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# Interaction of Nucleolin with an Evolutionarily Conserved Pre-ribosomal RNA Sequence Is Required for the Assembly of the Primary Processing Complex\*

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**The first processing event of the precursor ribosomal RNA (pre-rRNA) takes place within the 5' external transcribed spacer. This primary processing requires conserved *cis*-acting RNA sequence downstream from the cleavage site and several nucleic acids (small nucleolar RNAs) and proteins trans-acting factors including nucleolin, a major nucleolar protein. The specific interaction of nucleolin with the pre-rRNA is required for processing *in vitro*. *Xenopus laevis* and hamster nucleolin interact with the same pre-rRNA site and stimulate the processing activity of a mouse cell extract. A highly conserved 11-nucleotide sequence located 5–6 nucleotides after the processing site is required for the interaction of nucleolin and processing. *In vitro* selection experiments with nucleolin have identified an RNA sequence that contains the UCGA motif present in the 11-nucleotide conserved sequence. The interaction of nucleolin with pre-rRNA is required for the formation of an active processing complex. Our findings demonstrate that nucleolin is a key factor for the assembly and maturation of pre-ribosomal ribonucleoparticles.**

Ribosome biogenesis is a complex process that involves the transcription of a large pre-rRNA<sup>1</sup> precursor, its maturation, and assembly with ribosomal proteins (1, 2). Pre-rRNA undergoes multiple post-transcriptional nucleotide modification (3) and nucleolytic processing steps to yield the mature 18, 5.8, and 28 S rRNA species. The two first endonucleolytic cleavages occur in external transcribed spacers (ETS) and therefore do not lead to the formation of mature rRNA ends. The first cleavage also called early or primary processing occurs within the 5'-ETS (4–6) is conserved from yeast (7) to human (8) and can be found at various positions within the 5'-ETS (5, 7–11).

Despite its conservation, the role of this cleavage in ribosome biogenesis is still unknown, and only few factors involved in this process have been characterized. In yeast, the role of

Rnt1p, an RNase III homologue remains unclear (12, 13). In higher eukaryotes, the major nucleolar protein nucleolin (15), an endonuclease (14) and several small nucleolar RNA are also involved (15, 16), but their exact function remains to be elucidated. *In vitro* UV cross-linking has identified a small number of proteins, including nucleolin, that interact with the RNA substrate (17, 18). The different factors assemble in a large 20 S complex (18) that could be visualized at the terminal ends of nascent pre-rRNA (terminal balls) observed on Miller's Christmas tree by electron microscopy (19, 20). The formation of this complex seems necessary but not sufficient for processing (20, 21).

The sequence and RNA structural motif required for the processing have been extensively studied *in vitro* (21, 22). In mouse pre-rRNA, an evolutionary conserved 11-nt motif (+655 to +666) located just 5–6 nt downstream from the cleavage site is absolutely required for the processing (21) and for formation of the complex observed at the 5' end of nascent pre-rRNA (20). A 27-nt RNA (+645 to +672) is processed very inefficiently *in vitro*, but it seems to contain the minimal *cis*-acting sequence required for processing (21). The 200 nt that follow this motif are 80% conserved between mice and humans (5, 8, 23) and stimulate the processing (21). This RNA sequence can be the targets of several RNA-binding proteins or small nucleolar RNAs for the recognition of the cleavage site.

Nucleolin, one of the major nucleolar proteins in exponentially growing cells, is involved in several steps of ribosome biogenesis (24, 25). Nucleolin binds nascent pre-rRNA close to the transcription initiation site (26, 27), suggesting that nucleolin plays a role at an early step of pre-rRNA synthesis. We have previously shown that nucleolin is able to stimulate the primary processing *in vitro* (17). Specific interactions of nucleolin with the pre-rRNA substrate and with other cellular components like the U3snoRNP are required for the processing.

In this study, we show that an 11-nt evolutionary conserved sequence located just 5–6 nt downstream from the cleavage site is required for the interaction of nucleolin with the pre-rRNA substrate. This interaction is required for the assembly of the processing complex.

## MATERIALS AND METHODS

**Plasmids Constructs and *In Vitro* Transcription**—Mouse rDNA fragments (+541 to +1250 and +645 to +1250) were amplified by PCR using the following oligonucleotides: 5'-ETS-541 (5'-ggaagatcttgcctctgttctctctg-3') or 5'-ETS-645 (5'-ggaagatcttgcctctgttctctctg-3') and 5'-ETS-1250 (5'-ggaattcaacttccaacccagccgcg-3'). Underlined are the *Eco*RI and *Bgl*II sites used for the ligation in pSP72 (Promega) to give pSPETS<sub>541–1250</sub> and pSPETS<sub>645–1250</sub>. The conserved 11-nucleotide motif (ECM) was deleted by PCR. Two PCR reactions were performed with oligonucleotides 5'-ETS-541 and ΔM3A (5'-gagaactcggagcacaagagtgag-

