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Nuclear export and cytoplasmic processing of precursors to the 40S ribosomal subunits in mammalian cells

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It is generally assumed that, in mammalian cells, preribosomal RNAs are entirely processed before nuclear exit. Here, we show that pre-40S particles exported to the cytoplasm in HeLa cells contain 18S rRNA extended at the 3′ end with 20–30 nucleotides of the internal transcribed spacer 1. Maturation of this pre-18S rRNA (which we named 18S-E) involves a cytoplasmic protein, the human homolog of the yeast kinase Rio2p, and appears to be required for the translation competence of the 40S subunit. By tracking the nuclear exit of this precursor, we have identified the ribosomal protein Rps15 as a determinant of preribosomal nuclear export in human cells. Interestingly, inhibition of exportin Crm1/Xpo1 with leptomycin B strongly alters processing of the 5′-external transcribed spacer, upstream of nuclear export, and reveals a new cleavage site in this transcribed spacer. Completion of the maturation of the 18S rRNA in the cytoplasm, a feature thought to be unique to yeast, may prevent pre-40S particles from initiating translation with pre-mRNAs in eukaryotic cells. It also allows new strategies for the study of preribosomal transport in mammalian cells.

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Introduction

As is the case for other ribonucleoproteic particles (RNPs), the assembly and maturation of the pre-rRNPs begins cotranscriptionally, and thus takes place in the vicinity of the ribosomal genes. This activity leads to the buildup of the nucleolus, a subdomain of the nucleus, which emerges around the chromosomal clusters of the ribosomal transcription units. The major part of the production of the ribosomal subunits takes place within this structure: the processing and folding of the pre-rRNAs, in which the so-called external and internal transcribed spacers (ETS and ITS) are eliminated (see Figure 1), and the concomitant assembly with the ribosomal proteins imported from the cytoplasm. At some point during this process, the newly synthesized ribosomal subunits must exit the nucleus and reach the cytoplasm to ensure translation of mRNAs. Nuclear export must be tightly coordinated with pre-rRNA maturation. The mechanisms underlying the nuclear export of preribosomes in vertebrates and their coordination with preribosome maturation remain largely unknown.

Pre-rRNA maturation starts with the large 80–90S RNP particle which splits into the pre-40S and pre-60S particles after cleavage within the internal transcribed spacer 1 (ITS1). Proteomic analysis of these precursor rRNPs in yeast has revealed that their composition is highly dynamic and includes over 150 nonribosomal proteins putatively involved in ribosome biogenesis (Fatica and Tollervey, 2002; Milkereit *et al*, 2002; Fromont-Racine *et al*, 2003; Tschochner and Hurt, 2003). Many of these factors have homologs in vertebrates and some have been found in mammalian preribosomes (Takahashi *et al*, 2003). Through mutation analysis in yeast, it has been found that nuclear export of the pre-40S and pre-60S particles requires the GTPase Ran, a central component in nuclear–cytoplasmic exchanges, as well as the exportin Crm1p (Hurt *et al*, 1999; Moy and Silver, 1999; Gleizes *et al*, 2001). In the case of the pre-60S particles, the current model states that Crm1p is recruited by Nmd3p, a preribosomal factor associated with late pre-60S particles, that contains a leucine-rich nuclear export signal (NES) (Ho *et al*, 2000; Gadal *et al*, 2002). How Crm1p binds to the precursors of the small subunits has not been established yet. One ribosomal protein of the small subunit, Rps15p, has been shown to be required for the exit of the pre-40S particles from the nucleus, and may directly or indirectly interact with the nuclear transport machinery (Leger-Silvestre *et al*, 2004). Some preribosomal maturation factors accompany the precursors of the small subunit from the nucleus to the cytosol and may play a role in this transport process (Schafer *et al*, 2003; Oeffinger *et al*, 2004). Translocation through the nuclear pore complex (NPC) involves a subset of nucleoporins located on the cytoplasmic side of the pore, the so-called Nup82 complex, which includes Nup82p, Nup159p, Nsp1p, Nup116p and Gle2p (Hurt *et al*, 1999; Moy and Silver, 1999, 2002; Gleizes *et al*, 2001). These nucleoporins have overlapping but dissimilar functions in preribosome and mRNP nuclear export (Gleizes *et al*, 2001). In higher eukaryotes, the only factors required for preribosomal transport that have been characterized to date are Crm1 and Nmd3, whose roles in the production of the large subunit have been found to be conserved in HeLa cells and in *Xenopus laevis* oocytes (Thomas and Kutay, 2003; Trotta *et al*, 2003).

The main steps that lead to the production of the mature 18S, 5.8S and 28S rRNAs in vertebrates have been defined

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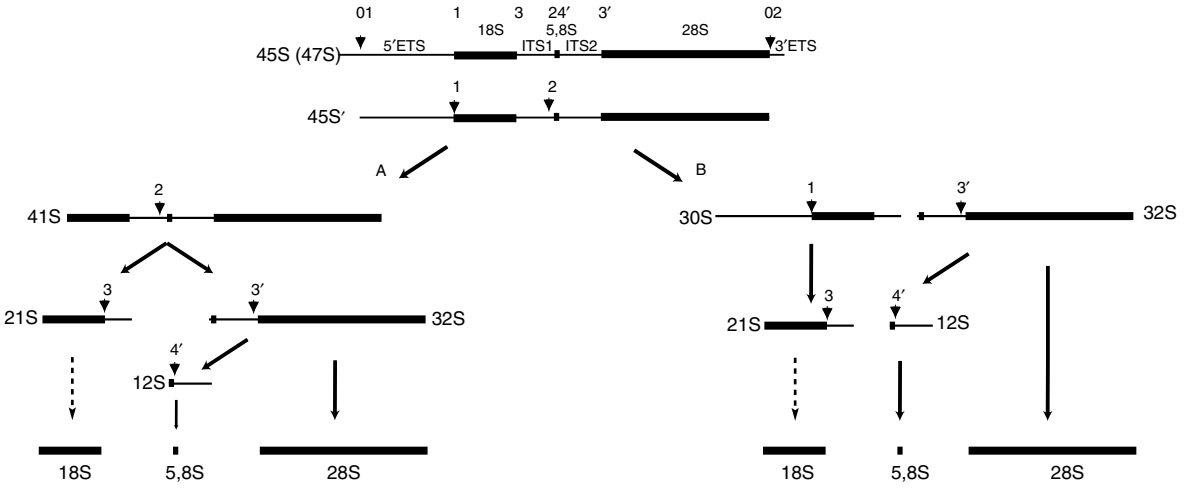


Figure 1 Pre-rRNA processing in HeLa cells. Schematic and nomenclature according to Hadjiolova *et al* (1993). Two alternative pathways are presented.

with varying degrees of precision; some have been thoroughly studied using both *in vitro* and *in vivo* assays, others are still poorly characterized (for reviews, see Hadjiolov, 1985; Eichler and Craig, 1994; Gerbi and Borovjagin, 2004). The overall scheme of this process is comparable to that which has been established in more detail for the yeast *Saccharomyces cerevisiae* (Venema and Tollervey, 1999). However, the sequences of the ETS and ITS diverge greatly between yeast and vertebrates, and more generally from one eukaryote to the other, which makes it difficult to predict the cleavage sites by direct sequence comparison. Noticeably, in vertebrates, the order of the endo- and exonucleolytic cleavages that eliminate the transcribed spacers seems to vary according to species, cell type or physiological state (Hadjiolova *et al*, 1993; Eichler and Craig, 1994; Gerbi and Borovjagin, 2004).

