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Nucleolin functions in the first step of ribosomal RNA processing

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The first processing step of precursor ribosomal RNA (pre-rRNA) involves a cleavage within the 5′ external transcribed spacer. This processing requires sequences downstream of the cleavage site which are perfectly conserved among human, mouse and *Xenopus* and also several small nucleolar RNAs (snoRNAs): U3, U14, U17 and E3. In this study, we show that nucleolin, one of the major RNA-binding proteins of the nucleolus, is involved in the early cleavage of pre-rRNA. Nucleolin interacts with the pre-rRNA substrate, and we demonstrate that this interaction is required for the processing reaction *in vitro*. Furthermore, we show that nucleolin interacts with the U3 snoRNP. Increased levels of nucleolin, in the presence of the U3 snoRNA, activate the processing activity of a S100 cell extract. Our results suggest that the interaction of nucleolin with the pre-rRNA substrate might be a limiting step in the primary processing reaction. Nucleolin is the first identified metazoan proteinaceous factor that interacts directly with the rRNA substrate and that is required for the processing reaction. Potential roles for nucleolin in the primary processing reaction and in ribosome biogenesis are discussed.

Keywords: nucleolin/ribosome biogenesis/rRNA processing/small nucleolar RNA

Introduction

Ribosome biogenesis is a complex process which involves the transcription of a large rRNA precursor, its maturation and assembly with ribosomal proteins (Hadjiolov, 1985; Eichler and Craig, 1994). Biogenesis of large amounts of ribosomes that are needed throughout the life of a cell requires an efficient coordination of different steps which take place in the nucleolus. In mammalian cells, rRNA is transcribed as a large precursor of 47S which undergoes multiple post-transcriptional nucleotide modifications (Maden, 1990) and nucleolytic processing steps to yield the mature 18S, 5.8S and 28S rRNA species (Eichler and Craig, 1994).

Two classes of processing events can be distinguished. In the first, the nucleolytic cleavages lead to the formation of the 5′ and 3′ extremities of the mature rRNA species. The second type of cleavages take place within the 5′ and 3′ external transcribed spacers (ETSs). After completion of rRNA transcription, a rapid processing occurs within the 5′ ETS of rRNA and it is followed by cleavages within the 3′ ETS (Miller and Sollner-Webb, 1981; Gurney, 1985).

The first processing step within the 5′ ETS, called the early or primary cleavage of pre-rRNA, has been the most studied. It occurs so fast that, for some time, it was believed to represent the transcription initiation site (Urano *et al.*, 1980; Bach *et al.*, 1981; Miller and Sollner-Webb, 1981). Although this processing does not lead directly to the formation of a mature rRNA species, it is well conserved in several species and can occur at various positions within the 5′ ETS: +650/+657 in mouse (Miller and Sollner-Webb 1981), +414/+419 in human (Kass *et al.*, 1987), +105 in *Xenopus laevis* (Mougey *et al.*, 1993a), +1700 in *Physarum polycephalum* (Blum *et al.*, 1986) and +609 in *Saccharomyces cerevisiae* (Hugues and Ares, 1991). Despite the conservation of this early processing event, its role in ribosome biogenesis is still unknown.

One interesting aspect of this cleavage reaction is that it can be reproduced accurately with an *in vitro* transcribed RNA and a cell extract. Deletional analyses on the mouse pre-rRNA have shown that a minimal RNA which contains five nucleotides upstream and 200 nucleotides downstream of the cleavage site can be processed efficiently (Craig *et al.*, 1987, 1991). Further deletions from the 3′ end of this RNA result in a progressive decrease of the cleavage efficiency (Craig *et al.*, 1987, 1991). Although the ETS nucleotide sequences have greatly diverged, this 200 nucleotide segment downstream of the first cleavage site is 80% conserved between mouse and human (Miller and Sollner-Webb, 1981; Miesfeld and Arnheim, 1982; Kass *et al.*, 1987) and can fold in a large stem–loop structure (Michot and Bachellerie, 1991). Even more strikingly, an 11 nucleotide sequence located at nt +658/+668 in the mouse pre-rRNA is perfectly conserved in mouse, human, *Xenopus borealis* and *X.laevis* (with one mismatch) (Kass *et al.*, 1987). Deletion of these conserved residues abolished the processing reaction, suggesting that they play an as yet undetermined but important role (Kass *et al.*, 1987). While a *X.laevis* pre-rRNA substrate is cleaved efficiently in a mouse cell extract, the converse is not true (Mougey *et al.*, 1993a). This suggests that some factors have been conserved between these two species that might be involved in the recognition of this conserved nucleotide sequence.

Unlike in yeast rRNA processing, for which numerous *trans*-acting factors have been identified by genetic analysis (Maxwell and Fournier, 1995; Tollervey, 1996), much less is known about the processing of higher eukaryotic pre-rRNAs. Processing-competent RNAs assemble in a large complex characterized by a sedimentation coefficient of ~20S (Kass and Sollner-Webb, 1990; Mougey *et al.*, 1993a). UV cross-linking

experiments identified a number of proteins whose identity and implication in the cleavage reaction remain to be determined.

Elegant experiments in *X.laevis* oocytes demonstrated that formation of a structure observed by electron microscopy at the terminal ends of the ribosomal transcripts (terminal balls) observed on Miller's Christmas trees (Miller and Beatty, 1969) was correlated directly to the presence of sequences required for the primary processing activity (Mougey *et al.*, 1993b). This suggests that these terminal balls might represent the primary processing complexes. Since these structures have been observed in all tissues and organisms examined, this further suggests that the primary processing, or the formation of this complex at the 5′ ETS, serves an important function.

Micrococcal nuclease treatment of a processing-competent extract abolishes the cleavage reaction, suggesting that, in addition to proteins, the reaction requires a nucleic acid component (Kass *et al.*, 1990). Several small nucleolar ribonucleoparticles (snoRNPs) are known to be involved in this reaction (Kass *et al.*, 1990; Enright *et al.*, 1996). In particular, the U3 small nucleolar RNA (snoRNA), one of the most abundant snoRNAs, has been found associated with both the pre-rRNA substrate and the cleavage product (Kass *et al.*, 1990). A U3-depleted extract processes an rRNA substrate inefficiently (Kass *et al.*, 1990; Mougey *et al.*, 1993a; Enright *et al.*, 1996), demonstrating that this snoRNA is important for the primary processing. Depletion of other snoRNAs (U14, U17 and E3) also significantly reduces the *in vitro* 5′ ETS processing (Enright *et al.*, 1996). The role of these snoRNAs in the processing reaction is still not understood. The observation that an almost complete depletion of U3 (98%) followed by the depletion of several other snoRNAs does not lower the primary processing activity below a basal level (Kass *et al.*, 1990; Enright *et al.*, 1996) suggests that these snoRNAs could have a stimulatory but not essential role in the processing. To understand this function better, it would be particularly interesting to know how U3 and other snoRNAs implicated in the processing reaction interact with the pre-rRNA, since no evolutionarily conserved complementarity exists between these snoRNAs and the rRNA.

Here we have identified nucleolin as one of the cross-linked proteins which interacts with the RNA substrate. Nucleolin is one of the major phosphoproteins of the nucleolus. We show that the interaction of nucleolin with the RNA substrate is required for the processing reaction *in vitro*. Moreover, increasing the level of nucleolin stimulates the processing activity of the S100 cell extract. This nucleolin-dependent activation requires the U3 snoRNA. Interestingly, we show that the N-terminal domain of nucleolin is required for an interaction with the U3 snoRNP. Nucleolin has been implicated in the regulation of different steps of ribosome biogenesis such as transcription and ribosome assembly. The requirement for an interaction between nucleolin and the pre-rRNA for the primary processing reaction opens up new insights into possible functions for this early cleavage in ribosome biogenesis.

