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Sharing of mitotic pre-ribosomal particles between daughter cells

Valentina Sirri1, Nathalie Jourdan2, Danièle Hernandez-Verdun3 and Pascal Roussel1,*

ABSTRACT

Ribosome biogenesis is a fundamental multistep process initiated by the synthesis of 90S pre-ribosomal particles in the nucleoli of higher eukaryotes. Even though synthesis of ribosomes stops during mitosis while nucleoli disappear, mitotic pre-ribosomal particles persist as observed in pre-nucleolar bodies (PNBs) during telophase. To further understand the relationship between the nucleolus and the PNBs, the presence and the fate of the mitotic pre-ribosomal particles during cell division were investigated. We demonstrate that the recently synthesized 45S precursor ribosomal RNAs (pre-rRNAs) as well as the 32S and 30S pre-rRNAs are maintained during mitosis and associated with the chromosome periphery together with pre-rRNA processing factors. Maturation of the mitotic pre-ribosomal particles, as assessed by the stability of the mitotic pre-rRNAs, is transiently arrested during mitosis by a cyclin-dependent kinase (CDK1–cyclin-B-dependent mechanism and can be restored by CDK inhibitor treatments. At the M→G1 transition, the resumption of mitotic pre-rRNA processing in PNBs does not induce the disappearance of PNBs; this only occurs when functional nucleolus reform. Strikingly, during their maturation process, mitotic pre-rRNAs localize in reforming nucleoli.

KEY WORDS: Nucleolus, PNB, Mitosis, Ribosomal RNA

INTRODUCTION

The synthesis of ribosomes is a highly complex multistep process requiring the RNA polymerases (Pol) I, Pol II and Pol III, and several hundred accessory factors in addition to the ribosomal proteins and ribosomal RNAs (rRNAs). In eukaryotes, ribosome biogenesis begins in the nucleolus with Pol-I-dependent transcription of a precursor ribosomal RNA (47S pre-rRNA) in vertebrates leading to the mature 18S, 5.8S and 28S rRNAs. Ribosomal and non-ribosomal proteins and small nucleolar ribonucleoproteins (RNPs) co-transcriptionally associate with 47S pre-rRNA and form 90S pre-ribosomal particles that then generate pre-40S and pre-60S ribosomal particles (Henras et al., 2008). These pre-ribosomal particles are further matured before being exported into the cytoplasm where the final processing steps generate mature ribosomal subunits. The progressive maturation of pre-ribosomal particles implies the stepwise assembly of ribosomal proteins and transient non-ribosomal processing factors, and occurs concomitantly with processing of the 47S pre-rRNA.

During mitosis, Pol-I-dependent transcription of ribosomal genes (rDNAs) is progressively repressed during prophase and maintained repressed in a cyclin-dependent kinase (CDK1–cyclin-B-dependent manner until telophase (Sirri et al., 2000). The resumption of rRNA transcription when CDK1–cyclin B is inactivated constitutes the first step in the formation of the nucleolus as well as in restoring ribosome biogenesis at the exit from mitosis (Sirri et al., 2000, 2002). Research in ribosome biogenesis has unraveled the pathways of 47S pre-rRNA processing (Mullineux and Lafontaine, 2012) and revealed how this complex process involves several hundred pre-rRNA processing factors (Tafforeau et al., 2013). Considering the large number of pre-rRNA processing factors, little is yet known of the fate of the pre-rRNA processing machinery during mitosis while ribosome biogenesis is turned off and nucleoli are no longer maintained. However, during cell division, several pre-rRNA processing factors, such as fibrillarin, nucleolin, Nop52 (also known as RRP1) and NPM (also known as B23), are co-localized at the periphery of chromosomes from prophase to telophase and then in pre-nucleolar bodies (PNBs) until the early G1 phase (Dundr et al., 2000; Gautier et al., 1992; Hügle et al., 1985; Savino et al., 1999). As reported recently (Booth et al., 2014), the localization at the chromosome periphery depends on the protein Ki-67. In addition to proteins, small nucleolar RNAs involved in pre-rRNA processing such as U3, U8, and U14 (Doust et al., 2000; Jiménez-García et al., 1994) are observed in PNBs. Furthermore, in addition to these non-ribosomal processing factors, pre-rRNAs (Boisvert et al., 2007; Carron et al., 2012; DiMario, 2004; Dundr et al., 2000; Fan and Pennman, 1971; Phillips, 1972) as well as ribosomal proteins (Carron et al., 2012; Gassmann et al., 2004; Hügle et al., 1985; Ohta et al., 2010) are observed on the chromosome periphery and in PNBs. It was also proposed that the association of pre-rRNAs with proteins in pre-rRNP complexes is maintained during mitosis (Piñol-Roma, 1999), and recently pre-ribosomal particles were reported in PNBs (Carron et al., 2012).

Despite extensive studies on nucleologenesis, it remains unclear how pre-rRNA processing is restored, and what role is played by PNBs. However time-lapse microscopy analyses have clearly shown the relationship between PNBs and reforming nucleoli (Dundr et al., 2000; Savino et al., 1999). Based on their ultrastructure and composition, PNBs were initially thought to be a step prior to the formation of nucleoli. Because PNBs differ in their components and lifetimes, it was proposed that different types of PNBs exist that are targeted to reforming nucleoli with different kinetics (Savino et al., 1999, 2001; Westendorf et al., 1998). Beyond the dynamics of postmitotic formation of nucleoli, PNBs themselves are highly dynamic structures (Dundr et al., 2000; Muro et al., 2010). Recently, PNBs were proposed to be the sites where maturation of the pre-rRNAs transiting through mitosis is restored in telophase. PNBs would be autonomous extra-nucleolar ribosome maturation sites whose disassembly in G1 phase is governed by processing and release of their pre-ribosome content (Carron et al., 2012).

To further understand the relationship between PNBs and nucleoli, we examined the fate of mitotic pre-ribosomal particles...
by mitotic pre-rRNAs (m-pre-rRNAs), during cell division. Recently synthesized 45S pre-rRNA, as well as 32S and 30S pre-rRNAs are maintained when nucleoli disappear. They associate with the chromosome periphery together with pre-rRNA processing factors. Maturation of mitotic pre-ribosomal particles is transiently arrested during mitosis and can be restored by CDK inhibitor treatments. At M–G1 transition, processing of m-pre-rRNAs does not induce dissolution of PNBs that depends on the reformation of functional nucleoli. During their maturation process, m-pre-rRNAs localize in reforming nucleoli and therefore might participate in the reformation of nucleoli in early G1 cells.