In vertebrates, although the assembly of the ribosomal subunits is only completed in the cytoplasm by addition of the last ribosomal proteins, it is widely assumed that processing of the preribosomal transcripts (pre-rRNAs) into mature 18S, 5.8S and 28S rRNAs takes place entirely in the nucleus. In contrast, in *S. cerevisiae*, the final maturation process of the small subunit occurs in the cytoplasm and includes the last cleavage at the 3′ end of the 18S rRNA (Udem and Warner, 1973; Vanrobays *et al*, 2001). This step, whose mechanism remains to be defined, requires the participation of various factors associated with the pre-40S particles, including the Rio proteins (Vanrobays *et al*, 2001, 2003). Here, we have addressed the nuclear transport of the pre-40S particles and the coordination of this process with the final steps of pre-18S rRNA maturation in mammalian cells. We show that cytoplasmic maturation of the 18S rRNA is not an exception restricted to yeast, but also takes place in mammalian cells. In HeLa cells, the pre-40S particles exiting the nucleus contain a precursor of the 18S rRNA whose final processing at the 3′ end occurs in the cytoplasm. Nuclear export of these pre-40S particles depends on the human ribosomal protein Rps15 and is also perturbed when exportin Crm1 is inhibited. These data suggest that, despite a strong divergence in the sequences of the transcribed spacers and differences in the pre-rRNA processing modes, the localization of the final cleavage of the pre-18S rRNA to within the cytoplasm is a common feature in eukaryotes. Nuclear export of the small ribosomal subunit as an incomplete form may prevent premature translation initiation on pre-mRNAs within the nucleus.

Results

Northern blot analysis of pre-rRNA processing in HeLa cells reveals a new precursor to the 18S rRNA

Pre-rRNA processing in HeLa cells has been proposed to occur through alternative pathways (Figure 1) (Hadjiolova *et al*, 1993). We first re-examined pre-rRNA processing in HeLa cells by Northern blot analysis of total RNA with probes complementary to the ETS1, the 5′-most nucleotides of the ITS1 (5′-ITS1 probe), or to the ITS2 (Figure 2A). The highest molecular weight precursor detected corresponds to a mixture of 45S and 45S′ pre-rRNAs, that is, before and after processing at the early cleavage site. The 30S pre-rRNA, produced by cleavage of the 45S/45S′ RNAs in the 3′ part of the ITS1 (site 2), was detected with the ETS1 and 5′-ITS1 probes. The complementary fragment generated by cleavage at site 2, namely the 32S pre-rRNA, was revealed by the ITS2 probe, along with the 12S pre-rRNA, which contains the 5.8S rRNA and the ITS2. A species migrating between the 45S/45S′ and the 30S pre-rRNAs did not contain the ETS1, but showed up with both probes complementary to the ITS1 and to the ITS2, as expected for the 41S pre-rRNA.

Two other major 18S rRNA precursors were detected with the 5′-ITS1 probe, but not with the ETS1- or ITS2-specific probes, indicating that they corresponded to precursors downstream of cleavage at sites 1 and 2. The slowest migrating precursor ran above the 18S rRNA, and corresponded to the 21S pre-rRNA, which is thought to result from cleavage at sites 1 and 2. In addition, we detected a new pre-18S species, which was as abundant as the 21S pre-rRNA and migrated with an electrophoretic mobility close to that of the mature 18S RNA. Treatment of the cells for 1 h with 40 ng/ml actinomycin D, which inhibits RNA polymerase I, resulted in the disappearance of this band as well as the other bands detected with the ITS1 probe, confirming that they all corresponded to pre-rRNAs (Figure 2B). As seen in Figure 2C, this small precursor was detected with a probe complementary to the first 20 nucleotides of the ITS1 and

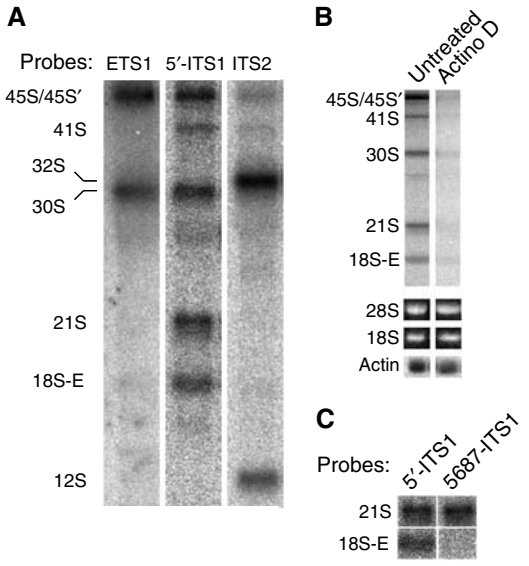
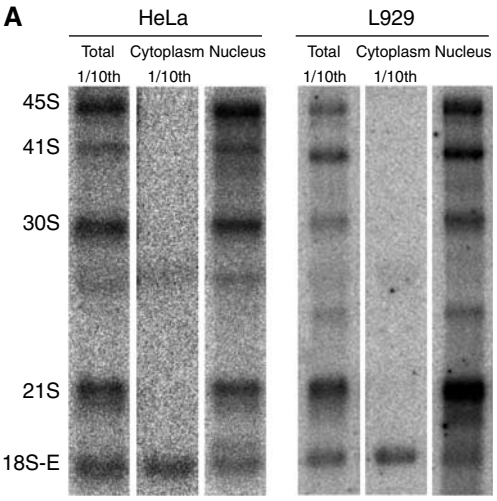


Figure 2 A new late precursor to the 18S rRNA in HeLa cells, the 18S-E pre-rRNA. (**A**) Northern blot analysis of total RNA extracts from HeLa cells. Northern blots probed with oligonucleotides complementary to the ETS1, the 5′ of the ITS1 (5′-ITS1) and the ITS2. The 5′-ITS1 probe hybridizes with a rapidly migrating precursor of the 18S rRNA, which we called 18S-E. (**B**) Synthesis of the 18S-E pre-rRNA is sensitive to treatment with actinomycin D. Analysis by Northern blot (probes: 5′-ITS1 and actin) and ethidium bromide staining (18S and 28S RNAs) of total RNA extracts from HeLa cells treated for 1 h with 40 ng/ml actinomycin D. (**C**) Northern blot analysis of total RNA extracts from HeLa cells probed with the 5′-ITS1 and 5687-ITS1 oligonucleotides.

spanning seven nucleotides of the 18S, but not with a probe hybridizing with nucleotides 159–183 of the ITS1 (probe ITS1-5687). We refined this data by performing 3′-RACE experiments on total RNAs and found a major cleavage site around position 24 in the ITS1 (Supplementary data). The intensity of this band did not parallel that of the 18S rRNA on Northern blots, as seen in the presence of actinomycin (Figure 2B) or after separation on polysome gradients (see Figure 3C); we can therefore exclude cross-hybridization of the 5′-ITS1 probe with the 18S rRNA on Northern blots. We named this uncharacterized RNA '18S-E', for extended and exported (see below).

18S rRNA precursors are exported from the nucleus in human and murine cells

To obtain further insight into the location of the 18S precursors in HeLa cells, we prepared nuclear and cytoplasmic fractions of these cells and analyzed their rRNA content by Northern blotting. As seen in Figure 3A, all the previously characterized 18S precursors were found exclusively in the nucleus, including the 21S pre-rRNA. Strikingly, the 18S-E species was unambiguously detected in the cytoplasm. Using quantitative phosphorimager analysis (Figure 3B), we estimated that the cytoplasm contained 70–80% of the 18S-E pre-rRNA, which indicates that it is efficiently exported from the nucleus. Under the same conditions, 5–8% of the signals detected with probes to the mature 18S or 28S rRNA was found in the nuclear fraction. Upon inhibition of rRNA synthesis in the nucleus with actinomycin D, this number dropped to 3%, which may be considered as an estimate of the contamination of the nuclear fraction with cytoplasmic ribosomes.



