snoRNA nuclear import and potential for cotranscriptional function in pre-rRNA processing

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ABSTRACT

Several snoRNAs are essential for the sequence of cleavage events required to produce the mature forms of 18S, 5.8S, and 28S rRNA from the large precursor molecule. In the absence of U22, mature 18S rRNA fails to accumulate; U8 snoRNA is essential for accumulation of both 5.8S and 28S rRNA. The mechanisms by which snoRNAs facilitate these cleavage events is not known and might include direct cleavage or assisting the rate or efficiency of ribosome assembly. To learn more about the mechanisms of snoRNA-mediated pre-rRNA processing, an examination of the kinetics of pre-rRNA processing in *Xenopus* oocytes was undertaken. Correct pre-rRNA processing can be restored in snoRNA-depleted oocytes following cytoplasmic injection of the corresponding in vitro-synthesized snoRNA. Analysis of the kinetics of pre-rRNA processing in these snoRNA-rescue experiments demonstrated that the rate of accumulation of mature rRNAs was slower than that seen in untreated oocytes. The snoRNAs were imported into the nucleus at a rate and overall efficiency less than that of U1 snRNA, used as a control for import. However, sufficient levels of snoRNA were present in the nucleus to yield a functional phenotype (rescue of rRNA processing) several hours before the snoRNAs were directly detectable in the nucleus via autoradiography. This indicated that very low amounts of the snoRNA in the nucleus were sufficient for rescue. Finally, transcriptional inhibitors were used to separate transcription and processing. Failure to rescue snoRNA-mediated processing of pre-accumulated precursors is consistent with a scenario in which U8 and U22 must be present during transcription of pre-rRNA.

Keywords: nuclear import, processing, ribosomal RNA, ribosome, snoRNA, U8 snoRNA, U22 snoRNA

INTRODUCTION

All eucaryotic cells contain a subnuclear compartment called the nucleolus where ribosomal genes are transcribed and much of ribosome biogenesis occurs (reviewed in Hadjiolov, 1985). The small nucleolar ribonucleoprotein particles (snoRNPs) play essential roles in various aspects of ribosome biogenesis, including base or ribose modification, processing, and folding of the rRNA molecule.

The majority of the snoRNAs are involved in directing site-specific modification of the pre-rRNA (reviewed in Peculis & Mount, 1996; Peculis, 1997a; Venema & Tollervey, 1999). The C/D box snoRNAs are responsible for 2' O-methylation of specific sites in rRNA (Cavaille et al., 1996; Kiss-Laszlo et al., 1996; Nicoloso et al., 1996). The H/ACA snoRNAs specify uridine residues that will be converted to pseudouridine (Ganot et al.,

1997; Ni et al., 1997). For both methylation and pseudouridylation, the nucleotides selected for posttranscriptional modification are specified by base pairing of the snoRNA with pre-rRNA; the modification is catalyzed by proteins present in the snoRNP (reviewed in Maden & Hughes, 1997; Venema & Tollervey, 1999).

A small subset of snoRNAs is thought to play a role in pre-rRNA processing that is distinct from base or ribose modification. Depletion of these snoRNAs results in failure to accumulate one or more mature rRNA species. Biochemical or genetic depletion experiments have demonstrated that U3, U14, U22, snR10, and snR30 affect 18S maturation; RNase MRP affects cleavage at the 5' end of 5.8S in yeast; and vertebrate U8 snoRNA is essential for accumulation of mature 5.8S and 28S rRNAs (reviewed in Maxwell & Fournier, 1995; Venema & Tollervey, 1999). Although these snoRNAs are clearly essential for ribosome biogenesis, little is known about how they facilitate processing on a molecular level and whether they direct cleavage or act by affecting the rate or efficiency of ribosome assembly. The work presented here is focused on measurement

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of the rates of pre-rRNA processing in *Xenopus* oocytes and the role of U8 and U22 snoRNA in these maturation events.

Previously, the rate of pre-rRNA processing has been examined, indirectly or directly, in several eukaryotes. In a yeast pulse-chase experiment, labeling with [³H-methyl] methionine for 2 min was followed by the appearance of mature 18S within the first minute of the unlabeled methionine chase (Beltrame et al., 1994; Henry et al., 1994; Zanchin & Goldfarb, 1999). Labeled mature 28S appears only slightly later, within 1 or 3 min of chase. In human cells, after a 2-h pulse of ¹⁴C-uridine, 18S is readily observed, but mature 28S is barely detectable (Hadjiolova et al., 1993). The appearance of ³H-uridine-labeled rRNAs has been examined at various stages of *Xenopus* embryogenesis (Shiokawa et al., 1987). The time of appearance for the mature rRNA species varies depending on the stage of embryogenesis. Mature 18S appears within 30 to 60 min, depending on developmental stage, but always before mature 28S is detected, between 50 and 80 minutes.