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<sup>1</sup> The abbreviations used are: pre-rRNA, precursor ribosomal RNA; ETS, external transcribed spacer; ECM, evolutionary conserved motif; nt, nucleotide(s); PCR, polymerase chain reaction; GST, glutathione S-transferase; CHO, Chinese hamster ovary; NS, non-specific.

caacgacgacgaatcgg-3') or 5'-ETS-1250 and  $\Delta$ M3B (5'-cgttgctcactcttat-gctcggagttctctctggccaggcc-3'). The last PCR used the first two purified PCR products and oligonucleotides 5'-ETS-541 and 5'-ETS-1250. This DNA fragment was ligated in the *Bgl*III and *Eco*RI sites of pSP72 to give pSPETS<sub>541-1250</sub> $\Delta$ ECM. The *Bgl*III site present in the resulting plasmids was removed by *Bgl*III digestion followed by T4 DNA polymerase exonuclease digestion and religation. The final plasmids were called pSPETS<sub>541-1250</sub> $\Delta$ *Bgl*III, pSPETS<sub>645-1250</sub> $\Delta$ *Bgl*III, and pSPETS<sub>541-1250</sub> $\Delta$ ECM $\Delta$ *Bgl*III. These plasmids were linearized by different enzymes *Dde*I (+656), *Bsp*EI (+677), *Bsp*120I (+764), and *Eco*RI (+1250) and used for *in vitro* transcription using T7 RNA polymerase to give the corresponding RNAs. Labeled RNA was synthesized using [ $\alpha$ -<sup>32</sup>P]CTP in the transcription reaction. Unincorporated nucleotides were removed by gel filtration (G50; Amersham Pharmacia Biotech), and then the RNA was ethanol precipitated. Unlabeled RNA competitors were synthesized as described previously (31).

For the construction of the GST-tagged CHO nucleolin a PCR reaction was performed with oligonucleotides KTNUC1 (5'-cgcgatccgt-gaagctcgaagaggtggcaaac-3') and KTNUC2 (5'-gctctagatcattat-tcaactctgtctctctctgtgg-3'; *Bam*HI and *Xba*I sites are underlined) and the cDNA of CHO nucleolin as template. The PCR product was subcloned in pEG(KG) plasmid (gift from Dr. Deschenes, Iowa City, Iowa) and give the pKTNUCFL plasmid.

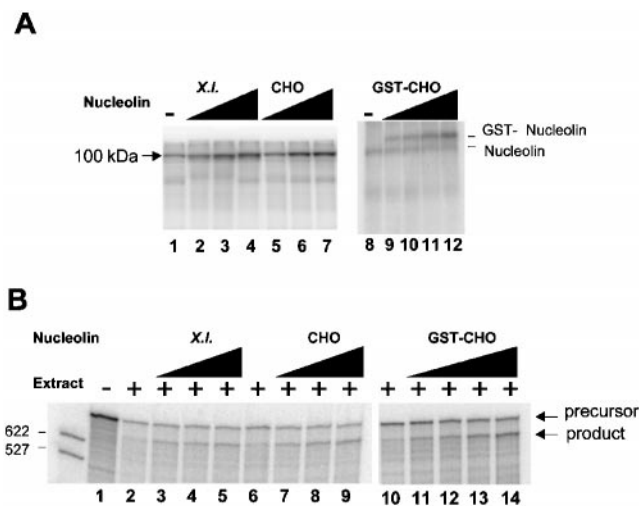
**Preparation of S100 Extracts, Production and Purification of Proteins, *In Vitro* Processing, and UV Cross-linking**—Extract, processing reaction, and UV cross-linking are performed as described previously (17). Hamster and *Xenopus* nucleolin were purified from exponentially CHO (Computer Cell Belgium), and A6 growing cells respectively. Recombinant p50 was expressed in *Escherichia coli* and purified as described previously (31, 32). The GST-tagged CHO nucleolin was expressed in *Saccharomyces cerevisiae* (JCW25 strain) (gift from Dr. Gartenberg, Piscataway, NJ) and purified using a glutathione-Sepharose column (Amersham Pharmacia Biotech) as described previously (39).

**Filter Binding Assay**—Filter binding assay using purified nucleolin protein and *in vitro* labeled RNA were performed as described previously (27, 31). Briefly, 10 fmol of labeled RNA were incubated with different concentration of purified nucleolin in a final volume of 50  $\mu$ l of binding buffer (200 mM KCl, 25 mM Tris, pH 7.5, 5 mM MgCl<sub>2</sub>, 20% glycerol, 50  $\mu$ g/ml tRNA, 10  $\mu$ g/ml bovine serum albumin) for 30 min at room temperature. Then the reaction mixtures were filtered on a nitrocellulose membrane and washed three times with binding buffer. The percentage of bound RNA was determined using a PhosphorImager. Native gel shift assay (see Fig. 5) was performed as described (16, 18). Briefly, after the processing reaction, half of the incubated reaction was directly loaded on a 0.8% TBE agarose gel. After a 5-h migration at 100 V, the gel was dried and autoradiographed.