B

Cell type	RNA	Cytoplasmic fraction(%) (mean ± s.d.)	Nuclear fraction(%) (mean ± s.d.)	*n*
HeLa	18S-E	76.8 ± 5.2	23.2 ± 5.2	6
L929	18S-E	84.8 ± 5.7	15.2 ± 5.7	4
HeLa	18S	93.1 ± 0.8	6.9 ± 0.8	4
	28S	94.0 ± 1.0	6.0 ± 1.0	4
HeLa + Actino D	18S	96.9	3.1	1
	28S	96.9	3.1	1

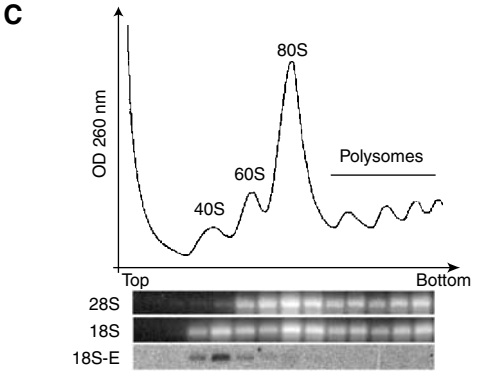


Figure 3 The 18S-E pre-rRNA is mostly located in the cytoplasm and does not participate in translation. (**A**) Northern blot analysis of total, cytoplasmic and nuclear RNA extracts from human HeLa cells and murine L929 cells shows localization of the 18S-E pre-rRNA to the cytoplasm. Hybridization was performed with probes complementary to the 5′-most nucleotides of the ITS1 and specific to each species. Only 1/10th of the total and cytoplasmic extracts was loaded on the gel. (**B**) Intracellular distribution of the 18S-E, 18S and 28S RNAs under various conditions was quantified on Northern blots by phosphorimaging. *n*: number of experiments. (**C**) Ribosome/polysome separation on sucrose gradient and Northern blot analysis of the fractions. HeLa cell cytoplasmic extracts were centrifuged on a 10–50% sucrose gradient. The 18S and 28S rRNAs were detected by ethidium bromide staining and the 18S-E species was revealed by Northern blotting with the 5′-ITS1 probe. The 18S-E RNA is found with the free 40S subunits.

We extended this observation to another mammalian species by repeating this experiment with murine L929 cells. As shown in Figure 3A, a probe directed to the

5′-most nucleotides of the murine ITS1 detected rRNA precursors in L929 total RNA migrating as in HeLa cells, including the 18S-E species. In addition, upon cell fractionation, this species was also found to be cytoplasmic in L929 cells; up to 90% of 18S-E rRNA was found in the cytoplasmic fraction (Figure 3B).

To more precisely establish the fate of the 18S-E species in the cytoplasm of HeLa cells, we next separated the cytoplasmic ribosomal particles on a sucrose gradient. Detection of the mature 18S and 28S rRNAs in the fractions clearly indicated the positions of the free 40S and 60S subunits, the 80S ribosome and the polysomes (Figure 3C). Hybridization with the 5′-ITS1 probe showed that the 18S-E species was restricted to particles migrating in the gradient with the 40S subunits, and was not incorporated into the polysomes, as expected for precursors to the 40S particles. These results confirm that the 18S-E species is exported in ~40S particles, and strongly suggest that it does not participate in translation.

Maturation of the 18S rRNA involves a cytoplasmic protein

The results above indicate that the 18S-E RNA is transported to the cytoplasm in pre-40S particles. In *S. cerevisiae*, cleavage at the 3′ end of the 18S rRNA takes place in the cytoplasm and requires several proteins associated with the pre-40S particle. We hypothesized that the processing of the 18S-E species in HeLa cells was an orthologous process involving the mammalian counterparts of these proteins. If this hypothesis is true, loss of function of one of these proteins should induce the accumulation of 18S-E RNA in the cytoplasm, without affecting the relative levels of the earlier 18S rRNA precursors, whereas the amount of 18S rRNA should decrease.

One of the best characterized proteins involved in the cytoplasmic maturation of the 18S rRNA in *S. cerevisiae* is Rio2p (*Sc*Rio2p), a protein kinase found in late pre-40S particles (Vanrobays *et al*, 2003). The human predicted counterpart (hRio2) displays 58% sequence similarity with *Sc*Rio2p over 419 amino acids and contains an additional C-terminal sequence. Rio2p in yeast is mostly detected in the cytoplasm and is exported from the nucleus via a Crm1p-dependent mechanism (Vanrobays *et al*, 2003; Leger-Silvestre *et al*, 2004). As shown in Figure 4A, cells expressing a cDNA encoding this protein fused to the green fluorescent protein (EGFP) displayed a fluorescent signal restricted to the cytoplasm when observed by confocal microscopy. There was no evidence of the presence of hRio2 in the nucleoli. Consistently, hRio2-EGFP was only detected in the cytoplasmic fraction by Western blot (Figure 4B). Upon treatment with leptomycin B (LMB), a drug inhibiting the exportin Crm1, hRio2-GFP shifted to the nucleus (Figure 4C), which indicates that it is actively exported from the nucleus by a Crm1-dependent mechanism, as previously observed for *Sc*Rio2p (Vanrobays *et al*, 2003). Nuclear export of hRio2 does not depend on association with pre-40S particles in the nucleus, since it was not perturbed upon inhibition of ribosomal biogenesis by actinomycin D (Figure 4C). As expected from a factor associated to pre-40S particles, a fraction of hRio2-EGFP cosedimented with free (pre-) 40S subunits in a sucrose gradient (Figure 4D). These results demonstrate that hRio2 is a cytoplasmic protein which is actively exported out of the nucleus through a Crm1-dependent mechanism, and suggest that it is associated with pre-40S particles.

We designed siRNAs targeting the hRIO2 mRNA. These siRNAs efficiently and specifically affected the stability of the endogenous hRio2 mRNA and inhibited the synthesis of hRio2-EGFP (Supplementary data). Northern blot analysis of total RNA with the 5′-ITS1 probe 72 h post-transfection with Rio2 siRNAs showed the expected phenotype, an increase in the 18S-E signal relative to that of the earlier precursors or of the 28S rRNA (Figure 5A). In parallel, the 18S/28S molar ratio, as measured from agarose gel stained with ethidium bromide or Northern blots, was found to drop by ~50%, reflecting a significant inhibition of the production of the 18S rRNA. Analysis of the intensity profiles on the autoradiography (normalized to the amount of 28S rRNA) indicated that accumulation of the 18S-E species was the major perturbation of pre-rRNA processing (Figure 5A). We also noticed a moderate accumulation of the 45S/45S′ precursor and of an undescribed species migrating just below the 28S rRNA, which we called '26S'. Increase in the levels of these precursors indicate retardation in processing of the 5′-ETS (see below the characterization of the 26S species, Figure 7). A similar phenotype was also observed in yeast upon depletion of Rio2p, and may be secondary to the strong defect in 40S subunit synthesis induced by Rio2 depletion (Vanrobays *et al*, 2003).

Consistent with a defect in the production of the 40S subunit, analysis of the translation machinery on sucrose gradients showed a decrease in the level of both free 40S subunits and 80S assembled ribosomes, together with a large buildup in the amount of free 60S particles (Figure 5B). Upon Northern blotting, the accumulating 18S-E pre-rRNA was mostly detected in the fractions corresponding to free (pre-) 40S subunits. A small part of it was also found in the 80S fraction, suggesting that, when accumulating in high amounts in the cytoplasm, these pre-40S particles could assemble with the 60S subunits into monosomes.

We next performed *in situ* hybridization with the 5′-ITS1 probe conjugated to Cy3 (Figure 5C). In untreated HeLa cells, we observed a strong labeling of the nucleolus and a weak signal in the cytoplasm, consistent with our cell fractionation data showing the presence of 18S rRNA precursors both in the nucleus and in the cytoplasm. Treatment with the siRNAs resulted in a marked increase in the cytoplasmic signal, as expected from the accumulation of 18S-E pre-rRNA in this compartment. To a lower extent, fluorescence in the nucleoli of hRIO2 siRNA-treated cells was more intense than in control cells, consistent with the higher levels of 45S/45S′ and 26S precursors observed by Northern blot. In parallel, the signal observed with a Cy5-conjugated probe complementary to the U2 snRNA decreased in cells treated with hRio2 siRNAs, indicating that the increase of the fluorescence observed with the 5′-ITS1 probe was not a mere artefact of the FISH procedure. A similar phenotype was found with two other siRNAs targeted against hRIO2 (not shown). After cell fractionation, more than 90% of the 18S-E RNA in hRio2-depleted cells was found in the cytoplasmic fraction (Figure 5D). The 3′-end of the 18S-E pre-rRNA accumulating under these conditions was identical to that observed in untreated cells when mapped by 3′ RACE analysis (Supplementary data).