Here I examined the rate of mature rRNA accumulation in *Xenopus* oocytes with or without prior depletion of U8 or U22 snoRNAs. Rescue of processing and the resulting time course were also examined by cytoplasmic microinjection of exogenous U8 or U22 snoRNAs. Because this rescue required relocation of the snoRNA from the site of injection in the cytoplasm to the site of processing in the nucleus, the rate of nuclear import of these snoRNAs was also examined. Within 4 to 6 h, snoRNAs injected into the cytoplasm could be directly detected within the nuclear compartment. Although the snoRNAs were imported at a rate and efficiency less than that of a U1 snRNA control, there were sufficient levels of snoRNA present to yield a functional phenotype (rescue of rRNA processing) several hours before the snoRNA had accumulated to a level directly detectable via autoradiography. Finally, transcriptional inhibitors were used to separate transcriptional-dependent events from posttranscriptional processing events. The observed failure to rescue processing of preaccumulated precursors is consistent with a requirement for the snoRNA to facilitate pre-rRNA processing via a cotranscriptional mechanism.

RESULTS

Previously, U22 snoRNA was shown to be necessary for maturation of 18S rRNA (Tycowski et al., 1994) whereas U8 snoRNA was demonstrated to be essential for accumulation of both 5.8S and 28S rRNAs (Peculis & Steitz, 1993). In these earlier analyses the "rescue" phenotype was observed after the oocytes had been incubated with the injected exogenous snoRNA for 12–18 h. Although this clearly demonstrates the ability of the snoRNAs to function in vivo, the design of these early experiments could address

neither the kinetics of pre-rRNA processing nor the effect of the snoRNA upon this processing rate.

Rate of accumulation of rRNA in vivo

I began by examining the rate at which mature 18S and 28S rRNAs appear in untreated oocytes. Microinjection of ³²P-UTP into *Xenopus* oocytes results in rapid labeling of all nascent transcripts. Because the rDNA genes are highly transcribed at this stage of oogenesis, mature rRNAs and their precursors are efficiently labeled by ³²P-UTP and easily detectable above the level of the other RNA transcripts (Peculis & Steitz, 1993). As a result, the rate of endogenous rRNA transcription and processing can readily be examined by monitoring the accumulation of in vivo-labeled precursors and mature rRNAs at various time points after injection of ³²P-UTP (see the schematic in Fig. 1A). Figure 1B shows the results from a time course of in vivo labeling RNA in oocytes. At the initial time point, (*t* = 0), ³²P-UTP was injected into the cytoplasm of oocytes. At each subsequent time point as indicated, 10 nuclei were isolated by manual dissection, RNA was extracted and resolved on denaturing agarose formaldehyde or polyacrylamide gels, and the rRNAs were visualized by autoradiography. In these experiments, each lane contained RNA from the equivalent of two nuclei. Figure 1B shows that precursor 40S rRNA was readily detectable within 20 min, indicating the rDNA was actively transcribed and full-length transcripts accumulated prior to processing (lane 1). Among processing intermediates, the 20S and 36S appeared first, accumulating to detectable levels within 60 min. Among the mature rRNA products, 18S was the first to appear, accumulating to detectable levels within 60 min. Mature 28S was readily seen only later in the time course (e.g., Fig. 1B, lane 5, 3 h). At these later time points, 12S, the long lived precursor to 5.8S, rRNA was also readily apparent (Fig. 1C, lane 5); mature 5.8S is not detected in the nucleus of oocytes (Peculis & Steitz, 1993).

Time course of U22-dependent pre-rRNA processing

Having determined the earliest time points at which an accumulation of newly synthesized rRNA could be detected in oocytes, I next examined whether the depleted snoRNAs were a rate-limiting component in pre-rRNA processing in the rescue experiments. Depletion of a specific snoRNA from oocytes can be accomplished by deoxyoligonucleotide-directed RNase H-mediated degradation (Savino & Gerbi, 1990; Peculis & Steitz, 1993). Pre-rRNA processing can subsequently be rescued by microinjection of an snoRNA produced by in vitro transcription (Peculis & Steitz, 1993).