RESULTS
In human cells, rDNA transcription gives rise to 47S pre-rRNA initially cleaved at both ends to generate 45S pre-rRNA, then processed by two alternative pathways to generate mature 18S, 5.8S and 28S rRNAs (Fig. S1). During mitosis, rDNA transcription is progressively repressed during prophase and restored in telophase (Fig. S2) but pre-rRNAs – thereafter designated m-pre-rRNAs – are maintained. Here, we have globally followed the fate of metabolically labeled m-pre-rRNAs during and at exit from mitosis. For this purpose, 5-ethynyl uridine (EU) was used as modified uridine because, in contrast to 5-fluorouridine (FU) (Wilkinson et al., 1975), it does not alter pre-rRNA processing.

The last pre-rRNAs synthesized localize at the chromosome periphery
To assess synthesis of m-pre-rRNAs, double metabolic labeling of RNAs in asynchronous HeLa cells was performed. To discriminate between the earlier- and later-synthesized RNAs, EU incorporation was allowed for 3 h, whereas FU incorporation was only allowed for the last 30 min before cell fixation. The localization of EU- and FU-labeled RNAs was compared with that of the nucleolar marker fibrillarin (Fig. 1A–F). In interphase cells, EU- and FU-labeled RNAs were observed mainly in nucleoli identified by fibrillarin (Fig. 1A) as well as in prophase cells, whereas rDNA transcription was progressively repressed (Fig. 1B). The EU-labeled RNAs were localized at the chromosome periphery in all transcriptionally inactive mitotic stages similarly to fibrillarin (Fig. 1C–F). This observation demonstrates that RNAs synthesized before transcription repression are maintained during mitosis and localize at the chromosome periphery. Because synthesis of such metabolically labeled RNAs is largely prevented by inhibition of rDNA transcription [through addition of the transcription inhibitor actinomycin D (AMD)] (Fig. 2A, +AMD), these mitotic RNAs are mostly m-pre-rRNAs. Interestingly, FU-labeled m-pre-rRNAs co-localized with fibrillarin in the early mitotic stages as observed in prometaphase (Fig. 1C) and in some metaphase cells (Fig. 1D,E) but not in later mitotic stages as illustrated for late anaphase (Fig. 1F). As (1) FU incorporation lasted only 30 min, (2) rDNA transcription is repressed in prophase and (3) the average time needed for HeLa cells to progress from the end of prophase (nuclear envelope breakdown) to metaphase (full chromosome alignment) is 20 min (Chen et al., 2008; Toyoda and Yanagida, 2006), the absence of FU-labeled m-pre-rRNAs was expected in later mitotic stages and showed the specificity of the in situ detections of FU- and EU-labeled RNAs. More interestingly, the presence of FU-labeled m-pre-rRNAs in early mitotic stages demonstrated that the most recently synthesized pre-rRNAs persist during mitosis and localize at the chromosome periphery.

m-pre-rRNAs determine the localization of nucleolar proteins at the chromosome periphery
To assess the relationship between m-pre-rRNAs and the localization of nucleolar proteins during mitosis, a double metabolic labeling of RNAs was performed on asynchronous HeLa cells (Fig. 2A). EU incorporation lasted 3 h before cell fixation whereas FU incorporation was limited to the last 1 h, a period sufficient to ensure FU labeling of m-pre-rRNAs in all metaphase cells. The cells were treated or not with AMD during the last 1 h of culture to specifically inhibit pre-rRNA synthesis. EU and FU incorporations were detected in metaphase cells and the localization of EU- and FU-labeled m-pre-rRNAs was most probably due to detection of pre-rRNAs synthesized during the 10 min before effective inhibition of rDNA transcription (Popov et al., 2013). More interestingly, the large decrease of these m-pre-rRNAs induced delocalization of fibrillarin from the
chromosome periphery to the cytoplasm (Fig. 2A, +AMD). This observation was reinforced by results obtained after siRNA depletion of nucleolin, known to play an important role in pre-rRNA synthesis (Fig. S3). As assessed by FU incorporation in nucleoli of interphase cells, rDNA transcription largely decreased in nucleolin-depleted HeLa cells and consequently, also the amount of m-pre-rRNAs present at the chromosome periphery during mitosis. The decrease of m-pre-rRNAs also induced delocalization of fibrillarin from the chromosome periphery to the cytoplasm. These results brought into question the relationship between the presence of m-pre-rRNAs and the localization of nucleolar proteins at the chromosome periphery. To further analyze the influence of m-pre-rRNAs on protein–chromosome association, western blots were performed on extracts prepared from HeLa cells accumulated in prometaphase for 4 h in the presence or absence of AMD, and from their isolated chromosomes (Fig. 2Ba,b). Nucleolar proteins involved in pre-rRNA processing and localized at the chromosome periphery, namely fibrillarin, nucleolin and Nop58, were analyzed and compared with the transcription factor UBF. Interestingly, the amounts of nucleolin, Nop58 and fibrillarin were lower in chromosome extracts prepared from AMD-treated cells compared with control cells. Conversely, UBF seemed enriched in chromosome extracts regardless of AMD treatment. Thus, the perichromosomal localization of these pre-rRNA processing factors depends on the presence of m-pre-rRNAs and most probably on direct and/or indirect interaction with m-pre-rRNAs.

Total RNAs were also prepared from HeLa prometaphase cells treated or not with AMD and from chromosomes (Fig. 2Bc) to further analyze the effects of AMD treatment on the presence of m-pre-rRNAs. Ethidium bromide (EtBr) staining of RNAs after electrophoretic separation showed first that the 45S and 32S pre-rRNAs were enriched in RNA extracts from chromosomes isolated from control cells (Fig. 2Bc, lane 3) in contrast to mature 28S and 18S rRNAs (Fig. 2Bc, lanes 1, 3), showing that 45S and 32S pre-rRNAs co-purified with chromosomes. The EtBr staining showed also that the 45S pre-rRNA disappeared after AMD-induced inhibition of pre-rRNA synthesis, whereas the 32S pre-rRNA only decreased slightly (Fig. 2Bc, lanes 1–4). The fact that m-pre-rRNAs present at the chromosome periphery not only correspond to pre-rRNA processing factors at the chromosome periphery of AMD-treated cells (Fig. 2Bb).

