JCB Article

Cyclin-dependent kinases govern formation and maintenance of the nucleolus

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In higher eukaryotic cells, the nucleolus is a nuclear compartment assembled at the beginning of interphase, maintained during interphase, and disorganized during mitosis. Even if its structural organization appears to be undissociable from its function in ribosome biogenesis, the mechanisms that govern the formation and maintenance of the nucleolus are not elucidated. To determine if cell cycle regulators are implicated, we investigated the putative role of the cyclin-dependent kinases (CDKs) on ribosome biogenesis and nucleolar organization. Inhibition of CDK1–cyclin B during mitosis leads to resumption of rDNA transcription, but is not sufficient to induce proper processing of the pre-rRNA and total relocalization of the processing machinery into rDNA transcription sites. Similarly, at the exit from mitosis, both translocation of the late processing machinery and pre-rRNA processing are impaired in a reversible manner by CDK inhibitors. Therefore, CDK activity seems indispensable for the building of functional nucleoli. Furthermore, inhibition of CDKs in interphasic cells also hampered proper pre-rRNA processing and induced a dramatic disorganization of the nucleolus. Thus, we propose that the mechanisms governing both formation and maintenance of functional nucleoli involve CDK activities and couple the cell cycle to ribosome biogenesis.

Introduction

The nucleolus is a model of an active and dynamic nuclear domain and plays a major role in compartmentalization of nuclear function. In higher eukaryotic cells, the nucleolus assembles at the exit from mitosis and is functionally active throughout interphase. Its major function, i.e., ribosome biogenesis, requires rDNA transcription, pre-rRNA processing, and assembly of the mature rRNAs with ribosomal proteins (Hadjiolov, 1985). The nucleolus was more recently reported to be a plurifunctional nuclear domain (Olson et al., 2000) involved in cell cycle control (Visitin and Amon, 2000), nuclear protein export (Zolotukhin and Felber, 1999), and the aging process (Guarente, 1997), and to contain components of signal recognition particles (Politz et al., 2000). Therefore, it is most likely that the existence of a fully active nucleolus is not only essential for ribosome production, but also for control of cell survival and cell proliferation (Carmo-Fonseca et al., 2000).

Nucleoli are generally composed of three morphologically distinct subdomains: the fibrillar centers (FCs),* the dense fibrillar component (DFC), and the granular component (GC) (Hadjiolov, 1985). The prevailing model is that the subdomains reflect the vectorial process integrating the 47S pre-rRNA in its maturation pathway, and consequently, the nucleolus is proposed to be "an organelle formed by the act of building a ribosome" (Mélèse and Xue, 1995). However, there is presently no information on the mechanisms controlling the coordination between the different steps of ribosome biogenesis, in particular the coordination between rDNA transcription and 47S pre-rRNA processing (Allmang et al., 1999), and what nucleolar organization actually reflects. It is well established that blockage of rDNA transcription induces nucleolar disassembly and segregation of the nucleolar machineries (Hadjiolov, 1985). However, we do not know if the maintenance of an organized nucleolar compartment throughout interphase is only dependent on pre-rRNA synthesis. Indeed, nucleolar fragmentation can be induced without direct interaction with rDNA transcription (Sinclair et al., 1997).

Ribosome biogenesis involves several machineries dedicated to rDNA transcription and processing of the 47S pre-rRNA into 18S, 5.8S, and 28S mature rRNAs (Scheer and Hock,

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Key words: rDNA transcription; cyclin-dependent kinase; pre-rRNA processing; inhibitor; nucleolus

*Abbreviations used in this paper: CDK, cyclin-dependent kinase; DFC, dense fibrillar component; ERK, extracellular signal-regulated kinase; ETS, external transcribed spacer; FC, fibrillar center; GC, granular component; GFP, green fluorescent protein; NOR, nucleolar organizer region; nt, nucleotide; PNB, prenucleolar body; pol, RNA polymerase; RT, room temperature; snoRNA, small nucleolar RNA; UBF, upstream binding factor.

1999; Shaw and Jordan, 1995). rDNA transcription is dependent on RNA polymerase (pol) I and requires at least two factors in addition to active pol I, i.e., the upstream binding factor (UBF) and the promoter selectivity factor, SL1 (Moss and Stefanovsky, 1995; Grummt, 1999). Processing of the 47S pre-rRNA is under the control of several RNP complexes involving small nucleolar RNAs (snoRNAs). This activity is ordered from the early step of processing in the 5′ external transcribed spacer (ETS) to the last steps, the internal transcribed spacer 2, and 5.8S processing (Tollervey, 1996).

During mitosis, the nucleolar activity is abolished and nucleoli are no longer maintained. The rDNA transcription machinery remains assembled in an inactive state at the level of nucleolar organizer regions (NORs), i.e., in chromosomal sites where rDNAs are also clustered (Roussel et al., 1996). Conversely, the processing machinery does not remain in the vicinity of the rDNAs. Indeed, proteins involved in pre-rRNA processing, such as fibrillarin, nucleolin, Nop52, and protein B23, are located at the periphery of chromosomes during mitosis and are recruited in prenucleolar bodies (PNBs) scattered throughout the nucleus in early G1 (Jiménez-Garcia et al., 1994; Savino et al., 1999; Dundr et al., 2000). In addition to proteins, PNBs contain snoRNAs involved in pre-rRNA processing such as U3, U8, and U14 snoRNAs (Gautier et al., 1994; Jiménez-Garcia et al., 1994; Dousset et al., 2000). Interestingly, it has been proposed that different types of PNBs exist, containing complexes dedicated to early or late processing events, and addressed to the forming nucleoli with different kinetics (Westendorf et al., 1998; Savino et al., 1999, 2001). These observations suggest a spatio-temporal order in the formation of PNBs and raise the possibility that at the M/G1 transition, the recruitment of the processing machinery to the forming nucleoli is regulated.

Even if a general linkage between nucleolar function and cell cycle regulation has not yet been established, rDNA transcription is known to be regulated in a cell cycle–dependent manner. On one hand, rDNA transcription is repressed at mitosis by the cyclin-dependent kinase (CDK)1–cyclin B-directed phosphorylation of components of the rDNA transcription machinery (Heix et al., 1998; Sirri et al., 2000). On the other hand, the increase of rDNA transcription during G1 progression appears to depend on phosphorylation of UBF by G1-specific CDK–cyclin complexes (Voit et al., 1999). Moreover, highly proliferating cancer cells with deregulated cell cycle controls also exhibit modified rDNA transcription activity. The retinoblastoma protein pRb which suppresses cell proliferation can also suppress the pol I activity by direct interaction with UBF (Hannan et al., 2000). Nevertheless, because rDNA transcription can be disconnected from pre-rRNA processing by in vivo inhibition of the CDK1–cyclin B pathway in mitotic cells (Sirri et al., 2000), it remains to be elucidated if ribosome production is regulated not only at the rDNA transcription level, but also through pre-rRNA processing. Here we investigate the role of CDKs on ribosome biogenesis, and on the formation and maintenance of the nucleolus. The results establish that pre-rRNA processing is controlled by CDKs throughout the cell cycle. Interfering with the pre-rRNA processing pathway induces the redistribution of nucleolar machineries and disorganization of the functional nucleolus.

Results

Inhibition of the CDK1–cyclin B pathway in mitotic cells induces the relocalization of fibrillarin at the level of NORs, but not that of Nop52

By inhibiting the CDK1–cyclin B pathway, we established that rDNA transcription may be restored in mitotic cells but not proper pre-rRNA processing (Sirri et al., 2000). Because the processing machinery does not remain associated with rDNAs during mitosis, the pre-rRNA processing defect could be due to the absence or abnormal targeting of the processing machinery to rDNA transcription sites. To test this hypothesis, we investigated the distribution of fibrillarin and Nop52 (Fig. 1) involved in early and late pre-rRNA processing events, respectively (Savino et al., 1999), using the experimental approach described previously (Sirri et al., 2000), i.e., colchicine-arrested mitotic HeLa cells treated or not with roscovitine, a highly selective inhibitor of CDKs (De Azevedo et al., 1997; Meijer et al., 1997). When CDK1–cyclin B was inhibited by roscovitine, fibrillarin, which is normally observed in the cytoplasm and enriched at the chromosome periphery (Fig. 1 b), was partly relocated in a few spots on the chromosomes (Fig. 1 e). Nop52 colocalized with fibrillarin in colchicine-arrested mitotic HeLa cells (Fig. 1, compare b with c). Roscovitine treatment also induced an important relocalization of Nop52 (Fig. 1, compare c with f) that now appeared as numerous small dots on the chromosome periphery which are most probably the PNBs observed in mitotic cells in telophase. To precisely define the relocalization of fibrillarin, UBF used as marker of NORs, and fibrillarin were localized in the same mitotic cells treated with roscovitine. The fluorescent patterns detected for fibrillarin and for UBF (Fig. 1, g–i) showed that fibrillarin relocalized at NORs, i.e., in chromosomal sites where rDNA transcription is restored after inhibition of the CDK1-cyclin B pathway. The comparison between the fibrillarin (Fig. 1 e) and Nop52 labellings (Fig. 1 f) showed that contrary to fibrillarin the relocalization of Nop52 in sites of resumption of rDNA transcription seemed not to only depend on the CDK1-cyclin B activity. Similar results were observed in HeLa cells overexpressing fibrillarin or Nop52 and in the parental HeLa cell line. The absence of relocalization of Nop52 at the level of sites where rDNA transcription restarts cannot be explained by different kinetics. Indeed, similar results to those shown in Fig. 1 obtained after 1 h of treatment were obtained in mitotic HeLa cells treated with roscovitine for 3 h (unpublished data).