## RESULTS

***Xenopus* and CHO Nucleolin Stimulates Processing in Mouse Cell Extract**—Although the 200 nt surrounding the cleavage site, known as the M3 box (28), are quite conserved between humans and mice, they have considerably diverged in *Xenopus*. To determine whether nucleolin from *Xenopus* and hamster cells were interchangeable, we tested and compared the interaction of *Xenopus laevis* and CHO nucleolin with the mouse 5'-ETS (Fig. 1A). A labeled RNA corresponding to nucleotides +541 to +1250 (RNA<sub>541/1250</sub>) from the mouse pre-rRNA was incubated in mouse S100 extract in presence of increasing amount of *X. laevis* (lanes 2–4) or CHO (lanes 5–7) nucleolin and subjected to an UV cross-linking experiment. These two proteins interacted with the same efficiency with the pre-rRNA substrate. To demonstrate that the exogenous nucleolin was actually binding to the pre-rRNA substrate and not competing away inhibitory proteins in the extract that would allow more endogenous nucleolin protein binding to the RNA, we used a recombinant GST-tagged protein (GST-CHO nucleolin) in this assay (Fig. 1A, lanes 8–12). This experiment clearly shows that exogenous GST-CHO nucleolin interacts with the mouse pre-rRNA and competes for binding with endogenous nucleolin.

Previous experiments (17) have established a correlation between an increase in specific interaction of nucleolin with the pre-rRNA substrate and an activation of the primary processing. We then determined whether the interaction of *X. laevis*

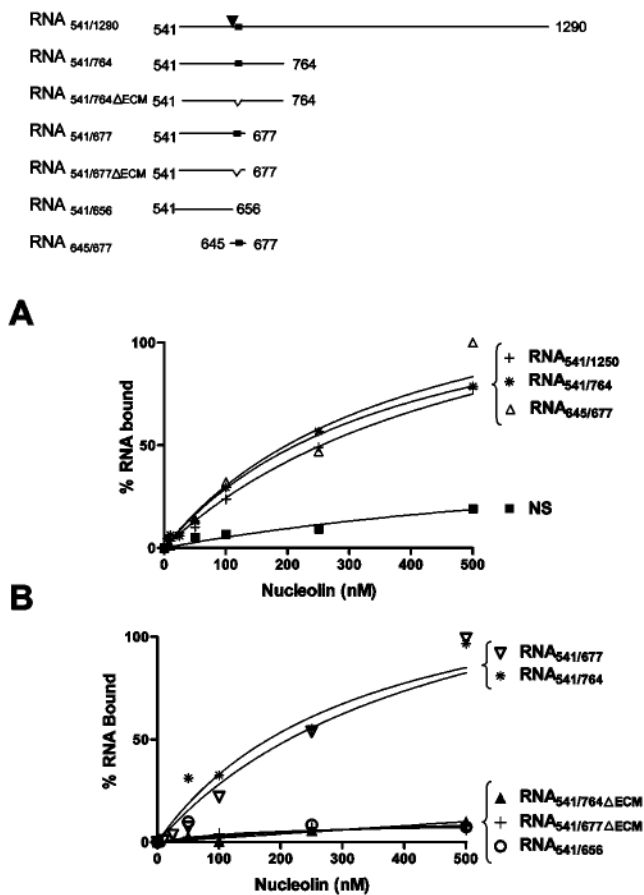


**FIG. 1. *X. laevis* and hamster nucleolin are interchangeable for the processing reaction in mouse cell extract.** A, increasing amount of purified *X. laevis* (X.l., lanes 2–4), CHO (lanes 5–7) or recombinant GST-CHO nucleolin (lanes 8–12) were added to the processing reaction that contained 10 fmol of radiolabeled RNA<sub>541/1250</sub>. Lanes 1 and 8, no exogenous nucleolin was added; lanes 2, 5, and 9, 5 pmol; lanes 3, 6, and 10, 10 pmol; lane 4, 7, and 11, 20 pmol; lane 12, 30 pmol. After incubation, an UV cross-linking was performed, and labeled proteins were resolved by SDS-polyacrylamide gel electrophoresis and autoradiography. B, processing of RNA<sub>541/1250</sub> in presence of *X. laevis* (lanes 3–5), CHO (lanes 7–9), or recombinant GST-CHO nucleolin (lanes 10–14). Lanes 1, 2, 6, and 10, no exogenous nucleolin was added; lanes 3, 7, and 11, 5 pmol; lanes 4, 8, and 12, 10 pmol; lanes 5, 9, and 13, 20 pmol; lane 14, 30 pmol.

nucleolin with the pre-rRNA was also able to stimulate the processing. In the presence of an excess of *X. laevis* (Fig. 1B, lanes 3–5), CHO (lanes 7–9), or recombinant GST-CHO nucleolin (lanes 10–14), the processing reaction is markedly activated. Altogether these data show that all these proteins are able to activate the processing reaction and that nucleolin-binding site on pre-rRNA is conserved between *X. laevis* and mouse despite the low sequence similarity within the 5'-ETS of these two species.

