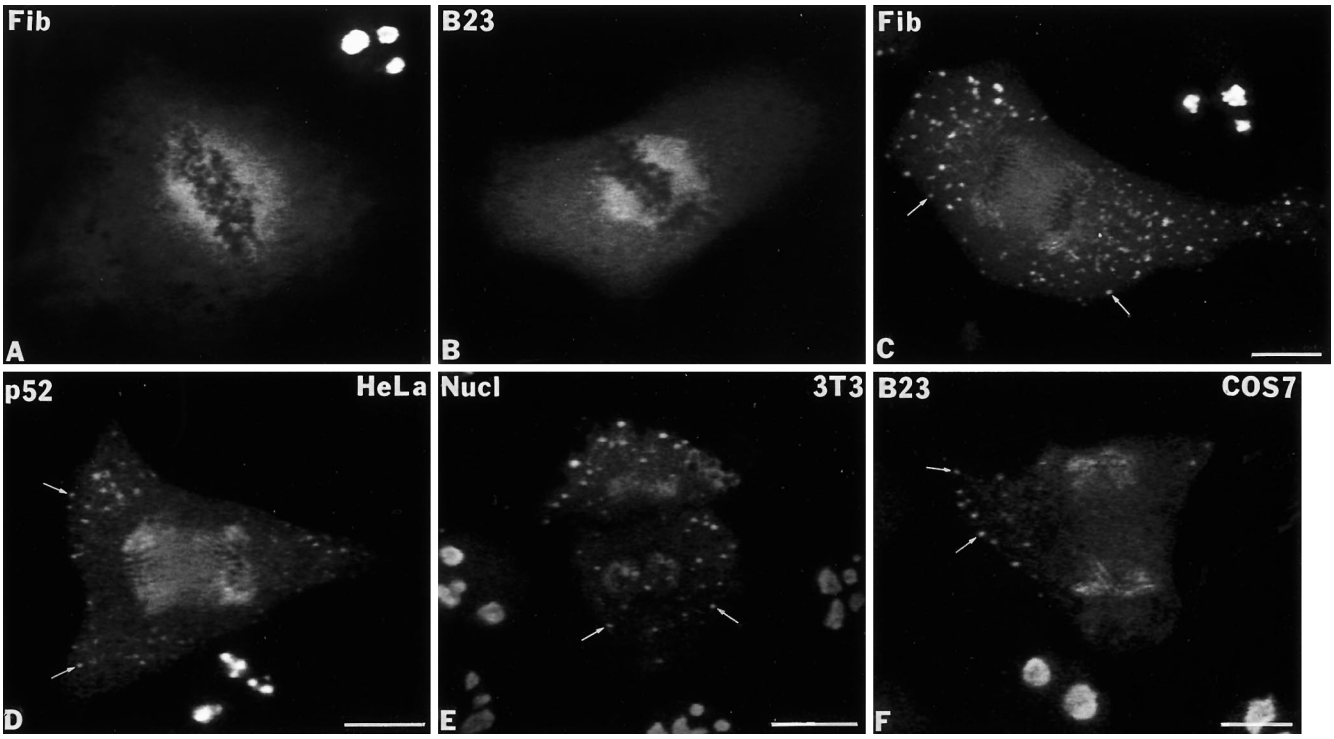


**Fig. 1A–F**. Nucleolar antigens are present in chromosome periphery and nucleolus-derived foci in several mammalian cell types. Cells were synchronized as described in Materials and methods and subjected to immunofluorescence confocal miscroscopy. **A–C** A5.9 CMT3 cells; **D–F** cell lines are indicated. In metaphase fibrillarin (**A**) labeled with an autoimmune serum and protein B23 (**B**) detected with anti-B23 mAb are associated with the periphery of chromosomes and with masses of material associated with the perichromosomal regions on both sides of the metaphase plate. In early anaphase fibrillarin (**C**) decorates the perichromosomal region and is also present in numerous nucleolus-derived foci (NDF) (*arrows*) which are variable in size and dispersed throughout the cytoplasm but excluded from the mitotic spindle region. The perichromosomal regions and numerous NDF (*arrows*) are labeled with autoimmune serum with specificity against nucleolar protein p52 during anaphase in human HeLa cells (**D**). Similarly, the periphery of chromosomes and many NDF (*arrows*) are labeled with polyclonal anti-nucleolin antibody during anaphase in mouse 3T3 cells (**E**) and protein B23 detected with anti-B23 mAb in monkey COS-7 cells (**F**). *Bars* 10 μm

focal images for each fluorochrome from the same focal plane were recorded independently and photographed on Kodak T-Max 400 film with a digital palette (Polaroid).

## Results

### *The NDF are present in various mammalian cell lines*

In previous studies we examined the location of the HIV-1 Rev protein and the nonribosomal nucleolar proteins fibrillarin, nucleolin, protein B23 and protein p52 throughout mitosis in Rev-expressing CMT3 cells (Dundr et al. 1996). These nucleolar proteins were relocated to the perichromosomal region when the nucleoli disintegrated in the Rev-expressing CMT3 cells during late prophase and prometaphase. At metaphase these proteins were located in the periphery of chromosomes in the metaphase plate but their locations were also extended over material associated with the perichromosomal region on both sides of the metaphase plate as seen with fibrillarin (Fig. 1A) and protein B23 (Fig. 1B). At anaphase they followed the chromatids and associated with their periphery during migration to opposite poles. However, in anaphase and telophase these proteins were also seen in numerous spherical particles (NDF) distributed throughout the cytoplasm but excluded from the mitotic spindle region as seen with fibrillarin (Fig. 1C) in A5.9 CMT3 cells.

