Repression of RNA Polymerase I Transcription by Nucleolin Is Independent of the RNA Sequence That Is Transcribed*

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Nucleolin is one of the most abundant non-ribosomal proteins of the nucleolus. Several studies in vitro have shown that nucleolin is involved in several steps of ribosome biogenesis, including the regulation of rDNA transcription, rRNA processing, and ribosome assembly. However, the different steps of ribosome biogenesis are highly coordinated, and therefore it is not clear to what extent nucleolin is involved in each of these steps. It has been proposed that the interaction of nucleolin with the rDNA sequence and with nascent pre-rRNA leads to the blocking of RNA polymerase I (RNA pol I) transcription. To test this model and to get molecular insights into the role of nucleolin in RNA pol I transcription, we studied the function of nucleolin in Xenopus oocytes. We show that injection of a 2–4-fold excess of Xenopus or hamster nucleolin in stage VI Xenopus oocytes reduces the accumulation of 40 S pre-rRNA 3-fold, whereas transcription by RNA polymerase II and III is not affected. Direct analysis of rDNA transcription units by electron microscopy reveals that the number of polymerase complexes/rDNA unit is drastically reduced in the presence of increased amounts of nucleolin and corresponds to the level of reduction of 40 S pre-rRNA. Transcription from DNA templates containing various combinations of RNA polymerase I or II promoters in fusion with rDNA or CAT sequences was analyzed in the presence of elevated amounts of nucleolin. It was shown that nucleolin leads to transcription repression from a minimal polymerase I promoter, independently of the nature of the RNA sequence that is transcribed. Therefore, we propose that nucleolin affects RNA pol I transcription by acting directly on the transcription machinery or on the rDNA promoter sequences and not, as previously thought, through interaction with the nascent pre-rRNA.

The synthesis of functional ribosomes is a major task for the cell. Ribosomal gene transcription can account for as much as 40% of all cellular transcription and ribosomal RNA for about 80% of the RNA content of living cells (1). The different steps of ribosome biogenesis take place in a subcompartment of the nucleolus called the nucleolus (2–4). The localization of the different steps of ribosome biogenesis in a single nucleolar compartment probably allows an efficient coordination and regulation of ribosome assembly. The formation of mature ribosomes is one of the most complex assembly of ribonucleoparticles involving the interaction of four different RNAs and about 80 ribosomal proteins (5). In addition, several nucleolar non-ribosomal proteins are required for this process (6–8). An ordered interaction of ribosomal and non-ribosomal proteins with pre-rRNA is probably required for the formation of functional ribosomes. The molecular details of this highly integrated process are still largely unknown.

The non-ribosomal proteins fibrillarin and nucleolin as well as some ribosomal proteins have been detected on nascent pre-rRNA (9–11) suggesting that they interact with the pre-rRNA during transcription. These abundant non-ribosomal proteins present in the nucleolus could be involved in the regulation and coordination of early steps of pre-rRNA packaging during transcription and also at later stages for ribosome assembly.

Nucleolin is one of the most abundant non-ribosomal proteins of the nucleolus (12–14). It is found within the dense fibrillar component at the site of rDNA transcription and within the peripheral granular component of the nucleolus where rRNA processing occurs (8, 15, 16). This localization suggests that nucleolin could be involved in different aspects of ribosome biogenesis. Indeed, it has been proposed that nucleolin can regulate: (i) transcription of the rDNA genes (17–21), (ii) maturation of the pre-rRNA (22, 23), and (iii) nucleocytoplasmic transport (24–26).

Because the N-terminal domain of nucleolin contains acidic regions that may interact with histones in chromatin and the central four RNA-binding domains interact with nascent pre-rRNA, it was proposed that nucleolin may form a bridge between the nascent transcript and chromatin, resulting in the blockage of transcription elongation (17, 18). We used the Xenopus oocyte system to test this model. We followed the transcriptional activity of the endogenous rDNA promoter or of minigenes and the maturation of rRNA in the presence of increased amount of nucleolin. We show that injection of nucleolin in stage VI oocytes specifically affects RNA pol I transcription and pre-rRNA maturation. Observation of ribosomal RNA transcription units by electron microscopy and the use of several chimeric minigenes provided direct evidence that an elevated amount of nucleolin down-regulates rDNA transcription independently of the RNA sequence that is transcribed.

MATERIALS AND METHODS

Constructs—The pol I′−rDNA plasmid (identical to pXLS108f (30)) was derived from pXLS108f (60). It contains a complete 6.6-kb Xenopus rDNA unit.