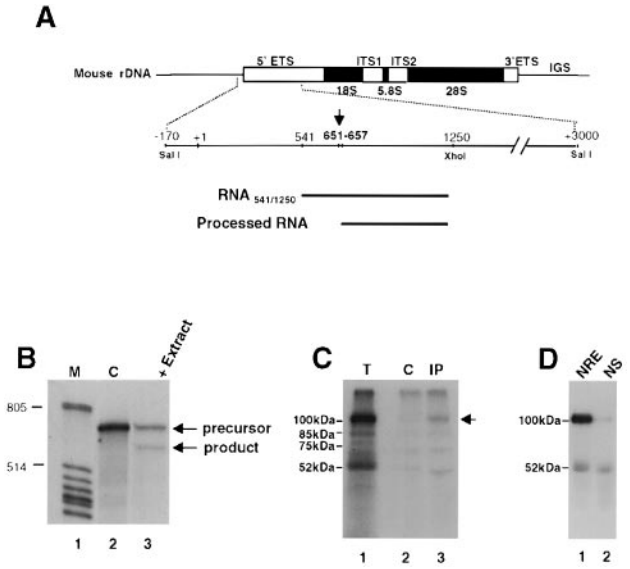

Fig. 1. Nucleolin interacts with an rRNA precursor in processing extracts. (**A**) Schematic representation of the mouse rDNA transcription unit and surrounding spacer regions. The region between –170 and +3000 is enlarged. The arrowhead indicates processing sites at +651 and +657. The T7 RNA polymerase-transcribed substrate $RNA_{541/1250}$ used in our experiments is indicated. (**B**) A standard processing assay is shown. Ten fmol of the radiolabeled $RNA_{541/1250}$ was added to the mouse cell extract (lane 3) and, following a 45 min reaction, RNA was extracted and electrophoretically resolved. In lane 2, the RNA precursor was incubated for the same period without extract. (**C**) Immunoprecipitation of UV cross-linked proteins. Cell extract was incubated for 30 min before the addition of 500 fmol of radiolabeled $RNA_{541/1250}$. After 45 min, samples were UV cross-linked as described in Materials and methods and analyzed by SDS–PAGE (lane 1) or after immunoprecipitation without (lane 2) or with (IP, lane 3) an anti-nucleolin antibody. In lane 3, the 52 kDa labeled protein appears to migrate slightly faster than in lane 1 because of the IgG present in the immunoprecipitation sample which migrates just above this protein. (**D**) Interaction of labeled NRE (lane 1) and NS (lane 2) RNAs with cellular proteins under processing reaction conditions. The NRE is a 68 nucleotide RNA that binds nucleolin with high affinity (Ghisolfi *et al.*, 1996). A single mutation within the consensus selected sequence drastically reduces nucleolin interaction and gives rise to the NS RNA (Serin *et al.*, 1997). RNAs were incubated in the extract for 45 min, then subjected to UV cross-linking performed as described in Materials and methods.

Results

Nucleolin interacts with a processing-competent rRNA substrate

Pre-rRNA undergoes a series of cleavages resulting in the production of mature 18S, 5.8S and 28S species. The first cleavage, called the early or primary processing cleavage, takes place within the 5′ ETS and can be reproduced efficiently in a cell extract system with an *in vitro* transcribed RNA. A radiolabeled RNA corresponding to nucleotides +541 to +1250 of the mouse rRNA ($RNA_{541/1250}$, Figure 1A) was *in vitro* transcribed using T7 RNA polymerase and incubated in a hamster S100 extract. This $RNA_{541/1250}$ is processed relatively efficiently to a 600 nucleotide product (Figure 1B, lane 3) corresponding to a cleavage at position +651/657 of the pre-rRNA. *In vivo* and *in vitro*, the primary processing occurs either at +651 or +657. Because of the low resolution of the analysis system used, only one band could be observed, but primer extension analysis confirmed that both sites

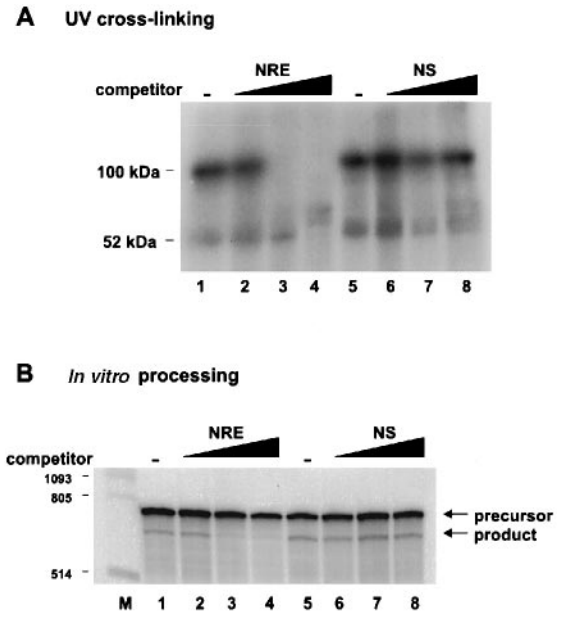

Fig. 2. Interaction of nucleolin with the rRNA substrate is required for the processing activity. Increasing amounts of RNA competitors were added to the cell extract 30 min before the addition of the RNA substrate ($RNA_{541/1250}$). The reaction was then allowed to proceed for 45 min. For each amount of competitor, a cross-linking assay (**A**) and a processing assay (**B**) were performed. In lanes 2–4 and 6–8, the NRE and NS RNAs were used as competitor, respectively. Lanes 1 and 5, no RNA competitor was added; lanes 2 and 6, 2 pmol of RNA competitor; lanes 3 and 7, 6 pmol; lanes 4 and 8, 20 pmol. The amount of nucleolin which is present in the extract for each reaction is ~2.5 pmol (data not shown).

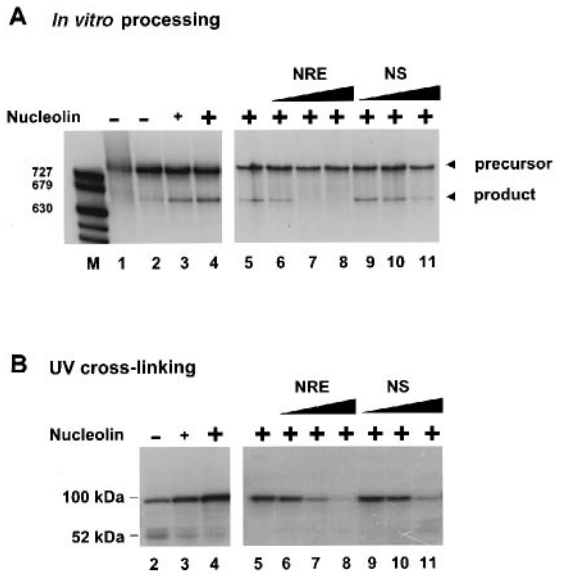

Fig. 3. Nucleolin stimulates the processing activity. (**A**) *In vitro* processing assay. Labeled $RNA_{541/1250}$ was processed in a mouse cell extract that had been pre-incubated without (lane 2) or with 5 (lane 3) or 10 pmol (lane 4) of purified nucleolin. Lane 1 shows the $RNA_{541/1250}$ substrate. In lanes 5–11, 10 pmol of purified nucleolin and increasing amounts of RNA competitors were added to the mouse cell extract. After 30 min of incubation, labeled $RNA_{541/1250}$ was added and incubated for 45 min. The amounts of specific (NRE) or non-specific (NS) RNA competitor added to the reaction are 20 (lanes 6 and 9), 40 (lanes 7 and 10) and 80 pmol (lanes 8 and 11). In lane 5, no competitor was added. (**B**) UV cross-linking experiment. Aliquots of the *in vitro* processing reaction shown in (A) were subjected to UV cross-linking and treated with RNase, and the labeled proteins were analyzed by SDS–PAGE.