Identification of m-pre-rRNAs associated with chromosomes and their fate at exit from mitosis

The identification of m-pre-rRNAs associated with chromosomes was performed by northern blot. Use of a 5.8S+ probe allowed...
with that of 45S and 32S pre-rRNA. The use of the 18S probe (Fig. 3B) showed that the amount of 30S pre-rRNA is low compared to that of 32S pre-rRNA (Fig. 3A) or of 30S pre-rRNA rRNAs with chromosomes. Noticeably, comparing the level of 45S presence of 45S pre-rRNA and revealed the association of 30S pre-rRNAs with chromosomes. The use of the ETS probe (Fig. 3B) confirmed the absence of 47S and 46S pre-rRNAs and the presence of 47S pre-rRNA was most probably processed and not degraded. This suggested also that the processing of 45S pre-rRNA occurs much more rapidly than that of 32S pre-rRNA. The slow processing of 45S pre-rRNA dramatically increased after CDK inhibitor treatments (Fig. 4Ca; AMD, AMD/ROSC and AMD/INDI). Global quantification of 47S–30S pre-rRNAs confirmed this observation (Fig. 4Cb; ROSC and INDI). Because in the absence of neosynthesized pre-rRNAs the primers ETS1 and ETS2 allow specific quantification of 45S and 30S pre-rRNAs, this also showed that the amount of 45S and 30S pre-rRNAs present in mitotic cells rapidly decreased after CDK inhibitor treatments (Fig. 4Ab; AMD, AMD/ROSC and AMD/INDI). Total RNAs were analyzed by RT-qPCR quantification (Fig. 4Aa–c; Fig. S1) using primers ETS1 and ETS2 to quantify unprocessed 47S pre-rRNA, ETS3 and ETS4 to quantify 47S–30S pre-rRNAs, i.e. 47S, 46S, 45S and 30S pre-rRNAs, and 5.8S1 and 5.8S2 to quantify 47S–125S pre-rRNAs, i.e. 47S, 46S, 45S, 43S, 41S, 32S and 12S pre-rRNAs. The quantification of the 47S pre-rRNA showed that both roscovitine and indirubin-3’-monoxime induce resumption of rDNA transcription. Indeed, the amount of 47S pre-rRNA dramatically increased after CDK inhibitor treatments (Fig. 4Aa; ROSC and INDI) whereas no increase was observed using AMD or AMD together with one of the CDK inhibitors (Fig. 4Aa; AMD, AMD/ROSC and AMD/INDI). Global quantification of 47S–30S pre-rRNAs confirmed this observation (Fig. 4Ab; AMD/ROSC and AMD/INDI). CDK inhibitors restore processing of m-pre-rRNAs To investigate the possible effects of the CDK inhibitors roscovitine and indirubin-3’-monoxime on m-pre-rRNAs, prometaphase HeLa cells were first treated for 2 h with either AMD or one of the CDK inhibitors, or both AMD and a CDK inhibitor. The simultaneous use of AMD and CDK inhibitors allowed discrimination between m-pre-rRNAs and neosynthesized pre-rRNAs.

Fig. 3. Identification of m-pre-rRNAs and their fate at exit of mitosis. (A,B) RNAs extracted from colcemid-blocked prometaphase HeLa cells (mitotic cells) and from purified chromosomes were resolved on gels (EtBr), blotted and hybridized with the 5.8S+ (A) and ETS (B) probes. (C) Nocodazole-blocked prometaphase HeLa cells were released into nocodazole-free medium containing (+) or not (−) AMD. RNAs were prepared from cells at 45, 60, 90 and 120 min, analyzed in a gel (EtBr), blotted and hybridized with the 5.8S+ probe.

detection of 47S, 46S, 45S, 43S, 41S, 32S and 12S pre-rRNAs and mature 5.8S rRNA (Fig. 3A; Fig. S1), whereas an ETS probe allowed detection of 47S, 46S, 45S and 30S pre-rRNAs (Fig. 3B; Fig. S1) and an 18S probe allowed detection of 47S, 46S, 45S, 43S, 41S, 30S, 26S, 21S and 18S-E pre-rRNAs and mature 18S rRNA (Figs S1, S4). The identification of 45S and 32S pre-rRNAs as being m-pre-rRNAs was confirmed using the 5.8S+ probe (Fig. 3A). As observed for mature 28S and 18S rRNAs, mature 5.8S rRNA was visible in mitotic cells but not associated with chromosomes (Fig. 3A, lanes 1, 2). In addition, the 5.8S+ probe did not reveal the presence of 47S, 46S, 45S, 41S and 12S pre-rRNAs in mitotic cells or in chromosome extracts. The use of the ETS probe (Fig. 3B) confirmed the absence of 47S and 46S pre-rRNAs and the presence of 45S pre-rRNA and revealed the association of 30S pre-rRNAs with chromosomes. Noticeably, comparing the level of 45S pre-rRNA with that of 32S pre-rRNA (Fig. 3A) or of 30S pre-rRNA (Fig. 3B) showed that the amount of 30S pre-rRNA is low compared with that of 45S and 32S pre-rRNA. The use of the 18S probe (Fig. S4) confirmed these results and did not reveal additional pre-rRNA quantitatively associated with chromosomes. To assess the fate of the major m-pre-rRNAs, namely 45S and 32S pre-rRNAs, when cells exit from mitosis, prometaphase-arrested HeLa cells were released from the mitotic block in the absence or presence of AMD and total RNAs analyzed by northern blot using the 5.8S+ probe (Fig. 3C). This analysis showed that the cells progressed and exited from mitosis as demonstrated by the restoration of 47S pre-rRNA synthesis (Fig. 3C, lanes 1, 2, 4, 6, 8). When pre-rRNA synthesis was prevented by AMD, 45S pre-rRNA (Fig. 3C, lane 1) progressively disappeared (Fig. 3C, lanes 1, 3, 5, 7, 9). Interestingly, 32S pre-rRNA was still clearly observed after 120 min (Fig. 3C, lanes 1, 3, 5, 7, 9), showing that 32S pre-rRNA possesses a longer half-life than 45S pre-rRNA.
32S pre-rRNA most probably explains the results obtained for quantifications of 47S–30S and 47S–12S pre-rRNAs after CDK inhibitor treatments (Fig. 4Ab,c).