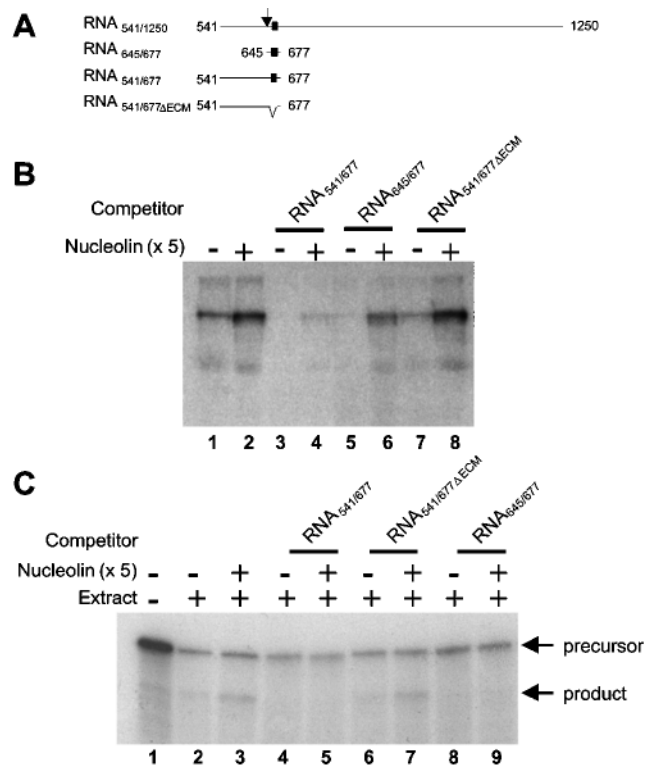
**The ECM Is Required for Nucleolin Interaction with the Pre-rRNA**—Interactions of nucleolin with different truncations of RNA<sub>541/1250</sub>, in absence of cellular extract, were studied to map the binding site of nucleolin (Fig. 2). We used a filter-binding assay with purified CHO nucleolin and labeled *in vitro* transcribed RNA (27). Deletion of the first 104 nt or of the last 573 nt of RNA<sub>541/1250</sub> did not affect nucleolin binding affinity ( $K_d$  of 180 nM  $\pm$  40 nM) (Fig. 2), indicating that nucleolin binding site is contained within the 32-nt RNA<sub>645/677</sub>. We used a previously characterized small 68-nt RNA (NS) which does not interact with nucleolin (15, 27, 28, 29) as a negative control for these interactions. The 32-nt RNA (RNA<sub>645/677</sub>) sufficient for the specific interaction of nucleolin contains an evolutionary conserved 11-nt motif (ECM), which is always located 5 or 6 nt after the processing site (9, 20, 29) (Fig. 2A). To determine whether this motif was required for nucleolin interaction, the 11-nt motif was deleted within RNA<sub>541/764</sub> and RNA<sub>541/677</sub> to give RNA<sub>541/764</sub> $\Delta$ ECM and RNA<sub>541/677</sub> $\Delta$ ECM respectively, and these RNA were used in the filter binding assay (Fig. 2B). The precise deletion of the 11-nt motif completely abolishes nucleolin interaction to a level comparable to a negative control for binding (NS and RNA<sub>541/656</sub>).

These RNAs were used as competitor in UV cross-linking and *in vitro* processing assays (Fig. 3, B and C). These competition assays have been previously used successfully to study the sequences and structural motifs required for the processing (9, 21). Labeled RNA<sub>541/1250</sub> was incubated in the extract and



**FIG. 2. Interaction of nucleolin with pre-rRNA.** *Top panel*, schematic representation of the different RNAs used. The *black square* indicates the position of the ECM, and the *arrow* indicates the primary processing site. Interaction of nucleolin in absence of cellular extract with these different RNAs was studied using a filter binding. These interactions were compared with interaction with a 68-nt RNA (*NS*) previously described (27–29) that does not interact significantly with nucleolin. *A*, deletion of nucleotide sequences at the 5' and 3' end of RNA<sub>541/1250</sub>. *B*, comparison of the interaction of nucleolin with wild type and  $\Delta$ ECM RNAs. Quantification was performed using a PhosphorImager. The key to the data points is shown on the figure.