To determine whether the NDF were also present in other mammalian cell lines and their presence was not an effect of the expression of the Rev protein, we examined human HeLa, mouse 3T3 and monkey COS-7 cells in parallel with monkey A5.9 CMT3 cells. Labeling of perichromosomal regions and numerous NDF during anaphase was clearly seen in HeLa cells during anaphase using an antibody to the p52 protein (Fig. 1D), in mouse 3T3 cells with nucleolin (Fig. 1E) and in COS-7 cells labeled with an anti-B23 antibody (Fig. 1F). Similarly, the NDF in progressively decreasing numbers were clearly detectable throughout telophase and occasionally in very limited numbers in early G1 phase (not shown). The results were essentially the same when we used different fixation and permeabilization methods. Although the NDF present in the untransfected cells were less abundant than in the A5.9 CMT3 cell line, they were present in all cell lines tested. Thus, these particles seem to be natural components which are not

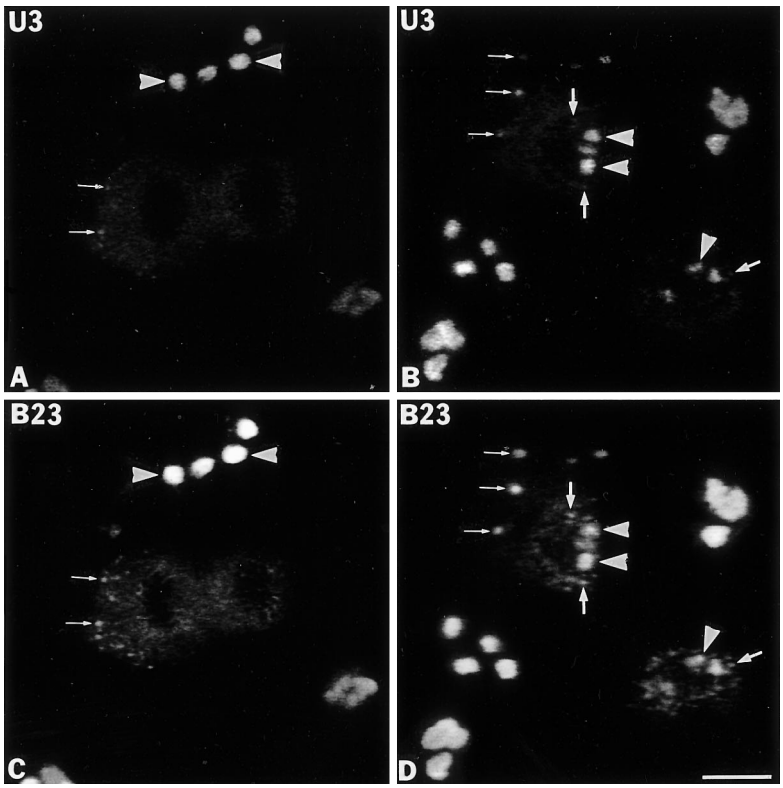


**Fig. 2A–D**. U3 snoRNA is present in NDF in A5.9 CMT3 cells during mitosis. U3 snoRNA was detected by fluorescence in situ hybridization as described in Materials and methods using a biotin-labeled anti-sense riboprobe. In interphase cells (*upper third* of **A** and **C**) U3 snoRNA is observed exclusively in multiple nucleoli (**A**, *arrowheads*). During anaphase U3 snoRNA labeling is dispersed in the cytoplasm with some signal found in the perichromosomal region of CMT3 cells (**A**). In addition, U3 snoRNA is found in the NDF (**A**, *small arrows*) where it colocalizes with protein B23 (**C**, *small arrows*). During late telophase U3 snoRNA is prominently visible in the reforming nucleoli of the two daughter cells (**B**, *arrowheads*), but it is also present in relatively weakly stained prenucleolar bodies (*large arrows*) and in several cytoplasmic NDF (**B**, *small arrows*) which are identically stained with anti-B23 antibody (**D**, *small arrows*). *Bar* 10 μm

cell-type dependent and they do not appear to be artifacts of transfection.

*The NDF contain U3 snoRNA*

As we showed above, the NDF contain fibrillarin which is associated directly or indirectly with U3 and several other snoRNPs (Maxwell and Fournier 1995) and the p52 protein which is also suggested to be a component of snoRNPs (Gautier et al. 1992). Therefore, we asked whether the NDF also contain the most abundant snoRNA, U3 snoRNA. The in situ hybridizations were performed with a biotin-labeled U3 snoRNA anti-sense riboprobe using A5.9 CMT3 and HeLa cells. The resulting RNA-RNA hybrids were visualized with avidin conjugated with Texas red. This was followed by immunolocalization using an antibody to visualize protein B23. The hybridization signal of U3 snoRNA was present exclusively in the multiple nucleoli of interphase CMT3 (Fig. 2A, arrowheads) and HeLa cells (not shown) with no signal for U3 in the cytoplasm. The corresponding sense U3 snoRNA riboprobe was used as a negative control and showed no specific labeling (not shown). From late prophase to early telophase the labeling for U3 snoRNA was dispersed in the cytoplasm with some signal found in the perichromosomal region as illustrated in Fig. 2A with a CMT3 cell in anaphase. This is in agreement with recent findings by other groups (Gautier et al. 1994; Weisenberger and Scheer 1995; Beven et al. 1996). In addition, the signal for U3 snoRNA was visible in the NDF in anaphase (Fig. 2A, small arrows), where it colocalized with protein B23 (Fig. 2C, small arrows). During late telophase, when the nuclear envelope is reformed and chromosomes are decondensed, the U3 snoRNA was still detectable in the NDF (Fig. 2B, small arrows) and it was also present in the PNBs (large arrows) and in reforming nucleoli (arrowheads) within the daughter cells. The U3 snoRNA signal in all three of these structures colocalized with protein B23 (Fig. 2D). However, at this stage the most intense labeling of U3 snoRNA was in reformed nucleoli, whereas the labeling of NDF and PNBs was relatively weak (Fig. 2B). The colocalization of U3 snoRNA with protein B23 in the NDF during anaphase and telophase confirmed the presence of U3 snoRNA in the same specific mitotic structures that are positive for nonribosomal perichromosomal proteins listed above. Consequently, the presence of U3 snoRNA together with its major associated protein, fibrillarin (see Fig. 1C), in NDF supports the view that mitotic NDF possibly contain mature assembled U3 snoRNP particles rather than nascent snoRNAs or unassembled snoRNP proteins.