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1 The abbreviations used are: pol I, polymerase I; rDNA, ribosomal DNA; CAT, chloramphenicol acetyltransferase; CMV, cytomegalovirus; ETS, external transcribed spacer; TRITC, tetramethylrhodamine isothiocyanate; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; RSA, bovine serum albumin; DAPI, 4,6-diamidino-2-phenylindole; MOPS, 4-morpholinepropanesulfonic acid; Pipes, 1,4-piperazine-dithanesulfonic acid; nt, nucleotide(s); UBF, upstream binding factor.
laevs rDNA spacer fragment with a truncated rDNA gene. A SalI linker was inserted into the 5'-ETS (position +116 to +136) to distinguish RNA transcribed from the plasmid and endogenous rRNA. pol II-CAT (pCMV-CAT) has a complete chloramphenicol acetyltransferase (CAT) gene cloned downstream the human cytomegalovirus (CMV) promoter (61).

pol I-CAT was constructed by inserting the CAT cDNA (PCR fragment obtained with primers 5-GAGCCCTTTTCTGTTTTCGAGCCCGGGAGCACTCATGC-3' and 5-AGGGGAGAAGCAGGGCCATGGAAAGACAACTCCTGATATAT-3') between the BssH restriction sites of pXL108f (6645-bp fragment, which contains the complete rDNA intergenic spacer with distal promoters and enhancer repeats and all the promoter sequences up to +143C). For the pol II-rDNA plasmid, the BssH 1745-bp fragment from pXL108f (rDNA sequence from +14 to +1758) was inserted into the Smal site of pspCMV-SV40poly(A) vector. This last plasmid was constructed by inserting the 590-bp XhoI-BglII fragment of the CMV promoter and the 900-bp EcoRI-BgIII fragment containing the SV40-poly(A) signal from the CMV-CAT plasmid (62) in the corresponding sites of psp72 (Promega).

pol I mini-CAT plasmid corresponds to the PolI-EcoRI fragment of pol I-CAT plasmid (−308 to +216) cloned between the BssH-EcoRI sites of pUC9. This minigene contains the pol I minimal promoter and the T3 and all the promoter sequences up to +216C before injection. Western blot analysis to detect the CHO-injected nucleolin was performed using a polyclonal antibody raised against a recombinant protein corresponding to the C-terminal domain of nucleolin (p50) (this antibody does not react with Xenopus nucleolin in Western blot analysis but reacts with the Xenopus protein in immunofluorescence studies). For the detection of endogenous nucleolin (immunofluorescence studies), we used a polyclonal antibody raised against a recombinant polypeptide corresponding to the four RBD of CHO-nucleolin proteins in Western blot and immunofluorescence analysis, respectively.

**RESULTS**

**Oocyte Injections—Ovaries from *X. laevis* female were surgically removed, cut into pieces, and treated with 2 mg/ml collagenase type IV before injection.** The preparations were then washed with PBS and resuspended in DAPI (0.2 μg/ml) for 30 min. Confocal microscopy was performed with a Zeiss confocal laser scanning microscope using a 63× oil immersion objective.

**RNA Analysis—For each experimental point, five oocytes were pooled and stored at −20 °C. Oocytes were homogenized in 500 μl of RNA extraction buffer (20 μM Tris, pH 7.5, 100 mM NaCl, 30 mM EDTA, 1% SDS, and 200 μg/ml proteinase K (Sigma)) and incubated at 37 °C for 1 h. Total RNA was extracted with phenol-water (3:7.5:1, v/v) and precipitated with 0.8 vol. of isopropanol. When oocytes were injected with a plasmid an additional step was added. After the isopropanol precipitation, nucleic acids were resuspended in 50 μl of sterile water and 25 μl of LiCl, 10 μM RNA was precipitated for 1 h on ice. After sedimentation, RNAs were resuspended with 70% ethanol in 4 μl of sterile water per oocyte. RNAs were resolved on 1% agarose-formaldehyde gels (6.5% formaldehyde, 20 μM MOPS, 5 mM sodium acetate, and 1 mM EDTA) for 12–14 h at 75 °C at 4.6% formaldehyde, 20 mM MOPS, 5 mM sodium acetate, and 1 mM EDTA as running buffer. For S1 nucleicase experiments, 1 equivalent oocyte RNA (4 μl) was precipitated with 10 ng of 5′ radiolabeled probe and then incubated in 20 μl of hybridization buffer (40 mM Pipes (pH 6.4), 400 mM NaCl, 1 mM EDTA, 80% formamide) for 10 min at 90 °C and overnight at 55 °C. S1 nuclease (120 units, Promega) and its reaction buffer were added to the hybridized sample and incubated for 1 h at 37 °C. After phenol-chloroform extraction and ethanol precipitation, protected RNA fragments were resolved on a denaturing 6% polyacrylamide–8 M urea gel.