This set of data is consistent with the 18S-E pre-RNA being a *bona fide* precursor to the 18S rRNA, whose conversion to mature rRNA takes place in the cytoplasm. This processing step involves the human structural homolog of *Sc*Rio2p,

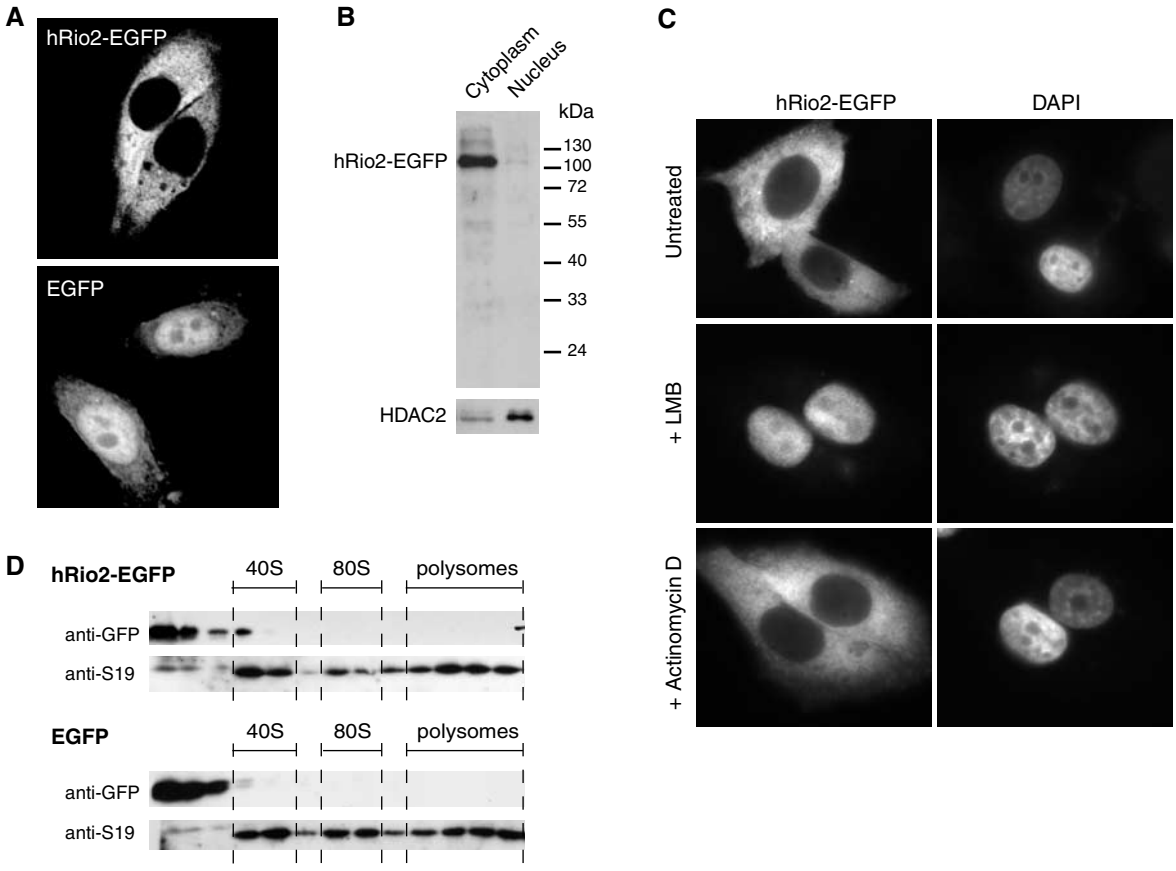


Figure 4 Localization of hRio2 in HeLa cells. (**A**) Visualization of HeLa cells expressing Rio2-EGFP and EGFP by laser-scanning confocal microscopy. (**B**) Western blot of the nucleus and cytoplasm fractions probed with anti-GFP antibodies shows that hRio2-GFP is a cytoplasmic protein. As a control, the histone deacetylase HDAC2 is detected in the nuclear fraction. (**C**) hRio2-GFP is actively exported from the nucleus through a mechanism depending on Crm1, but not on preribosome synthesis. At 48 h after transfection with the hRio2-EGFP expression vector, cells were treated with 10 nM LMB for 2 h, or with 0.04 μg/ml actinomycin D for 1 h. (**D**) A fraction of hRio2-GFP cosediments with (pre-)40S particles on a sucrose gradient. Lysates from cells expressing hRio2-EGFP or EGFP were fractionated by ultracentrifugation on sucrose gradient as in Figure 3B. Fractions were analyzed by SDS–PAGE and Western blot with antibodies against GFP and against S19, a protein of the 40S subunit which is used as a marker of the free (pre-)40S subunits, the 80S monosomes and the polysomes. Note that expression of EGFP was much higher than that of hRio2-EGFP.

which is located to the cytoplasm and appears to be the true functional homolog of the yeast protein.

Nuclear export of the pre-40S particles in mammalian cells requires the ribosomal protein Rps15

We next moved on to the identification of molecular determinants of the nuclear export of pre-40S particles in HeLa cells. A defect in the nuclear export of the pre-40S particles should translate into retention of the 18S-E RNA in the nucleoplasm together with its disappearance from the cytoplasm, which can be assessed by FISH or by Northern blot analysis of subcellular fractions.

Our work on yeast has recently led us to identify the ribosomal protein Rps15 as a new factor required for nuclear export of the pre-40S particles (Leger-Silvestre *et al*, 2004). The high degree of homology of this protein between *S. cerevisiae* and mammals suggests that its function in preribosomal transport may be conserved. To test this hypothesis, we used siRNAs to block Rps15 synthesis and, consequently, its assembly into preribosomes. The siRNAs effectively downregulated Rps15 mRNA without affecting the mRNAs encoding other ribosomal proteins, including Rps19 (Figure 6A) and Rps17 (not shown). Analysis of ribosomes on sucrose gradients 48 h after transfection showed a profile clearly corresponding to a defect in the production of the small subunits, with the complete disappearance of the peak corresponding to the 40S subunit (Figure 6A). Consistent with these observations, electrophoretic analysis of the gradient fractions (which are prepared from cytoplasmic extracts) showed a dramatic decrease of the levels of 18S and 18S-E RNAs, whereas the amount of 28S rRNA remained unaffected (Figure 6A). In contrast, the 18S-E pre-rRNA was more abundant in siRNA-treated cells as compared to control cells, when detected by Northern blot analysis of total RNAs, which includes nuclear RNAs (Figure 6B). Indeed, Northern blot analysis of subcellular fractions showed a conspicuous redistribution of the 18S-E pre-rRNA into the nuclear fraction (Figure 6C). When these cells were submitted to FISH with the 5′-ITS1 probe, the pre-40S particles appeared to be trapped in the nucleoplasm, which was clearly labeled, with almost no signal being detected in the cytoplasm (Figure 6D). This defect in nuclear export was not paralleled by strong alterations in the early steps of pre-rRNA processing, except for a moderate increase in the level of the 45S/45S′ precursor, as seen on Northern blot (Figure 6B). Taken together, these results indicate that Rps15 is not required for pre-rRNA processing upstream of the 18S-E species, but is