*The NDF contain nucleolar protein No55*

A recently-characterized nucleolar protein designated No55 was found to be associated with the perichromosomal region from late prophase to early telophase and in the PNBs during telophase (Ochs et al. 1996). To confirm our previous findings that the nonribosomal nucleolar proteins associated with the perichromosomal region during mitosis are also present in the NDF, we per-

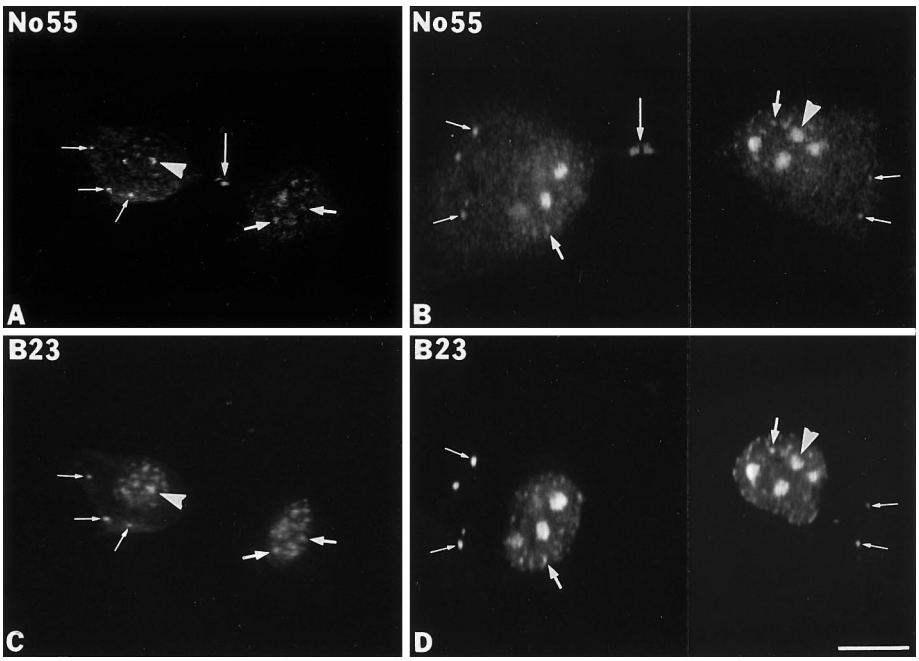


**Fig. 3A–D.** Nucleolar protein No55 is present in the NDF in CMT3 cells. At telophase, protein No55 (**A**) as visualized with specific autoimmune sera is associated with numerous prenucleolar bodies (*large arrows*) and reforming nucleoli (*arrowhead*) in daughter nuclei where it colocalizes with protein B23 (**C**) stained by anti-B23 mAb. In addition, both nucleolar proteins colocalize in cytoplasmic NDF (*small arrows*). Later in telophase, protein No55 (**B**) colocalizes with protein B23 (**D**) in prominently stained reformed nucleoli (*arrowheads*) and prenucleolar bodies (*large arrows*). Both proteins also colocalize in several cytoplasmic NDF (*small arrows*). Interestingly, the anti-No55 autoimmune serum stains structures in the cleavage furrow (*long arrows*) during cytokinesis (**A, B**) which is completely negative for protein B23 (**C, D**). *Bar* 10 μm

formed double-labeling experiments using anti-No55 autoimmune sera and antibodies against protein B23 and nucleolin. In agreement with Ochs et al. (1996), we observed that protein No55 is associated with the perichromosomal region from late prophase to early telophase with diffuse staining throughout the cytoplasm. During anaphase protein No55 was also seen in numerous NDF, where it colocalized with the other nonribosomal nucleolar proteins mentioned above (not shown). In telophase (Fig. 3A,C), No55 was present in PNBs (large arrows) and in reforming nucleoli (arrowheads) as well as in the NDF (small arrows), where it colocalized with protein B23. Curiously, labeling for No55 was also found in structures in the cleavage furrow (Fig. 3A, long arrow), which are negative for protein B23 (Fig. 3C) and other nonribosomal nucleolar proteins immunolocalized in this study. As the cells progressed further into telophase the signal for No55 was more prominent in nucleoli (Fig. 3B, arrowheads), but it was also present in numerous PNBs (large arrows) and NDF (small arrows). In all three of these components protein No55 colocalized with protein B23 (Fig. 3D). In the late telophase cells the labeling of the structures in the cleavage furrow (Fig. 3B, long arrow) was even more prominent than in early telophase (Fig. 3A).