For primer extension experiments, 1 equivalent oocyte RNA (4 μl) was incubated with 1 ng of 5′ radiolabeled primer in 7.5 μl of hybridization buffer (25 mM Heps (pH 7), 50 mM KCl) for 3 min at 90 °C and allowed to cool down slowly to 45 °C. Reverse transcriptase (MMLV, 200 units, Promega) was added with 5 μl of extension buffer (50 mM Tris (pH 8), 10 mM dithiothreitol, 10 mM MgCl2, 200 μM each dNTP) and incubated for 1 h at 42 °C. The reaction was stopped with 30 μl of the STOP buffer (300 mM NaCl, 1 mM EDTA), ethanol-precipitated, and loaded on a denaturing 6% polyacrylamide–8 M urea gel.

Gels were dried, exposed on a PhosphorImager screen for quantification, and autoradiographed.

Two rDNA probes were used for S1 nuclease experiments. The first correspond to the 441-bp PolI-SolI fragment of pBswt and contains the first 125 nt of *Xenopus* 5′-ETS and the 305-nt upstream sequence corresponding to the 40 S promoter and adjacent terminator. The protected fragment is 125 nt and is specific to the pol I-rDNA minigenes. The second probe corresponds to the 491-bp PolI-NatI fragment of the pXCR7 plasmid. It contains the first 171 nt of the *Xenopus* 5′-ETS and the 316-nt upstream sequence corresponding to the 40 S promoter adjacent terminator. This probe is specific to the endogenous rDNA genes and protects a fragment of 171 nt. The DNA fragments were dephosphorylated and 32P end-labeled with T4 polynucleotide kinase (Promega) and [γ32P]ATP. The CAT primers sequence for primer extension experiments were 5′-ATCAACCGGTGGTATATACTCAGTG-3′ for the pol II-CAT plasmid and 5′-ACATGATATTGGTCGTGACG-3′ for the pol I-CAT and pol I mini-CAT plasmids, which hybridize to the 5′-end of the CAT RNA.

**RESULTS**

**Microinjection of Purified Nucleolin Affects Pre-rRNA Transcription and Maturation—Western blot analysis indicates that about 20 ng of nucleolin is present in stage VI oocytes (data not shown). Because of this large stock of endogenous nucleolin, we were unable to over-express significantly the amount of nucleolin by microinjection in the oocyte of nucleolin mRNA or of an expression vector (data not shown). To overcome this difficulty, we injected highly purified nucleolin from...
hamster (CHO) or \( X. laevis \) cells (Fig. 1). After the injection of nucleolin (4-fold increase of nucleolin/oocyte) into the oocyte nucleus, RNA was labeled by the injection of \( [\alpha-\text{32P}]\text{CTP} \). Total RNA was then extracted at various times and run on an agarose gel (Fig. 1, B and C). With both nucleolins, a drastic effect on 40 S accumulation and on its processing was observed. The amount of 40 S pre-rRNA produced in presence of exogenous nucleolin was reduced about 3–4-fold with both proteins. Furthermore, the 40 S pre-rRNA produced in the presence of excess nucleolin was not mature but instead was apparently simply degraded, producing a smear below the 40 S pre-rRNA (Fig. 1, B, lanes 10 and 12, and C, lanes 6 and 8). The smear below the 40 S pre-rRNA produced in oocytes injected with \( X. laevis \) nucleolin is not as apparent as with CHO nucleolin, however; and even with a longer labeling time, no significant accumulation of mature 28 S RNA was observed (data not shown), indicating that the 40 S pre-rRNA was degraded. However, in some experiments (see Fig. 1C) some 18 S mature RNA could be observed only when \( X. laevis \) nucleolin was injected. Incubation of purified proteins (from \( X. laevis \) or CHO cells) with \( \text{in vitro} \) transcribed \( 5' \)-ETS RNA did not show any degradation of the RNA, indicating that these proteins are not contaminated by a ribonuclease activity (data not shown). To determine whether these effects were specific for the nucleolin proteins, mock injection of buffer or other proteins such as the nucleolar Nop10 protein (27) or BSA was performed (Fig. 2). Injection of a large molar excess of Nop10 and BSA compared with nucleolin had no effect either on the production of 40 S or on its processing. These experiments demonstrated that excess of nucleolin in the oocyte has a profound and specific effect on the production of mature rRNA species. All experiments that follow have been performed with hamster and \( X. laevis \) nucleolin. For clarity of the figures, only one set of data is shown for each experiment.