To further understand the processing of m-pre-rRNAs and to compare the effects of roscovitine on the processing of neosynthesized pre-rRNAs with those of indirubin-3'-monoxime, northern blot analyses were performed on extracts prepared from mitotic cells treated as above for 2 or 6 h using the 5.8S+ (Fig. 5A) and ETS (Fig. 5C) probes. The results obtained by simultaneously using the CDK inhibitors and AMD confirmed that both CDK inhibitors most probably trigger processing of m-pre-rRNAs (Fig. 5A–D). Similarly to what was seen in asynchronous cells (Fig. 5A, lanes 8, 9), mitotic 32S pre-rRNA slowly decreased and was still observed after 6 h of CDK inhibitor treatments (Fig. 5A,B). Even if degradation cannot be completely ruled out, this argues in favor of processing of m-pre-rRNAs. Interestingly, roscovitine and indirubin-3'-monoxime affect differently the processing of neosynthesized 47S pre-rRNA. Indeed, as we reported previously (Sirri et al., 2000), roscovitine led to the accumulation of unprocessed neosynthesized pre-rRNAs (Fig. 5A, lanes 2, 3, 8; Fig. 5C, lanes 2, 6). This processing defect results also in low levels of 32S, 30S and 12S pre-rRNAs (Fig. 5A–D). Conversely, indirubin-3'-monoxime induced both rDNA transcription and processing of transcripts as suggested when comparing the patterns obtained from extracts prepared from prometaphase cells treated with indirubin-3'-monoxime for 6 h and from untreated asynchronous cells and their quantifications (Fig. 5A–D). The processing of transcripts in mitotic cells treated with indirubin-3'-monoxime was most probably close to that occurring in interphase cells. Indeed, the percentage of 47S–45S pre-rRNAs and of either 32S and 12S pre-rRNAs (Fig. 5Ea) or 30S pre-rRNA (Fig. 5Eb) were similar in indirubin-3'-monoxime-treated prometaphase cells (Fig. 5Ea,b, INDI) and in untreated asynchronous cells (Fig. 5Ea,b, Async), contrary to what was observed in roscovitine-treated prometaphase cells (Fig. 5Ea,b, ROSC).

The CDK inhibitor indirubin-3'-monoxime induces relocation of m-pre-rRNAs in nucleoli

The fact that indirubin-3'-monoxime induced both rDNA transcription and processing of transcripts (Fig. 5A–E) that normally occur in nucleoli prompted us to verify the formation of nucleoli in indirubin-3'-monoxime-treated prometaphase cells. For
this, the location of the nucleolar markers Nop52 and fibrillarin was analyzed in colcemid-arrested prometaphase Nop52–GFP HeLa cells treated with roscovitine (Fig. 6Aa), indirubin-3′-monoxime (Fig. 6Ab), both AMD and roscovitine (Fig. 6Ac) or with both AMD and indirubin-3′-monoxime (Fig. 6Ad) for 6 h. As already reported (Sirri et al., 2002), roscovitine treatment did not induce
formation of nucleoli and Nop52 localized in dots far from fibrillarin in roscovitine-treated cells (Fig. 6Aa). Conversely, formation of nucleoli was observed in indirubin-3′-monoxime-treated cells (Fig. 6Ab) as assessed by the gathering of both nucleolar markers in the same sites (Fig. 6Ab, merge). Noticeably, no formation of nucleoli was detected in cells treated with both AMD and CDK inhibitors (Fig. 6Ac,d), i.e. in conditions where rDNA transcription was impaired but not processing of m-pre-rRNAs (Fig. 5A–D), and Nop52-containing dots were observed.

Because CDK inhibitors exhibited different effects on nucleologenesis, we were intrigued by the fate of m-pre-rRNAs in roscovitine- or indirubin-3′-monoxime-treated mitotic cells. For this, Nop52–GFP expressing and metabolically EU-labeled prometaphase HeLa cells were untreated (Fig. 6Ba) or treated with roscovitine (Fig. 6Bb) or indirubin-3′-monoxime (Fig. 6Bc) for 2 h. EU-labeled prometaphase cells were washed and treated with CDK inhibitors in an EU-free medium containing nocodazole so as to prevent EU uptake and therefore limit the synthesis of new EU-labeled pre-rRNAs, whereas the CDK inhibitor treatments triggered resumption of rDNA transcription. The cells were processed to visualize Nop52 (Fig. 6B, Nop52) or to reveal EU-labeled m-pre-rRNAs (Fig. 6B, EU) and to carry out in situ detection of rDNA transcription. As expected (Sirri et al., 2002), Nop52 localized at the chromosome periphery in control cells (Fig. 6Ba, Nop52) and in small dots in roscovitine-treated cells (Fig. 6Bb, Nop52). The fact that Nop52-containing small dots were observed far from the sites of resumption of rDNA transcription (Fig. 6Bb, merge) confirmed that roscovitine impairs nucleologenesis. Similarly to Nop52 distribution, EU-labeled RNAs were detected at the chromosome periphery in control cells (Fig. 6Ba, EU) and as small dots in roscovitine-treated cells far from the sites of rDNA transcription (Fig. 6Bb, EU).

In contrast to roscovitine, indirubin-3′-monoxime treatment induced the formation of nucleoli (Fig. 6Bc, Nop52) as argued by the gathering of Nop52 close to the sites of Pol I activity. Strikingly, EU-labeled RNAs were then observed in reformed nucleoli (Fig. 6Bc, EU). It should be noted that even if CDK inhibitor treatments were performed in an EU-free medium, we could not exclude the possibility that neosynthesized EU-labeled pre-rRNAs were also detected. However, because in roscovitine-treated cells, EU labeling was not or only weakly detected in the sites of resumption of rDNA transcription (Fig. 6Bb), neosynthesized EU-labeled pre-rRNAs were most probably negligible and therefore the EU-labeled RNAs observed in reformed nucleoli of indirubin-3′-monoxime-treated cells mainly corresponded to EU-labeled m-pre-rRNAs.
m-pre-rRNAs relocate in nucleoli at exit from mitosis