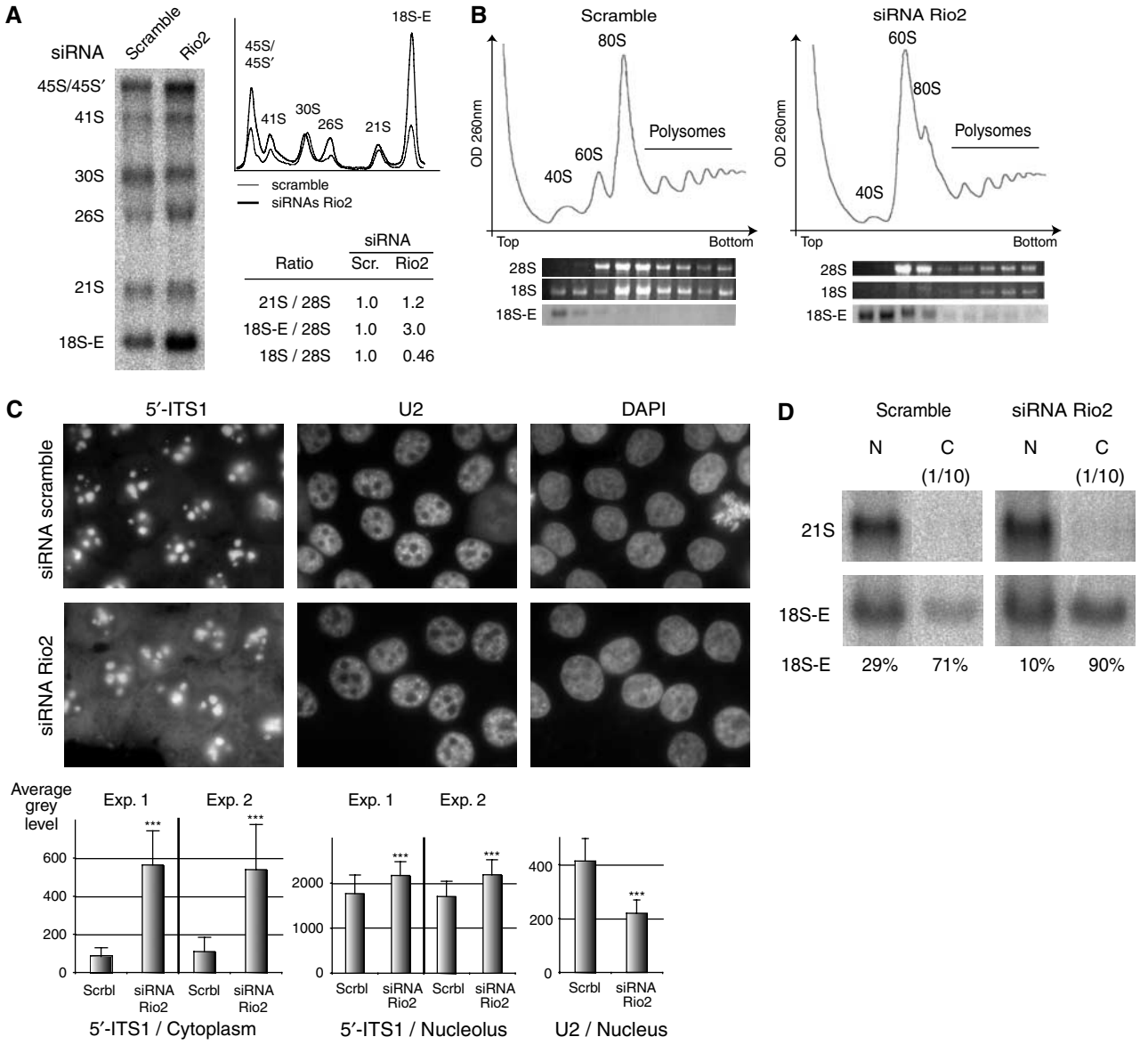


Figure 5 Analysis of pre-rRNA processing in HeLa cells after knockdown of hRio2 expression with siRNAs. (**A**) Northern blot analysis with the 5′-ITS1 probe of total RNAs shows accumulation of the 18S-E pre-rRNA in HeLa cells treated with hRio2 siRNAs. A scrambled siRNA with no defined target was used as a control. Equal amounts of RNA were loaded in each lane. The intensity profiles were determined by phophorimaging and normalized to the amount of 28S rRNA. The levels of 18S and 28S rRNAs were measured by reprobing the blot with complementary oligonucleotides (not shown). The values of the ratios were arbitrarily set to 1.0 in control cells. (**B**) Polysome analysis of HeLa cells treated with scrambled or hRio2 siRNAs and detection by Northern blot of the 18S-E pre-rRNA. Depletion of hRio2 leads to a strong imbalance between the free 60S and 40S subunits, indicating a defect in 40S subunit production. The 18S and 28S rRNAs were visualized with ethidium bromide. (**C**) FISH with the 5′-ITS1 probe conjugated to Cy3 shows accumulation of 18S-E pre-rRNA in the cytoplasm. In parallel, the U2 snRNA was detected with a Cy5-labeled probe. Quantification of the fluorescence was performed as described in Materials and methods. For the 5′-ITS1 probe, the results of two separate experiments are shown (***$P<0.001$, Student's *t*-test). (**D**) Northern blot analysis of subcellular nuclear and cytoplasmic fractions of HeLa cells with the 5′-ITS1 probe reveals accumulation of the 18S-E pre-rRNA in the cytoplasm upon treatment with hRio2 siRNA for 72 h. Only 1/10th of the cytoplasmic fraction was loaded on the gel. The levels of 18S and 28S were determined on the same blot with specific probes. The subcellular distribution of the 18S-E, 18S and 28S RNAs was quantified by phosphorimaging. N: nucleus; C: cytoplasm.

essential for efficient nuclear export of the pre-40S particles. These experiments were carried out in L929 cells with similar results (data not shown). We conclude that the role of Rps15 in preribosomal trafficking has been conserved in evolution.

Role of Crm1 in maturation and nuclear exit of the pre-40S particles in HeLa cells

We next examined the role of the exportin Crm1 in nuclear exit of the pre-40S particles. Crm1 was proposed to be a nuclear export factor of preribosomes in vertebrates (Thomas and Kutay, 2003; Trotta *et al*, 2003), as in yeast. As shown in Figure 6D, a 2 h treatment of HeLa cells with LMB, a potent inhibitor of Crm1, was sufficient to induce redistribution of the 5′-ITS1 FISH signal from the cytoplasm to the nucleoplasm, with the nucleoli remaining strongly labeled. Northern blot analysis of RNAs after fractionation of cells treated with LMB confirmed redistribution of the 18S-E pre-rRNA from the cytoplasm to the nucleus (Figure 6C),