The reduction in the 40 S pre-rRNA pool could be the result of a reduction of rRNA transcription by RNA pol I, an increase of 40 S turnover, or both. To distinguish between these possibilities, the stability of the 40 S pre-rRNA was measured in the presence or absence of injected nucleolin. CHO nucleolin was first injected into oocyte nuclei, and then the RNA was labeled for 1 h before the inhibition of transcription with actinomycin D. Under these conditions, we observed that the level of rRNA transcribed in the presence of nucleolin was reduced about 3 times (Fig. 3A, compare lane 8 with lane 1). Total RNA was isolated at various times after drug treatment and analyzed on an agarose gel (Fig. 3). Quantification of the disappearance of 40 S pre-rRNA in the absence (lanes 1–7) and presence (lanes 8–14) of injected nucleolin indicated that nucleolin did not promote a faster decay of the 40 S pre-rRNA (Fig. 3C). Instead, a slight increase in the half-life of this precursor was observed. In control oocytes, the stability of the 40 S is determined mainly by its rate of processing because the disappearance of the 40 S is quantitatively correlated with the appearance of the mature 18 and 28 S RNA. In injected oocytes, the half-life of the 40 S seems to be determined by its rate of degradation because no intermediate precursors were detected. Because the half-life of 40 S rRNA is identical in control and injected oocytes, the decrease in 40 S pre-rRNA accumulation must have been the direct consequence of a reduction in RNA pol I transcription.
Nucleolin Specifically Represses RNA Polymerase I Transcription—to further characterize the role of nucleolin on RNA pol I transcription, we first tested the efficiency with which nucleolin repressed RNA pol I transcription. Different amounts of CHO nucleolin were injected into the oocyte nuclei, and after 12 h RNA was labeled for 4 h before analysis (Fig. 4A). Quantification of 40 S pre-rRNA synthesized in the presence of nucleolin showed a linear and rapid decrease of RNA pol I transcription (Fig. 4B). A 3-fold repression of transcription was obtained with the injection of as little as 0.5 pmol of nucleolin (corresponding to a 2–3-fold increase in nucleolin in the oocyte). The experiment also indicated that the effect of nucleolin on rRNA maturation is very efficient. The injection of as little as 0.4 pmol (2-fold increase in nucleolin in the oocyte) is sufficient to inhibit the maturation of all newly synthesized rRNA. Western blot analysis of nuclear and cytoplasmic fraction of control and injected oocytes showed that injected CHO nucleolin (1.15 pmol) is retained in the nucleus and is stable during the experiment (data not shown).

To further characterize the specificity of the repression of RNA pol I transcription mediated by nucleolin, we examined the effect of nucleolin on RNA pol III transcription. Oocytes were injected with nucleolin as described previously, and RNA was labeled for 12 h. Analysis of these RNAs on a 1.0% agarose gel (Fig. 5A) indicated that the level of 40 S in nucleolin-injected oocytes was reduced 3-fold compared with buffer-injected oocytes (compare lanes 1 and 2). The same RNAs were also analyzed on a 6% acrylamide-urea gel to detect the low molecular weight 5 S and tRNA RNA pol III transcripts. In control oocytes the rRNA 12 and 5.8 S maturation products and the RNA pol III 5 S and tRNAs transcripts were clearly detected (Fig. 5B, lane 3). In contrast, in oocytes injected with nucleolin (lane 4) only, the RNA pol III transcripts (5 S and tRNAs) were observed and found at a level similar to that in control oocytes, indicating that neither their transcription nor their maturation had been affected by nucleolin. The absence of 12 and 5.8 S rRNA in nucleolin-injected oocytes (lane 4) further demonstrated that the 40 S pre-rRNA was not mature but was instead degraded, in agreement with previous experiments. RNA polymerase II transcription is weak in stage VI oocytes, and transcripts are generally stable.
on this graph.