m-pre-rRNAs were still present, at least as 32S pre-rRNAs, in HeLa cells 2 h after release of the nocodazole block (Fig. 3C), and in colcemid-arrested mitotic HeLa cells treated for 2 or 6 h with CDK inhibitors (Fig. 4B,C; Fig. 5A,B) and they were detected in reformed nucleoli in indirubin-3′-monoxime-treated prometaphase cells (Fig. 6Bc). The localization of m-pre-rRNAs in reforming nucleoli at exit from mitosis was further investigated by using the fact that roscovitine treatment impairs formation of nucleoli in a reversible manner (Sirri et al., 2002). Metaphase-synchronized Nop52-GFP HeLa cells were untreated or treated with roscovitine for 90 min, i.e. when cells proceeded through M–G1 transition (Fig. 7, Control and +Rosc 90 min). Cells appearing as early G1 cells were cultured for an additional 90 min after removal (+Rosc 90 min) or without removal (+Rosc 180 min) of roscovitine. The cells were then treated (+Rosc 90 min) or not (Control) with roscovitine for 90 min as early G1 cells (Fig. 7, Control and +Rosc 90 min). In control cells, Nop52 and EU-labeled pre-rRNAs mainly localized in the reformed nucleoli with a minor proportion still localized in PNBs. When reformation of nucleoli was prevented by roscovitine treatment, both Nop52 and EU-labeled pre-rRNAs were detected in the same small dots in the nucleus. After reversion of the effects of roscovitine on nucleologenesis (Fig. 7, +Rosc 90 min −Rosc 90 min), both Nop52 and EU-labeled pre-rRNAs localized in reformed nucleoli. When cells were maintained in roscovitine-containing culture medium (Fig. 7, +Rosc 180 min), both Nop52 and EU-labeled pre-rRNAs were still observed in the same small nuclear dots. Therefore, remarkably, EU-labeled pre-rRNAs were detected localized similarly to Nop52 in cells treated or not treated with roscovitine, i.e. in PNBs and reforming nucleoli.

However, even if the experiment was performed with excess uridine to minimize incorporation of EU after resumption of rDNA transcription, we could not be absolutely certain that nucleolar EU labeling was not the result of post-mitotic synthesis as rDNA transcription restarted in reforming nucleoli. To exclude this possibility, experimental conditions that render post-mitotic synthesis undeflectable were first defined. Post-mitotic synthesis could be detected as FU or EU incorporation in reforming nucleoli of early G1 cells as shown in Fig. 8Aa,b by comparing the detection of FU- or EU-labeled RNAs and the nucleolar marker fibrillarin. For this, nocodazole-arrested prometaphase HeLa cells were maintained for 1 h in medium containing EU or FU (Fig. 8Ab, −Uridine). After this uptake step, the cells were washed and released for 2 h in nucodazole-free medium containing excess uridine. Conversely, post-mitotic synthesis was no longer detected when the uptake step was carried out in the presence of excess uridine in addition to EU or FU (Fig. 8Ab, +Uridine). Similar experiments were then performed except that the cells were synchronized in prometaphase in the presence of EU to also detect the pre-mitotically-labeled pre-rRNAs (Fig. 8Ba,b). The prometaphase cells were then washed and maintained for 1 h in medium containing FU alone (Fig. 8Bb, −Uridine) or FU together with excess uridine (Fig. 8Bb, +Uridine) before being washed and finally released for 2 h in nocodazole-free medium containing excess uridine where they progressed to early G1. When post-mitotic synthesis was detectable (Fig. 8Bb, −Uridine), EU and FU labeling mainly co-localized. When post-mitotic synthesis was no longer detectable (Fig. 8Bb, +Uridine), EU labeling, corresponding only to pre-mitotic synthesis, was clearly observed in reforming nucleoli and in PNBs of early G1 cells as demonstrated by the detection of fibrillarin. Consequently, m-pre-rRNAs synthesized before repression of rDNA transcription during prophase, actually localize in reforming nucleoli at the exit from mitosis.

DISCUSSION

So far, cell biology studies on m-pre-rRNAs, principally based on the fluorescence in situ hybridization (FISH) approach, have brought a wealth of information. However, the FISH approach has limitations, especially for RNAs such as rRNAs synthesized as precursors. Indeed, except for probes specifically hybridizing the 47S pre-rRNA, no probe highlights a single mature or pre-rRNA. Disappearance of FISH signals, which occurs as the processing events take place, does not allow tracking of the resulting mature or pre-rRNA. Moreover, it is impossible to know when pre-rRNAs were synthesized and therefore impossible to discriminate between m-pre-rRNAs transiting through mitosis and neosynthesized pre-
rRNAs. To overcome these limitations, we have globally followed metabolically labeled m-pre-rRNAs during and at exit from mitosis. Even if rDNA transcription is progressively repressed during prophase and restored at telophase, m-pre-rRNAs are maintained and transit through mitosis. We clearly observed that 45S, 32S and 30S pre-rRNAs remain associated with chromosomes. Other pre-rRNAs were not observed, showing that they are not maintained or maintained in very low amounts. These results agree with previous studies showing that 45S pre-rRNAs localize at the chromosome periphery in contrast to 47S and 46S pre-rRNAs (Carron et al., 2012). The arrest of pre-rRNA processing is supported by the presence of m-pre-rRNAs, especially the presence of the short-lived 45S pre-rRNA in HeLa cells blocked in mitosis for several hours. Because mitotic 45S pre-rRNA rapidly decreased in a manner unlikely related to degradation in colcemid-blocked mitotic HeLa cells after CDK inhibitor treatments, it may be proposed that as for rDNA transcription (Sirri et al., 2000), inhibition of pre-rRNA processing could be established at G2–M transition and/or

![Fig. 8. m-pre-rRNAs relocate in nucleoli at M–G1 transition.](image)