were used (data not shown). Cleavage efficiency (20–50% of the input rRNA substrate) varies slightly from one extract to another (see, for example, Figures 1B, 2B, 3A, 4A, 5C and 6A). This might indicate that a limiting component required for the cleavage reaction is present in these different extract preparations. In order to identify proteins involved in the primary processing, $RNA_{541/1250}$ was incubated in the cell extract, then subjected to UV cross-linking. After RNase digestion, cross-linked proteins were revealed by SDS–PAGE. This processing-competent rRNA associates with a defined number of proteins (100, 85, 75 and 52 kDa) (Figure 1C, lane 1) and is consistent with previous cross-linking experiments which used 4-thiouridine-substituted RNA (Kass and Sollner-Webb, 1990). This pattern of cross-linked proteins strikingly resembles the detection of nucleolin and its *in vivo* maturation products by Western blot analysis (Bugler *et al.*, 1982; Bourbon *et al.*, 1983b), and led Kass and Sollner-Webb (1990) to suggest that nucleolin might be the 100 kDa protein which interacts with this RNA. An immunoprecipitation of UV cross-linked proteins with an anti-nucleolin antibody was performed to test this hypothesis (Figure 1C, lane 3). This experiment shows that the anti-nucleolin antibody could precipitate the 100 kDa cross-linked protein, identifying it as nucleolin. The 52 kDa protein was also significantly retained, and may represent either a degradation product of nucleolin or an associated protein which is co-immunoprecipitated. These data establish that nucleolin interacts with this processing-competent rRNA substrate.

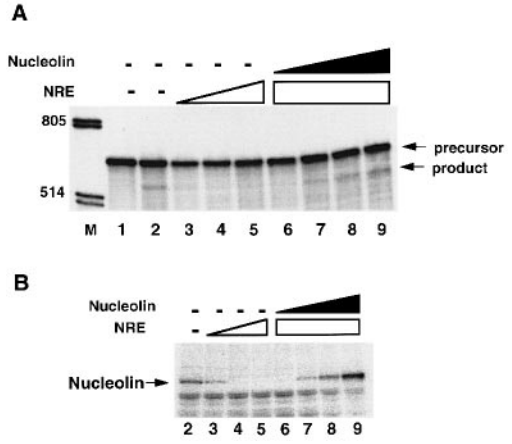

Fig. 4. Exogenous nucleolin can restore the processing activity of an NRE-treated extract. (**A**) *In vitro* processing assay. Ten (lane 3), 20 (lane 4) and 40 pmol (lanes 5–9) of NRE RNA competitor, and 2.5 (lane 6), 5 (lane 7), 10 (lane 8) and 20 pmol (lane 9) of nucleolin were incubated for 15 min in the extract before the addition of labeled precursor $RNA_{541/1250}$. After a 60 min incubation, the reaction was stopped and RNA analyzed on a 6% polyacrylamide gel. (**B**) UV cross-linking experiment. Aliquots of the *in vitro* processing reaction shown in (A) were subjected to UV cross-linking, treated with RNase and the labeled proteins were analyzed by SDS–PAGE.

Nucleolin–rRNA interaction is required for the primary rRNA processing

The RNA-binding properties of nucleolin have been studied extensively (Olson *et al.*, 1983; Bugler *et al.*, 1987; Ghisolfi *et al.*, 1996; Serin *et al.*, 1996, 1997). Up

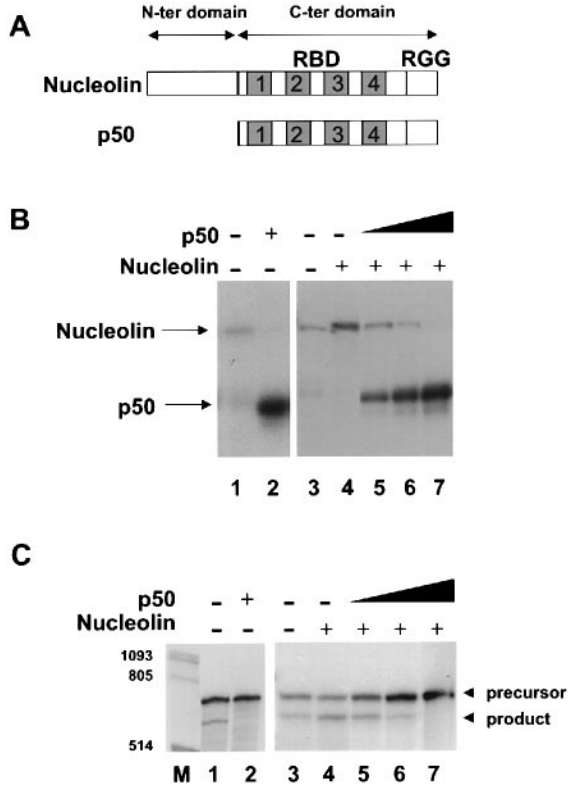

Fig. 5. The N-terminal domain of nucleolin is required for the processing activity. (**A**) Schematic representation of the structure of nucleolin and p50. p50 is a recombinant protein where the N-terminal domain of nucleolin has been deleted. The four RNA-binding domains (RBD 1–4) and the glycine/arginine-rich (RGG) domain of nucleolin are represented. (**B**) UV cross-linking assay. p50 (lane 2, 20 pmol; lane 5, 10 pmol; lane 6, 20 pmol; lane 7, 40 pmol) and 5 pmol of purified nucleolin (lanes 4–7) were incubated for 30 min in the mouse cell extract before the labeled $RNA_{541/1250}$ was added. After the reaction, the samples were UV cross-linked as described in Materials and methods and resolved by SDS–PAGE. (**C**) *In vitro* processing. The same experimental protocol and the same amount of purified proteins were used in this processing assay as in the UV cross-linking assay shown in (B). After the reaction, RNA was extracted and loaded on a 6% denaturing polyacrylamide gel and then autoradiographed.