For these reasons we have been unable to find a suitable system to determine whether the injection of nucleolin affects pol II transcription of the oocyte genes. To overcome this difficulty, we used the RNA pol II minigene, pol II-CAT, encoding the CMV promoter in fusion with the CAT gene (Fig. 6A). This plasmid, which is commonly used to study regulation of pol II transcription in Xenopus oocytes, was injected in the oocyte 1 h after nucleolin. Transcription from the CMV promoter was assayed by primer extension 8 and 22 h after plasmid injection. Nucleolin had no effect on the steady state level of CAT RNA after nucleolin. Transcription from the CMV promoter was solely at the level of RNA pol I transcription. Injected Nucleolin Co-localizes with UBF and Fibrillarin—To determine whether the injection of exogenous nucleolin alters nucleolar structures, the localization of different nucleolar proteins, UBF and fibrillarin, involved in rDNA transcription and pre-rRNA maturation, respectively, was studied by confocal microscopy in control and injected oocytes. After micronucleoli spreading, preparations were stained with DAPI (data not shown) and then probed with an anti-UBF (A17) and anti-fibrillarin (72B9) antibodies followed by the secondary fluorescent antibody (Fig. 7). Injection of Xenopus or CHO nucleolin had no effect on nucleolin number, size, or shape (data not shown). To localize the injected protein, we labeled nucleolin with TRITC before injection. The micronucleoli of injected oocytes were then spread and observed by confocal microscopy. The transcription factor UBF localizes as small dots (which can be seen in DAPI), whereas the maturation protein fibrillarin localizes as rings around UBF dots (Fig. 7). Injected labeled nucleolin co-localizes at the same time with UBF and fibrillarin, as would be expected for a protein implicated in the transcription and maturation of the pre-rRNA. Furthermore, the localization of UBF and fibrillarin was not affected. These results show that the repression of RNA pol I transcription and the degradation of the 40 S pre-rRNA observed in this study are not the consequence of an alteration of nucleolar structure or a relocalization of a transcription factor. These effects, rather, are the consequences of a direct or indirect interaction of nucleolin with the transcription and maturation machinery.

Electron Microscopic Study of rDNA Transcription Units—To look more closely at the effect of injected nucleolin on RNA pol I transcription, we performed chromatin spreading on control or injected oocytes (Fig. 8). Ribosomal gene transcription units visualized by electron microscopy give rise to structures called “Christmas trees,” first described by Miller and Beatty (28). These structures correspond to active transcription units spaced by intergenic sequences. Normal trees show 88 ± 10 transcription complexes/gene associated with nascent pre-rRNA fibrils (Fig. 8A). Each of these lateral fibrils ends with a 5’ terminal structure called a “terminal ball,” which is believed to represent the pre-rRNA processing complex (29). In oocytes injected with nucleolin, Christmas trees can be detected, but their structure is strongly altered (Fig. 8, B–E); the number of transcription complexes/tree, the “wild type” structures (i.e., with about 90 transcription complexes/unit) is rarely observed (<1% of the trees that can be observed). Most trees present an altered phenotype ranging from an almost total lack of transcription complexes and rRNP fibrils (Fig. 8, C and D), corresponding to a total inactivation of the transcription unit, to a more moderate phenotype with trees lacking 50–85% of the transcription complexes (Fig. 8, B and E). rDNA transcription units or intergenic spacer lengths are not affected by nucleolin injection. In the presence of injected...
Nucleolin—Plasmids carrying an entire rDNA unit or various truncations (RNA pol I minigenes) have been extensively used to study cis- and trans-acting factors involved in the regulation of RNA pol I transcription. We examined the ability of nucleolin to repress RNA pol I transcription from a pol I-rDNA minigene (pX1S108f (30)) that contains an entire rDNA unit truncated between the 18 and 28 S sequences (Fig. 9A). This minigene contains all of the intergenic spacer, but a SalI linker has been inserted into the 5′-ETS to distinguish the minigene transcripts from endogenous rRNA (30). Nucleolin was injected into oocyte nuclei 1–12 h before the co-injection of pol I-rDNA and pol II-CAT plasmids. Trial experiments indicated that injected nucleolin was stable during this period and that the length of this pre-incubation (between 1 and 12 h) gave the same results. Total RNA was extracted and then analyzed by S1 nuclease protection (RNA pol I minigene) and reverse transcription (RNA pol II minigene).