(Aa) Schematic of the timing of the experiment. (Ab) Nocodazole-arrested prometaphase HeLa cells were resuspended in nocodazole-containing medium in the presence of EU or FU alone (−Uridine), or with excess uridine (+Uridine) for 1 h. The cells were then washed and cultured in nocodazole-free medium containing excess uridine for 2 h before being processed to observe fibrillarin and to reveal EU or FU post-mitotic incorporations. (Ba) Schematic of the timing of the experiment. (Bb) Nocodazole-arrested prometaphase HeLa cells accumulated in the presence of EU were washed and resuspended in nocodazole-containing medium in the presence of FU alone (−Uridine) or with excess uridine (+Uridine) for 1 h. The cells were then washed and cultured in nocodazole-free medium containing excess uridine for 2 h before being processed to detect EU pre-mitotic and FU post-mitotic incorporations, or to observe fibrillarin and reveal EU pre-mitotic incorporation. Arrowheads indicate the reforming nucleoli. Scale bars: 10 µm.
maintained during mitosis by a general CDK1–cyclin B-kinase-dependent mechanism. However, specific mechanisms might directly or indirectly inhibit specific steps of pre-rRNA processing. In particular, because 32S pre-rRNA processing depends on the presence of 5S rRNA (Dechampesme et al., 1999; Donati et al., 2013; Zhang et al., 2007), shutdown of 32S pre-rRNA processing might be linked to the regulation of Pol III transcription and/or any step involved in recruiting the RPL5–RPL11–5S rRNA precursor complex. In addition, as suggested by the presence of only 45S, 32S and 30S pre-rRNAs in mitotic cells (this study; Carron et al., 2012; Dundr and Olson, 1998), inhibition operates in a targeted manner on these pre-rRNA processing steps and logically excludes most pre-rRNA processing factors (Tafforeau et al., 2013) as potential targets.

Concerning the resumption of m-pre-rRNA processing, our results agree with the fact that maturation of m-pre-rRNAs is restored in PNBs at telophase (Carron et al., 2012) when CDK1–cyclin-B kinase is inhibited. However, as observed here, processing of mitotic 32S pre-rRNA occurs slowly and lasts during the M–G1 transition. Because the presence of 5S rRNA is required for 32S pre-rRNA processing (Dechampesme et al., 1999; Donati et al., 2013; Zhang et al., 2007) and as 5S RNP regulates the tumor suppressor p53 (Donati et al., 2013; Sloan et al., 2013), processing of mitotic 32S pre-rRNA could be correlated to cell-cycle progression.

m-pre-rRNAs determine the location of proteins at the chromosome periphery

Previous studies have shown that 45S pre-rRNA, in addition to small nucleolar RNAs and nucleolar proteins involved in the processing and modification of pre-rRNAs as well as in the assembly of mature rRNAs with ribosomal proteins, localize at the chromosome periphery during mitosis (Boisvert et al., 2007; Carron et al., 2012; DiMario, 2004; Douset et al., 2000; Gautier et al., 1992; Hüge et al., 1985; Jiménez-Garcia et al., 1994; Savino et al., 1999). Here, we show that 45S, 32S and 30S pre-rRNAs are maintained at the chromosome periphery and determine the location of pre-rRNA processing factors as demonstrated for fibrillarin, Nop58 and nucleolin. As pre-rRNP complexes were shown to be present in mitotic cells (Piñol-Roma, 1999), this observation indicates that m-pre-rRNAs are most probably the RNA moiety of pre-rRNP complexes present at the chromosome periphery. Beyond the presence of pre-rRNP complexes, the presence of pre-ribosomal particles is strongly suggested by the fact that processing of mitotic 45S pre-rRNAs is rapidly reactivated after CDK inhibitor treatments (this study) as is also the case of the Pol I transcription machinery (Sirri et al., 2000). Moreover, in addition to the effects on rDNA transcription and m-pre-rRNA processing, CDK inhibitor treatments induce the formation of PNBs (Sirri et al., 2002) where rDNA transcription and m-pre-rRNA processing, CDK inhibitor machinery (Sirri et al., 2000). Moreover, in addition to the effects on transcription and processing of neosynthesized pre-rRNAs in the incipient nucleoli (Carron et al., 2012). The disassembly of PNBs would result from maturation and release of their pre-ribosome content and progressive release of processing factors. From loss-of-function experiments, the authors concluded that PNB disappearance depends on the processing of m-pre-rRNAs (Carron et al., 2012).

However, such experiments alter processing of m-pre-rRNAs in PNBs and of neosynthesized pre-rRNAs in incipient nucleoli and, therefore, do not allow actual discrimination between direct and indirect effects. In agreement with the model, m-pre-rRNAs are processed in cells without functional nucleoli as in prometaphase cells treated with roscovitine, with AMD and roscovitine, or with AMD and indirubin-3′-monoxime. Nevertheless, processing of m-pre-rRNAs occurs without dissolution of PNBs in these cells. Conversely, dissolution of PNBs occurs as cells progress through M–G1 transition and in prometaphase cells treated with indirubin-3′-monoxime, i.e. in cells exhibiting functional nucleoli as assessed by rDNA transcription and processing of neosynthesized pre-rRNAs. Consequently, processing of m-pre-rRNAs is not sufficient to induce disappearance of PNBs that seems to require the presence of functional nucleoli.

Even though processing of m-pre-rRNAs can occur in PNBs, there is no evidence that m-pre-rRNAs are entirely processed in PNBs and exported to the cytoplasm. Conversely, we observed the presence of rRNAs that originate from m-pre-rRNAs in reforming nucleoli at the M–G1 transition. In addition, processing of m-pre-rRNAs exhibits a marked difference in timing between pre-40S and pre-60S ribosomal particles (this study; Carron et al., 2012; Dundr et al., 2000); especially, the duration of 32S pre-rRNA processing, which is so long that it could appear somewhat difficult to reconcile with the lifetime of PNBs.

Even though further experiments are needed to uncover their roles, it is tempting to propose that mitotic pre-rRNP might be involved in reorganizing functional nucleoli. This hypothesis is corroborated by results obtained using CDK inhibitors. Indeed, the CDK inhibitor roscovitine impairs relocation of mitotic pre-rRNP from PNBs to the sites of resumption of rDNA transcription as assessed by the pre-rRNA processing factor Nop52 (Yoshikawa et al., 2015) and the m-pre-rRNAs, and prevents formation of functional nucleoli as evidenced by the processing defect of neosynthesized pre-rRNAs. Conversely, the CDK inhibitor indirubin-3′-monoxime impairs neither relocalization of mitotic pre-rRNP nor formation of functional nucleoli, suggesting that both events are related. Moreover, the nucleolus localization of the 40S and/or 60S ribosomal subunit precursors generated from the mitotic pre-rRNP might be a prerequisite for their export.