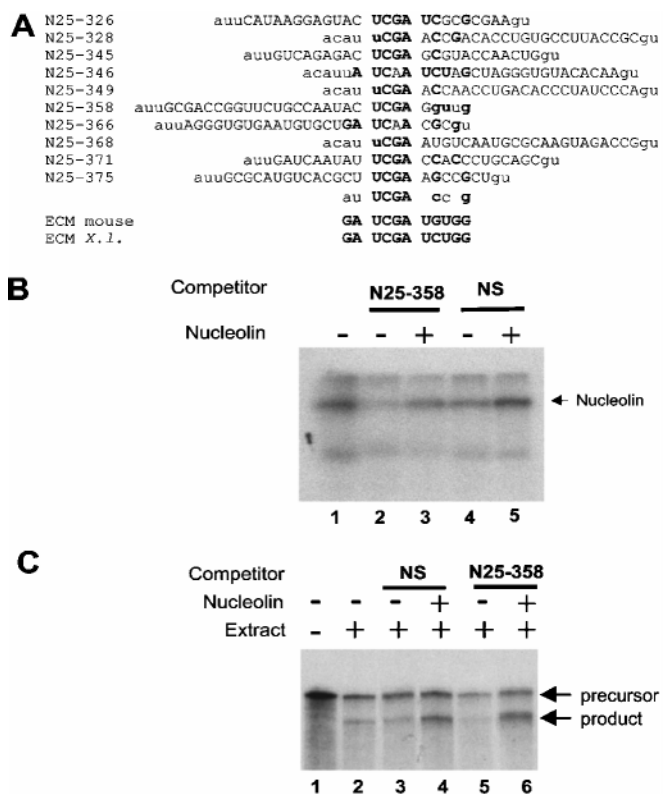
subjected to UV cross-linking (Fig. 3*B*, lane 1) and processing reaction (Fig. 3*C*, lane 2). Addition of RNA competitors that contain the ECM (RNA<sub>541/677</sub> and RNA<sub>645/677</sub>) were able to compete nucleolin interaction with labeled RNA<sub>541/1250</sub> (Fig. 3*B*, lanes 3 and 5) and abolished processing of the RNA substrate (Fig. 3*C*, lanes 4 and 8). In contrast, the RNA competitor that does not contains this 11-nt motif (RNA<sub>541/677 $\Delta$ ECM</sub>) did not significantly compete for nucleolin interaction (Fig. 3*B*, lane 7) nor RNA<sub>541/1250</sub> processing (Fig. 3*C*, lanes 6). To demonstrate that RNA<sub>541/677</sub> and RNA<sub>645/677</sub> and not RNA<sub>541/677 $\Delta$ ECM</sub> titrated nucleolin, a 5-fold excess of nucleolin was added to the UV cross-linking and processing experiments. An increase of nucleolin cross-linking and a stimulation of RNA<sub>541/1250</sub> processing were observed when no competitors (Fig. 3, *B* and *C*, lanes 2 and 3, respectively) or when RNA<sub>541/677 $\Delta$ ECM</sub> was used (Fig. 3, *B* and *C*, lanes 8 and 7, respectively). In contrast, nucleolin cross-link was only partially restored (Fig. 3*B*, lanes 4 and 6), and no activation of RNA processing was observed (Fig. 3*C*, lanes 5 and 9) when ECM containing RNA competitors were added. Even in presence of added nucleolin, RNA competitor and nucleolin are present in about equimolecular amount, and therefore, unlabeled RNA<sub>541/677</sub> and RNA<sub>645/677</sub> are still efficiently competing nucleolin. Furthermore, these two RNAs are sufficient for formation of proc-



**FIG. 3. The 11-nucleotide ECM is required for the interaction of nucleolin with the pre-rRNA and nucleolin activation of the primary processing.** *A*, schematic representation of the different RNAs used. The *black box* indicates the position of the ECM, and the *arrow* indicates the primary processing site. In RNA<sub>645/677 $\Delta$ ECM</sub> the conserved 11 nucleotides have been deleted. *B*, cross-linking of nucleolin with <sup>32</sup>P-labeled RNA<sub>541/1250</sub> in presence of different RNA competitors. Labeled RNA<sub>541/1250</sub> was incubated in cell extract in presence of 40 pmol of unlabeled RNA competitors, RNA<sub>541/677</sub> in lanes 3 and 4, RNA<sub>645/677</sub> in lanes 5 and 6, RNA<sub>645/677 $\Delta$ ECM</sub> in lanes 7 and 8. A 5-fold excess (compared with nucleolin present in the extract) of exogenous CHO nucleolin (10 pmol) was added in lanes 2, 4, 6, and 8. In lanes 1 and 2, no RNA competitor was added in the extract. After UV cross-linking, labeled proteins were analyzed by SDS-polyacrylamide gel electrophoresis, and the gel was autoradiographed. *C*, *in vitro* processing reaction in presence of different competitors. Labeled RNA<sub>541/1250</sub> was incubated with (lanes 2–9) or without (lane 1) cell extract in presence of different unlabeled RNA competitors (20 pmol), RNA<sub>541/677</sub> in lanes 4 and 5, RNA<sub>645/677 $\Delta$ ECM</sub> in lanes 6 and 7, and RNA<sub>645/677</sub> in lanes 8 and 9. An excess of nucleolin (10 pmol) was added in lanes 3, 5, 7, and 9. After the processing reaction, RNA was extracted and analyzed on a 6% polyacrylamide gel.