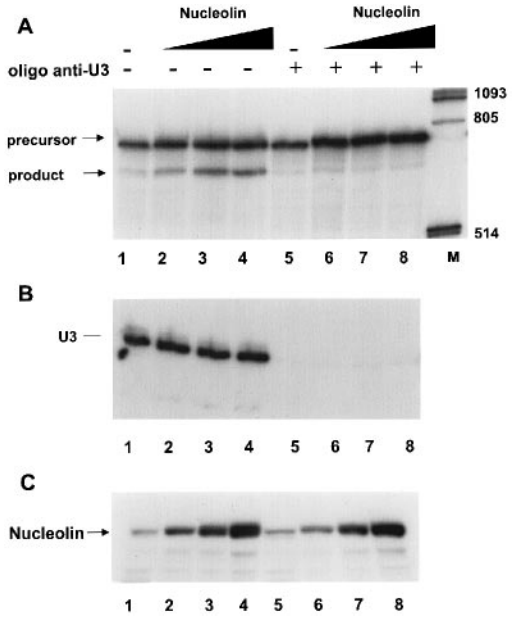

Fig. 6. Nucleolin and U3 are both required for processing activity. (**A**) Processing assay in mouse U3-depleted cell extract. Cell extract was incubated for 30 min in the absence (lanes 1–4) or presence (lanes 5–8) of an oligonucleotide $U3_{64–79}$ complementary to the U3 snoRNA. Half of the reaction was then removed and used for a Northern blot analysis (B). To the remaining reactions, increasing amounts of nucleolin were added and incubated for an additional 30 min. Then, labeled $RNA_{541/1250}$ was added and the reaction was allowed to proceed for 45 min. The amounts of nucleolin added were 5 (lanes 2 and 6), 10 (lanes 3 and 7) and 20 pmol (lanes 4 and 8). (**B**) Northern blot analysis. To verify that U3 snoRNA was depleted in the cell extract, a Northern blot was performed using the labeled $U3_{64–79}$ oligonucleotide as a probe. (**C**) UV cross-linking on the non-depleted (lanes 1–4) or depleted (lanes 5–8) extract with the labeled $RNA_{541/1250}$ was performed in the presence of increasing amounts of nucleolin. Lanes 1–8 correspond to the same lanes as in (A) and (B).

to now, only one nucleolin RNA target constituted by a small stem–loop structure (nucleolin recognition element: NRE, Ghisolfi *et al.*, 1996) has been identified. The first two RNA-binding domains of nucleolin are required for this specific interaction (Bouvet *et al.*, 1997; Serin *et al.*, 1997). To determine if the NRE RNA interacts with nucleolin of the S100 extract, labeled NRE was incubated in the extract then subjected to a UV cross-linking experiment (Figure 1D). As a control, we used an NRE mutant (NS) with a single point mutation within the RNA loop (Serin *et al.*, 1997). This mutant shows a reduced affinity for nucleolin (Bouvet *et al.*, 1997; Serin *et al.*, 1997). The NRE RNA is strongly cross-linked with a 100 kDa protein (lane 1) compared with the NRE mutant (lane 2). The p52 protein previously found cross-linked to $RNA_{541/1250}$ interacts with the same efficiency with the NRE and NS RNAs, suggesting that this is probably an abundant RNA-binding protein which interacts non-specifically with RNA. The simple cross-linking pattern obtained with NRE (compare lanes 1 of Figure 1C and D) and the fact that a single point mutation in this RNA abolishes cross-linking with the p100 suggest that the interaction between this protein and the NRE RNA is highly specific.

To test if the interaction of the 100 kDa protein with the processing-competent substrate $RNA_{541/1250}$ observed in Figure 1C was related to the nucleolin RNA-binding specificity, an increasing amount of competitor RNA (NRE) was added to the processing reaction and an aliquot of this reaction was subjected to UV cross-linking (Figure 2A). Addition of nucleolin RNA target, NRE, results in a loss of the 100 kDa protein cross-link (lanes 2–4), whereas the 52 kDa signal remains mostly unchanged. In the same competitor range, NS RNA is unable to prevent the interaction of the 100 kDa protein with the rRNA substrate. Altogether, these results show that this protein possesses nucleolin RNA-binding specificity, which is in agreement with the immunoprecipitation experiment (Figure 1C) identifying the 100 kDa protein as nucleolin. These results also indicate that it is unlikely that the 52 kDa protein is a nucleolin degradation product which contains nucleolin RNA-binding domains since it is cross-linked with both the NRE and NS RNA (Figure 1D), and its interaction with the $RNA_{541/1250}$ is not competed efficiently by the NRE RNA (Figure 2A).

We next asked whether the interaction of nucleolin with the $RNA_{541/1250}$ substrate was required for the processing reaction. RNA was extracted from the second half of the processing reaction used in Figure 2A and analyzed on a

denaturing gel (Figure 2B). In the presence of an excess of nucleolin RNA target (lanes 2–4), the processing reaction is reduced drastically, whereas in the presence of the non-specific RNA (lanes 6–8), the efficiency of the cleavage reaction is unchanged. These results demonstrate that the interaction of nucleolin with the rRNA substrate is correlated with the ability of the extract to support the cleavage reaction.

Exogenous nucleolin stimulates the primary processing

If the interaction of nucleolin with the pre-rRNA substrate is a limiting step in the primary processing reaction, then addition of an excess of nucleolin to the extract theoretically would stimulate processing. To test this hypothesis, an increasing amount of purified nucleolin protein was added to the extract before the addition of radiolabeled precursor $RNA_{541/1250}$. After the incubation period, analysis of the cleavage efficiency showed that the addition of exogenous nucleolin stimulated the processing reaction (Figure 3A, lanes 2–4). The level of stimulation depends on the basal level of activity present in the extract (see, for example, reactions with other extract preparations in Figures 5C and 6A), but in each case addition of exogenous nucleolin increases the processing activity of the extract (2- to 5-fold). Remarkably, this nucleolin-induced processing activity is correlated with an increased cross-linking of $RNA_{541/1250}$ substrate with nucleolin (Figure 3B, lanes 2–4). The specificity of this nucleolin-dependent activation was again tested with the nucleolin NRE RNA target. In the presence of an increasing amount of NRE, both the nucleolin–$RNA_{541/1250}$ substrate interaction and the processing activity are progressively lost (Figure 3A and B, lanes 6–8) whereas even in the presence of high levels of non-specific RNA competitor (NS), cleavage activity and nucleolin rRNA interaction are still observed (Figure 3A and B, lanes 9–11). In agreement with the results presented above, these data indicate that nucleolin interaction with the precursor rRNA is required for the processing reaction. Quantification of the amount of nucleolin present in the extract (~2.5 pmol, data not shown) indicates that it is in excess relative to the added rRNA substrate. However, most of this nucleolin is engaged in large complexes (>20S, data not shown). Therefore, the 'free' nucleolin available for the interaction with the $RNA_{541/1250}$ is likely to be limiting. The addition of purified nucleolin increases the pool of nucleolin available for the interaction with the rRNA substrate, and thus increases the primary processing reaction of this added RNA.

Exogenous nucleolin can restore the processing activity of an NRE-treated extract

The interaction between the NRE RNA sequence and nucleolin is highly specific *in vitro* (Ghisolfi *et al.*, 1996; Bouvet *et al.*, 1997; Serin *et al.*, 1997), and within the S100 processing extract (Figures 1D, 2 and 3). However, up to now, we could not exclude that the addition of the NRE RNA to the extract titrates a protein other than nucleolin. To demonstrate conclusively that the interaction of nucleolin with the pre-rRNA substrate is required for the processing reaction, we added increasing amounts of purified nucleolin to an NRE-treated extract (Figure 4). As shown previously (Figure 2), addition of increasing amounts of NRE RNA to the extract prevents the cleavage reaction (Figure 4A, lanes 3–5) and the interaction of endogenous nucleolin with the RNA substrate (Figure 4B, lanes 3–5). When increasing amounts of purified nucleolin are added to this processing-deficient extract, the full processing activity of the extract is progressively restored (Figure 4A, lanes 6–9). Addition of an excess of nucleolin is still able to stimulate the processing activity above the efficiency of the untreated extract (Figure 4A, compare lanes 2 and 9). This cleavage activity is again accompanied by an increase of nucleolin cross-linking with the RNA substrate (Figure 4B, lanes 6–9). This addback experiment is in agreement with data shown in Figures 2 and 3 where, in the presence of an excess of nucleolin, more NRE competitor is required for the inhibition of the processing reaction. Altogether, these experiments show that the inhibitory effect of the NRE RNA is related directly to nucleolin and strengthen our previous data (Figures 2 and 3) that the interaction of nucleolin with the pre-rRNA substrate is required for the processing reaction.