In cells proceeding through M/G1 transition, CDK inhibitor-treatment does not interfere with relocalization of fibrillarin at the level of rDNA transcription sites, but blocks relocalization of Nop52 and impairs formation of nucleoli

To determine the effect of roscovitine on the localization of fibrillarin and Nop52 at the exit from mitosis, metaphase-synchronized HeLa cells were treated or not treated with 75

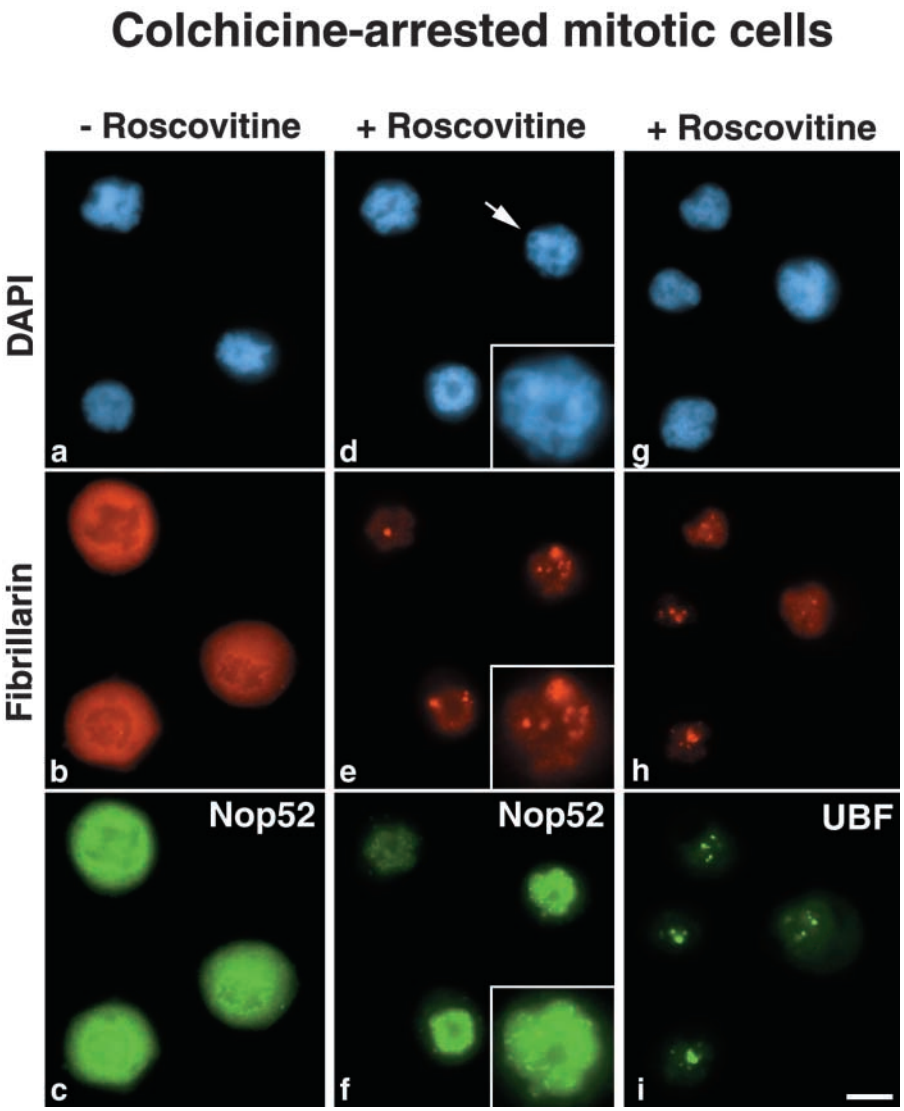

Figure 1. **Roscovitine treatment induces relocalization of fibrillarin at the level of NORs but not that of Nop52 in colchicine arrested mitotic HeLa cells.** Stably transfected Nop52-GFP HeLa cells blocked in mitosis by colchicine treatment were treated (d–f) or not (a–c) with 75 μM roscovitine for 1 h. The cells were processed to label fibrillarin (b and e) and to observe Nop52-GFP (c and f). To precisely locate fibrillarin in roscovitine-treated mitotic cells, both fibrillarin (h) and UBF (i) were simultaneously labeled in untransfected HeLa cells treated with 75 μM roscovitine for 1 h (g–i). Fibrillarin and Nop52 which are normally observed in the cytoplasm and enriched at the chromosome periphery in mitotic cells (b and c) were largely relocated after roscovitine treatment (compare b with e, and c with f). Fibrillarin was then mainly localized in spots (e and h) corresponding to the NORs, where UBF is localized (compare h with i). Nop52 was observed in numerous small dots on the chromosome periphery (f). Arrow (d) indicates the cell for which enlargements are shown (d–f, insets). Bar, 10 μm.

μM roscovitine for 90 min, i.e., when cells proceeded through and exited from mitosis. Search for both proteins was then undertaken and transcription revealed by in situ transcription assays. As shown in Fig. 2, independently of roscovitine, cells proceeded through mitosis and appeared after 90 min as actively transcribing early G1 cells. As already published (Savino et al., 1999, 2001), in early G1 control cells, fibrillarin (Fig. 2, a–d) translocated to rDNA transcription sites (Fig. 2, compare c with d) in the reformed nucleoli (Fig. 2 a), and Nop52 (Fig. 2, i–l) appeared mainly relocalized in the reformed nucleoli (Fig. 2, compare i, k, and l) with a minor part still localized in PNBs (Fig. 2 l). When cells exited from mitosis in the presence of roscovitine, part of the fibrillarin (Fig. 2, e–h) relocalized at the level of rDNA transcription sites (Fig. 2, compare g with h), whereas Nop52, absent from rDNA transcription sites, appeared in small dots in the nucleus (Fig. 2, compare o with p). Moreover, in the roscovitine-treated cells, no reformed nucleolus was visible (Fig. 2, compare e with g, and m with o), showing that rDNA transcription is not sufficient to induce formation of nucleoli visible by phase contrast, and that resumption of rDNA transcription and formation of nucleoli may be uncoupled. As assessed by microscope observations and localization of fibrillarin and Nop52 (unpublished data), this effect on the formation of nucleoli could be induced by treatment with other CDK inhibitors, olomoucine, alsterpaullone, and purvalanol. Conversely, the negative control iso-olomoucine and U0126, a selective and potent inhibitor of mitogen-activated protein kinase kinase and consequently of extracellular signal-regulated kinase (ERK)1/2, caused no obvious effect on nucleologenesis.

To ascertain that CDK inhibitors actually impair the formation of nucleoli at the exit from mitosis and to rule out the possibility that the absence of formation of nucleoli occurred as a consequence of an effect of CDK inhibitors on late mitotic events, metaphase-synchronized HeLa cells were treated (Fig. 3, e–h) or not treated (Fig. 3, a–d) with 75 μM roscovitine for 90 min. Cells appearing as early G1 cells were cultured for an additional 90 min after removal (Fig. 3, i–l) or without removal (Fig. 3, m–p) of roscovitine. The observation by phase contrast and the detection of fibrillarin and Nop52, proteins known to be localized in nucleoli during interphase, in the same cells make it possible to follow the formation of nucleoli and the correct localization of these

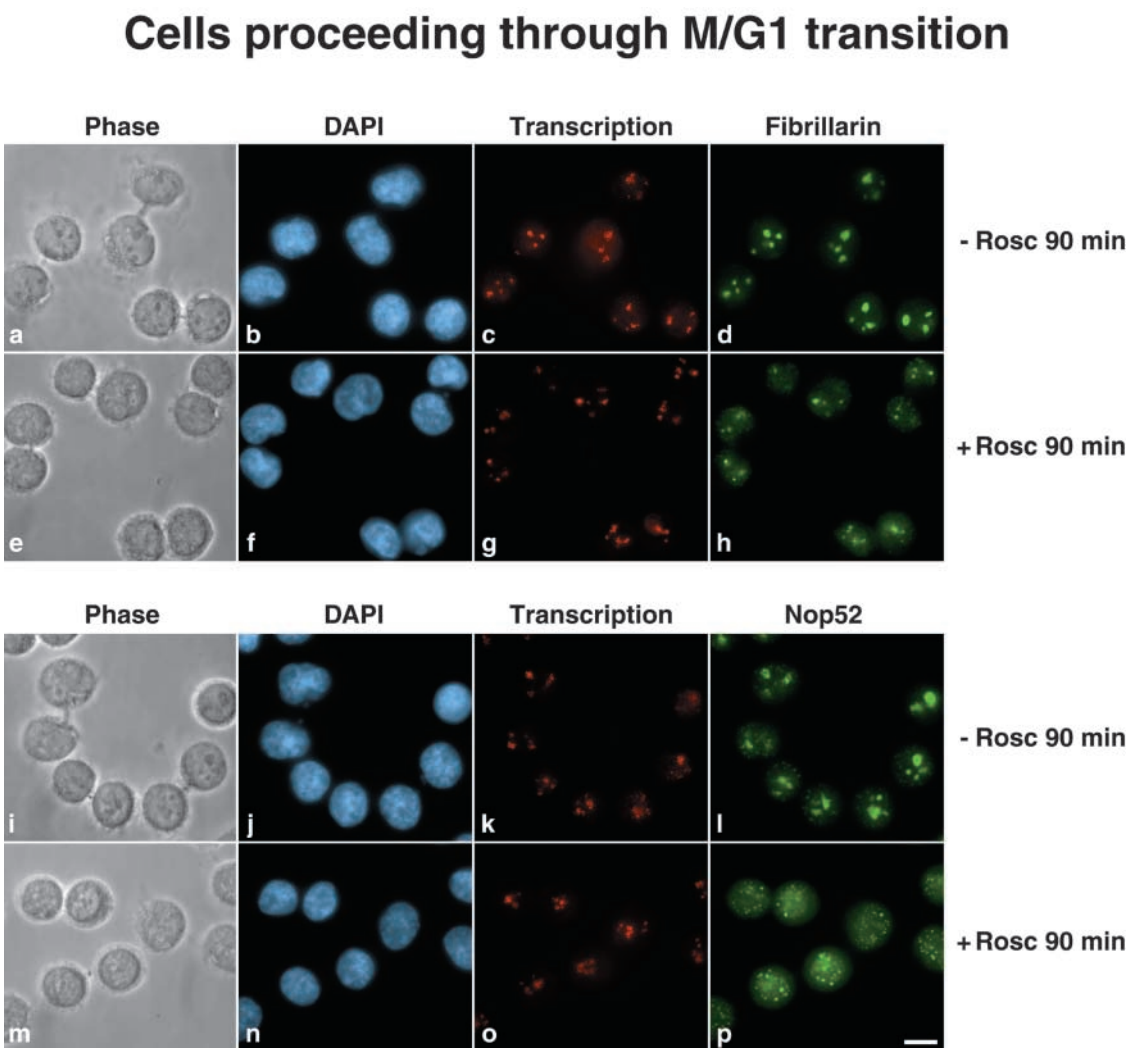

Figure 2. **Roscovitine does not interfere with relocalization of fibrillarin at the level of rDNA transcription sites but blocks relocalization of Nop52 and impairs formation of nucleoli at the exit from mitosis.** Metaphase-sychronized HeLa cells were treated (e–h and m–p) or not treated (a–d and i–l) with 75 μM roscovitine for 90 min. The cells were processed to detect transcription activity (c, g, k, and o), and to label fibrillarin (d and h) and Nop52 (l and p). They were analyzed by phase-contrast (a, e, i, and m) and DAPI staining (b, f, j, and n). Cells treated or not with roscovitine proceeded through mitosis and appeared after 90 min as actively transcribing early G1 cells. (a–d) In early G1 control cells, fibrillarin relocalized at rDNA transcription sites (compare c with d) in the reformed nucleoli (a), and (i–l) Nop52 appeared mainly relocalized in the reformed nucleoli (compare i, k, and l) with a minor part still localized in PNBs (l). In roscovitine-treated cells, part of the fibrillarin relocalized at rDNA transcription sites (compare g with h), whereas Nop52, absent from rDNA transcription sites, appeared as small dots in the nucleus (compare n, o, and p). In the roscovitine-treated cells no reformed nucleolus was visible (e and m). Bar, 10 μm.

two nucleolar proteins. As expected from results shown in Fig. 2, contrary to control cells (Fig. 3, a–d) for which both Nop52 and fibrillarin were translocated to reformed nucleoli (Fig. 3, compare a and c with d), cells treated by roscovitine exited from mitosis without apparent formation of nucleoli (Fig. 3, compare a with e). The absence of formation of nucleoli is also argued by the fact that Nop52 and fibrillarin did not localize in the same sites (Fig. 3, compare g with h). More interestingly, the effect caused by the roscovitine treatment was reversible. Indeed, 90 min after the removal of roscovitine, it was possible to observe reformed nucleoli in which both Nop52 and fibrillarin were localized (Fig. 3, i–l). Reversion was not observed when cells were maintained for 90 min more in roscovitine containing culture medium (Fig. 3, m–p) and therefore appeared actually to be due to the removal of the CDK inhibitor.