To validate the S1 nuclease assay and to detect the level of pol I transcription in the absence or presence of exogenous nucleolin, we checked if we were able to detect the same level of repression by nucleolin on the endogenous rDNA genes by this assay (Fig. 9D). Using a probe complementary to the first 171 nt of Xenopus 5′-ETS, the amount of protected fragment was reduced by about 3-fold in the presence of exogenous nucleolin. The co-injection of the pol I-CAT plasmid had no effect on the transcription level of the endogenous gene. Interestingly, this repression level is very similar to that observed on the accumulation of the 40 S pre-rRNA, showing that this nuclease S1 assay can be used to monitor the nucleolin effect on rDNA transcription. When we looked at the effect of nucleolin on the transcription of the pol I-rDNA plasmid, we observed that in the presence of nucleolin (Fig. 9B, lanes 3 and 5) the level of pol I-rDNA transcription was about 3-fold lower than in control oocytes (lanes 2 and 4). This level of repression was comparable with that of the endogenous rRNA genes (Figs. 1, 3, 5, and 9D).

Expression from the CMV promoter in the same oocytes (Fig. 9C) was not affected by nucleolin. Additional controls were performed to demonstrate the specificity of this transcription repression by nucleolin. Mock injection of buffer or other proteins (same as in Fig. 2) (Fig. 9C) had no effect on the transcription level from the pol I-rDNA plasmid, demonstrating that the effect of nucleolin on this plasmid is specific. This experiment also validates the minigene approach that we used to dissect the molecular mechanism of this repression.

To further characterize the molecular mechanism by which nucleolin affects transcription of rDNA genes, we then determined whether this RNA pol I repression was dependent on DNA sequences present in the RNA pol I promoter or on the nucleolin, the few transcription complexes present on the gene are distributed all along the transcription unit, and some of them do not seem to be associated with an RNP fibril. In the same cluster of ribosomal genes, transcription units can be affected differently. For example, in Fig. 8B the three consecutive transcription units show 25, 43, 49 transcription complexes, respectively. This low frequency of transcription complexes on Christmas trees of injected oocytes indicates a strong decrease of the RNA pol I transcriptional activity. Pre-rRNP fibrils in injected oocytes also seem to be shorter in many of these transcription units (Fig. 8, C and D, for example), suggesting that the corresponding RNA molecules are badly packaged. Many fibrils (25%) in injected oocytes also lack the terminal ball structure (see arrowhead in Fig. 8B), which has been proposed to represent the early processing complex (29). The shorter length of the RNP fibrils and sometimes the apparent absence of the terminal balls might be the result of a default in the co-transcriptional packaging of the nascent RNA with proteins or of a break in the weakened RNP fibers during chromatin spreading because of a lower amount of proteins associated with the RNA.

Pol II-CAT plasmid had no effect on the transcription level from the pol I-rDNA plasmid, demonstrating that the effect of nucleolin on this plasmid is specific. This experiment also validates the minigene approach that we used to dissect the molecular mechanism of this repression.
FIG. 8. Christmas trees structure is severely affected by the injection of exogenous nucleolin. Spread preparations were prepared from control oocytes (A) or 1 h after the injection of 23 nl of purified Xenopus nucleolin (1 mg/ml) (B–E). Typical normal Christmas trees shown in A show ~88 ± 10 polymerase complexes associated with an RNP fibril. A terminal ball is visible at the extremity of each fibril. After injection of nucleolin, the amount of polymerase complex/tran-
scription unit is drastically reduced (36 ± 13). C–E show representative structures with 22, 32, and 43 polymerase com-
plexes/gene. About 25% of the RNP fibrils lack the characteristic terminal balls (see arrowheads in B for example). It is import-
tant to note that in the injected oocyte, it is extremely rare to observe normal Christmas trees as shown in A. Bar = 0.3 µm. F, graph corresponding to the quantification of transcription complexes/tran-
scription units in control oocytes and injected oocytes. These numbers represent an average from the observation of 45 repre-
sentative Christmas trees from control and injected oocytes.

RNA sequence that is transcribed. For these experiments plas-
mids carrying a CAT gene under the control of a pol I promoter (pol I-CAT) or an rDNA gene under the control of a CMV promoter (pol II-rDNA) were constructed (Fig. 10A). We then tested the ability of exogenous nucleolin to repress transcription from these plasmids (Fig. 10, B and C). The injection of exogenous nucleolin had no effect on pol II-rDNA mimigene transcription (Fig. 10B, lanes 4 and 5), whereas it efficiently repressed transcription from the pol I-CAT minigene (Fig. 10B, lanes 7 and 8). The level of repression (3-fold) was again compar-
able with the effect on endogenous rDNA genes (Fig. 1) or on Pol I-rDNA minigenes (Figs. 9B and 10B). This experiment shows that the repression induced by exogenous nucleolin is not dependent upon transcribed RNA sequences but rather requires the pol I promoter.

