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” (Mélesé 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 nucleolus 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,

*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.
RNA. This activity is ordered from the early step of several RNP complexes involving small nucleolar RNAs (sno-RNAs). 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 (CDK1–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 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 relocation 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 relocation 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 relocation 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 relocated 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 relocation 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 relocation 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 relocation of fibrillarin at the level of rDNA transcription sites, but blocks relocation 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
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-synchronized 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.
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
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 trabeculae. 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 [32P]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 [32P]-labeled 47-45S pre-rRNAs and [32P]-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 [32P]-labeled 28S and 18S mature rRNAs only slightly increased (compare B with A, lanes e–h).

Figure 4. Roscovitine treatment leads to accumulation of 46S pre-rRNA at exit from mitosis. (A) Metaphase-synchronized HeLa cells were metabolically labeled with [32P]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 [32P]-labeled unprocessed 47S pre-rRNA appeared and increased concomitantly with the [32P]-labeled partially processed or mature rRNAs (A, lanes e–h). In roscovitine-treated cells, the newly synthesized 46S pre-rRNA accumulated and no [32P]-labeled 45S pre-rRNA was detectable (B, lanes e–h). The levels of [32P]-labeled mature 28S and 18S rRNAs only slightly increased (compare B with A, lanes e–h).
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, interphase HeLa cells were metabolically labeled for 2 h with $^{32}$Porthophosphate 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-
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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 \(\mu M\) roscovitine and the \(^{32}P\)-labeled RNAs analyzed after a chase in nonradioactive medium containing (Fig. 9 B) or not containing 75 \(\mu M\) 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.
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)
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.

Figure 8. Roscovitine treatment leads to accumulation of 46S pre-rRNA. (A) Interphasic HeLa cells were metabolically labeled for 2 h with [32P]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 32P-labeled 5′-ETS core probe recognizing the 47–45S pre-rRNAs (5′ETS core), a 32P-labeled 3′-ETS probe recognizing both 47S and 46S pre-rRNAs (3′-ETS), and a 32P-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.

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-
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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 build ribosomes (Mélèse and Xue, 1995). Consequently, nucleolar disorganization is induced when transcription is inhibited during interphase or during nucleologenesis (Doussset 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 nucleolus. 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 and resuspended in nocodazole-free medium for 30 min where they proceeded into metaphase in a semisynchronous manner. Cells were also blocked in mitosis by colchicine treatment (0.02 μg/ml for 1.5 h) and mitotic cells 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 1.5 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 (NO97) was provided by L. Meijer (Station Biologique, Roscoff, France). 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 then postfixed in 2% (wt/vol) paraformaldehyde for 20 min at room temperature (RT) and permeabilized with 0.5% Triton X-100 for 5 min. Cells were then fixed in 2% (wt/vol) paraformaldehyde for 20 min at RT and permeabilized with 0.5% Triton X-100 for 5 min. Metaphase-synchronized 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 anti-fading 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 [32P]orthophosphate at a final concentration of 125 μCi/ml (ICN Biomedicals) in the presence or absence of 75 μM rosocivitine and RNA synthesis analyzed every 30 min up to 120 min. Interphasic HeLa cells were metabolically labeled with [32P]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 [32P]orthophosphate (125 μCi/ml) in phosphate-free MEM for 2 h in the presence of 75 μM rosocivitine and chased in nonradioactive medium with excess phosphate for various times up to 4 h in the presence or absence of 75 μM rosocivitine.

For 32P-labeling experiments, total RNAs were isolated using TRI REAGENT (Sigma-Aldrich), and RNAs corresponding to 4 × 105 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 32P-labeled RNAs using NIH Image software.

For Northern blot analyses, total RNAs were isolated from interphasic cells treated with 75 μM rosocivitine for various times from 0 to 2 h using TRI REAGENT. RNAs corresponding to 5 × 106 cells were separated by electrophoresis on 0.75% agarose formaldehyde gels and transferred to positively charged membranes. The rDNA probes were labeled with [α-32P]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 rosocivitine 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.

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References