### *Nopp140 is not associated with chromosome periphery and NDF during mitosis*

For all the nonribosomal nucleolar proteins discussed above the pattern of localization during mitosis was similar, i.e., movement from disintegrating nucleoli to chromosomal periphery during late prophase, to NDF in anaphase, to PNBs in telophase and finally to reformed nucleoli. Therefore, we searched for an example of a nonribosomal nucleolar protein that did not fit this pattern of distribution during mitosis. A candidate for this was Nopp140, which is a highly phosphorylated nucleolar protein that shuttles on tracks between the nucleolus and the cytoplasm (Meier and Blobel 1992) and distributes throughout the cytoplasm during metaphase (Meier and Blobel 1990). In prophase, Nopp140 was primarily associated with disintegrating nucleoli (Fig. 4A, arrowhead). During prometaphase the protein became dispersed in the cytoplasm which was essentially its only location in metaphase (Fig. 4B) and early telophase (Fig. 4C). Some signal for Nopp140 remained in the cytoplasm during telophase, although substantial portions of the protein had entered reforming nucleoli by this stage (Fig. 4D, arrowheads). From anaphase through telophase the NDF which contained protein B23 were clearly visible (Fig. 4E,F, small arrows). However, in the same cells Nopp140 did not colocalize with protein B23 in the NDF (Fig. 4C,D). During early telophase protein B23 was localized with decondensing chromosomes in the proximity of the reassembling nuclear envelope (Fig. 4E), whereas at this stage Nopp140 localized exclusively in the cytoplasm (Fig. 4C). Nopp140 was not detected in numerous small dot-like PNBs positive for fibrillarin, protein p52, protein B23, protein No55 and nucleolin (not shown) which began to form in the proximity of decondensing chromosomes during this stage of mitosis. After completion of nuclear envelope formation in late telophase Nopp140 was prominently visible in newly reforming nucleoli (Fig. 4D, arrowheads), as was protein B23 (Fig. 4F, arrowheads). There was no detectable labeling of Nopp140 in numerous PNBs present in the nucleoplasm (Fig. 4D) which were positive for protein B23 (Fig. 4F, large arrows) and other nonribosomal nucleolar proteins examined in this study (not shown). Only on rare occasions did we detect tiny Nopp140-positive dots (one to three

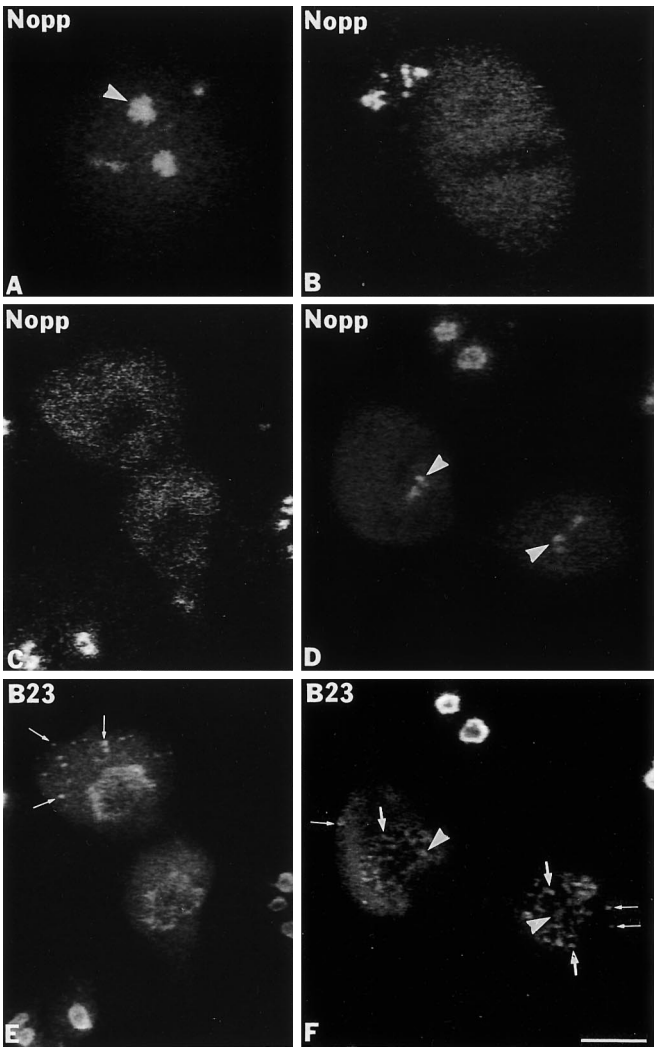


**Fig. 4A–F**. Nucleolar shuttling phosphoprotein Nopp140 is not present in the perichromosomal region or in NDF in CMT3 cells. In early prophase Nopp140 visualized with a polyclonal anti-Nopp140 antibody is predominantly localized in disintegrating nucleoli (*arrowhead*) (**A**). During metaphase Nopp140 is dispersed in the cytoplasm (**B**). In early anaphase Nopp140 remains in the cytoplasm (**C**) in contrast with protein B23 which is associated with the decondensing chromosomes (**E**). Nopp140 is not present in the NDF (**C**) which are positive for protein B23 (**E**, *small arrows*). In late telophase Nopp140 is predominantly localized in reforming nucleoli (*arrowheads*) within daughter nuclei with weak labeling in the cytoplasm (**D**). Nopp140 colocalizes with protein B23 in reforming nucleoli (*arrowheads*) but does not colocalize with protein B23 in numerous prenucleolar bodies (*large arrows*) and cytoplasmic NDF (*small arrows*) (**F**). *Bar* 10 μm

per daughter cell) which were reminiscent of PNBs in close proximity to reforming nucleoli in early stages of nucleologenesis. Virtually identical results we observed also for NAP57 protein (not shown). Thus, Nopp140 appears to follow a different pathway into reforming nucleoli compared to several nonribosomal perichromosomal nucleolar proteins: rather than associating with perichromosomal regions and NDF in early stages of mitosis and PNBs during nucleologenesis, it enters reforming nucleoli directly from its dispersed cytoplasmic location during telophase.