Several nucleolin domains are required for its activity

A recombinant protein, p50 (Figure 5A), encoding the four RNA-binding domains and the RGG motif of nucleolin is sufficient to account for the RNA-binding affinity and specificity of the full-length protein (Serin *et al.*, 1997). We therefore tested if this domain was sufficient to stimulate the processing reaction. The p50 protein was added to the cleavage reaction before addition of the $RNA_{541/1250}$ precursor, then half of the sample was subjected to UV cross-linking (Figure 5B, lanes 1–2), and the remaining reaction sample analyzed for the processing activity (Figure 5C, lanes 1–2). As the p50 protein interacts with the rRNA substrate, a decrease of nucleolin cross-linking is observed. An 8-fold excess of p50 over the nucleolin present in the extract is sufficient to prevent nucleolin interaction with the RNA substrate (Figure 5B, lane 2). Remarkably, this decrease of nucleolin binding is perfectly correlated with a decrease of rRNA processing (Figure 5C, lane 2). When exogenous nucleolin is added to the extract at the same time as the p50 protein, the two proteins compete for the interaction with $RNA_{541/1250}$, showing again that the interaction of nucleolin with the rRNA substrate is specific and saturable. As p50 interaction with $RNA_{541/1250}$ increases, a simultaneous decrease of nucleolin–rRNA interaction and of the processing reaction is observed (Figure 5B and C, lanes 3–7). Therefore, these results not only confirm that nucleolin interaction with the rRNA substrate is required for the processing reaction, but also demonstrate that the C-terminal RNA-binding domains of nucleolin are not sufficient to support the processing reaction. The interaction of the p50 protein with the pre-rRNA, by impeding the binding of full-length nucleolin to the substrate, could prevent the correct assembly of the processing complex. The N-terminal end of nucleolin that is characterized by stretches of acidic residues might be required for the interaction of nucleolin with other components of the processing complex.

U3 snoRNA is required for nucleolin activity

The primary processing reaction requires not only the formation of a large protein complex (Kass and Sollner-

Webb, 1990) but also several snoRNAs (Enright *et al.*, 1996). The U3 snoRNA has been shown to be associated with the rRNA processing complex and also to be required for the processing reaction (Kass *et al*., 1990). U3 snoRNA depletion can be achieved easily by oligonucleotide-directed RNase H degradation (Figure 6B, lanes 5–8). This U3-depleted extract shows a reduced rRNA processing activity (Figure 6A, compare lanes 1 and 5; Kass *et al.*, 1990). It recently has been suggested that snoRNPs might be stimulatory but not essential for the processing reaction (Enright *et al.*, 1996). To determine if nucleolin was still able to stimulate the processing activity in the absence of U3 snoRNA, increasing amounts of nucleolin were added to a U3-depleted extract. Although nucleolin interaction with the rRNA substrate is not affected by the absence of the U3 snoRNA (Figure 6C), no processing activation is observed in this depleted extract (Figure 6A, lanes 5–8). Therefore, nucleolin interaction with the pre-rRNA is not sufficient to support the processing reaction. Since nucleolin interacts with the rRNA substrate independently of the presence of U3 snoRNA, one function of nucleolin might be to recruit different factors that are required for the formation of the processing complex or for the stimulation of the cleavage reaction.

Nucleolin interacts with the U3 snoRNP

Apart from nucleolin, which is shown here to be involved in the primary processing reaction, the U3, E3, U14 and U17 snoRNAs are the only other known factors implicated in this maturation process *in vitro*. Although U3 snoRNA can be cross-linked to several regions of the rRNA substrate downstream of the processing site (Maser and Calvet, 1989; Stroke and Weiner, 1989; Beltrame and Tollervey, 1992; Tyc and Steitz, 1992), it is not obvious how the U3 snoRNA interacts with it since no significant evolutionarily conserved complementarities between the U3 snoRNA and the rRNA substrate can be found. It is possible that interaction of U3 snoRNA is either mediated or stabilized by its associated protein, which could interact directly or indirectly with the pre-rRNA substrate. Nucleolin has not been described amongst the proteins forming the U3 snoRNP (Parker and Steitz, 1987; Lübben *et al*., 1993), and we found that nucleolin was not able to interact with an *in vitro* transcribed U3 snoRNA (data not shown). The requirement for both nucleolin and several snoRNAs for the processing reaction prompted us to test if nucleolin could interact with some snoRNPs. Biotinylated nucleolin (biotinylation does not affect the ability of nucleolin to interact with the rRNA substrate and to activate the processing reaction; data not shown) was incubated in the extract for a 30 min period, then nucleolin-associated RNAs were recovered. After 3′ end labeling, they were analyzed on a denaturing polyacrylamide gel (Figure 7A). Comparison of the RNAs recovered on streptavidin beads alone and with nucleolin shows that a 217 nucleotide RNA was specifically retrieved and strongly enriched with nucleolin (compare lanes 2 and 3). When the same experiment was performed with biotinylated p50 (lane 4), the 217 nucleotide RNA was not recovered, and the overall pattern of labeled RNA was identical to what was obtained with the beads alone. From the RNA size, we suspected the 217 nucleotide RNA to represent the U3 snoRNA. Two experiments were performed to identify this RNA unambiguously. RNAs recovered with streptavidin beads alone, nucleolin and p50 were either subjected to an immunoprecipitation with an anti-trimethyl-CAP antibody (anti-TMG) (Figure 7A, lanes 5–7), or analyzed by Northern blotting (Figure 7B, lanes 3–5). Immunoprecipitation with anti-TMG antibody clearly indicates that the 217 nucleotide RNA is trimethylated, as is U3 snoRNA. Northern blotting with a U3 snoRNA-specific probe (Figure 7B) further shows that the U3 snoRNA is recovered specifically with nucleolin and not p50. Other abundant snRNAs (U1, U2, U4 and U5 identified by their size) were pulled down non-specifically with the streptavidin beads (Figure 7A, lanes 2–4) and were also immunoprecipitated with the anti-TMG antibodies (lanes 5–7). Altogether, these experiments show that nucleolin is able to interact with the U3 snoRNP and that this interaction requires the N-terminal end of nucleolin. The U3 snoRNA is almost the only RNA species pulled down with nucleolin, showing that the interaction between nucleolin and the U3 snoRNA is specific. Since nucleolin is still able to interact with the U3 snoRNP in the presence of an excess of the NRE RNA sequence (data not shown) and U3 snoRNA cannot be recovered with the p50 protein which binds to the pre-rRNA substrate, it is unlikely that the interaction between nucleolin and the U3 snoRNP is mediated through an RNA intermediate present in the extract. These experiments suggest rather a direct interaction between the N-terminal domain of nucleolin and one or several U3 snoRNA-associated proteins.