Like the disappearance of PNBs that requires the presence of functional nucleoli, nucleolar localization of m-pre-rRNAs reflects the close relationship between PNBs and nucleoli. Given that the 5S RNP can regulate the tumor suppressor p53 (Donati et al., 2013; Sloan et al., 2013) and that it plays a crucial role in 32S pre-rRNA processing (Dechampesme et al., 1999; Donati et al., 2013; Zhang et al., 2007), the processing of m-pre-rRNAs, disappearance of
PNBs, M–G1 progression and the formation of functional nucleoli are very likely correlated. Thus once formed, nucleoli can regulate cell cycle progression (Tsai and Pederson, 2014).

**MATERIALS AND METHODS**

**Antibodies**

The human autoimmune serum with specificity against fibrillarin (O61; 1:3000) was described previously (Sirri et al., 2002). The anti-fibrillarin [anti-FBL (126-140); 1:10,000], anti-BrdU (clone BU-33; 1:500), anti-UBF (clone 6B6; 1:10,000) antibodies were from Sigma-Aldrich. The anti-nucleolin (MS-3; 1:1000) and anti-Nop58 (C-20; 1:1000) antibodies were from Santa Cruz Biotechnology. The alkaline phosphatase-conjugated anti-digoxigenin antibodies were from Roche (11093 274 910; 1:2000). The secondary antibodies coupled to Alexa Fluor 488 (109-545-088 and 115-545-166; 1:500), Alexa Fluor 594 (109-585-088; 1:500) and Alexa Fluor 647 (709-605-149; 1:500) were from Jackson ImmunoResearch Laboratories, Inc. and those coupled to horseradish peroxidase were from Sigma-Aldrich (A5420; 1:10,000) and Jackson ImmunoResearch Laboratories (111-035-003 and 115-035-003; 1:3000).

**Primers**

PCRs and/or real-time PCRs were performed using oligonucleotides corresponding to human rDNA (see Fig. S1). The forward oligonucleotides were ETS1: 5′-GAGGTGTGGCCCTCCGGATGC-3′, ETS2: 5′-CCTCTAGGGCCGCAAAGGACG-3′, ETS5: 5′-GTCTGGTGGTGGTTGCGAGC-3′, 5′-85.S1: 5′-CCTCTAGGACGCACCTTTGCCG-3′ and 18S1: 5′-GTCTTAAAGCGACCAGCGGC-3′; and the reverse oligonucleotides were ETS2: 5′-ACCAGCAAGGAACAGCAGG-3′, ETS4: 5′-CTCCAGGAGCGACCAGG-3′, ETS6: 5′-CCACCGGAGCTGCTCACAC-3′, 5′-85.S2: 5′-CTGGAGGGAACACCCACAGC-3′ and 18S2: 5′-AGGGGAGCATTACGATGCG-3′.

**Probes**

The ETS (nt +935/+1082), 18S (nt +4477/+4610) and 5.8S (nt +6718/+6845) probes corresponding to human rDNA were generated as digoxigenin-labeled using primers ETS5 and ETS6, primers 18S1 and 18S2, and primers 5.8S1 and 5.8S2, respectively, and the PCR DIG probe synthesis kit (Roche).

**Cell culture and synchronization, inhibitor treatments and siRNA transfections**

Stably transfected Nop52-GFP (Savino et al., 2001) and untransfected HeLa cell lines were cultured in MEM supplemented with 10% FCS and 2 mM L-glutamine (GIBCO BRL). The cells blocked in prometaphase by nocodazole (0.04 µg/ml, Sigma-Aldrich) or colcemid (KaryoMax colcemid solution at 0.02 µg/ml, GIBCO BRL) treatments for 4 h or overnight were selectively harvested by mechanical shock. For metaphase synchronization, the cells were arrested in prometaphase by nocodazole treatment for 4 h, washed and resuspended in nocodazole-free medium for 30 min. For chromosome preparations, the cells were blocked in prometaphase by nocodazole treatment for 4 h, washed and resuspended in nocodazole-free medium for 30 min. For chromosome preparations, the cells were blocked in prometaphase by colcemid treatment for 4 h in the presence or absence of AMD (0.05 µg/ml, Sigma-Aldrich) added 1 h before the colcemid solution. 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. The cells were treated with AMD (0.05 µg/ml) to specifically inhibit rDNA transcription and with the CDK inhibitors roscovitine (75 µM) or indirubin-3′-monoxime (25 µM) obtained from Sigma-Aldrich.

The cells were transfected with the siControl RISC-negative siRNA (Dharmacon) and the nucleolin targeting siRNA (SI02654925; Qiagen) using INTERFERin™ (Polyplus-transfection) the day of cell seeding and grown for 48 h before the experiments.

**RNA metabolic labeling**

To analyze the timing of synthesis of m-pre-rRNAs, asynchronous cells were cultured in medium containing 100 µM EU for 3 h to which 1 mM FU was added for the last 30 or 60 min of culture. To analyze the fate of m-pre-rRNAs, prometaphase cells were collected in the presence of 100 µM EU, extensively washed and resuspended in EU-free medium or in EU-free medium containing uridine (1 mM). To evaluate the influence of post-mitotic RNA synthesis on EU labeling observed after mitosis, the cells were accumulated by nocodazole treatment, selectively harvested and resuspended in nocodazole-containing medium in the presence of 100 µM EU or 10 µM FU alone or with 1 mM uridine, extensively washed before being resuspended in nocodazole-free medium containing 1 mM uridine. To determine the influence of nucleolin on RNA synthesis, HeLa cells treated with siRNAs were cultured in medium containing 1 mM FU for the last 1 h.

**In situ detection of rDNA transcription, of FU- and/or EU-labeled RNAs and immunofluorescence labeling**

rDNA transcription was detected in fixed cells as previously described (Roussel et al., 1996) and BrUTP incorporation was detected using anti-BrdU antibodies revealed by the Alexa-Flour-488- or 594-conjugated anti-mouse antibodies. For the detection of FU-labeled RNAs and/or EU-labeled RNAs and/or fibrillarin, the cells were fixed with methanol for 20 min at −20°C, air-dried for 5 min and rehydrated with PBS for 5 min. FU incorporation was detected similarly to BrUTP incorporation. EU incorporation was detected using the Click-iT RNA Imaging Kit (Invitrogen) introducing the Alexa Fluor 594 dye. Fibrillarin was detected using the O61 serum revealed by Alexa-Flour-647-conjugated anti-human antibodies. When EU-labeled RNAs were to be detected simultaneously with BrUTP incorporation, FU-labeled RNAs or fibrillarin, the cells were fixed in 4% paraformaldehyde for 15 min at room temperature after the incubations of antibodies before EU detection. All preparations were mounted with the Fluoroshield antifading solution containing 4,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich). The cells were imaged by fluorescence microscopy performed using a Leica upright SPS confocal microscope with a 63× objective and version 2.7.3 of the Leica Application Suite Advanced Fluorescence software. Multiple fluorophores were recorded sequentially at each z-step. Projections of z-series and merge images were performed using ImageJ software (NIH).