### *Mitotic coiled bodies and NDF disappear from the cytoplasm of telophase cells at approximately the same time*

Recent studies suggest that there is a close, but ill-defined, relationship between the coiled body (CB) and the nucleolus (Bohmann et al. 1995a,b), i.e., some nucleolar components are shared between nucleoli and CBs: fibrillarin and U3 snoRNA, Nopp140 and NAP57, ribosomal protein S6 (Raška et al. 1991; Jiménez-García et al. 1994; Meier and Blobel 1994). It is also known that when cells enter mitosis CBs disintegrate and p80 coilin which is specifically located in CBs is dispersed in the cytoplasm. However, the remnants of mitotic CBs (MCBs) persist during mitosis in constant numbers until late telophase (Andrade et al. 1993; Carmo-Fonseca et al. 1993; Ferreira et al. 1994). It is interesting that there appears to be a greater number of MCBs than interphase coiled bodies, on a per cell basis. Therefore, we investigated the relationship between NDF and MCBs and the possibility that material from the NDF and from MCBs entered postmitotic nuclei at similar times during mitosis. For this, we examined the location of p80 coilin which is specifically located in CBs, relative to the location of protein B23 which is present in the NDF but is not present in CBs (Raška et al. 1991) and the p52 protein which is present in NDF but whose presence in CBs has not been examined. During metaphase and anaphase (Fig. 5A) p80 coilin was largely dispersed throughout the cytoplasm, although some of the p80 coilin signal was present in several MCBs (long arrows). In the same cells, numerous protein B23-containing NDF were clearly visible (Fig. 5E, small arrows): these did not colocalize with the MCBs observed in Fig. 5A. No signal for p80 coilin was found in the NDF (Fig. 5A,E). Similarly, in early telophase when the nuclear envelope is reforming, the MCBs (Fig. 5B, long arrows) remained in the cytoplasm, although some of the dispersed p80 coilin signal was present in the newly forming nuclei. Interestingly, during metaphase and anaphase some MCBs were also visible inside the mass of chromosomes in the metaphase plate or moving to opposite poles (Fig. 5A). However, when the chromosomes decondense and the nuclear envelope is reassembling in early telophase the MCBs are not present in the nuclear interior but some MCBs are near the cytoplasmic side of the nuclear envelope (Fig. 5B, long arrows). At the same time, protein B23 was present in the nuclear interior and in the NDF (Fig. 5F, small arrows) which did not colocalize with the MCBs (Fig. 5B). In late telophase/early G1 phase cytoplasmic MCBs completely disappeared (Fig. 5C), as did the NDF (Fig. 5G). In most late telophase cells p80 coilin had entered the nuclei, where it was present only as dispersed staining, but in some late telophase/early G1 cells a larger number of small newly reformed CBs was visible (Fig. 5C, long arrows). In cells at this stage, p80 coilin in newly reformed CBs did not colocalize with