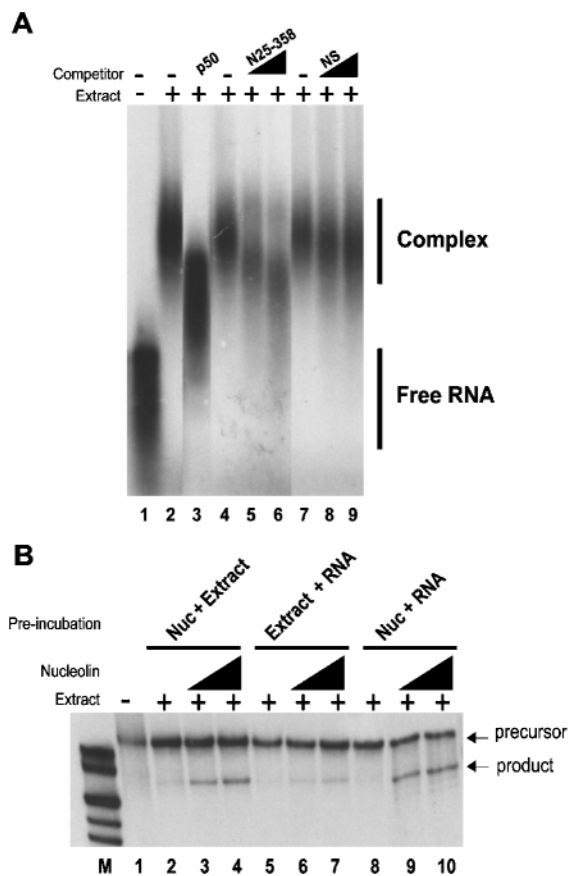
essing complex (18, 21). Nucleolin could therefore form a stable complex with these competitors and titrate other factors involved in the processing reaction. Altogether, these results demonstrate that the ECM is required for nucleolin interaction with the RNA.

*In Vitro Selection with Nucleolin Identifies an UCGA Motif Contained in the ECM*—A SELEX experiment performed with nucleolin has characterized several RNA binding sequences (27). Half of these sequences form a small stem-loop structure composed of a short stem (5 base pairs) and a 7–10-nt loop containing the motif (U/G)CCCGA. This motif interacts with high affinity ( $K_d$  of 5–20 nM) with nucleolin (27, 30). The other sequences interact with lower affinity ( $K_d$  of 100 nM) and show much higher sequence diversity (Fig. 4*A*). Alignment of these SELEX sequences highlight an UCGA motif contained in the ECM (Fig. 4*A*), indicating that this motif could be a key determinant of nucleolin RNA-binding specificity. One of the selected sequences (N25–358) was used in competition experiments (Fig. 4, *B* and *C*). When unlabeled N25–358 was added to the cell extract, the interaction of nucleolin with RNA<sub>541/1250</sub>



**FIG. 4. Nucleolin SELEX selected sequences titrates nucleolin-processing activity.** *A*, alignment of RNA sequences identified in a SELEX experiment with CHO purified nucleolin. Lowercase letters represent nucleotides that are part of the flanking constant sequence of the oligonucleotide used in the SELEX experiment. These sequences represent 50% of the selected sequences. The remaining sequences contained a conserved motif previously described (27). SELEX sequences are compared with the mouse and *X. laevis* evolutionary conserved motif found 5–6-nt downstream from the processing site (respectively, *ECM mouse* and *ECM X.l.*). *B*, cross-linking of nucleolin with  $^{32}$ P-labeled RNA<sub>541/1250</sub> in presence of different RNA competitors. Labeled RNA<sub>541/1250</sub> was incubated in cell extract in presence of 20 pmol of unlabeled RNA competitors, RNA NS in lanes 4 and 5 and RNA N25–358 in lanes 2 and 3. NS RNA has been previously studied in detail (27, 30, 31) and does not interact with nucleolin. In lanes 3 and 5, 10 pmol of purified CHO nucleolin were added to the reaction. *C*, *in vitro* processing reaction in presence of different competitors.  $^{32}$ P-Labeled RNA<sub>541/1250</sub> was incubated with (lanes 2–6) or without (lane 1) cell extract in presence of unlabeled RNA competitors, RNA NS in lanes 3 and 4 and RNA N25–358 in lanes 5 and 6. Nucleolin (10 pmol) was added in lanes 4 and 6. After the processing reaction, RNA was extracted and analyzed on a 6% polyacrylamide gel.