CDK inhibitor-treatment interferes with the restoration of pre-rRNA processing at the exit from mitosis and leads to the accumulation of the 46S pre-rRNA

To analyze the possible effect of roscovitine on processing of the newly synthesized pre-rRNA when mitotic repression of rDNA transcription is normally abolished, rRNA synthesis was followed in synchronized cells during exit from mitosis. Metaphase-synchronized HeLa cells were metabolically labeled with [^{32}P]orthophosphate in the absence (Fig. 4 A) and presence (Fig. 4 B) of 75 μM roscovitine, and the newly synthesized RNAs were analyzed every 30 min up to 120 min, i.e., when cells proceeded through and exited from mitosis. As assessed by microscope observations, independently of roscovitine, most cells appeared in telophase at 30 min (unpublished data) and the mitotic repression of rDNA transcription was being released (Fig. 4, A and B, lane e). In

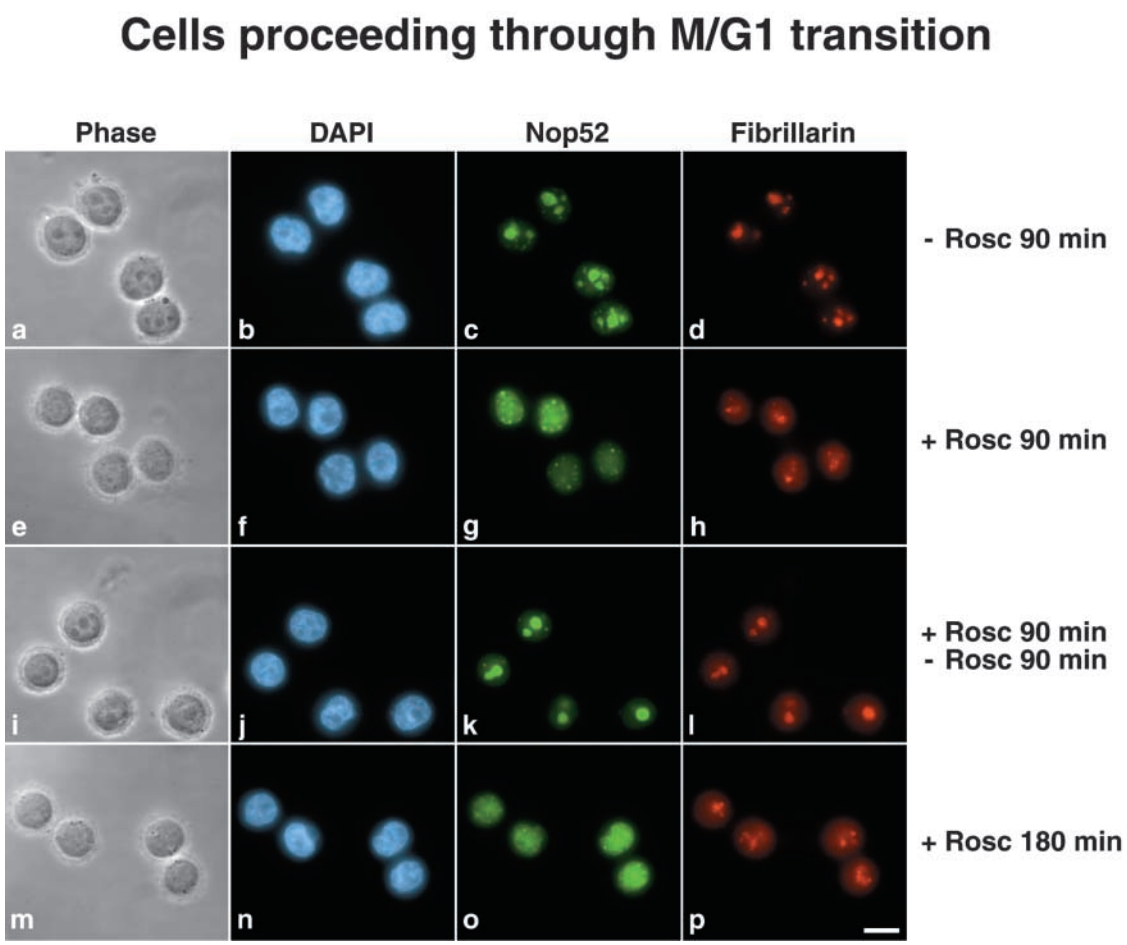

Figure 3. **The roscovitine effects on the relocalization of Nop52 and on the formation of nucleoli at the exit from mitosis are both reversible.** Stably transfected Nop52-GFP HeLa cells synchronized in metaphase were treated (e–h) or not treated (a–d) with 75 μM roscovitine for 90 min. Roscovitine-treated cells were cultured for an additional 90 min without (i–l) or with roscovitine (m-p). The cells were analyzed by phase-contrast (a, e, i, and m), DAPI staining (b, f, j, and n), Nop52-GFP observation (c, g, k, and o), and fibrillarin labeling (d, h, l, and p). In untreated cells (a–d), Nop52 and fibrillarin were mainly localized in the reformed nucleoli (compare a, c, and d). Roscovitine-treated cells exited from mitosis without apparent formation of nucleoli (compare a with e) and without gathering of Nop52 and fibrillarin in the same sites (compare g with h). Interestingly, 90 min after removal of roscovitine, nucleoli were formed, in which both Nop52 and fibrillarin were localized (i–l). Reversion was not observed when cells were cultured for an additional 90 min in roscovitine containing medium (m–p). Bar, 10 μm.

untreated cells, the ^{32}P-labeled unprocessed 47S pre-rRNA (reflecting resumption of rDNA transcription) appeared and increased concomitantly with the partially processed or mature ^{32}P-labeled rRNAs (reflecting pre-rRNA processing) (Fig. 4 A, e–h). Consequently, processing of the newly synthesized pre-rRNA appears not to be delayed with respect to its synthesis when cells exit from mitosis. Conversely, in the presence of roscovitine, resumption of rDNA transcription at the exit from mitosis led to the accumulation of 46S pre-rRNA (Fig. 4 B). The strong defect in pre-rRNA processing in the presence of roscovitine was also observed by the absence of the ^{32}P-labeled 45S pre-rRNA and the weak increase of the levels of ^{32}P-labeled mature 28S and 18S rRNAs (Fig. 4, compare A with B, lanes e–h).

Interphase nucleoli are no longer maintained after CDK inhibitor-treatment

The formation of nucleoli could be prevented by CDK inhibitor treatments, and therefore appeared as a regulated process. Consequently, it may be asked whether the same treatments could modify interphasic nucleoli. To answer this question, the localization of both fibrillarin and UBF and of both Nop52 and UBF was performed in asynchronous interphasic HeLa cells treated or not for 2 h with 75 μM roscovitine (Fig. 5). In control cells, the detection of both fibrillarin and UBF (Fig. 5, a–e), and Nop52 and UBF (Fig. 5, k–o) showed that these proteins are localized in nucleoli easily identifiable by phase contrast (Fig. 5, e and o). Roscovitine treatment led to dramatic modifications of the nucleoli as observed by phase contrast (Fig. 5, compare e with j and o with t) whatever the stage of interphase of the cells. Nucleolar proteins were now detected in at least two kinds of nuclear bodies as visible by phase contrast. UBF and fibrillarin appeared mainly in the same small nuclear bodies (Fig. 5, f–j), whereas Nop52 appeared as components of larger nuclear bodies (Fig. 5, p–t). The two kinds of nuclear bodies were easily distinguishable by comparing the localizations of Nop52 and UBF (Fig. 5, q–s). Interestingly, this disorganization of nucleoli obtained after roscovitine treatment was completely reversed 2 h after removal of roscovitine and