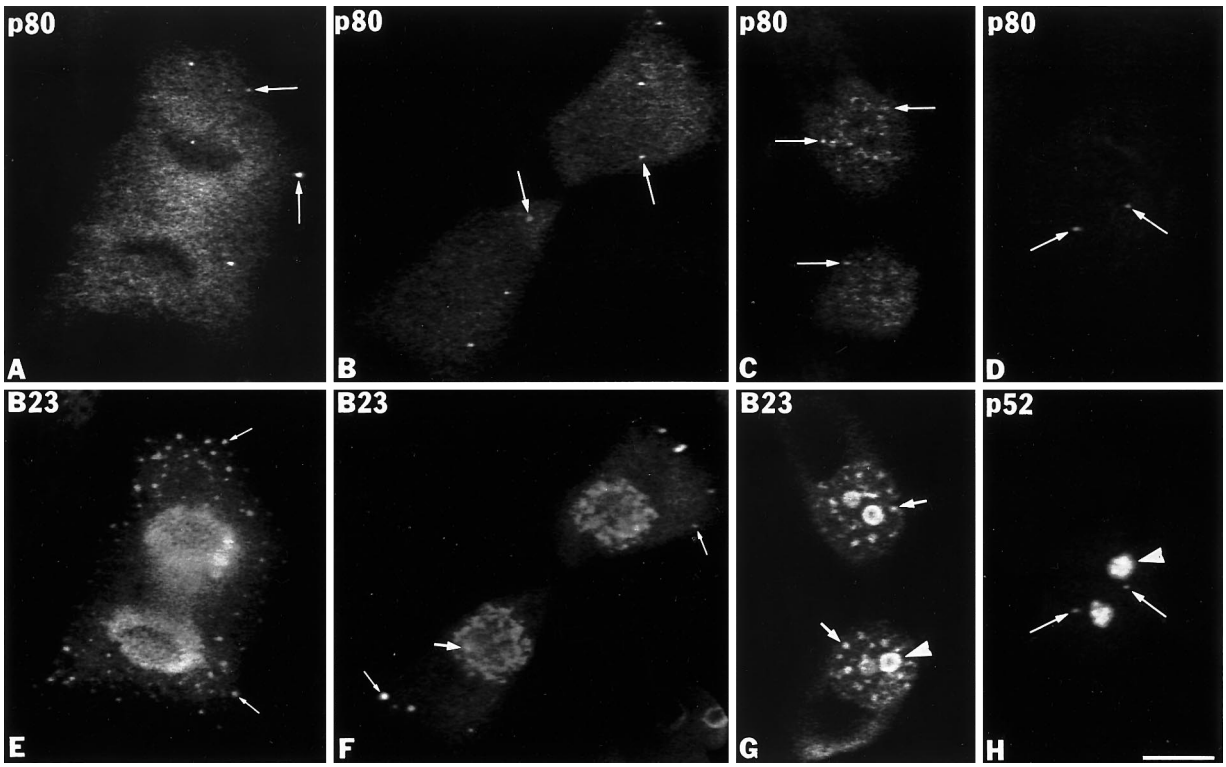


**Fig. 5A–H**. Mitotic coiled bodies (MCBs) and NDF disappear from postmitotic daughter cells at the same time in CMT3 cells. During anaphase the specific marker of coiled body, p80 coilin as detected by a polyclonal anti-p80 coilin antibody, is predominantly dispersed in the cytoplasm but it is also present in several residual MCBs (*long arrows*) (**A**). In contrast, protein B23 stained with anti-B23 mAb decorates perichromosomal region and numerous NDF (*small arrows*) (**E**) which do not colocalize with MCBs (**A**). In early telophase when the nuclear envelope begins re-assembly p80 coilin is present in the cytoplasm and in several cytoplasmic MCBs (*long arrows*) (**B**). In contrast, protein B23 is still associated with decondensing chromosomes and prenucleolar bodies (PNBs) (*large arrows*), but is also present in a few cytoplasmic NDF (*small arrows*) (**F**). In late telophase/early G1 phase p80 coilin diffusively labels the nucleoplasm, but small newly forming CBs (*long arrows*) also become visible (**C**). Protein B23 is present in reforming nucleoli (*arrowhead*) and many persisting PNBs (*large arrows*) (**G**) which do not colocalize with newly reforming CBs (**C**). During interphase p80 coilin is present in nuclear CBs (*long arrows*) (**D**) where it colocalizes with the p52 protein (*long arrows*) (**H**) which is also found in nucleoli (*arrowhead*). *Bar* 10 μm

protein B23 in PNBs (Fig. 5G, large arrows) and in reformed nucleoli (arrowhead). The superimposition of separate images showing the simultaneous localization of p80 coilin (Fig. 5C) and protein B23 (Fig. 5G) did not show any overlap between the two signals (not shown). Interestingly, in interphase cells there was colocalization of the p52 protein with p80 coilin in nuclear CBs (Fig. 5D,H, long arrows). This adds one more nucleolar protein to the list of proteins that are shared between NDF and CBs. These results indicate that the NDF and the MCBs disperse and disappear from the cytoplasm at approximately the same time during mitosis.

### *The HIV-1 Rev protein disperses in the cytoplasm of late telophase cells*

Previous studies indicated that the HIV-1 wild-type Rev protein followed a pattern of location similar to that of several other nucleolar proteins until early telophase, i.e., it associated with chromosomal periphery prior to metaphase and appeared in NDF in anaphase (Dundr et al. 1996). In late telophase when the nuclear envelope has reformed and the NDF are greatly reduced in number the wild-type Rev protein is excluded from the daughter nuclei to cytoplasm (Fig. 6A) when other nucleolar proteins such as protein B23 enter nuclei and associate with PNBs (Fig. 6C, large arrow) and then enter reforming nucleoli (arrowhead). In contrast, the *trans*-dominant negative Rev protein (TD Rev) which has a defective nuclear export signal migrates to PNBs (Fig. 6B, large arrows) and to the reformed nucleoli (arrowhead), where it colocalizes with protein B23 (Fig. 6D). Unlike the wild-type Rev protein there is no signal for the TD Rev in the cytoplasm in late telophase (Fig. 6B). These results suggest that the disintegration of the NDF is programmed to proceed regardless of the ability of their components to be imported into nuclei.

## Discussion

In this study, we have shown that the nonribosomal nucleolar proteins fibrillarin, nucleolin, protein B23, No55 and protein p52 have similar patterns of location throughout mitosis in human HeLa, monkey COS-7 and