decreased significantly (Fig. 4*B*, compare lane 2 with lanes 1 and 4) and reduced the basal processing activity of the extract (Fig. 4*C*, compare lanes 5 and 2). When excess of nucleolin and RNA competitor are added at the same time, the interaction of nucleolin with labeled RNA<sub>541/1250</sub> and processing activity of the extract was restored (Fig. 4, *B*, lanes 2 and 3, and *C*, lanes 5 and 6, respectively). An RNA sequence, NS, which does not interact with nucleolin (17, 31) was neither able to compete nucleolin cross-linking with RNA<sub>541/1250</sub> nor processing activity (Fig. 4, *B* and *C*, lanes 4 and 5 and lanes 3 and 4, respectively). This add-back experiment demonstrated that N25–358 titrated specifically nucleolin and strongly suggests that the UCGA motif present in the ECM interacts with nucleolin. It is also interesting that full activity of the extract was restored when nucleolin is added to N25–358 treated extracts, whereas this was not possible with RNA<sub>541/677</sub> and RNA<sub>645/677</sub> competitors. These two RNAs are competent for processing complex formation, although this is not the case of N25–358 (data not shown). Therefore, the selected sequence titrates specifically nucleolin,



**FIG. 5. RNA binding activity and the N-terminal domain of nucleolin are required for the formation of a specific complex.**

*A*, labeled RNA<sub>541/1250</sub> was incubated without (lane 1) or with (lanes 2–9) cell extract and with unlabeled RNA competitors, N25–358 (lanes 5 and 6) and NS (lanes 8 and 9), 40 pmol (lanes 5 and 8), or 80 pmol (lanes 6 and 9). In lane 3, there was a 20-fold excess (20 pmol) of recombinant p50 (nucleolin without the N-terminal domain (17)). After incubation of 60 min at 30° C, the reaction mixture was loaded on a 0.8% agarose gel. *B*, interaction of nucleolin with the pre-rRNA is required at an early step of the processing reaction. The activation of the processing of RNA<sub>541/1250</sub> by nucleolin was studied in three different experimental conditions in which the order of addition of the three components (nucleolin, cell extract, and labeled RNA<sub>541/1250</sub>) varies. Preincubation, nucleolin + cell extract (lanes 2–4), cell extract + labeled RNA<sub>541/1250</sub> (lanes 5–7), and nucleolin + labeled RNA<sub>541/1250</sub> (lanes 8–10) were performed during 30 min before addition of the third component. Increasing amounts of nucleolin were added: 5 pmol (lanes 3, 6, and 9) and 10 pmol (lanes 4, 7, and 10). After 45 min of incubation, RNAs were extracted and analyzed on a 6% polyacrylamide gel.

whereas RNA<sub>541/677</sub> and RNA<sub>645/677</sub> might titrate other factors required for the processing.

*Interaction of Nucleolin with the Pre-rRNA Substrate Is Required for the Formation of the Processing Complex*—Previous studies have shown that processing activity is correlated with the formation of a large ribonucleoprotein complex (9, 18, 20, 22). To study the role of the interaction of nucleolin with the pre-rRNA in complex formation, the RNA N25–358 was added to the processing competent extract with labeled RNA<sub>541/1250</sub> and directly loaded on a native agarose gel (Fig. 5*A*). Addition of increasing amount N25–358 was able to prevent the formation of the correct complex (lanes 5 and 6), whereas the NS RNA had no effect (lanes 8 and 9). When RNA<sub>541/677</sub> $\Delta$ ECM is used as competitor, the formation of the complex is not affected (data not shown) as previously published (9). These results demonstrate that nucleolin interaction with the pre-rRNA is required for correct complex formation. A recombinant protein (p50) containing the RNA-binding domains of nucleolin com-

petes with full-length nucleolin for the interaction with the pre-rRNA and inhibit the processing reaction (17). Addition of p50 in the cell extract (lane 3) prevents complex formation. Altogether these results demonstrate that the interaction of nucleolin with the ECM is required for the correct assembly of the processing complex on the pre-rRNA. The N-terminal domain of nucleolin (missing in the recombinant p50 protein) is also required, suggesting that nucleolin can recruit other components of the processing complex.

**Nucleolin Is Required at an Early Step of the Processing Complex Formation**—To get an insight on the role of nucleolin in complex formation, the protein was added at different times in the processing reaction (Fig. 5B). In all previous experiments, cell extracts and exogenous nucleolin were preincubated 30 min before the addition of  $^{32}\text{P}$ -labeled RNA<sub>541/1250</sub> (lanes 3 and 4). Under these conditions, addition of nucleolin stimulates the processing activity (compare lanes 3 and 4 with lane 2). If nucleolin is preincubated with the RNA substrate before addition of cell extract (lanes 9 and 10), the same strong processing activation is observed. In contrast, when cell extract and pre-rRNA are incubated for 30 min before the addition of nucleolin, only a weak activation was observed (lanes 6 and 7). These results suggest that the activation of the processing is more efficient when nucleolin interacts with the pre-rRNA substrate first. This first step might be required for an efficient recruitment of the processing complex.