FIGURE 1. Rate of accumulation of mature rRNA in *Xenopus*. **A:** Schematic of injection procedure. At time = 0, 100 nCi of ^{32}P -UTP was injected into *Xenopus* oocytes. At the times indicated, the nuclei from 10 oocytes were hand dissected in 5:1 PO_4 buffer (see Materials and Methods), total RNA was isolated, and RNA from the equivalent of two nuclei were resolved on an agarose-formaldehyde gel (**B**) or on a denaturing polyacrylamide gel (**C**). The mobility of the pre-rRNA intermediates and mature species are indicated with the identity shown schematically.

To examine the time course for U22 rescue, the endogenous pool of U22 snoRNA was depleted from oocytes by injection of the U22-specific deoxyoligonucleotide described previously (Tycowski et al., 1994; see Fig. 2A). After the overnight in vivo labeling period in the absence of endogenous U22, in vitro-synthesized precursor U22 snoRNA was injected into the cytoplasmic compartment of oocytes (U22 rescue samples). At the time points indicated (Fig. 2B), nuclei from six oocytes were isolated, pooled, and total RNA was extracted. RNA from the equivalent of three oocytes was resolved on a denaturing agarose-formaldehyde gel and the in vivo labeled RNAs examined by autoradiography. Figure 2B, lane 1, contains RNA from oocytes receiving only ^{32}P -UTP. This is the typical in vivo-labeled rRNA pattern. Figure 2B, lane 2, contains RNA from oocytes that received both ^{32}P -UTP and the U22-specific deoxyoligonucleotide. The characteristic U22-depletion pattern is seen (Tycowski et al., 1994); there was a slight overall decrease in the amount of all precursors, and while precursors and intermediates were observed, no mature 18S rRNA was detected. Fig-

ure 2B, lanes 3–9, contain the U22 rescue samples. Detectable amounts of 18S were present within 40 min of the U22 snoRNA injection. Accumulation of labeled precursors and mature rRNA continued throughout the time course with restoration of the pattern obtained for untreated oocytes (Fig. 2B, lane 1) achieved in an overnight rescue sample (Fig. 2B, lane 9).

Time course of U8-dependent pre-rRNA processing

U8 snoRNA is essential for the accumulation of both 5.8S and 28S rRNA in oocytes (Peculis & Steitz, 1993). In U8-depleted oocytes, no mature 28S is seen, but there is an accumulation of two abnormal intermediates designated 32*S and 36*S (Peculis & Steitz, 1993). Both intermediates are precursors to 28S rRNA and contain additional sequence at their 3' ends that is not correctly removed in the absence of U8 (Peculis & Steitz, 1993). To examine the rate of U8-mediated processing, ^{32}P -UTP and a U8-specific deoxyoligonucleotide were injected into *Xenopus* oocytes that were then incu-

FIGURE 2. Time course of U22 rescue. **A:** Schematic of injection procedure. To examine the rate of U22-dependent accumulation of 18S rRNA, *Xenopus* oocytes were injected with a U22-specific deoxyoligonucleotide plus ^{32}P -UTP and incubated for 12–18 h. At time = 0, in vitro-synthesized uncapped U22 snoRNA was injected into the cytoplasmic compartment of these U22-depleted oocytes and at the time points indicated, nuclei were hand-isolated, and total RNA harvested and resolved on denaturing agarose-formaldehyde gels. **B:** Autoradiograph indicating the in vivo-labeled rRNAs present in the nucleus. Lane 1: in vivo-labeled RNA from an untreated oocyte; lane 2: RNA from a U22-depleted oocyte; lanes 3–9, RNA isolated from oocytes at the times indicated after a cytoplasmic injection of in vitro-synthesized, uncapped U22 snoRNA. The mobility of the pre-rRNA intermediates and mature species are indicated with the identity shown schematically to the right. The RNA in lanes 1 and 9 was isolated from oocytes that were incubated for 36 h with ^{32}P -UTP.

bated for 12–18 h to accumulate a pool of labeled pre-rRNAs (Fig. 3A). Following this incubation, in vitro-synthesized U8 RNA was injected into the cytoplasm of oocytes (U8 rescue samples). At the time points indicated (Fig. 3B), nuclei were harvested and total RNA was isolated. Figure 3B, lane 1, contains RNA from oocytes that received both ^{32}P -UTP and the U8-specific deoxyoligonucleotide. The typical U8-depletion pattern was seen (Peculis & Steitz, 1993); no mature 28S rRNA accumulated, and the abnormal 32S and 36S intermediates appeared. Figure 3B, lanes 2–5, contain RNA from the U8 rescue samples. At 2–3 h after introduction of U8 RNA, the normal form of 32S began to accumulate, but no mature 28S was detected. By 4 h, both mature 28S and the normal form of 32S were easily detectable.