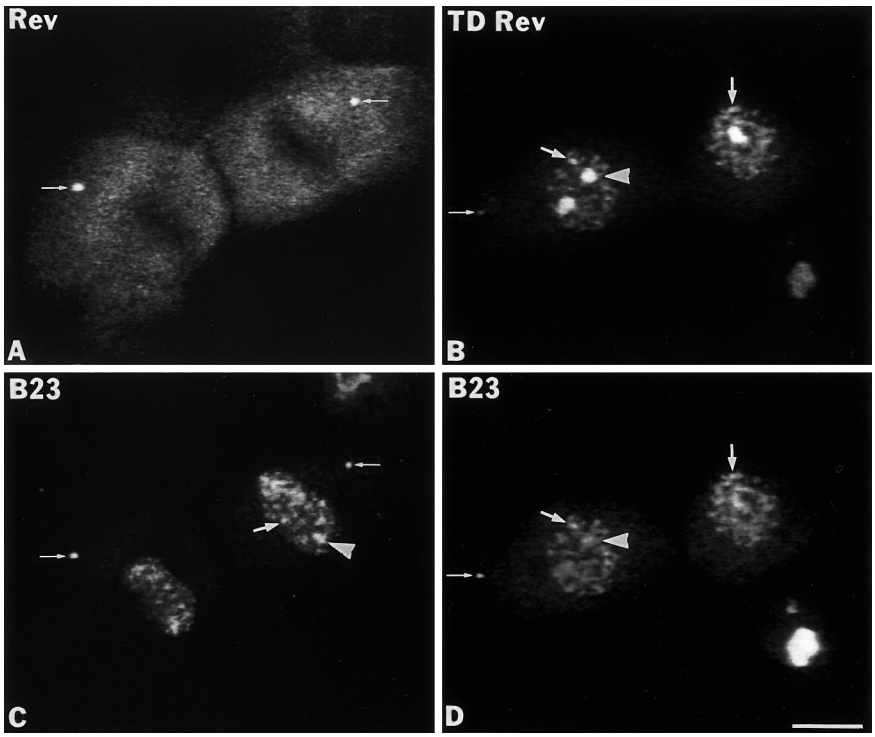


**Fig. 6A–D.** Localization of the wild-type Rev protein and *trans*-dominant negative Rev protein (TD Rev) in late telophase Rev-expressing A5.9 CMT3 cells. The wild-type Rev protein as detected with a polyclonal anti-Rev antibody is present in the cytoplasm of daughter cells in late telophase (**A**). In contrast, protein B23 labeled with anti-B23 mAb is present in reforming nucleoli (*arrowhead*) and numerous PNBs (*large arrows*) (**C**). Both proteins colocalize in cytoplasmic NDF (*small arrows*) (**A, C**). In contrast, the non-shuttling mutant TD Rev is localized in reformed nucleoli (*arrowhead*) and in PNBs (*large arrows*) (**B**) as is protein B23 (**D**) in late telophase. The wild-type Rev protein (**A**), TD Rev (**B**) and protein B23 (**C,D**) are also localized in a few persistent cytoplasmic NDF (*small arrows*). *Bar* 10 μm

mouse 3T3 cells. The pattern we observed for this group of proteins is an initial association with the perichromosomal region after the disintegration of nucleoli in late prophase followed by accumulation in numerous NDF during anaphase and telophase. Work presented here and in previous studies (Dundr et al. 1996) also showed that a *trans*-dominant negative mutant HIV-1 Rev protein has a distribution during mitosis in CMT3 cells nearly identical to that of the above-mentioned proteins; however, the wild-type Rev protein deviated from this pattern in late telophase. Although the NDF are less abundant and slightly smaller in all the cell types tested as compared to Rev-expressing CMT3 cells, they share other characteristics that are virtually identical: they contain the same nonribosomal perichromosomal nucleolar proteins listed above, appear in early anaphase, progressively decline in number during early telophase and completely disappear in late telophase/early G1 phase. The greater size and larger number of the NDF in the CMT3 cells may be due to the larger cell size and consequent presence of more nucleolar material per cell. In any event, these mitotic structures seem to be present in all mammalian cells and they contain natural nucleolar components.

The nucleolar proteins that coat the perichromosomal region from prometaphase until early telophase seem to be involved in the nascent pre-rRNA processing and formation of preribosomal subunits during ribosome biogenesis and they do not appear to be related to RNA pol I transcriptional events (Hernandez-Verdun et al. 1993). Several of the nucleolar proteins examined in this study which are perichromosomal and also accumulate in the NDF have been implicated in preribosomal RNA processing and ribosome assembly. For example, nucleolin is associated with nascent preribosomal RNA (Herrera and Olson 1986) and it has recently been found in processing complexes (Ghisolfi-Nieto et al. 1996). In addition, protein B23 is present in the dense fibrillar and granular components of the nucleolus (Biggiogera et al. 1990) which contain preribosomal particles in various stages of assembly and processing. The ribonuclease activity of protein B23 also suggests its involvement in pre-rRNA processing (Herrera et al. 1995). Finally, U3 snoRNA and one of its associated proteins, fibrillarin, were recently observed in the perichromosomal region (Gautier et al. 1994; Weisenberger and Scheer 1995; Beven et al. 1996) and in the NDF (this work). Both of these components are implicated in preribosomal RNA processing (Maxwell and Fournier 1995), which further supports the idea that material from the chromosome periphery and NDF is involved in the processing and assembly stages of ribosome biogenesis. The presence of both U3 snoRNA and fibrillarin in these structural mitotic elements is in agreement with the observation that snRNPs retain their mature overall structure during mitosis (Reuter et al. 1985). It will be interesting to determine whether other snoRNAs and snoRNPs follow the same pathway through mitosis as they return to the nucleolus. In addition, it will be important to know whether pre-rRNA is located in the NDF, since it has been observed in perichromosomal regions by at least two research groups (Medina et al. 1995; Beven et al. 1996).