**Chromosome preparation**

The procedure used to isolate chromosomes was adapted from Paulson (1982). The solutions contained RNaseOUT™ recombinant ribonuclease inhibitor (Invitrogen) and Protease Inhibitor Cocktail (Sigma-Aldrich). For each preparation, about 2×10⁶ prometaphase cells were pelleted at 100 g for 10 min, suspended in 10 ml 0.5 mM Tris-HCl pH 7.4, 0.2 mM spermine, 0.5 mM spermidine, 2 mM EDTA and 80 mM KCl and incubated at room temperature for 10 min. Swollen cells were collected by centrifugation at 4°C (200 g for 15 min) and resuspended in 5 ml 0.5% chilled buffer A containing 0.1% NP40. All subsequent steps were carried out at 4°C. Cells were then disrupted by passage through a G26 needle. To discard contaminating cells and nuclei, the chromosome suspension was centrifuged on a 0.25 M sucrose cushion in buffer A containing 0.1% NP40. All subsequent steps were carried out at 4°C, air-dried for 5 min, and the supernatant re-centrifuged on a 0.5 M sucrose cushion in buffer A containing 0.1% NP40 for 200 g for 5 min, and the supernatant re-centrifuged on a 0.5 M sucrose cushion in buffer A containing 0.1% NP40 for 2000 g for 20 min. The purity of the chromosome pellet was verified by fluorescence microscopy after DAPI staining and the pellet resuspended in SDS-PAGE sample buffer for protein analysis or in NucleoSpin RNA Lysis buffer (Macherey-Nagel) for RNA analysis.

**Immunoblotting**

Protein extracts were prepared by resuspending pellets containing whole mitotic cells or purified chromosomes in SDS-PAGE sample buffer, sonicated, boiled for 5 min and centrifuged. Whole mitotic cell (20 µg) and purified chromosome (10 µg) protein extracts were resolved by SDS-PAGE, transferred to nitrocellulose membranes (Protran, Schleicher and Schuell). The membranes were incubated with anti-UBF, anti-nucleolin, anti-Nop58 or anti-fibrillarin antibodies revealed by suitable horseradish peroxidase-conjugated secondary antibodies and immunoreactivity detected by chemiluminescence (GE Healthcare).

**RNA analysis**

RNAs were extracted from cells and from purified chromosomes using NucleoSpin RNA and separated (4 µg for cells and 400 ng for
chromosomes) in 0.8% agarose formaldehyde gels. For northern blot analyses, RNAs were transferred to positively charged membranes (Roche). Hybridization was carried out using DIG Easy Hyb buffer (Roche) at 50°C. After washes, the probe was revealed using the DIG Wash and Block Buffer Set (Roche) and the alkaline-phosphatase-conjugated anti-digoxigenin antibodies. Alkaline phosphatase activity was detected using the chemiluminescent substrate CDP-Star (Roche) and quantified with the ImageJ software.

**RT-qPCR**

Total RNA was quantified, electrophoresed to verify its quality and reverse-transcribed (2 μg) with the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen) using random hexamers as primers (125 ng). Real-time PCR was performed using LightCycler 480 SYBR Green I Master and run on a LightCycler 480 II device (Roche). For each reaction, 1.5% synthesized cDNA and 1 μM of a pair of specific primers, either ETS1 and ETS2, ETS3 and ETS4, or 5.8S1 and 5.8S2, were used. Normalization was performed using primers 18S1 and 18S2, which primarily amplify the cDNAs corresponding to mature 18S rRNA. Amplification efficiency for each assay was determined by running a standard dilution curve. Cycle threshold values and relative quantifications were calculated by the LightCycler 480 software.

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**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

V.S. designed, performed and analyzed the experiments. N.J. performed all analyses by confocal microscopy. P.R. supervised the project, designed, performed and analyzed the experiments, and co-wrote the manuscript with D.H.-V.

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**Supplementary information**

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**References**


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**Fig. S1. Scheme of the two pre-rRNA processing pathways in human cells.** Pre-rRNA processing involves numerous cleavages (arrows) following two alternative pathways. The primers used for RTqPCR experiments are indicated as double arrows in blue (ETS1, ETS2), red (ETS3, ETS4), green (18S1, 18S2) and purple (5.8S1, 5.8S2) as well as the ETS (closed rectangle), 18S (hatched rectangle) and 5.8S+ (open rectangle) probes used for northern blots (open rectangle).
Fig. S2. Pol I transcription is progressively repressed during prophase and restored in telophase. HeLa cells were observed at different stages of the cell cycle identified by DNA staining. The *in situ* detection of Pol I transcription revealed that transcription is progressively repressed in prophase (arrowheads) and maintained repressed during mitosis until telophase when Pol I transcription was restored. Bar, 10 µm.
Fig. S3. m-Pre-rRNAs determine the location of fibrillarin at the chromosome periphery. Hela cells treated with a control siRNA or a nucleolin targeting siRNA were cultured in FU- (1 mM) containing medium for 1 h. The FU-labeled RNAs and fibrillarin were analyzed in interphase (A) and metaphase (B) cells. Bar, 10 μm.
Fig. S4. Identification of m-pre-rRNAs associated with chromosomes. (A, B) RNAs were isolated from colcemid-blocked prometaphase HeLa cells (mitotic cells), from purified chromosomes (chromosomes) and from asynchronous Hela cells (asynchronous cells). After separation in a gel (EtBr), RNAs were blotted and hybridized with the 18S probe (18S probe). (A) The hybridized probe was revealed for a short exposure time. (B) The probe hybridized on the upper part of the blot was revealed for a long exposure time.