In X. laevis, all intergenic repeated sequences have been shown to be enhancers of RNA pol I transcription (31). To determine whether these sequences where required, we tested the ability of nucleolin injection to repress transcription from a pol I minigene (~308 to +14) lacking these sequences (pol I mini-CAT) (Fig. 11A). The injection of exogenous nucleolin was also able to repress transcription from such a plasmid (Fig. 11, B and C), indicating that this pol I minimal promoter carries all of the information required for this repression and that nucleo-
in does not act on the enhancer sequences located within the intergenic spacer.

DISCUSSION

Nucleolin is a major component of the nucleolus and can represent as much as 10% of the nucleolar protein in prolifer-
ating cells (12). In vitro studies have suggested various roles for nucleolin in several steps of ribosome biogenesis (7, 8, 32). In this report we studied the molecular mechanism by which nucleolin affects pol I transcription. We show, contrary to the model proposed several years ago (17, 18), that nucleolin does not repress transcription through interaction with the nascent pre-rRNA transcript. Our results rather suggest an interaction of nucleolin with the RNA pol I machinery or the rDNA pro-
moter sequence. Microinjection of purified hamster or Xenopus

nucleolin into Xenopus oocytes induced a strong and specific repression of transcription from the endogenous rDNA genes and from microinjected rDNA minigenes (Figs. 1–5 and 9–11). The 40 S rRNA precursor produced in the presence of injected nucleolin was not mature but instead was degraded. Electron microscopic observations of Christmas trees (Fig. 8) show that in presence of an excess of nucleolin, the structure of the nascent RNP is altered; i.e., the length of the fibrils is irregular, some terminal balls are missing, and the RNP fibers are consistently less contrasted that in the control oocytes. These characteristics suggest that the nascent RNA is associated with fewer proteins or that the quality of the packaging is altered. Structural analysis of the nucleolin-RNA complex and bio-
chemical studies suggest that nucleolin could act as an RNA chaperone (33–35), which could help the formation of pre-rRNP during transcription by interacting with pre-rRNA and some ribosomal proteins (26). The injection of exogenous nucleolin in Xenopus oocytes may modify the transient association of endo-
genous nucleolin with pre-rRNA, or titrate factors involved in the maturation or assembly of pre-rRNA, resulting in the degradation of the 40 S pre-RNA. The role of nucleolin on pre-rRNA packaging will be described elsewhere.2

The repression of RNA pol I transcription observed in pres-
ence of an excess of nucleolin was specific because RNA pol III (Fig. 5) and RNA pol II (Figs. 6, 9, and 10) transcription were not affected. In our experiments, nucleolin was injected 1–12 h before rRNA labeling or plasmid injection. Under these condi-
tions nucleolin was stable, and its localization was restricted to the nucleus. The localization of the nucleolar proteins UBF and fibrillarin (Fig. 7) was not affected by the injection of exogenous nucleolin. Therefore it is unlikely that the repression of RNA pol I transcription and the absence of pre-rRNA maturation that we observed is the result of a major alteration of pre-
existing nucleoli structures, but rather it suggests that nucleo-
in is recruited on the endogenous rDNA genes and on micro-

2 B. Roger, A. Moisand, F. Amalric, and P. Bouvet, manuscript in preparation.
injected minigenes. The colocalization of injected nucleolin (Nucleolin-TRITC, Fig. 7) with UBF and fibrillarin in oocyte micronucleoli further supports this hypothesis. The direct observation of rDNA transcription units by electron microscopy shows a strong decrease in RNA pol I transcriptional activity in the injected oocytes (Fig. 8). Polymerases are not as densely packed as in control oocytes but are distributed all along the rDNA units. It is remarkable that the reduction in polymerases per transcription unit compared with control oocytes (2–5-fold) is similar to the level of repression of transcription of the 40 S pre-rRNA (3–4-fold). It seems therefore that an increased level of nucleolin affects RNA pol I transcription by decreasing the number of transcripts per gene rather than decreasing the number of active rDNA genes. These data also demonstrate that the reduction in the production of pre-rRNA in the presence of an elevated amount of nucleolin is a direct consequence of a diminution of RNA pol I transcription efficiency and is not a consequence of an increased degradation of the nascent transcripts.