One example of a nonribosomal protein that does not follow this pattern of behavior during mitosis is the rat nucleolar shuttling phosphoprotein Nopp140. This protein was found in the nucleolar dense fibrillar compo-

nents in interphase cells and dispersed throughout the cytoplasm at metaphase (Meier and Blobel 1990). The same location was found for a human homolog of Nopp140, the p130 protein which shares 74% sequence identity with rat Nopp140 (Pai et al. 1995). Our results confirm the cytoplasmic distribution of Nopp140 from prometaphase to early telophase in CMT3 and HeLa cells. In addition, Nopp140 was not found in the NDF in anaphase and telophase cells visualized in colocalization experiments with nucleolar perichromosomal proteins. The same results were obtained for the nucleolar protein NAP57 which is associated with Nopp140 (Meier and Blobel 1994): NAP57 is dispersed in the cytoplasm from prometaphase to early anaphase with no detectable staining of the NDF (not shown). These results are also consistent with our previous finding that ribosomal protein S6 which is also dispersed in the cytoplasm during mitosis, is not present in the NDF (Dundr et al. 1996). Thus, of the nucleolar proteins examined to date, those proteins that have a dispersed cytoplasmic location during most of mitosis, none have been found in the NDF.

Since the NDF have not been extensively characterized, it is not known whether the NDF are the same as or analogous to previously described nucleolar remnants (NRs). The NRs as the persisting nucleolar structure throughout mitosis were originally observed by Hsu et al. (1965) and later by Noel et al. (1971) in Chinese hamster tissues. More recently, NRs in Chinese hamster ovary (CHO) cells were shown to contain nucleolar perichromosomal proteins and U3 snoRNA (Azum-Gélade et al. 1994; Gautier et al. 1994). Interestingly, the CHO cells used in above studies contained just one prominent NR during mitosis until late telophase. In contrast, the number of NDF during anaphase in HeLa, 3T3 and COS-7 cell is sometimes as many as 50, but in Rev-expressing CMT3 cells the number of NDF is occasionally over 100. The CHO cells are characterized by high nucleolar activity in exponentially growing phase (Gautier et al. 1994), as are all the cell types tested in this study. Therefore, this discrepancy in numbers of NRs in CHO cells and of NDF in other cell types cannot be explained by different amounts of available nucleolar material.

How the NDF arise can only be speculated on at this time. It is interesting that the same group of proteins and U3 RNA are distributed among the chromosome periphery and the NDF simultaneously during anaphase and telophase. One possible explanation for this is that nucleolar material composed of those components is loosely associated with the chromosomes prior to and during metaphase. However, after metaphase there may be alterations of chromosome structure so that fewer binding sites are available and less nucleolar material adheres to the chromosomal periphery. In this case, a portion of the chromosomal peripheral material moves to the cytoplasm, where it forms large particles which serve as storage reservoirs until it is needed. A second possible mechanism would involve the formation of the NDF as part of an orderly process to deliver the material from the chromosome periphery to the newly forming nucleolus. This process would begin after metaphase and be completed in late telophase. Indeed, the number of the NDF seems to be maximal in mid- to late- anaphase, after which it steadily declines as the nucleolar material is incorporated into PNBs and nucleoli. There appears to be a stage in late telophase when the nuclear envelope has formed and when chromosomes have decondensed, but only a few of the NDF remain in the cytoplasm (Fig. 3B,D). These remaining NDF could represent the last stage of the process when all of the nucleolar material is removed from the chromosomes and the NDF provide the final reservoir before it enters the nucleus. This latter scenario would suggest that the appearance and disappearance of the NDF represents a highly regulated program of events that delivers nucleolar material back to the nucleolus within a defined time frame.

The final question is: What is the function of the NDF? Possible answers to this question are largely in the realm of speculation. The first possibility is that nucleolar components of a certain class have a high affinity for each other whether they are in the nucleolus or outside of it. These components would naturally aggregate if not attached to another structure. In this case there would be no extraordinary function for the NDF. A second function suggested by Benavente et al. (1989) and Hernandez-Verdun et al. (1993) for the perichromosomal layer is that it may be a storage site that permits equal redistribution of nucleolar components between the daugher cells. This could also be the case for the NDF. Contrary to this notion, we have observed that the distribution of the NDF in the cell plasm does not always result in the equal apportioning of the NDF in the daughter cells. Thus, it is not likely that the purpose of the NDF is to ensure uniform dispersal of the nucleolar components during mitosis. The third possibility has already been alluded to above, i.e., the NDF may provide an orderly process for delivery of nucleolar material to reforming nucleoli. The reason for this may have to do with timing of events in nucleolar reformation. In late stages of mitosis nucleoli are reformed around NORs. Transcription must take place initially before the pre-rRNA processing apparatus is added. There is evidence to suggest that the NDF contain pre-rRNA processing components rather than constituents of the RNA pol I transcriptional apparatus (see above). Thus, the NDF may be a means of keeping the processing components separated from the transcriptional complex until there is a need for processing of the preribosomal RNA.

A curious phenomenon exists with components that should not normally be found in the nucleolus such as the HIV-1 Rev protein. As shown here and in previous studies (Dundr et al. 1996) Rev behaves like the perichromosomal/NDF proteins until telophase when it is completely excluded from the nucleus and does not reenter until G1 phase. However, a *trans*-dominant negative mutant Rev with a defective nuclear export signal behaves like the other proteins described here. The probable reasons for delayed entry of the wild-type Rev protein have been discussed previously (Dundr et al. 1996); however, the important point related to the current study is that complete coordination of the entry of the perichromosomal/NDF proteins into nucleoli is not obligatory. In other words, all proteins found in the NDF need

not reenter nuclei and nucleoli at the same time. This latter observation would argue for a simple storage role for the NDF rather than a more complex regulatory one. In any event, a great deal more characterization of these foci must be done before their precise functions can be determined.

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