Rate of snoRNA import

The rate at which mature 18S and 28S rRNAs accumulate in oocytes is clearly different. 18S rRNA accumulates with faster kinetics, so perhaps it is not surprising that U22-mediated 18S processing was detectable sooner than U8-mediated 28S processing (compare Figs. 2B and 3B). However, the slower rate observed for U8-rescued processing in these experiments could also be due to a slower rate of U8 import, relative to U22. To examine this possibility, the rate of nuclear import of the snoRNAs was examined.

The method used to measure snoRNA nuclear import was as follows. Labeled snoRNA transcripts were microinjected into the cytoplasmic compartment of oocytes. After various lengths of time the oocytes were

FIGURE 3. Time course of U8 rescue. **A:** Schematic of injection procedure. To examine the rate of U8-dependent accumulation of 28S rRNA, *Xenopus* oocytes were injected with a U8-specific deoxyoligonucleotide plus ^{32}P -UTP and incubated for 12–18 h. At time = 0, in vitro-synthesized GpppG-capped U8 snoRNA was injected into the cytoplasmic compartment of these U8-depleted oocytes and at the time points indicated, nuclei were hand-isolated, and total RNA harvested and resolved on denaturing agarose-formaldehyde gels. **B:** Autoradiograph indicating the in vivo-labeled rRNAs present in the nucleus. Lane 1: in vivo-labeled RNA from a U8-depleted oocyte; lanes 2–5: RNA isolated from oocytes at the times indicated following a cytoplasmic injection of in vitro-synthesized U8 snoRNA. The mobility of the pre-rRNA intermediates and mature species are indicated with the identity shown schematically to the left.

dissected into nuclear and cytoplasmic fractions, total RNA was extracted, and the distribution of labeled RNAs was examined by electrophoresis and autoradiography. The reliability of this method depends on accurate injection and on clean separation of nuclear and cytoplasmic contents. In these experiments, coinjection of a labeled, intron containing tRNA precursor (pre-tRNA^{Phe}; Haselbeck & Greer, 1993) was used to monitor accurate cytoplasmic delivery and clean recovery of nuclear fractions free of cytoplasmic contamination. The pre-tRNA splicing enzymes in *Xenopus* oocytes are nuclear limited (Mattoccia et al., 1988). Thus, nuclear injection of pre-tRNA results in the rapid appearance of a spliced product (Fig. 4A, left panel) whereas cytoplasmic injection does not yield spliced product (Fig. 4A, right panel). In the nuclear import experiments described below, the absence of spliced tRNA provides evidence of accurate cytoplasmic delivery and the ab-

sence of labeled pre-tRNA in nuclear fractions provides evidence of clean fractionation of nuclear and cytoplasmic contents. In addition to pre-tRNA^{Phe}, U1 snoRNA was injected as a positive control for import (Mattaj et al., 1993). The U1 snoRNA is injected into the cytoplasm where it becomes hypermethylated and is imported into the nucleus. Once in the nucleus, there is a nuclear activity which removes 2 nt from the 3' terminus of trimethyl-G cap-containing U1 snoRNAs (Yang et al., 1992).

Here I chose to assay for the import of U8 and U22 snoRNAs, and in addition, examined U3, a very abundant snoRNA involved in 18S maturation. However, because of the similarities in size of U8 and U22, and the presence of a U3 nuclear degradation product that migrates very near U8 RNA, U8 RNA was examined separately, coinjected with its U1 and pre-tRNA^{Phe} controls (Fig. 4C). Labeled U3 and U22 were examined to-