Several hypotheses can explain the repression of RNA pol I transcription by nucleolin: (i) repression through interaction with the nascent rRNA transcripts, (ii) repression through interaction with chromatin/rDNA sequences, and (iii) repression through interaction with/titration of a component of the transcription machinery. Nucleolin is clearly not required for basal RNA pol I transcription in vitro, but this does not exclude its being an important factor to consider for the regulation of rDNA expression in vivo. The recent identification of nucleolin as a glucocorticoid receptor interacting-protein (36) suggests that it might be an important factor involved in glucocorticoid effects on rRNA synthesis (37, 38).
A model for an RNA-dependent transcriptional elongation arrest induced by nucleolin had been proposed (17). It is known that nascent RNA could regulate RNA chain elongation either directly or indirectly (39, 40). The coordinate interaction of nucleolin RNA-binding domains with specific sequences/structures in the nascent rRNA transcript (11, 41) and with factors of the RNA pol I transcriptional machinery could mediate transcriptional pausing or premature transcript release. However, our data are not in favor of this model. Analysis of rDNA transcription using an S1 nuclease experiment with a probe complementary to the very 5′-end of the 40 S pre-rRNA (first 100 nt) also shows a 3-fold lower accumulation in the presence of an excess of nucleolin (Figs. 9 and 10). Therefore, it is unlikely that the smear observed below the 40 S pre-rRNA results from the release of pre-rRNA chains all along the rDNA gene. Moreover, when the CAT gene replaces the rDNA sequence downstream of the pol I promoter, exogenous nucleolin efficiently represses the transcription from the DNA template. Therefore, our results argue rather that the pol I promoter contains all of the information required for the repression by nucleolin. It has been shown that nucleolin binds tightly to chromatin (42, 43), and to DNA fragments containing the region of the nontranscribed rDNA spacer upstream of the initiation site (19). Furthermore, nucleolin is able to interact with histone H1 and to modulate chromatin structure (20, 21) suggesting that nucleolin could mediate repression of transcription through an interaction with the chromosomal intergenic spacer. However, our data with the pol I mini-CAT plasmid, which contains only a minimal promoter, show that intergenic sequences are not required for the repression of transcription by exogenous nucleolin (Fig. 11). Therefore the repression of transcription that we observed is not the result of binding of nucleolin with the intergenic rDNA spacer. These sequences are composed of several repeated elements that act to enhance ribosomal transcription (31, 44–47). The enhancer sequences does not affect elongation rate but rather causes more genes to be actively transcribed (47). The binding of the transcription factor UBF to the repetitive enhancer sequence is believed to mediate the transcriptional enhancement despite the low sequence selectivity of UBF binding (48, 49). If we compare the level of repression of RNA pol I transcription between pol I-CAT (containing the enhancer sequences) and the pol I mini-CAT (does not contains the enhancers), we can see that the repression on pol I mini-CAT is about 2-fold stronger than on...
Fig. 11. A minimal pol I promoter is sufficient for the repression induced by nucleolin. A, schematic representation of pol I-CAT and pol I mini-CAT plasmids. pol I mini-CAT minigene has a minimal pol I promoter (~308±14) upstream of a truncated CAT gene. B, 23 nl of plasmids (50 ng/μl) were injected in control or Xenopus nucleolin-injected oocytes. After 24 h, total RNA was extracted and analyzed using the reverse transcription assay. Extended products were quantified, and values were reported on graphs (C). RT, reverse transcription; NI, noninjected oocytes.

the pol I-CAT (Fig. 11). This suggests that the enhancer sequences are partially able to release the repression by nucleolin.

Interestingly, a transcription terminator precedes all vertebrate rDNA promoters known so far. In Xenopus, this T3 terminator (46), similar to the T 0 sequences in mouse (50), have been shown to augment transcription from the adjacent rDNA promoter (30, 51–53). The interaction of TTF-1 (transcription terminator factor) with terminator elements (54–56) in a chromatin- and ATP-dependent fashion (57–59), activates RNA pol I transcription by a mechanism that remains to be determined. This T3 terminator is present in the minigene construct (pol I mini-CAT) that we used in this study. An attractive model to explain nucleolin function would be that nucleolin, instead of acting directly on promoter sequence or transcription machinery, could affect the function of the upstream terminator on the adjacent rDNA promoter. As demonstrated for TTF-1, nucleolin function could be dependent on chromatin structure, which could explain why studies in vitro with rDNA templates were not able to show any role of nucleolin in polymerase I transcription.

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