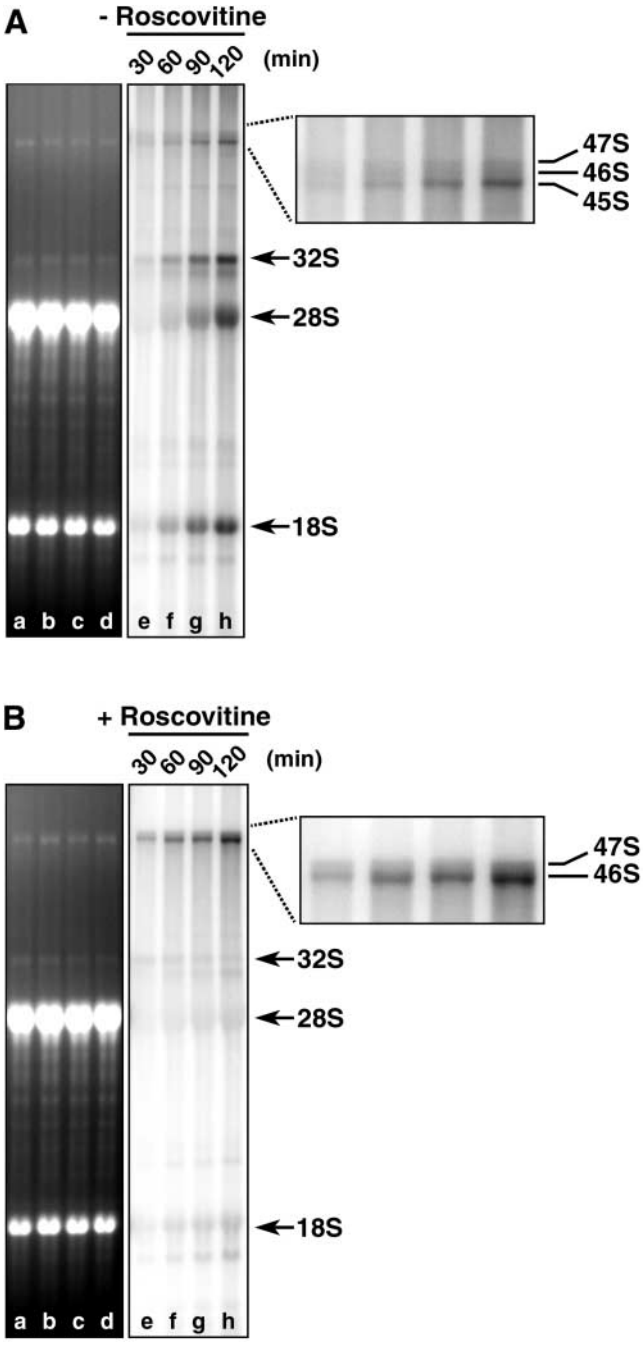

Figure 4. **Roscovitine treatment leads to accumulation of 46S pre-rRNA at exit from mitosis.** (A) Metaphase-synchronized HeLa cells were metabolically labeled with [^{32}P]orthophosphate in the absence and (B) in the presence of 75 μM roscovitine and the RNAs isolated and analyzed at 30 min (lanes a and e), 60 min (lanes b and f), 90 min (lanes c and g), and 120 min (lanes d and h), i.e., during the M/G1 transition. (A and B, lanes a–d) RNAs were resolved in parallel on the same 1.2% agarose formaldehyde gel to discriminate between 47S, 46S, and 45S pre-rRNA and detected by ethidium bromide staining. (A and B, lanes e–h) Newly synthesized RNAs detected after autoradiography. In the absence of roscovitine the ^{32}P-labeled unprocessed 47S pre-rRNA appeared and increased concomitantly with the ^{32}P-labeled partially processed or mature rRNAs (A, lanes e–h). In roscovitine-treated cells, the newly synthesized 46S pre-rRNA accumulated and no ^{32}P-labeled 45S pre-rRNA was detectable (B, lanes e–h). The levels of ^{32}P-labeled mature 28S and 18S rRNAs only slightly increased (compare B with A, lanes e–h).

could be induced by treatment with other CDK inhibitors (unpublished data). Indeed, olomoucine, alsterpaullone, and purvalanol caused similar effects on the nucleoli as assessed by microscope observations and localization of fibrillarin and Nop52, but not U0126 used at concentrations up to 200 μM for 3 h and the negative control iso-olomoucine used at the same concentration as olomoucine.

The organization of the interphase nucleoli is rapidly modified after roscovitine treatment

The nucleolar organization in asynchronous interphasic HeLa cells treated with roscovitine was examined by electron microscopy. Already after 30 min of roscovitine treatment, the arrangement of the three main nucleolar subdomains visible by electron microscopy was strongly modified (Fig. 6 A) when compared with control cells (Fig. 6 B). After roscovitine, FCs were only partly surrounded by the DFC, and the disorganization of the GC was visible. Interestingly, new structures formed in continuity with the remaining DFC (Fig. 6 A, arrow) and close to the FC. These new structures corresponded to densely packed granules distributed in trabaeculae. When the roscovitine treatment was prolonged for 2 h, these new dense structures were still visible in the proximity of FCs, but also far from FCs indicating a dispersion of these structures in the nucleus (unpublished data).

CDK inhibitor treatment reduces the generation of mature rRNAs by interfering with both rDNA transcription and pre-rRNA processing in interphasic cells

To determine the effects of CDK inhibitor treatment on nucleolar activities, asynchronous interphasic HeLa cells were metabolically labeled with [^{32}P]orthophosphate in the absence or presence of a CDK inhibitor (Fig. 7 A). Cells were treated without CDK inhibitor (Fig. 7 A, lanes a′–c′), with 75 or 25 μM roscovitine (Fig. 7 A, lanes d′–f′ and g′–i′, respectively), with 250 μM olomoucine (Fig. 7 A, lanes j′–l′), or with 250 μM iso-olomoucine used as negative control (Fig. 7 A, lanes m′–o′), and the newly synthesized RNAs analyzed after increasing times up to 3 h. The ^{32}P-labeled 47-45S pre-rRNAs and ^{32}P-labeled 28S and 18S mature rRNAs were quantified to compare the levels of the unprocessed pre-rRNAs and processed rRNAs (Fig. 7 B). Considering that the inhibitory activity of roscovitine is about 10 times greater than that of olomoucine, similar results were obtained with both CDK inhibitors (Fig. 7 A, compare lanes g′–i′ with j′–l′). It was first noticeable that the levels of ^{32}P-labeled 28S and 18S mature rRNAs decreased in CDK inhibitor–treated interphasic cells (Fig. 7, A and B), showing that synthesis of mature rRNAs was partially inhibited. Interestingly, the variations observed after CDK inhibitor treatment were not explainable only by a decrease in rDNA transcription. Indeed, the level of ^{32}P-labeled 47-45S pre-rRNAs was not significantly affected and did not vary in the same proportion as the ^{32}P-labeled mature rRNAs as particularly obvious after 75 μM roscovitine treatment (Fig. 7, A and B, compare Control with 75 μM Rosc). Therefore, the decrease in the generation of mature rRNAs most likely involved not only a partial inhibition of rDNA transcription but also a defect in pre-rRNA processing.

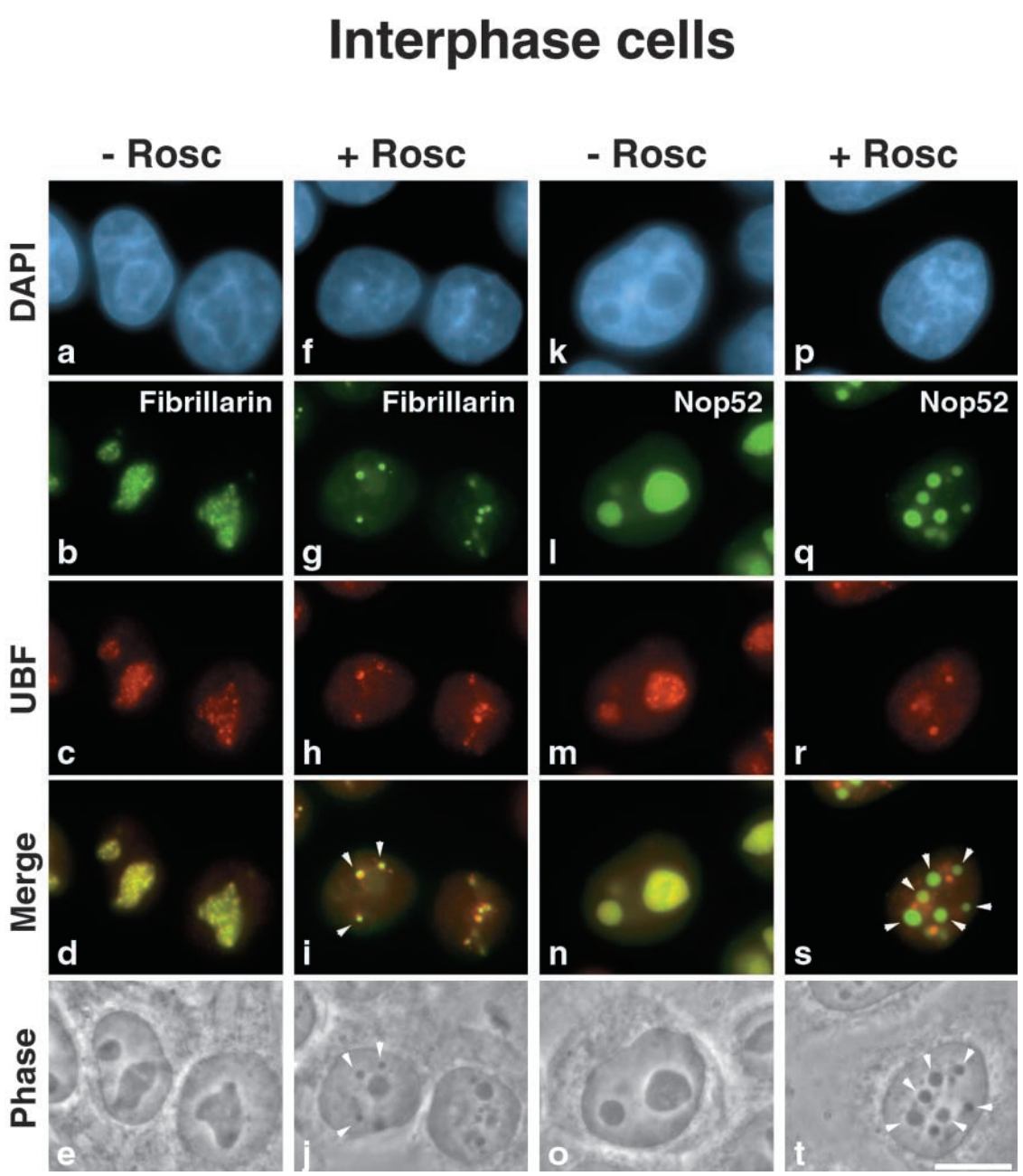

Figure 5. **Interphase nucleoli are no longer maintained after roscovitine treatment.** Stably transfected fibrillarin-GFP and Nop52-GFP HeLa cells were treated (f–j and p–t, respectively) or not (a–e and k–o, respectively) with 75 μM roscovitine for 2 h. Interphasic cells were analyzed by DAPI staining (a, f, k, and p), fibrillarin-GFP (b and g) or Nop52-GFP observation (l and q), UBF labeling (c, h, m, and r), and phase-contrast (e, j, o, and t). In control cells, fibrillarin (b), Nop52 (l), and UBF (c and m) were localized in nucleoli easily identifiable by phase contrast (compare b–e and l–o). The roscovitine treatment led to dramatic modifications of the nucleoli as observed by fibrillarin labeling (compare b with g), Nop52 labeling (compare l with q), UBF labelling (compare c with h, and m with r), and phase contrast (compare e with j, and o with t). Fibrillarin and UBF were now mainly localized in small nuclear bodies (i–j, arrowheads) but not colocalized as seen by merging both labellings (i). Nop52 and UBF were localized in different nuclear bodies as seen by comparing superimposition of both labellings to phase contrast (s and t, arrowheads). Bar, 10 μm.