#### DISCUSSION

In these experiments we show that the interaction of nucleolin with an evolutionary conserved RNA sequence (ECM) in the pre-rRNA 5'-ETS is required for processing and formation of a specific complex. Nucleolin is well conserved from human to *Xenopus* (24, 25), and we show that nucleolin from *X. laevis* and hamster interact with the same pre-rRNA sequence and activate processing activity of a mouse cell extract (Fig. 1). The N-terminal domain of nucleolin that is not required for RNA binding (31, 32) is needed for complex formation (Fig. 5A) and processing activation (17). The interaction of the N-terminal domain of nucleolin with other factors involved in the processing, like the U3snoRNP (17), seems therefore well conserved between mouse and *Xenopus*. A SELEX experiment with hamster nucleolin characterized more precisely the RNA sequence required for nucleolin interaction with the pre-rRNA and highlighted the UCGA motif contained in ECM (Fig. 4). The SELEX selected sequences efficiently compete the interaction of nucleolin with the pre-rRNA and the formation of specific complexes. This suggests that nucleolin interacts with the UCGA motif present in the ECM and that this interaction is required for the formation of a processing complex. However, this small motif cannot explain by itself the RNA binding specificity of nucleolin toward the pre-rRNA primary processing binding site. Phylogenetic and mutagenesis studies suggest that this sequence is needed in a single-stranded conformation for processing and specific complex (21, 29). No strong secondary structure could be found in the SELEX sequences, indicating that the UCGA motif is also probably in a single-stranded conformation. Future work should determine the importance of the surrounding sequence for the specific interaction of nucleolin. The ECM is highly conserved, and its deletion or point mutation completely inhibits processing (21, 22). Furthermore, this sequence is required for the presence of the terminal balls at the 5' end of nascent pre-rRNA (20). Therefore, the interaction of nucleolin with this highly conserved sequence implies that nucleolin plays an important role in the processing and formation of an active processing complex.

The minimal pre-rRNA sequence requirement (+645 to +672) for processing and specific complex formation (21) in-

cludes the ECM. The formation of a ribonucleoprotein complex on this RNA motif was reported to be necessary but not sufficient for processing (21). The U3snoRNP, which is absolutely required for processing (16) is only present in complex formed on larger RNA (9, 16, 18). Sequences downstream of the ECM together with the interaction between the U3snoRNP and the N-terminal domain of nucleolin (17) might cooperate for the activation of the processing reaction. The complex formed on the minimal RNA sequence is very stable and cannot be displaced (18). If nucleolin is added after complex formation on the pre-rRNA substrate, it is not able to stimulate the processing (Fig. 5B). However, when nucleolin competitors (Fig. 5A; p50, N25–358) are added at the beginning of the reaction, the formation of the complex is prevented. This suggests that the early interaction of nucleolin with the ECM is required for the formation of an active processing complex.

The ECM is required for the formation of the terminal balls at the 5' ends of nascent pre-rRNA transcript in *Xenopus* oocyte (20). However, in *Xenopus* oocytes, the primary processing is not detected (9, 20, 33). Because the formation of these structures is conserved in all tissues and organisms examined, the formation of this complex rather than the primary processing may serve an important function. Our results indicate that nucleolin does not activate the processing when a stable complex is preformed on the pre-rRNA substrate, indicating that the ordered formation of a stable complex that could include nucleolin is necessary for the formation of an active complex. The regulation of nucleolin, like the phosphorylation of the N-terminal domain of nucleolin during the cell cycle (34–37), could modulate the formation of this complex and therefore of the processing.

The N- and C-terminal domains of nucleolin are involved in protein-protein interactions (17, 38), whereas the central domain mediates specific interaction with the pre-rRNA (17, 27, 31, 32, 38). The function of nucleolin in this processing is likely the result of these different properties. The specific interaction of nucleolin with the ECM on nascent pre-rRNA might represent the first step in the assembly of a large ribonucleoparticle involved in this processing. Nucleolin could then recruit, by protein-protein interaction, other factors involved in the cleavage, like the U3snoRNP. Nucleolin interaction with the pre-rRNA could also play a role of a chaperone for the formation of the correct RNA tertiary structure of the nascent transcript. The evolutionary conservation of (i) nucleolin, (ii) the interaction of nucleolin with pre-rRNA sequences involved in the cleavage, and (iii) the formation of a specific complex at the 5' end of nascent pre-rRNA indicates that this primary processing event plays an important and still unknown function for ribosome biogenesis.

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**Interaction of Nucleolin with an Evolutionarily Conserved Pre-ribosomal RNA Sequence Is Required for the Assembly of the Primary Processing Complex**

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