Discussion

In this study, we show that nucleolin, one of the major phosphorylated RNA-binding proteins of the nucleolus, participates in the first processing step of rRNA. Our data demonstrate that the interaction of nucleolin with the rRNA substrate is required for the primary processing reaction (Figures 2–5). The addition of purified nucleolin to a processing-competent extract stimulates the processing reaction (Figure 3). This is the first demonstration that a metazoan proteinaceous factor interacts directly with the rRNA substrate and that it is required for the processing reaction. Obviously, the mere interaction of nucleolin with the pre-rRNA substrate is not sufficient to support this processing, since the C-terminal domain of nucleolin which bears the nucleolin RNA-binding domains interacts with the rRNA substrate but inhibits rather than stimulates the reaction (Figure 5). Furthermore, in the absence of U3 snoRNA, increased levels of nucleolin do not promote the processing reaction (Figure 6).

The U3 snoRNA was the first and best characterized snoRNA shown to be required for the processing *in vitro* (Kass *et al.*, 1990). *In vivo* psoralen cross-linking studies have shown in human (Maser and Calvet, 1989), rat (Stoke and Weiner, 1989) and yeast (Beltrame and Tollervey, 1992) that the U3 snoRNA can be associated with a region of the 5′ ETS including or adjacent to the primary processing site. However, the U3 snoRNP cannot be recovered associated with a minimal RNA substrate encompassing just five nucleotides upstream and ~200 nucleotides downstream of the cleavage site (Kass *et al.*, 1990), suggesting that the stable interaction of U3 snoRNA with the rRNA requires sequences far downstream of the

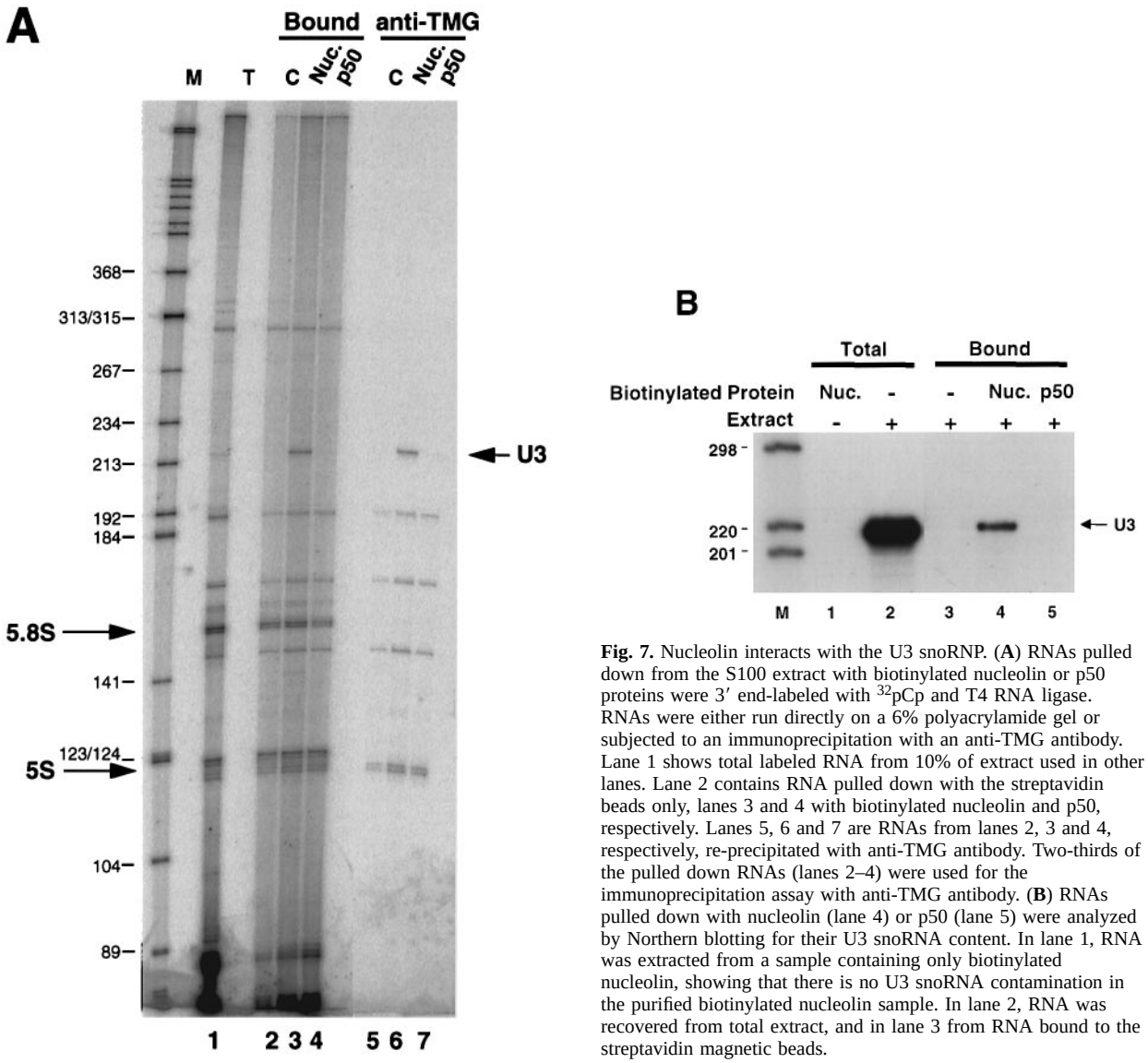

Fig. 7. Nucleolin interacts with the U3 snoRNP. (**A**) RNAs pulled down from the S100 extract with biotinylated nucleolin or p50 proteins were 3′ end-labeled with ^{32}pCp and T4 RNA ligase. RNAs were either run directly on a 6% polyacrylamide gel or subjected to an immunoprecipitation with an anti-TMG antibody. Lane 1 shows total labeled RNA from 10% of extract used in other lanes. Lane 2 contains RNA pulled down with the streptavidin beads only, lanes 3 and 4 with biotinylated nucleolin and p50, respectively. Lanes 5, 6 and 7 are RNAs from lanes 2, 3 and 4, respectively, re-precipitated with anti-TMG antibody. Two-thirds of the pulled down RNAs (lanes 2–4) were used for the immunoprecipitation assay with anti-TMG antibody. (**B**) RNAs pulled down with nucleolin (lane 4) or p50 (lane 5) were analyzed by Northern blotting for their U3 snoRNA content. In lane 1, RNA was extracted from a sample containing only biotinylated nucleolin, showing that there is no U3 snoRNA contamination in the purified biotinylated nucleolin sample. In lane 2, RNA was recovered from total extract, and in lane 3 from RNA bound to the streptavidin magnetic beads.

cleavage site. Because no proteins of the U3 snoRNP have been found to interact with the pre-rRNA substrate (Parker and Steitz, 1987; Kass and Sollner-Webb, 1990), the stable association of this snoRNP with the rRNA is probably mediated through protein–protein interactions. It would be interesting to determine if the interaction between the N-terminal end of nucleolin and the U3 snoRNP (Figure 7) affects the interaction of U3 with the pre-rRNA. More recently, E3, U14 and U17 have also been implicated (Enright *et al.*, 1996). Whether these small RNAs interact directly with the precursor RNA or through their associated proteins remains to be determined.