The CDK inhibitor–induced defect in pre-rRNA processing leads to an increase in 46S pre-rRNA and to the disappearance of the 45S pre-rRNA in a fully reversible manner

To better define the effects of CDK inhibitor treatment on pre-rRNA processing, interphasic HeLa cells were metabolically labeled for 2 h with [^{32}P]orthophosphate in the absence (Fig. 8 A, lanes a and d) or presence of 25 μM (Fig. 8 A, lanes b and e) or 75 μM (Fig. 8 A, lanes c and f) roscovitine, and the RNAs resolved on a 1.2% agarose formaldehyde gel to discriminate between 47S, 46S, and 45S pre-rRNA. As mentioned above, roscovitine altered the generation of 28S and 18S mature rRNAs. The decrease of the newly synthesized mature rRNAs could not be explained only by partial inhibition of rDNA transcription. Indeed, analysis of the ^{32}P-labeled rRNAs showed that with both inhibitor concen-

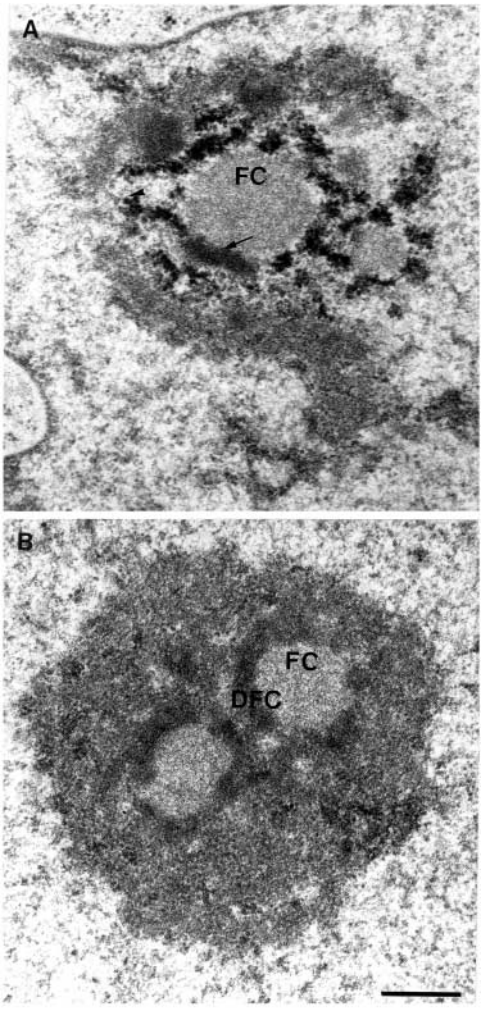

Figure 6. **Ultrastructural organization of nucleolus in HeLa cells treated with roscovitine (A) or not treated (B).** After 30 min in the presence of 75 μM roscovitine, the DFC is only partly visible (arrow) and replaced by new densely packed and highly contrasted material that surrounds the FC (A). In this highly contrasted material, granules are visible (A, arrowhead). Compare in B the distribution of DFC around the FC in normal conditions when rDNA transcription and pre-rRNA processing occur. Bar, 0.5 μm.

trations the ^{32}P-labeled 47S and 46S pre-rRNAs were not decreased in the same proportions as the 28S and 18S rRNAs. In particular, the ^{32}P-labeled 46S pre-rRNA increased (Fig. 8 A, lanes e and f) showing that processing of 46S pre-rRNA into 45S pre-rRNA occurred less efficiently. Logically, the partially processed 45S pre-rRNA present at a high level in control cells was no longer detectable in treated cells (Fig. 8 A, compare lane d with lanes e and f). The pre-rRNA processing defect was also visible by the modification in the ratio between the 32S and 30S pre-rRNAs (Fig. 8 A, compare lane d with lanes e and f). These results were confirmed by Northern blot analyses carried out on interphasic HeLa cells treated for increasing times up to 2 h with 75 μM roscovitine (Fig. 8 B). RNAs were analyzed using ^{32}P-labeled rDNA probes recognizing the 45-47S pre-rRNAs (Fig. 8 B, 5′-ETS core), the 46S-47S pre-rRNAs (Fig. 8 B, 3′-ETS), and the unprocessed 47S pre-rRNA (Fig. 8 B, 5′-ETS leader). The results obtained with the 5′-ETS core probe showed that the 45-47S pre-rRNA species globally increased with the duration of roscovitine treatment and that the 45S pre-rRNA detected by the 5′-ETS core probe (Fig. 8 B, 5′-ETS core, *) but not detected with the 3′-ETS and 5′-ETS leader probes rapidly disappeared. The global increase detected using the 5′-ETS core probe was due to the increase of the 46S pre-rRNA. Indeed, the labeling obtained with the 3′-ETS probe recognizing both 46S and 47S pre-rRNA rapidly increased after roscovitine treatment (Fig. 8 B, 3′-ETS), whereas the level of the unprocessed 47S pre-rRNA revealed specifically by the 5′-ETS leader probe (Fig. 8 B, 5′-ETS leader) did not significantly increase as assessed by quantification (unpublished data). Therefore, it seems likely that roscovitine interferes with pre-rRNA processing in interphasic cells and leads to a rapid increase of the 46S pre-rRNA and to the disappearance of the 45S pre-rRNA.

However, to definitively establish that pre-rRNA processing is impaired in the presence of roscovitine and to verify the reversibility of the pre-rRNA processing defect, interphasic HeLa cells were pulse-labeled for 2 h with [^{32}P]orthophosphate in the presence of 75 μM roscovitine and the ^{32}P-labeled RNAs analyzed after a chase in nonradioactive medium containing (Fig. 9 B) or not containing 75 μM roscovitine (Fig. 9 A) for increasing times up to 4 h. In the absence of roscovitine during the chase, restoration of proper pre-rRNA processing, and residual ^{32}P incorporation rapidly led to the appearance of ^{32}P-labeled 32S pre-rRNA (Fig. 9 A, lane b′, arrowhead) and to progressive accumulation of 28S and 18S mature rRNAs (Fig. 9 A, lanes a′–e′). Conversely in the presence of roscovitine (Fig. 9 B), proper pre-rRNA processing was not restored and consequently residual ^{32}P incorporation led to an increase of ^{32}P-labeled 47-46S pre-rRNAs (Fig. 9 A and B, compare lanes a′–e′). The residual pre-rRNA processing observed in roscovitine-treated cells generated weak and unequal amounts of 28S and 18S rRNAs, showing once again that proper pre-rRNA processing was impaired.

Discussion

Inhibition of CDK1–cyclin B is sufficient to induce the first events of nucleologenesis

Nucleoli are dynamic structures undissociable from rDNA transcription, pre-rRNA processing and ribosome assembly. They disassemble concomitantly with repression of rDNA transcription, most probably caused by CDK1–cyclin B-directed phosphorylation of components of the rDNA transcription machinery (Heix et al., 1998; Sirri et al., 1999). As for pre-rRNA processing, it is as yet unknown whether its arrest occurring at the onset of mitosis (Fan and Penman, 1971) takes place as a consequence of the arrest of pre-rRNA synthesis or whether it is also regulated. Conversely, rDNA transcription is restored in telophase at the level of competent NORs (Roussel et al., 1996). As reported here, when cells exit from mitosis restoration of pre-rRNA processing is concomitant with restoration of rDNA transcription. Interestingly, in vivo inhibition of the CDK1–cyclin B is sufficient to restore rDNA transcription in mitotic cells but not pre-rRNA processing

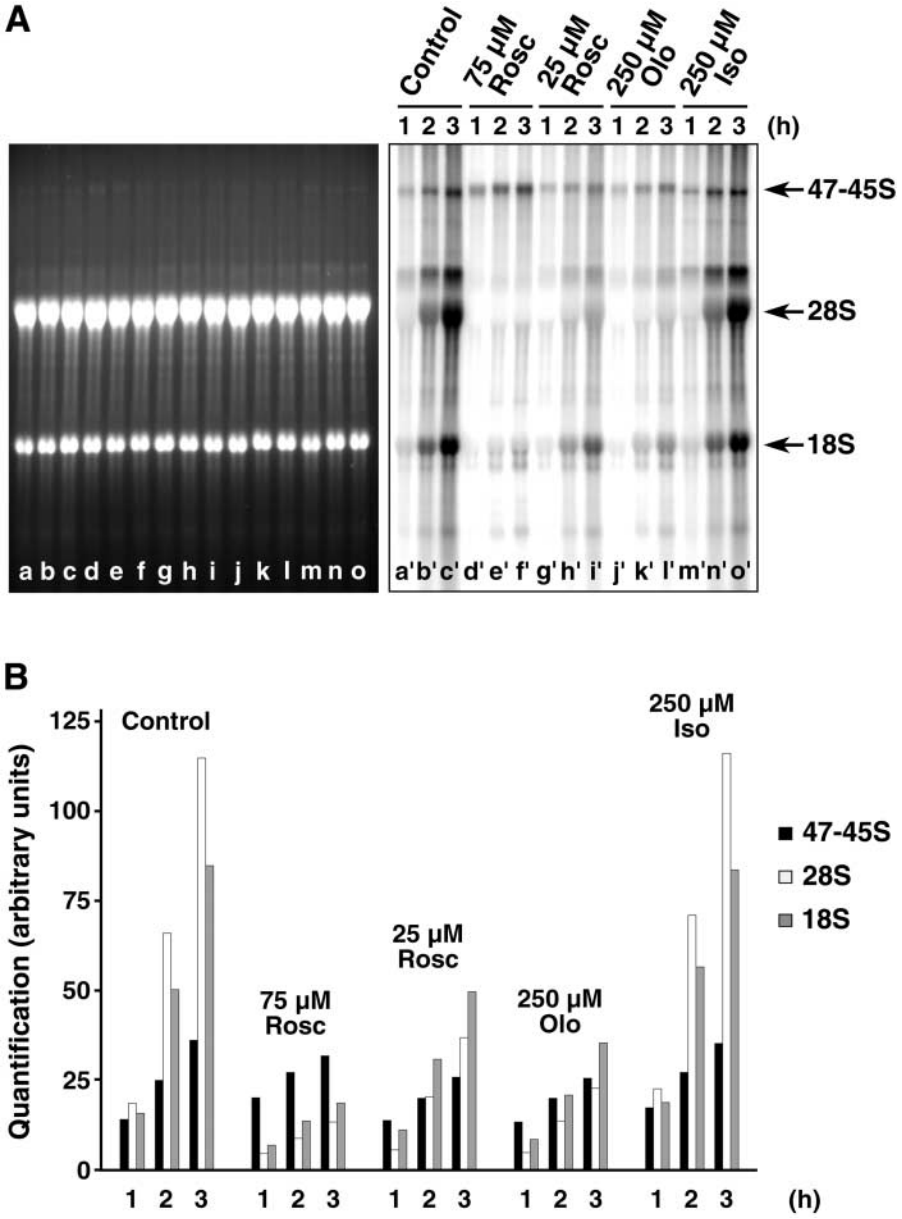

Figure 7. **CDK inhibitor treatment reduces the generation of mature rRNAs.** Asynchronous interphasic HeLa cells were metabolically labeled with [^{32}P]orthophosphate for increasing times up to 3 h in the absence or presence of CDK inhibitors. RNAs were isolated and analyzed. (A) Lanes a–o, RNAs resolved on a 1% agarose formaldehyde gel and detected by ethidium bromide staining; lanes a′–o′, RNAs detected after autoradiography; lanes a–c and a′–c′, cells not treated; lanes d–f and d′–f′, cells treated with 75 μM roscovitine; lanes g–i and g′–i′, cells treated with 25 μM roscovitine; lanes j–l and j′–l′, cells treated with 250 μM olomoucine; lanes m–o and m′–o′, cells treated with 250 μM iso-olomoucine. (B) Quantification of the ^{32}P-labeled partially processed 47–45S pre-rRNAs and ^{32}P-labeled mature 28S and 18S rRNAs corresponding to the representative experiment shown in A. The levels of ^{32}P-labeled mature rRNAs decreased after roscovitine and olomoucine treatments compared with control cells but not when cells were treated with iso-olomoucine. The level of ^{32}P-labeled 47–45S pre-rRNAs appeared less affected by CDK inhibitor-treatments especially in 75 μM roscovitine-treated cells (A, compare lanes a′–c′ and d′–f′, and B, compare Control and 75 μM Rosc).