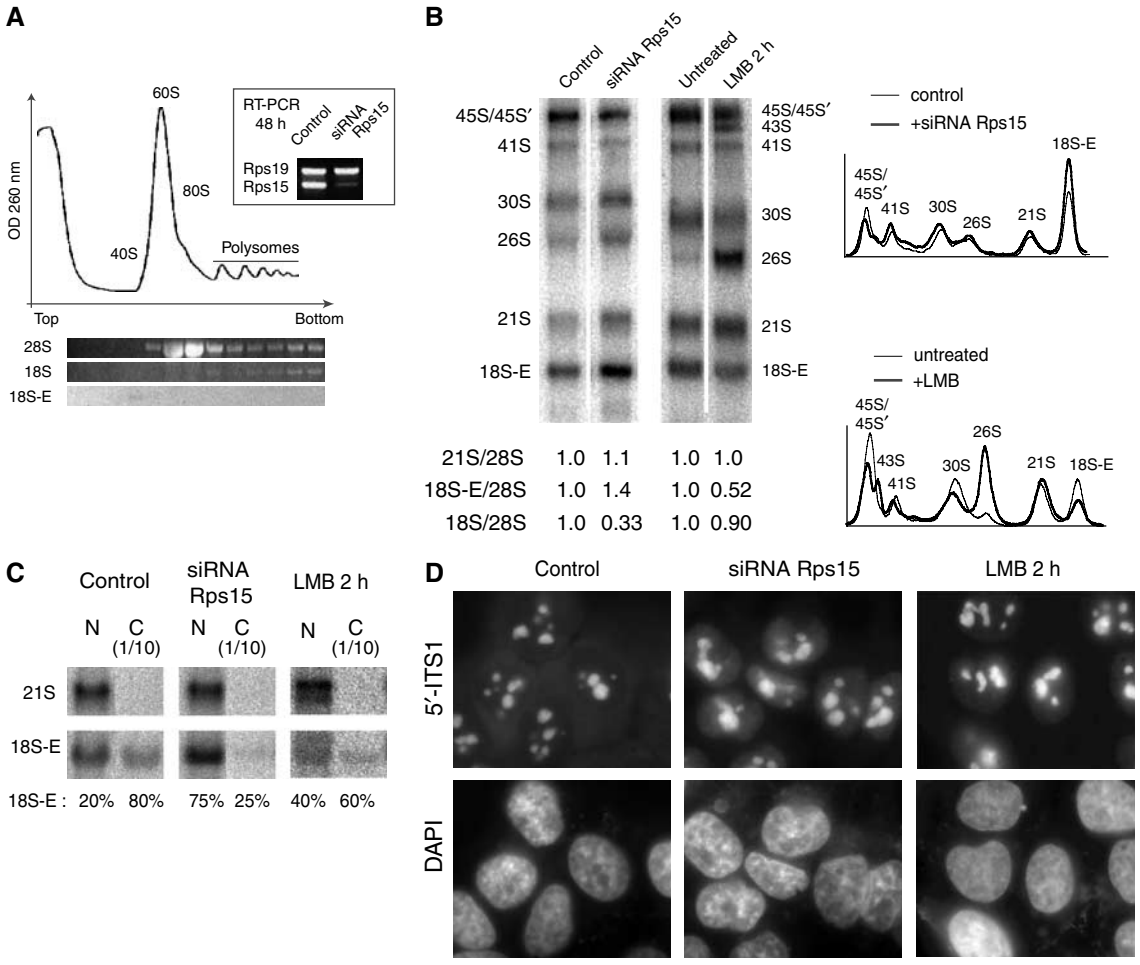


Figure 6 Characterization of nuclear export factors for the pre-40S particles. (**A**) Rps15 siRNAs specifically downregulate the level of Rps15 mRNA and efficiently block the production of the 40S subunit. RT–PCR analysis showed that treatment with Rps15 siRNAs for 48 h strongly affected the level of the Rps15 mRNA, but not that of the Rps19 mRNA (inset). As seen by polysome analysis on sucrose gradients, knockdown of Rps15 expression resulted in the disappearance of the free 40S subunit pool and in a strong increase in the level of free 60S subunits (compare with the control profiles in Figures 3 and 5). The 18S-E pre-rRNA was detected at very low levels in the fractions by Northern blot. The 18S and 28S rRNAs were visualized by ethidium bromide staining. (**B**) Northern blot analysis of total RNAs from HeLa cells treated with siRNAs directed against *h*Rps15 or with LMB. Hybridization performed with the 5′-ITS1 probe. The intensity profiles were established as in Figure 5A. The levels of 18S and 28S rRNAs were measured by reprobing the blot with complementary oligonucleotides (not shown). The values of the ratios were arbitrarily set to 1.0 in control cells. (**C**) Detection by Northern blot of the 18S-E pre-rRNA in subcellular fractions of HeLa cells treated with siRNAs against Rps15 or with LMB. Distribution of the 18S-E pre-rRNA was quantified by phosphorimaging. N: nucleus; C: cytoplasm. (**D**) FISH with the 5′-ITS1 probe on HeLa cells treated with siRNAs against Rps15 or with LMB.

although not as marked as in Rps15-depleted cells. But, more strikingly, Northern blot analysis of pre-rRNAs with the 5′-ITS1 probe showed that, unlike in cells treated with Rps15 siRNAs, pre-rRNA processing was strongly altered in LMB-treated cells (Figure 6B). The level of 18S-E RNA was decreased when compared to untreated cells and two undescribed precursors accumulated: the 26S pre-rRNA, already detected in lower amount in hRio2-depleted cells, and a high-molecular-weight precursor which we called 43S. As expected, the level of 18S rRNA did not significantly vary over the 2-h course of the LMB treatment. These results indicate that Crm1 is necessary, directly or indirectly, for ribosome biogenesis upstream of nuclear export.

Alteration of processing of the 5′-ETS in Crm1-deficient cells

In order to get insight into the alteration of pre-rRNA processing upon loss of function of Crm1, we performed a time-course analysis by metabolic labeling of RNAs with [^{32}P]orthophosphate in cells treated or not with LMB. Total RNAs were then isolated and fractionated by electrophoresis. As seen in Figure 7A after autoradiography, LMB treated cells displayed a deficit in (pre-)rRNA synthesis or stability. Production of the mature rRNAs was strongly reduced, whereas an RNA running just below the 45S pre-rRNA (most probably the 43S species) accumulated as on Northern blots. Variation of the level of the 26S species, which migrates very close to the 28S rRNA, was difficult to assess. This result indicates that inhibition of Crm1 function is detrimental to synthesis or stability of the pre-rRNA and to its processing.

We next characterized the 26S intermediate. Hybridization on Northern blot with various probes showed that its 5′-end was located between positions 1600 and 1732 in the 5′-ETS (Figure 7B). By primer extension, we mapped this extremity around position G1643 (Figure 7C). This cleavage site was

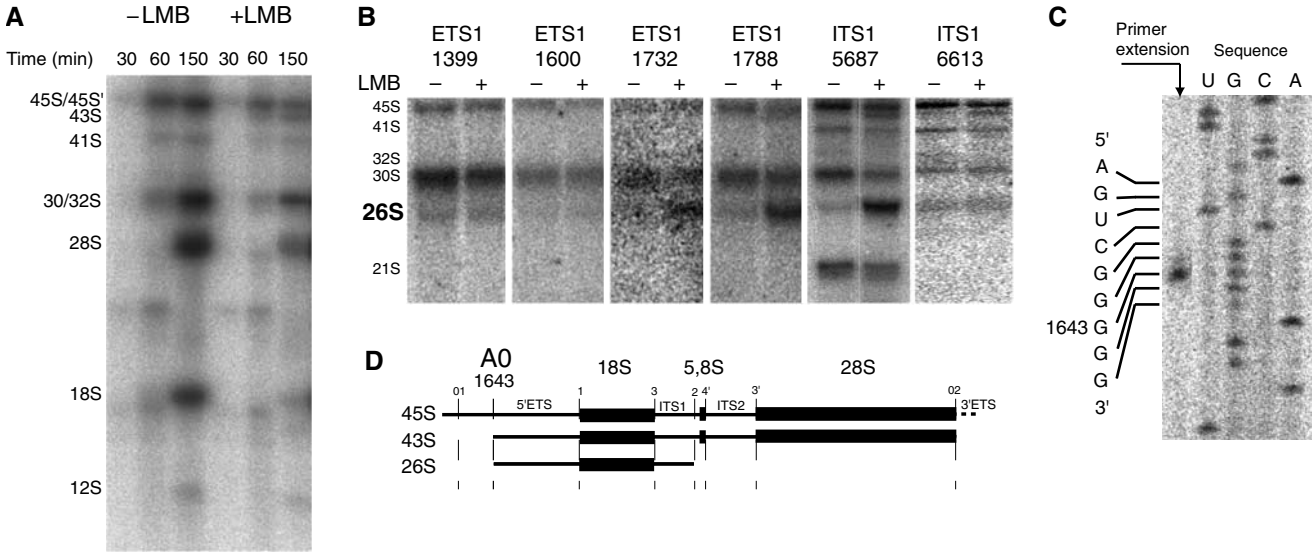


Figure 7 Characterization of the pre-rRNAs accumulating in LMB-treated cells. (**A**) Metabolic labeling of RNAs in HeLa cells with [^{32}P]orthophosphate. Time after addition of [^{32}P]orthophosphate to the medium is indicated (see Materials and methods). Total RNAs were extracted, separated by electrophoresis, and revealed by autoradiography. (**B**) Mapping of the extremities of the 26S RNA with probes complementary to the 5′-ETS (ETS1) and to the ITS1. Comparison of total RNAs from untreated and LMB-treated cells facilitates visualization of the 26S RNA. (**C**) Determination of the 5′-end of the 26S RNA by primer extension. Reverse transcription was performed with oligonucleotide ETS1-1722 on nuclear RNAs from LMB-treated cells. (**D**) Proposed boundaries for the 26S and 43S pre-rRNAs.

not reported before in mammalian cells and may correspond to the A0 site described in *Xenopus* oocyte (Borovjagin and Gerbi, 2001). The 26S RNA migrates in agarose gel with an apparent size of 4900–5000 nucleotides, which suggests that its 3′-end results from cleavage at site 2 in the ITS1. Indeed, it was detected on Northern blot (Figure 7B) with a probe complementary to nucleotides 159–178 in the ITS1 (probe ITS1 5687), but not with a probe spanning the ITS1/5.8S junction (probe ITS1 6613). The 43S species likely corresponds to the precursor of the 26S RNA, before cleavage at site 2. These results indicate that inhibition of Crm1 function strongly affects processing of the 5′-ETS at site 1 (5′-end of the 18S rRNA) and reveal a new cleavage site in the 5′-ETS in human cells.