The number of proteins that can be cross-linked to the rRNA substrate (Figure 1C; Kass and Sollner-Webb, 1990; Mougey *et al.*, 1993a) and the size of the processing complex suggest that this primary processing involves a limited number of factors. The two major cross-linked proteins are p100 (nucleolin) and p52. The other minor proteins could represent cleavage products of nucleolin or non-specific RNA-binding proteins since, when exogenous nucleolin is incubated in the extract, more nucleolin becomes cross-linked whereas the other minor proteins disappear (Figures 3 and 5), suggesting that the interaction of these proteins with the pre-rRNA substrate is not absolutely required for the primary processing reaction. The size of the processing complex (sedimentation coefficient of 20S and a diameter of 250 Å) (Miller and Bakken, 1972; Puvion-Dutilleul *et al.*, 1977; Kass and Sollner-Webb, 1990) suggests the presence of a relatively small number of factors compared with a spliceosome complex.

It has been proposed that in *Xenopus* oocytes, the terminal balls that decorate nascent transcripts on Christmas trees (Miller and Beatty, 1969) represent the processing complex because the same rRNA nucleotides are required both for the *in vitro* processing and for formation of terminal balls (Mougey *et al.*, 1993b). However, since no significant primary processing is observed in *Xenopus* oocytes (Savino and Gerbi, 1991; Mougey *et al.*, 1993a,b), these terminal balls are not likely to represent active processing complexes. Fibrillarin, one loosely associated U3 snoRNP protein, has been detected at the 5′ end of the rRNA, presumably within the terminal balls (Scheer and Benavente, 1990). Whether nucleolin and the U3 snoRNA are also present within these terminal balls remains to be determined. In yeast, a double strand-specific endoribonuclease, Rnt1p, analogous to the bac-

terial RNase III, was shown to be required for rRNA processing at the A_0 site (Abou Elela *et al.*, 1996). This cleavage also requires the U3 snoRNA *in vivo*, but purified recombinant Rnt1p can faithfully reproduce it *in vitro* without other components. The relationship between the yeast A_0 and the mouse primary processing site is not clear. The A_0 site is located in a double-stranded region, whereas the mouse site is in a single-stranded conformation (Craig *et al.*, 1991; Michot and Bachellerie, 1991). Alteration of this single-stranded structure blocks the processing, suggesting that it is recognized by a single strand-specific ribonuclease (Craig *et al.*, 1991). A nucleolar endoribonuclease activity able to cleave the mouse rRNA substrate at nucleotide +650 has been described (Eichler and Eales, 1982; Shumard and Eichler, 1988). The 50–52 kDa protein found within the fraction which contains the endoribonuclease activity might be the 52 kDa protein that we observe in our UV cross-linking experiment. Further purification of this RNase activity and cloning of its gene would be required to determine if this ribonuclease is involved in this processing and if it interacts directly with the rRNA precursor.

How does nucleolin stimulate the primary processing event?

By immunoelectron microscopy on ribosomal transcription units, nucleolin was detected to be associated with nascent rRNA, close to the transcription initiation start point (Ghisolfi *et al.*, 1996). This localization, as well as data from other binding studies, are in agreement with an interaction of nucleolin at a very early step after rRNA transcription (Bourbon *et al.*, 1983b; Herrera and Olson, 1986). Nucleolin possesses four RNA-binding domains, suggesting that it could interact with several RNA targets. Our previous work demonstrated that the first two RNA-binding domains are involved in the recognition of a small RNA stem–loop structure (NRE) (Ghisolfi *et al.*, 1996; Serin *et al.*, 1996, 1997; Bouvet *et al.*, 1997). The RNA-binding specificity of the two last RNA-binding domains remains to be determined. The NRE sequence is an efficient competitor for the interaction of nucleolin with the rRNA substrate (Figures 2–4). However, no typical NRE could be found within the sequence of this rRNA substrate ($RNA_{541/1250}$). Preliminary data show that nucleolin interacts with a specific sequence, different from the NRE motif, downstream of the cleavage site (data not shown). Binding studies with different nucleolin peptides revealed that nucleolin interaction with this sequence requires several RNA-binding domains (unpublished data). Competition of nucleolin binding to the $RNA_{541/1250}$ with the NRE RNA can be explained by the fact that both interactions require the first RNA-binding domain. Further work is in progress to determine the precise mechanism of nucleolin interaction with the pre-rRNA substrate.

Nucleolin interaction with $RNA_{541/1250}$ does not require the U3 snoRNA, and kinetic cross-linking experiments indicate that it is one of the first proteins that interacts with the processing-competent RNA (Kass and Sollner-Webb, 1990). These results suggest that the specific interaction of nucleolin with the rRNA substrate might be the first step of the primary processing event. Nucleolin could then recruit other factors (including the U3 snoRNP) required for the cleavage reaction to form the processing complex visualized as the terminal balls on Miller's Christmas trees. The U3 snoRNA and nucleolin protein are very abundant in the nucleolus; they are present at roughly the same molar concentration (data not shown). Their high concentration supports the idea that these factors participate in the formation of the processing complex (terminal balls) present on all nascent rRNA transcripts. A possible function of nucleolin and its associated factors could be to confer a proper folding to the pre-rRNA, which would then allow site-specific cleavage.

The multiple functions of nucleolin

The identification of nucleolin as one of the factors involved in this processing step sheds new light on the possible regulation and function of the primary processing event. The phosphorylation sites of nucleolin reside exclusively within the N-terminal domain of the protein. Extensive phosphorylation by a casein kinase (CKII) occurs in interphase and by $p34^{cdc2}$ during mitosis (Caizergues-Ferrer *et al.*, 1987; Belenguer *et al.*, 1990; Peter *et al.*, 1990). This suggests that phosphorylation may be a mechanism for regulating nucleolin function during the cell cycle. Correlations have been established between phosphorylation of the protein and its maturation into defined subfragments (Bourbon *et al.*, 1983a; Suzuki *et al.*, 1985; Warrener and Petryshyn, 1991). The cell cycle-dependent phosphorylation of the N-terminal domain of nucleolin may have several consequences for the primary processing activity: (i) it could affect directly the interaction of nucleolin with other components of the processing complex; or (ii) the phosphorylation-dependent maturation of nucleolin could result in the production of a C-terminal domain that is still able to interact with the pre-rRNA substrate but is inefficient in supporting the processing reaction (analogous to the p50 used in this study). The phosphorylation of nucleolin and its consequent maturation could, therefore, greatly affect the processing reaction.

Numerous reports have implicated nucleolin in the regulation of nucleolar structure and transcription (Bouche *et al.*, 1984, 1994; Escande *et al.*, 1985; Suzuki *et al.*, 1985; Egyhazi *et al.*, 1988; Erard *et al.*, 1988; Belenguer *et al.*, 1989; Ohmori *et al.*, 1990; Peter *et al.*, 1990; Kharrat *et al.*, 1991; Yang *et al.*, 1994; Sirri *et al.*, 1995; Bharti *et al.*, 1996; Hanakahi *et al.*, 1997). The nucleolin maturation process was also suggested to be involved in the regulation of rDNA transcription (Bouche *et al.*, 1984; Suzuki *et al.*, 1985).

Our finding that nucleolin is implicated in the first processing step of rRNA does not exclude that it can play a role in other steps of ribosome biogenesis. The involvement of nucleolin in both the transcriptional regulation and processing of the rRNA suggests that these two events might even be linked. This regulatory process would be reminiscent of the coupled transcription–splicing–polyadenylation of mRNAs mediated through interaction of cleavage–polyadenylation factors (CPSF, CstF) and splicing factors with the polII C-terminal domain (Yuryev *et al.*, 1996; Bourquin *et al.*, 1997; Kim *et al.*, 1997; McCracken *et al.*, 1997). This possible coordination between rRNA processing and transcription could be an efficient way for the cell to regulate the production of the large amount of ribosomes needed during the cell's life.