(Sirri et al., 2000). Therefore, even if rDNA transcription and pre-rRNA processing are restored concomitantly at the exit from mitosis, both are regulated by distinct mechanisms.

Using CDK inhibitors to uncouple restoration of rDNA transcription and pre-rRNA processing, we tested the prevailing model (Scheer and Weisenberger, 1994), according to which transcriptionally active rDNAs, serving as nucleation sites, possess by themselves the ability to organize the nucleoli. Our results show that rDNA transcription is not sufficient to induce the formation of nucleoli as demonstrated by phase contrast observations and by the fact that two nucleolar marker proteins, fibrillarin and Nop52 do not overlap either in mitotic cells induced to transcribe or in actively transcribing early G1 cells previously treated with a CDK inhibitor. In both cases, fibrillarin is only partly localized at the level of rDNA transcription sites where nucleoli are expected to form, and Nop52 appears in bodies corresponding most probably to PNBs and therefore absent from rDNA transcription sites. At the exit from mitosis, localization of Nop52 close to the rDNA transcription sites, i.e., in the reforming nucleoli, is therefore not (or not entirely) dependent on rDNA transcriptional activity. As to the partial localization of fibrillarin at the rDNA transcription sites, this is also most probably not due to rDNA transcriptional activity. Indeed, fibrillarin and nucleolin, both implicated in the early stages of the pre-rRNA processing pathway, partly relocalized at NORs at the exit from mitosis even if rDNA transcription is inhibited by actinomycin D (Dousset et al., 2000). Because fibrillarin and nucleolin localize at NORs in the absence of rDNA transcription, and because Nop52 does not localize at NORs after CDK inhibitor treatment in mitotic cells and early G1 cells (albeit they actively transcribe), we conclude that the formation of functional nucleoli at the exit from mitosis is not governed solely by the resumption of rDNA transcription.

Our previous results demonstrating that rDNA transcription could be reactivated in mitotic cells (Sirri et al., 2000)

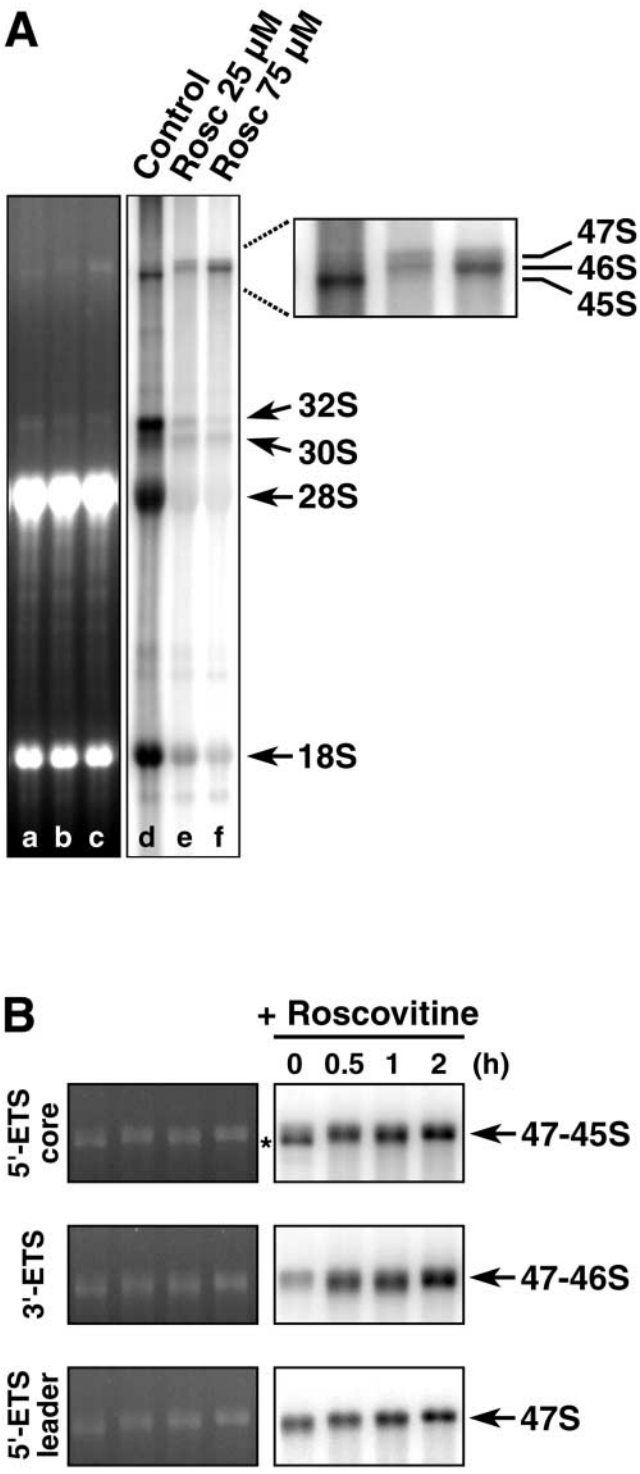

Figure 8. **Roscovitine treatment leads to accumulation of 46S pre-rRNA.** (A) Interphasic HeLa cells were metabolically labeled for 2 h with [^{32}P]orthophosphate in the absence of roscovitine (lanes a and d), and in the presence of 25 μM (lanes b and e) or 75 μM roscovitine (lanes c and f). RNAs were isolated and analyzed. (A) Lanes a–c, RNAs detected in 1.2% agarose formaldehyde gel by ethidium bromide staining; lanes d–f, RNAs detected after autoradiography. In a dose-dependent manner, roscovitine treatment increased the level of 46S pre-rRNA and reduced the levels of 32S pre-rRNA and mature 28S and 18S rRNAs. (B) Interphasic HeLa cells were treated for increasing times up to 2 h with 75 μM roscovitine. RNAs were isolated, resolved on 0.75% agarose formaldehyde gels and blotted. The blots were hybridized with a ^{32}P-labeled 5′-ETS core probe recognizing the 47–45S pre-rRNAs (5′ETS core), a ^{32}P-labeled 3′-ETS probe recognizing both 47S and 46S pre-rRNAs (3′-ETS), and a ^{32}P-labeled 5′-ETS leader probe specific for the unprocessed 47S pre-rRNA (5′ETS leader). The 45–47S pre-rRNAs globally increased with increasing time of treatment and the 45S pre-rRNA, only detected by the 5′-ETS core probe (*), rapidly disappeared. This increase was also observed with the 3′-ETS but not with the 5′ETS leader probe.

and the present results obtained in the same experimental conditions suggest that roscovitine treatment makes it possible to reproduce the first events of nucleologenesis. This corresponds to release from mitotic silencing of rDNA transcription, targeting in transcription sites of early pre-rRNA processing components such as fibrillarin, and recruitment of late pre-rRNA processing components such as Nop52, in PNBs. These first events of nucleologenesis are most probably regulated by inactivation of the CDK1–cyclin B. Indeed, among the CDKs known to be substantially inhibited by roscovitine when tested in vitro, the CDK1-cyclin B is the only kinase active during mitosis (De Azevedo et al., 1997; Meijer et al., 1997). Moreover, these events normally take place in telophase when CDK1–cyclin B is inactivated.

A CDK activity is indispensable to form a functional nucleolus at the exit from mitosis

In addition to inactivation of CDK1–cyclin B, another CDK activity seems to be indispensable to promote the last events of nucleologenesis and to form a functional nucleolus at the exit from mitosis. Indeed in early G1 phase, cells proceeding through mitosis in the presence of a CDK inhibitor exhibit no relocalization of Nop52 to rDNA transcription sites, no formation of nucleoli, and a defect in pre-rRNA processing leading to accumulation of 46S pre-rRNA. Moreover, the CDK inhibitor effects are fully reversible. Consequently, at the exit from mitosis, a CDK activity seems indispensable to promote relocalization of the late pre-rRNA processing components from PNBs to rDNA transcription sites and to promote formation of functional nucleoli. Nevertheless, further investigations are necessary to establish if restoration of pre-rRNA processing only depends on correct localization of the processing machinery at the level of rDNA transcription sites or if the processing machinery must also to be activated. A third possibility would be that relocalization of late pre-rRNA processing components from PNBs to rDNA transcription sites would depend on activation of early pre-rRNA processing components.

Concerning the identity of the kinase(s) involved in the formation of functional nucleoli, in addition to CDKs, i.e., CDK1–cyclin B, CDK2–cyclin A, CDK2–cyclin E, and CDK5-p35, the different CDK inhibitors used in this study inhibit ERK1/2 to a lesser extent. Because U0126, an inhibitor of mitogen-activated protein kinase kinase and consequently of ERK1/2, did not affect formation of nucleoli, the kinase implicated is probably a CDK. Because CDK1–cyclin B is inactivated when this nucleologenesis-related kinase is to be activated, the remaining candidates are CDK2–cyclin A, CDK2–cyclin E, and CDK5-p35. Even if CDK2 has not yet been reported as active at the beginning of inter-