Discussion

Maturation of the ITS1 in mammals is a multi-step process that ends in the cytoplasm

Our data support the view that, in mammalian cells, processing of the 3′ end of the 18S rRNA is achieved through at least three cleavage steps within the ITS1: one in the 3′ part of the ITS1 (cleavage 2), which yields the 21S pre-rRNA, one in its 5′ part (around nucleotide 24 of the ITS1), which forms the 18S-E pre-rRNA, and, finally, cleavage at the 3′ end of the 18S rRNA. These results echo those of Raziuddin *et al* (1989), who examined the fate of mouse pre-rRNA domains transcribed from plasmids in hamster cells and showed processing of the mouse ITS1 55 nucleotides downstream of the 18S 3′-end. This processing step was reproduced *in vitro* with an endoribonuclease isolated from the nucleoli of Erhlich ascites tumor cells (Eichler and Eales, 1982; Shumard *et al*, 1990). From the cell fractionation experiments presented here, we can infer that the 18S-E pre-rRNA is generated in the nucleus and rapidly exported to the cytoplasm. Consistent with a quick transfer to the cytoplasm of the newly synthesized pre-40S particles, the granular component of the nucleolus, where late preribosomes accumulate before transport to the cytoplasm, has been shown in HeLa cells to contain a low proportion of pre-40S particles relative to pre-60S particles by *in situ* hybridization and electron microscopy (Puvion-Dutilleul *et al*, 1991).

Our data strongly support a model according to which the 18S-E pre-rRNA is matured into 18S rRNA in the cytoplasm. Consistent with this last conversion step taking place after nuclear export, we show that a cytoplasmic protein (hRio2) is required for production of the 18S rRNA. In yeast, Rio2p associates late with the pre-40S particles and is located in the cytoplasm. Depletion of this protein leads to cytoplasmic accumulation of pre-40S particles containing 20S pre-rRNA, the last precursor to the 18S rRNA, which is extended at its 3′ end by a stretch of the ITS1. Similarly, our data show that knockdown of hRio2 by siRNAs results in an increase in the 18S-E pre-rRNA level in the cytoplasm of human HeLa cells, paralleled by a strong decrease in the amount of mature 40S subunits. Thus, a cytoplasmic protein in human cells is involved in a major pathway of 18S rRNA synthesis. Like the 20S pre-rRNA in *S. cerevisiae*, the 18S-E pre-rRNA in mammals is the only pre-18S RNA found in the cytoplasm, and thus the only potential substrate for the processing step that involves hRio2. These results strongly argue for a role of hRio2 in the conversion of the 18S-E pre-rRNA to 18S rRNA in the cytoplasm (Figure 8). Our data do not exclude, however, that 18S rRNA is also produced through an alternative pathway in the nucleus.

Interestingly, hRio2, like its yeast homolog, is exported from the nucleus by a Crm1-dependent pathway. One may hypothesize that hRio2 is a shuttling protein which associates with pre-40S particles shortly before they exit nucleus. However, neither the pre-rRNA maturation steps upstream of the 18S-E intermediate nor the translocation of the pre-40S particles to the cytoplasm is affected by depletion of hRio2.

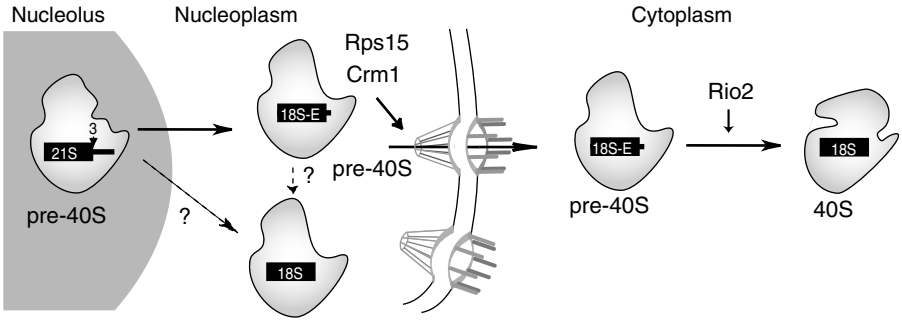


Figure 8 Transport and maturation of the 18S-E pre-rRNA. Crm1 may be implicated in nuclear export of the pre-40S subunits, but is also required for pre-18S rRNA maturation. Nuclear export of the pre-40S subunits requires Rps15, whereas hRio2 is necessary for conversion to mature 40S subunits.

In addition, nuclear export of hRio2 does not require binding to preribosomes (it is still efficient in actinomycin D-treated cells). A role for hRio2 in a nuclear step of ribosome biogenesis remains to be demonstrated. Alternatively, hRio2 may be constitutively excluded from the nucleus by Crm1 to prevent association with pre-40S particles before they reach the cytoplasm, as already discussed for yeast Rio2p (Leger-Silvestre *et al*, 2004).

The ribosomal protein Rps15 is required for nuclear exit of the pre-40S particles

The demonstration that pre-rRNAs are exported to the cytoplasm offers new ways to follow ribosomal transport in mammalian cells by *in situ* hybridization and cell fractionation. Hence, we show that transport of the pre-40S particles requires the ribosomal protein Rps15; knockdown of Rps15 expression does not affect pre-rRNA processing, but causes nuclear retention of the small subunit precursors, which strongly recalls our previous observations in yeast. Rps15 is the homolog of the ribosomal protein S19 in bacteria and archeons, and is located in the head of the small subunit. Conservation of the function of Rps15 between yeast and humans is supported by the high degree of homology of these proteins (60% identity and 73% similarity). Putative roles for Rps15 in rendering the pre-40S particles competent for nuclear export have been discussed elsewhere (Léger-Silvestre *et al*, 2004), including chaperoning pre-rRNA folding, recruiting preribosomal factors or directly mediating interaction with the nuclear export machinery. Rps15 is the only ribosomal protein known to date to be involved in the transport of pre-40S particles to the cytoplasm. Further characterization of the protein composition of preribosomes blocked in the nucleus may bring further insight into the mechanisms of the late maturation steps and the nuclear export of the pre-40S particles in mammals.

Crm1 is required for maturation of the 5′-ETS in HeLa cells

Our data also confirm that pre-40S particles accumulate in the nucleus upon inhibition of Crm1 by LMB (Thomas and Kutay, 2003). However, this treatment also leads to a severe defect in pre-rRNA processing at site 1, the 5′-end of the 18S rRNA. Inhibition of Crm1 leads to accumulation of an undescribed intermediate, which we called 26S. The 26S pre-rRNA is present in low amount in untreated cells and appears to result from cleavage at position G1643 in the 5′-ETS. We propose that this processing site corresponds to the A0 site characterized in budding yeast, trypanosomes and *X. laevis* (Gerbi and Borovjagin, 2004). In these organisms, cleavage at this site is coordinated with processing at sites 1 and 2 (A1 and A2 in yeast) and involves the U3 snoRNA (Venema and Tollervey, 1999; Borovjagin and Gerbi, 2001). The low proportion of 26S pre-rRNA in HeLa cells suggests that this cleavage is closely linked to processing at site 1. Interestingly, treatment with LMB appears to uncouple processing at site 1 from cleavage at site 0. Whether site 2 is correctly processed in LMB-treated cells remains to be clearly established. Mutations in U3 or in U3-associated factors block processing at site 1 and 2, but not at site A0 in yeast and *X. laevis* (Hughes and Ares, 1991; Borovjagin and Gerbi, 2001). Recent results indicate that Crm1 is required for targeting of the U3 snoRNP to the nucleolus in HeLa cells (Boulon *et al*, 2004). Since the U3 snoRNP is involved in the processing of the 5′-ETS, it is tempting to hypothesize that the alteration of the 18S rRNA production seen in LMB-treated cells may result from the mislocalization of U3 to the nucleolus. The observation that Crm1 function is required upstream of nuclear export in human ribosome biogenesis makes it difficult to draw conclusions on the direct involvement of Crm1 in preribosome transport in HeLa cells, since the nuclear export defect observed in LMB-treated cells could be secondary to the pre-rRNA processing failure.