Materials and methods

Plasmid constructs and in vitro RNA transcription

The mouse rDNA fragment (+541 to +1250) which contains the primary processing sites (+651/+657) was PCR amplified using the following oligonucleotides: 5′ETS-541 5′ggaagatcttcgctcgttgtgttctcttg3′ and 5′ETS-1250 5′ggaattcaaactttccaaccccagccgcg3′ (the *Eco*RI and *Bgl*II sites introduced at the 5′ and 3′ ends of the PCR product are underlined). The PCR product was subcloned in pSP72 (Promega) to give $pSPETS_{541–1250}$. The plasmid was linearized by *Eco*RI and used for *in vitro* transcription using T7 RNA polymerase. For the synthesis of radiolabeled RNA, 50 µCi of [α-^{32}P]CTP were included in the transcription reaction. Unincorporated nucleotides were removed by gel filtration (G50, Pharmacia) then the RNA was ethanol precipitated. The 68 nucleotide RNA competitor NRE and NS were synthesized as previously described (Serin *et al.*, 1997).

Preparation of the S100 extracts and in vitro processing

The S100 extract was prepared from exponentially growing L1210 mouse cells as described (Miller and Sollner-Webb, 1981). Processing assays (50 µl) were performed using 5 µl of extract and 10 fmol of rRNA substrate. Proteins, RNA competitor and extract were mixed to achieve a final buffer concentration of 20 mM HEPES pH 7.9, 120 mM KCl, 0.5 mM $MgCl_2$, 10% glycerol, 2 mM dithiothreitol (DTT), 0.05 mM EDTA. After a 30 min pre-incubation at 30°C, radiolabeled $RNA_{541/1250}$ was added and the reaction left for 45 min at 30°C. The reaction was stopped by addition of one volume of 0.3 M NaCl, 100 mM Tris–HCl pH 7.5, 0.6 M sodium acetate pH 5.5, 1% SDS, 1 mM EDTA, 0.2 mg/ml tRNA and 10 µg/ml proteinase K (Sigma), and incubated for 30 min at 37°C. RNA was phenol extracted before being ethanol precipitated and loaded on a 6% denaturing polyacrylamide gel.

UV cross-linking and immunoprecipitation

The extract, purified proteins and RNA competitors were incubated under the same conditions as for the processing assay, except that the amount of radiolabeled $RNA_{541/1250}$ was 500 fmol. After the 45 min reaction at 30°C, samples were UV cross-linked for 2 min (Stratalinker, Stratagene). RNase A (Sigma) was then added at a final concentration of 0.5 µg/µl and incubated at 37°C for 60 min. Samples were then subjected to a 10% SDS–PAGE and autoradiographed.

For the immunoprecipitation experiment, after cross-linking and RNase A digestion, 5 µg of a polyclonal antibody raised against purified nucleolin (Ab 134) was added and incubated for 2 h in 300 µl of RIPA buffer [150 mM NaCl, 50 mM HEPES pH 7.5, 0.1% SDS, 1% Triton, 5 mM EDTA, 0.1% bovine serum albumin (BSA), 50 µg/ml tRNA] at room temperature. Then 15 µl of pre-washed packed beads of protein A–Sepharose (Pharmacia) were added and incubated for 30 min at room temperature. After three washes with RIPA buffer and one with 50 mM HEPES pH 7.5, 150 mM NaCl, 5 mM EDTA, the beads were loaded on a 10% SDS–PAGE; 5–10% of the total cross-linked, and all of the immunoprecipitated proteins were loaded.

U3 snoRNA depletion and Northern blot analysis

The U3 snoRNA was depleted using an antisense oligonucleotide $U3_{64–79}$ (5′GTGGTTTCGGGTGCTC3′) (Kass *et al.*, 1990) and RNase H which is present in the extract. The antisense oligonucleotide (1 µg) was added to 10 µl of S100 cell extract in the following buffer: 20 mM HEPES pH 7.9, 120 mM KCl, 0.5 mM $MgCl_2$, 10% glycerol, 2 mM DTT, 0.05 mM EDTA, and incubated for 5 min at 37°C and 25 min at 30°C. Total RNA was then extracted from half of the reaction for a Northern blot analysis. The other half was used for a processing or UV cross-linking experiment as previously described.

For the Northern blot analysis, RNAs were loaded on a denaturing polyacrylamide gel and transfered to a HYBOND-N^+ membrane (Amersham). The transfered RNA was UV cross-linked to the membrane (Stratalinker, Stratagene), then incubated in the following buffer: 5× SSPE, 5× Denhardt's solution, 0.5% SDS and 20 µg/ml sonicated salmon sperm DNA, for 1 h at 42°C. The labeled $U3_{64–79}$ oligonucleotide (5 pmol) was added and incubated overnight at 42°C. The membrane was washed successively twice in 2× SSPE, 0.1% SDS for 30 min at 42°C, once in 1× SSPE and once in 0.1× SSPE, 0.1% SDS for 30 min each at room temperature before autoradiography.

Purification of nucleolin and p50

Nucleolin was purified from exponentially growing CHO cells (Computer Cell Culture Center, Belgium), and recombinant p50 protein was expressed in *Escherichia coli* and purified as described previously (Ghisolfi *et al.*, 1996; Serin *et al.*, 1997). Biotinylation of these proteins on a cysteine residue was performed using iodoacetyl-LC-biotin according to the manufacturer's instructions (Pierce). Only one cysteine is present in nucleolin and p50 (within RNA-binding domain 3). The level of biotinylation of these proteins was roughly identical, as determined by Western blot analysis.

Nucleolin–U3snoRNP interaction

Biotinylated nucleolin or p50 (20 pmol) was incubated for 1 h at 30°C with 5 µl of S100 cell extract in the following buffer: 20 mM HEPES pH 7.9, 120 mM KCl, 0.5 mM $MgCl_2$, 10% glycerol, 2 mM DTT, 0.05 mM EDTA. Pre-washed (in 20 mM HEPES pH 7.9, 120 mM KCl, 0.5 mM $MgCl_2$, 10% glycerol, 2 mM DTT, 0.05 mM EDTA, 0.1% BSA, 50 µg/ml tRNA) streptavidin magnetic beads (Dynal) were added for 30 min at room temperature. After three washes with the same buffer, bound RNA was recovered and ethanol precipitated. RNA was either loaded directly on a 6% denaturing polyacrylamide gel for a Northern blot analysis, using the labeled $U3_{64–79}$ oligonucleotide as a probe, or 3′ end-labeled with [^{32}P]cytidine 3′, 5′-biphosphate (^{32}pCp)and T4 RNA ligase (Biolabs). A fraction of the 3′ end-labeled RNA was used for an immunoprecipitation with anti-TMG antibodies (generous gift from R.Lührmann). For each immunoprecipitation, 2.5 µg of anti-TMG antibodies were bound to protein A–Sepharose in NET2 buffer (Tris–HCl 50 mM pH 7.5, NaCl 150 mM, NP-40 0.05%). Labeled RNA was then added and incubated for 1 h at 4°C. After five washes with NET2 buffer, RNA was recovered and ethanol precipated.

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