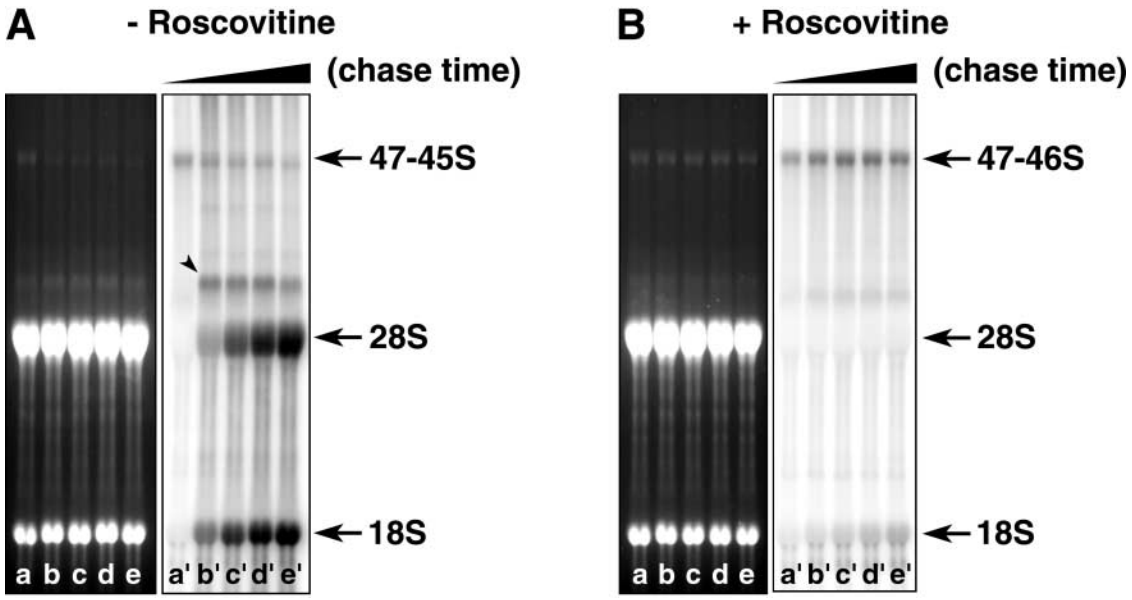

Figure 9. **Roscovitine-induced defect in pre-rRNA processing is fully reversible.** (A and B) Asynchronous interphasic HeLa cells were pulse-labeled with [^{32}P]orthophosphate in the presence of 75 μM roscovitine for 2 h. After a chase in nonradioactive medium in the absence (A) or presence (B) of 75 μM roscovitine for 0 h (A and B, lanes a and a′), 1 h (A and B, lanes b and b′), 2 h (A and B, lanes c and c′), 3 h (A and B, lanes d and d′), and 4 h (A and B, lanes e and e′), RNAs were isolated and resolved on a 1% agarose formaldehyde gel. (A and B, lanes a–e) Ethidium bromide staining. (A and B, lanes a′–e′) Autoradiography. In the absence of roscovitine during the chase, the defect in pre-rRNA processing induced by roscovitine was rapidly reversed as shown by the high increase of the ^{32}P-labeled 32S pre-RNA (A, lane b′, arrowhead) and mature 28S and 18S rRNAs (A, lane b′). Consequently, the residual synthesis of ^{32}P-labeled pre-rRNAs led to accumulation of the ^{32}P-labeled mature 28S and 18S rRNAs (A, compare a′–e′). Conversely, when the chase was applied in the presence of roscovitine (B), the residual synthesis of ^{32}P-labeled pre-rRNAs led to an increase of ^{32}P-labeled 47–46S pre-rRNAs (compare A to B, lanes a′–e′). Only a slight increase of the 28S and 18S rRNAs was observed.

phase and CDK5 has no known function in proliferative cells, further investigations are necessary to test the possible key role played by these CDKs in the formation of nucleoli. However, it is also possible that the kinase implicated is not yet characterized as a target of these CDK inhibitors.

A CDK activity is also indispensable to maintain a functional nucleolus throughout interphase

Treatment with the highly selective CDK inhibitors, roscovitine, olomoucine, purvalanol, or alsterpaullone modify both rDNA transcription and pre-rRNA processing, and induce a dramatic but reversible disorganization of active nucleoli, whatever the interphase stage of the cells. As expected from previous results showing that the transcription factor UBF is regulated by CDKs among which CDK2–cyclin E (Voit et al., 1999), rDNA transcription decreases after CDK inhibitor treatment but remains active. Remarkably, in addition to the decrease in rDNA transcription, these treatments impair pre-rRNA processing. Even if additional experiments are necessary to identify the kinase(s) implicated, it seems most likely that maintaining functional nucleoli implies CDK(s). Indeed, inhibition of ERK1/2, the only kinase in addition to CDKs sensitive to each CDK inhibitor used here, when tested in vitro, caused no obvious effect on nucleoli.

CDK inhibitor treatments do not lead to the accumulation of incorrectly processed rRNAs, but hamper the first processing events leading to accumulation of 46S pre-rRNA and disappearance of 45S pre-rRNA. It is noteworthy that even if rDNA transcription is lowered, the level of 47S pre-rRNA remains constant. Therefore, the cleavage leading to the 46S pre-rRNA from the 47S pre-rRNA is most probably also hampered. Possibly, the complexes involved in the first cleavages are inhibited or partly inhibited in the presence of CDK inhibitors and this inhibition might prevent or disturb subsequent pre-rRNA cleavages by hindering subsequent protein–protein and/or protein–RNA interactions. It may also be hypothesized that the cleavages of pre-rRNA are prevented because the pre-rRNA undergoes structural rearrangements in the presence of CDK inhibitors or conversely does not undergo the structural rearrangements required to be properly and efficiently processed.

How is a nucleolus maintained as a functional domain?

The nucleolus is a highly dynamic domain generated by the processes necessary to built ribosomes (Mélèse and Xue, 1995). Consequently, nucleolar disorganization is induced when transcription is inhibited during interphase or during nucleologenesis (Dousset et al., 2000). This is characterized by typical features corresponding to segregation of the nucleolar components, FCs, DFC, and GC (Hadjiolov, 1985). CDK inhibitor treatment results in reorganization of the DFC, where the first pre-rRNA processing events occur normally a few seconds after transcription termination (Allmang et al., 1999), and in the absence of typical GC. These features suggest that pre-rRNA processing must be achieved to establish typical DFC and the transition between DFC and GC. Therefore, the structural organization of nucleoli is not only determined by rDNA transcription activity, and

obviously both rDNA transcription and pre-rRNA processing are required to maintain a nucleolus. It is noteworthy that CDK inhibition disrupts the highly organized nucleolus but does not scatter the nucleolar machineries in the nucleus. The rDNA transcription machinery is still active and the processing machinery is delocalized in nuclear bodies distributed in the nucleoplasm. Since CDK inhibitor–induced nucleolus disorganization is rapidly reversible, it is tempting to propose that the active nucleolus constitutes a functional nuclear domain whose organization is determined by the dynamics of the processes necessary to produce ribosomes, i.e., rDNA transcription, pre-rRNA processing and ribosome assembly. The CDK inhibitors could interfere with the steady state of these processes by blocking key factor(s) involved in pre-rRNA processing. Another possibility would be to consider a decrease of rDNA clustering modifying the local concentration of molecules which normally interact with the processing machinery and thus locally diminishing the retention of these complexes.

Whatever the mechanism blocked by the CDK inhibitors, the presence of a fully active nucleolus clearly depends on cell cycle regulators. Conversely, the important question concerning the possible role of active nucleoli in the control of cell cycle progression warrants further investigation.

Materials and methods

Cell culture and synchronization

Stably transfected fibrillarin–green fluorescent protein (GFP) and Nop52-GFP HeLa cell lines previously reported (Savino et al., 2001) and untransfected HeLa cell line were cultured in MEM supplemented with 10% FCS and 2 mM L-glutamine (GIBCO BRL). For synchronization in metaphase, the cells were accumulated in prometaphase by nocodazole treatment (0.04 μg/ml for 4 h), selectively harvested by mechanical shock, washed, and resuspended in nocodazole-free medium for 30 min where they progressed into metaphase in a semisynchronous manner. Cells were also blocked in mitosis by colchicine treatment (0.02 μg/ml for 14 h) and mitotic cells harvested by mechanical shock. For immunofluorescence and in situ transcription assays, asynchronous cells were grown as monolayers on glass slides, and synchronized mitotic cells were transferred onto poly-L-lysine-coated glass slides.

Kinase inhibitor treatments

Roscovitine, olomoucine, and iso-olomoucine were obtained from Calbiochem, U0126 from Promega, and alsterpaullone, and purvalanol (NG97) was provided by L. Meijer (Station Biologique, Roscoff, France). Synchronized mitotic cells and asynchronous cells were treated with 25 or 75 μM roscovitine, or with 250 μM olomoucine or iso-olomoucine for various times up to 3 h. Alsterpaullone and purvalanol were used on asynchronous cells at a concentration of 75 μM for various times up to 3 h. U0126 was used on asynchronous cells at concentrations up to 200 μM for 3 h.

Antibodies and probes

Antibodies. The human autoimmune sera with specificity against UBF (A17) and Nop52 (C13) were described previously (Roussel et al., 1996; Savino et al., 1999). The human autoimmune serum with specificity against fibrillarin (O61) was characterized during this study and the mouse monoclonal anti-fibrillarin was 72B9 (Reimer et al., 1987). The anti-BrdU antibody was obtained from Sigma-Aldrich. Texas Red–conjugated secondary antibodies specific for human and mouse IgGs, and FITC-conjugated goat anti–human antibodies were obtained from Jackson ImmunoResearch Laboratories, Inc.

Probes. The 5′-ETS leader and the 3′-ETS probes correspond to fragments of human rDNA, respectively, 409 nucleotides (nt) downstream from the initiation site (nt +1/+410) and 309 nt into the 3′-ETS (nt +13040/+13348). These fragments were first amplified by PCR as BamHI-EcoRI fragments using pB_{ES} (Wilson et al., 1982) and pD_{ES} (Erickson and Schmickel, 1985) as template DNAs and inserted into the BamHI/EcoRI sites of pBluescript SK(+). The 5′-ETS leader and 3′-ETS probes were then generated by BamHI/EcoRI digestions. The 5′-ETS core probe was a SalI-SalI fragment of human rDNA (nt +693/+2921) prepared from pB_{SS} (Wilson et al., 1982).

Assay of pol activity in situ and immunofluorescence labeling

Assay of pol activity in situ was performed as previously described in conditions set up to reveal pol I and pol II transcription (Roussel et al., 1996). Cells were then postfixed in 2% (wt/vol) paraformaldehyde for 20 min at room temperature (RT) and permeabilized with 0.5% Triton X-100 at RT for 5 min. Bromo-uridine 5′-triphosphate incorporation was detected by immunofluorescence labeling using a mouse monoclonal anti-BrdU antibody revealed by Texas Red–conjugated goat anti–mouse antibodies. In addition to bromo-uridine 5′-triphosphate incorporation, fibrillarin and Nop52 were simultaneously detected using sera O61 and C13, respectively, and revealed by FITC-conjugated goat anti–human antibodies.

For immunofluorescence labeling, cells were also fixed in 2% (wt/vol) paraformaldehyde for 20 min at RT and permeabilized with 0.5% Triton X-100 for 5 min. Cells were incubated with sera at RT for 45 min, and the antibodies were revealed with suitable Texas Red– and/or FITC-conjugated secondary antibodies. DNA was visualized with DAPI. All preparations were mounted with the antifading solution Citifluor (Canterbury, UK). Fluorescent microscopy was performed using a CCD camera Leitz DMRB and images assembled using Adobe Photoshop.