Conclusion

It has been known for a long time that a set of ribosomal proteins associate with the particles in the cytoplasm of mammals, completing the assembly of the ribosome subunits (Hadjiolov, 1985). The present study establishes that final maturation after nuclear export also includes processing of the pre-18S rRNA. Most, if not all, the factors shown to date to associate with late pre-40S particles in *S. cerevisiae* have mammalian counterparts. The conservation of Rio2's function leads us to hypothesize that these factors perform parallel functions in the production of mammalian 18S rRNA and accompany the precursors from the nucleus to the cytoplasm. The irreversibility of this final cytoplasmic cleavage of the pre-18S RNA, conserved in yeast and mammals, may contribute to the unidirectionality of preribosome translocation through the NPCs in eukaryotes by hampering their reassociation with transport factors. In addition, the necessity of this final step for rendering the small subunit competent for translation would

prevent the activity of these particles in the nucleus, together with other mechanisms like nuclear exclusion of translation factors (Bohnsack *et al*, 2002).

Materials and methods

Cells, reagents, antibodies and probes

HeLa, U2OS and L929 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), penicillin and streptomycin, at 37°C with 5% CO_2. LMB was kindly provided by Dr Barbara Wolff (Novartis), and used at 10 nM for 2 h. Actinomycin D (Sigma) was used at a concentration of 40 ng/ml for 1 h. Antibodies against human S19 were produced by injecting the recombinant protein in rabbit (Eurogentec), the anti-GFP monoclonal antibodies were purchased from Roche Applied Science, and the anti-HDAC1/2 antibody (Transduction Laboratories) was kindly provided by Didier Trouche (LBME-CNRS, Toulouse). The sequences of the probes used for Northern blot and FISH are listed in Table I.

Knockdown of gene expression with siRNAs

The siRNA duplexes were purchased from Eurogentec and designed as follows: RPS15, UCACCUACAAGCCCGUAAAdTdT; hRIO2 (GenBank accession number NM_018343), ACAUGGUGGCUGUAAUAA AdTdT. In some experiments, HeLa cells were transfected with siRNAs using oligofectamine (Invitrogen) according to Legube *et al* (2004). In most experiments, electrotransformation was used with similar results. In this case, the cells were washed once in serum-free DMEM medium (no antibiotics) and resuspended in the same medium at a density of 5×10^7 cells/ml. In all, 200 µl of the cell suspension was added to a 4 mm cuvette (Eurogentec), along with 10 µl of a 100 µM stock solution of siRNA, and incubated on ice for 5 min. The cells were then electrotransformed with a Bio-Rad Gene Pulser (250 V, 960–975 µF), incubated for an additional 5 min on ice, resuspended and added to 20 ml of medium supplemented with 10% FBS and penicillin–streptomycin in a 150-cm^2 flask.

Fluorescence in situ hybridization

Cells grown on coverslips were fixed with 4% paraformaldehyde in PBS, washed twice with PBS and permeabilized for 16 h at 4°C in 70% ethanol. After two washes with 2 × SSC, 10% formamide to rehydrate the cells, hybridization was performed for 3 h at 37°C in a buffer consisting of 10% formamide, 2 × SSC, 0.5 µg/µl tRNA, 10% dextran sulfate, 50 µg/ml BSA and 10 mM ribonucleoside vanadyl complexes containing 0.5 ng/µl of probe. The coverslips were then washed twice with 2 × SSC, 10% formamide and once with PBS, and then mounted in mowiol/DAPI (0.1 µg/ml). Images were captured with a 12-bit CoolSnap ES camera (Roper Scientifics) mounted on a DMRB microscope (Leica) using the Metavue software (Universal Imaging). Fluorescence in the cytoplasm was quantified with Metavue by measuring the mean fluorescence intensity in 10-μm^2 squares randomly distributed over the cells with an average number of three squares per cells. Measures were performed in at least 100 cells per sample. To quantify the signal in the nucleolus (5′-ITS1 probe) or in the nucleus (U2 probe), these compartments were selected by automatic thresholding and the mean fluorescence intensity in each of these domains was measured in at least 80 cells.

Expression of hRio2-EGFP

To construct pEGFP-RIO2, a human RIO2 cDNA was generated by RT–PCR and cloned (*Pst*I; *Bam*HI) in-frame with the sequence encoding EGFP in plasmid pEGFP-C2 (Bioscience Clontech). EGFP alone was expressed from pEGFP-C2. Transfection of HeLa cells was performed by electrotransformation with 20 µg of plasmid, as described above for siRNAs. Fluorescence was observed by laser-scanning confocal microscopy (Leica SP2) at the microscopy facility of the IEFG (IFR 109-CNRS) or by wide-field microscopy as for FISH.

Cell fractionation

After treatment with siRNAs or with drugs as indicated, the cells were washed successively with DMEM, PBS and Buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl). Mechanical disruption was then performed with a Dounce homogenizer in Buffer A with 0.5 mM DTT. After centrifugation at 1000 *g* for 10 min at 4°C, the supernatant was collected and considered as the cytoplasmic fraction. The pellet, containing the nuclei, was washed with 10 mM Tris, pH 7.5, 33 mM $MgCl_2$, 250 mM sucrose. The nuclear fraction was resuspended in 10 mM $MgCl_2$, 250 mM sucrose and further purified by centrifugation for 10 min at 500 *g* on a sucrose cushion (0.5 mM $MgCl_2$, 350 mM sucrose). The nuclei were then lysed in 50 mM Na acetate, pH 5.1, 140 mM NaCl, 0.3% SDS, and the RNAs were isolated with two phenol extractions. Total and cytoplasmic RNAs were prepared with RNAzol according to standard protocols, or with Trizol (Invitrogen).

Analysis of ribosomes by sucrose density gradient centrifugation

Cells were treated 10 min before and during fractionation with 100 µg/ml cycloheximide. Cytoplasmic extracts, prepared as described above, were centrifuged twice for 5 min at 12 000 *g*. The equivalent of 1 mg of proteins was layered on a 10–50% sucrose gradient and centrifuged for 105 min at 40 000 *g* in a SW41 rotor (Beckman). The gradient was then analyzed at $OD_{260\,nm}$ and fractions were collected. The RNAs of each fraction were extracted after the addition of SDS (0.6% final), with two phenol–chloroform extractions.

RNA analysis

Northern blots were performed as described previously (Léger-Silvestre *et al*, 2004). The 3′RACE analysis was adapted from Kiss and Filipowicz (1993). The forward primer used for PCR amplification spanned the 18S/ITS1 junction (CGCGAATTCGATCA TTAACGGAGCCCGGAG). The amplified fragments were subcloned and automatically sequenced. Primer extension was performed according to Beltrame and Tollervey (1992) with primer 1732-ETS1.

Table I Probes used for FISH and RNA analysis

Probe	Sequence
ETS1	GT*GAGCACGACGT*CACCACATCGATCGAAGAT*C
1399-ETS1	CGCTAGAGAAGGCTTTTCTC
1600-ETS1	CACAGTAGGCGAGAGC
1732-ETS1	TCACGCGCCGGACAGAG
1788-ETS1	ACCGGTCACGACTCGGCA
5′-ITS1	CCT*CGCCCTCCGGGCT*CCGTTAATTGAT*C
5687-ITS1	TCTCCCTCCCGAGTTCTCGGCTCT
6613-ITS1	CTAAGAGTCGTACGAGGTCG
ITS2-d/e	GCGCGACGGCGGACGACACCGCGGCGT*C
ITS2-b	CT*GCGAGGGAACCCCCAGCCGCGCA
18S	TT*TACTTCCTCTAGATAGTCAAGTTCGACC
28S	CCCGTTCCCTTGGCTGTGGTTTCGCTAGAT*A
Actin	AGGGATAGCACAGCCTGGATAGCAAC
U2	AT*ACTGATAAGAACAGATACT*ACACTTGATCTTAGCCAT*A
5′-ITS1 (*Mus musculus*)	GCT*CCTCCACAGTCT*CCCGTTAATGAT*C

T* represents C6-amino-modified desoxythymidines, which were conjugated to Cy3 or to Cy5 for FISH.

Metabolic labeling

To label RNAs, HeLa cells were preincubated for 1 h in phosphate-free DMEM supplemented with 5% dialyzed FCS, and then for 1 h with or without LMB (10 ng/μl) in the same medium. [^{32}P]orthophosphate was added to the medium (10 μCi/μl) for 30 min to label the intracellular pool of nucleotides. The medium was then replaced with cold DMEM supplemented with 10% SVF, and cells were immediately collected or further cultured for 30 and 120 min. Total RNAs were extracted with RNAzol, separated by electrophoresis, and revealed by autoradiography.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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