Metabolic labeling and RNA analysis

For the analysis of rRNA synthesis in cells proceeding through M/G1 transition, cells were synchronized in metaphase in phosphate-free MEM. Metaphase-synchronized cells were then metabolically labeled with [^{32}P]orthophosphate at a final concentration of 125 μCi/ml (ICN Biomedicals) in the presence or absence of 75 μM roscovitine and RNA synthesis analyzed every 30 min up to 120 min. Interphasic HeLa cells were metabolically labeled with [^{32}P]orthophosphate (125 μCi/ml) in phosphate-free MEM for various times up to 3 h in the absence or presence of kinase inhibitor.

For pulse-chase experiments, interphasic cells were pulse labeled with [^{32}P]orthophosphate (125 μCi/ml) in phosphate-free MEM for 2 h in the presence of 75 μM roscovitine and chased in nonradioactive medium with excess phosphate for various times up to 4 h in the presence or absence of 75 μM roscovitine.

For ^{32}P-labeling experiments, total RNAs were isolated using TRI REAGENT (Sigma-Aldrich), and RNAs corresponding to 4×10^5 cells were separated by electrophoresis on 1–1.2% agarose formaldehyde gels. The RNAs were transferred to positively charged membranes (Roche). After UV cross-linking, autoradiographies were performed with a PhosphorImager (Molecular Dynamics, Inc.) and quantifications of ^{32}P-labeled rRNAs using NIH Image software.

For Northern blot analyses, total RNAs were isolated from interphasic cells treated with 75 μM roscovitine for various times from 0 to 2 h using TRI REAGENT. RNAs corresponding to 5×10^5 cells were separated in 0.75% agarose formaldehyde gels and transferred to positively charged membranes. The rDNA probes were labeled with [α^{32}P]dCTP by nick translation (GIBCO BRL). Hybridization with the radiolabeled probes was carried out as previously described (Sirri et al., 2000). Autoradiographies were performed with a PhosphorImager and quantifications using NIH Image software.

Transmission electron microscopy

Cells treated or not with 75 μM roscovitine for 0.5, 1, or 2 h were fixed in 2.5% (vol/vol) glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, at 4°C for 1 h, rinsed, postfixed in 1% (wt/vol) osmium tetroxide in the same buffer for 30 min, dehydrated, and embedded in Epon 812. After resin polymerization, ultrathin sections were conventionally contrasted with uranyl acetate (30 min) followed by lead citrate (10 min) and examined in a Philips CM12 electron microscope.

The authors thank J.E. Sylvester (Nemours Children's Clinic, Jacksonville, FL) and L. Meijer for the gift of rDNA-containing plasmids and CDK inhibitors, respectively, T.M. Savino (Institut Jacques Monod, Paris, France) for stably transfected fibrillarin-GFP and Nop52-GFP HeLa cell lines, S. Boeuf (Institut Jacques Monod, Paris, France) for cell culture, and A.L. Haenni for critical reading of the manuscript.

This work was supported in part by grants from the Centre National de la Recherche Scientifique and the Association pour la Recherche sur le Cancer (Contracts 4290 and 5304).

Submitted: 4 January 2002
Revised: 29 January 2002
Accepted: 29 January 2002

References

Allmang, C., E. Petfalski, A. Podtelejnikov, M. Mann, D. Tollervey, and P. Mitchell. 1999. The yeast exosome and human PM-Scl are related complexes of 3′-5′ exonucleases. *Genes Dev.* 13:2148–2158.

Carmo-Fonseca, M., L. Mendes-Soares, and I. Campos. 2000. To be or not to be in the nucleolus. *Nat. Cell Biol.* 2:107–112.

De Azevedo, W.F., S. Leclerc, L. Meijer, L. Havlicek, M. Strnad, and S.-H. Kim. 1997. Inhibition of cyclin-dependent kinases by purine analogues: crystal structure of human cdk2 complexed with roscovitine. *Eur. J. Biochem.* 243: 518–526.

Dousset, T., C. Wang, C. Verheggen, D. Chen, D. Hernandez-Verdun, and S. Huang. 2000. Initiation of nucleolar assembly is independent of RNA polymerase I transcription. *Mol. Biol. Cell.* 11:2705–2717.

Dundr, M., T. Misteli, and M.O.J. Olson. 2000. The dynamics of postmitotic reassembly of the nucleolus. *J. Cell Biol.* 150:433–446.

Erickson, J.M., and R.D. Schmickel. 1985. A molecular basis for discrete size variation in human ribosomal DNA. *Am. J. Hum. Genet.* 37:311–325.

Fan, H., and S. Penman. 1971. Regulation of synthesis and processing of nucleolar components in metaphase-arrested cells. *J. Mol. Biol.* 59:27–42.

Gautier, T., N. Fomproix, C. Masson, M.C. Azum-Gélade, N. Gas, and D. Hernandez-Verdun. 1994. Fate of specific nucleolar perichromosomal proteins during mitosis: cellular distribution and association with U3 snoRNA. *Biol. Cell.* 82:81–93.

Grummt, I. 1999. Regulation of mammalian ribosomal gene transcription by RNA polymerase I. *Prog. Nucleic Acid Res. Mol. Biol.* 62:109–154.

Guarente, L. 1997. Link between aging and the nucleolus. *Genes Dev.* 11:2449–2455.

Hadjiolov, A.A. 1985. The nucleolus and ribosome biogenesis. Springer-Verlag, Wien, New York. 268 pp.

Hannan, K.M., R.D. Hannan, S.D. Smith, L.S. Jefferson, M. Lun, and L.I. Rothblum. 2000. Rb and p130 regulate RNA polymerase I transcription: Rb disrupts the interaction between UBF and SL-1. *Oncogene.* 19:4988–4999.

Heix, J., A. Vente, R. Voit, A. Budde, T.M. Michaelidis, and I. Grummt. 1998. Mitotic silencing of human rRNA synthesis: inactivation of the promoter selectivity factor SL1 by cdc2/cyclin B-mediated phosphorylation. *EMBO J.* 17:7373–7381.

Jiménez-Garcia, L.F., M.d.L. Segura-Valdez, R.L. Ochs, L.I. Rothblum, R. Hannan, and D.L. Spector. 1994. Nucleologenesis: U3 snRNA-containing prenucleolar bodies move to sites of active Pre-rRNA transcription after mitosis. *Mol. Biol. Cell.* 5:955–966.

Meijer, L., A. Borgne, O. Mulner, J.P.J. Chong, J.J. Blow, N. Inagaki, M. Inagaki, J.-G. Delcros, and J.-P. Moulinoux. 1997. Biochemical and cellular effects of roscovitine, a potent and selective inhibitor of the cyclin-dependent kinases cdc2, cdk2 and cdk5. *Eur. J. Biochem.* 243:527–536.

Mélèse, T., and Z. Xue. 1995. The nucleolus: an organelle formed by the act of building a ribosome. *Curr. Opin. Cell Biol.* 7:319–324.

Moss, T., and V.Y. Stefanovsky. 1995. Promotion and regulation of ribosomal transcription in eukaryotes by RNA polymerase I. *Prog. Nucleic Acid Res. Mol. Biol.* 50:25–65.

Olson, M.O.J., M. Dundr, and A. Szebeni. 2000. The nucleolus: an old factory with unexpected capabilities. *Trends Cell Biol.* 10:189–196.

Politz, J.C., S. Yarovoi, S. Kilroy, K. Gowda, C. Zwieb, and T. Pederson. 2000. Signal recognition particle components in the nucleolus. *Proc. Natl. Acad. Sci. USA.* 97:55–60.

Reimer, G., K.M. Pollard, C.A. Pennig, R.L. Ochs, M.A. Lischwe, H. Busch, and E.M. Tan. 1987. Monoclonal autoantibody from NZB/NZW F1 mouse and some human scleroderma sera target a Mr 34000 nucleolar protein of the U3-ribonucleoprotein particle. *Arthritis Rheum.* 30:793–800.

Roussel, P., C. André, L. Comai, and D. Hernandez-Verdun. 1996. The rDNA transcription machinery is assembled during mitosis in active NORs and absent in inactive NORs. *J. Cell Biol.* 133:235–246.

Savino, T.M., R. Bastos, E. Jansen, and D. Hernandez-Verdun. 1999. The nucleolar antigen Nop52, the human homologue of the yeast ribosomal RNA processing RRP1, is recruited at late stages of nucleologenesis. *J. Cell Sci.* 112:1889–1900.

Savino, T.M., J. Gébrane-Younès, J. De Mey, J.-B. Sibarita, and D. Hernandez-Verdun. 2001. Nucleolar assembly of the rRNA processing machinery in living cells. *J. Cell Biol.* 153:1097–1110.

Scheer, U., and D. Weisenberger. 1994. The nucleolus. *Curr. Opin. Cell Biol.* 6:354–359.

Scheer, U., and R. Hock. 1999. Structure and function of the nucleolus. *Curr. Opin. Cell Biol.* 11:385–390.

Shaw, P.J., and E.G. Jordan. 1995. The nucleolus. *Annu. Rev. Cell Dev. Biol.* 11: 93–121.

Sinclair, D.A., K. Mills, and L. Guarente. 1997. Accelerated aging and nucleolar fragmentation in yeast sgs1 mutants. *Science.* 277:1313–1316.

Sirri, V., P. Roussel, and D. Hernandez-Verdun. 1999. The mitotically phosphorylated form of the transcription termination factor TTF-1 is associated with the repressed rDNA transcription machinery. *J. Cell Sci.* 112:3259–3268.

Sirri, V., P. Roussel, and D. Hernandez-Verdun. 2000. In vivo release of mitotic silencing of ribosomal gene transcription does not give rise to precursor ribosomal RNA processing. *J. Cell Biol.* 148:259–270.

Tollervey, D. 1996. Trans-acting factors in ribosome synthesis. *Exp. Cell Res.* 229: 226–232.

Visitin, R., and A. Amon. 2000. The nucleolus: the magician's hat for cell cycle tricks. *Curr. Opin. Cell Biol.* 12:372–377.

Voit, R., M. Hoffmann, and I. Grummt. 1999. Phosphorylation by G1-specific cdk-cyclin complexes activates the nucleolar transcription factor UBF. *EMBO J.* 18:1891–1899.

Westendorf, J.M., K.N. Konstantinov, S. Wormsley, M.-D. Shu, N. Matsumoto-Taniura, F. Pirollet, F.G. Klier, L. Gerace, and S.J. Baserga. 1998. M phase phosphoprotein 10 is a human U3 small nucleolar ribonucleoprotein component. *Mol. Biol. Cell.* 9:437–449.

Wilson, G.N., L.L. Szura, C. Rushford, D. Jackson, and J. Erickson. 1982. Structure and variation of human ribosomal DNA: the external transcribed spacer and adjacent regions. *Am. J. Hum. Genet.* 34:32–49.

Zolotukhin, A.S., and B.K. Felber. 1999. Nucleoporins Nup98 and Nup214 participate in nuclear export of human immunodeficiency virus type 1 Rev. *J. Virol.* 73:120–127.