FIGURE 4. Rate of snoRNA import into the nucleus. **A:** To demonstrate the role of pre-tRNA^{Phe} as a control for the site of injection, oocytes were injected with in vitro-synthesized, ³²P-labeled pre-tRNA^{Phe} in the nucleus (left panel) or cytoplasm (right panel). After incubation for the various lengths of time indicated, nuclei were hand-isolated, total RNA was harvested, and the RNA equivalent of two nuclei was resolved on a denaturing acrylamide gel. The migration of pre-tRNA^{Phe} and mature tRNA is indicated. **B:** To examine nuclear import of snoRNAs under different conditions, snoRNAs were coinjected with pre-tRNA^{Phe} as a negative control and U1 snoRNA as a positive control. The effects of coinjection with blue dextran and isolation of nuclei in two different buffers was examined. Mixtures of U1, U3, pre-U22, and pre-tRNA^{Phe} were injected into the cytoplasm of *Xenopus* oocytes with or without the addition of blue dextran mixed with the RNA. At the time points indicated, the nuclei six oocytes were isolated in either 5:1 PO₄ buffer or J buffer. Total RNA was isolated and the equivalent of two nuclei was resolved on an 8% acrylamide gel. U3 snoRNA has a stable degradation fragment seen in the nucleus (Baserga et al., 1992). U1 snoRNA undergoes processing, removal of 2 nt are seen (Baserga et al., 1992; Yang et al., 1992). U22 snoRNA is injected as an uncapped message, containing 5' and 3' additional sequences (polylinker); the mature form is seen in the nucleus. The mobility of each species is indicated at the right edge. The 2 h sample in J buffer plus blue dextran shows cytoplasmic contamination. **C:** Mixtures of U1, U8, and pre-tRNA^{Phe} were injected in the cytoplasm of *Xenopus* oocytes. At the time points indicated, the nuclei of six oocytes were isolated in 5:1 PO₄ buffer. Total RNA was isolated and the equivalent of two nuclei was resolved on a denaturing polyacrylamide gel. Mobility of each RNA is indicated at the right edge.

gether in coinjection experiments with pre-tRNA and U1 controls (Fig. 4B). Note that the pre-U22 snoRNA was injected as an uncapped transcript containing flanking 5' and 3' sequences because it is intron encoded in vertebrates (Tycowski et al., 1994).

A mix of the U3, pre-U22, U1, and pre-tRNA^{Phe} RNAs (with or without blue dextran marker dye) was injected into the cytoplasm of oocytes. Following incubation times as indicated, nuclei were isolated in either 5:1 PO₄ plus 10 mM Mg²⁺ or J buffer (see Materials and Methods). The enucleated cytoplasms were also collected. Total RNA was harvested from each of the resulting fractions and the RNAs were resolved by de-

naturing acrylamide gel electrophoresis. Figure 4B shows a typical time course of import under the four conditions used.

In these and other import experiments, the fraction of snoRNA imported was determined as the quantity of RNA in the nucleus, divided by the sum of cytoplasmic plus nuclear sample for each time point. For each sample, the percentage of pre-tRNA present in the nuclear fraction was used as a measure of the amount of cytoplasmic contamination and this amount was subtracted from all samples. These results indicated that there was no significant effect on import due to the presence of blue dextran, nor does the isolation buffer have an

effect on measured import efficiency. Nuclear accumulation of U3 snoRNA was barely detectable at 2 h after injection, but after a 20-h incubation, 20–25% of the injected U3 snoRNA was present in the nucleus. The U1 control was imported at a faster rate and a higher efficiency; up to as much as 60% of the U1 RNA was nuclear after 20 h. The pre-U22 was unstable in the cytoplasm. However, RNA corresponding to mature U22 was imported into and apparently stable in the nucleus. After 20 h, 20–25% of the U22 present in the oocyte was nuclear. Figure 4C shows comparable data for U8. Up to 20% of the injected U8 snoRNA accumulated in the nucleus after 20 h. These results clearly establish that snoRNAs injected into the cytoplasm are imported into the nucleus.

Is U22 required cotranscriptionally?

The relationship between transcription and snoRNA-facilitated processing was examined using transcriptional inhibitors. These experiments were designed to look for evidence of U8- and U22-dependent processing in the absence of new rRNA transcription in oocytes. Actinomycin D (AMD) is known to inhibit pre-rRNA synthesis, but allows processing of previously transcribed rRNA intermediates (Puvion-Dutilleul et al., 1992, 1997). AMD was used as an inhibitor of pre-rRNA synthesis to examine whether the snoRNAs could process the pool of previously transcribed pre-rRNAs.

Actinomycin D is typically administered to cells by addition to the culture media at various concentrations, although direct delivery via microinjection has also been described (Adamson & Woodland, 1977). In this work, the effects of externally applied versus injected AMD were indistinguishable. To examine whether the AMD inhibition of transcription was complete, oocytes were coinjected with AMD (23 ng per oocyte) and ^{32}P -UTP. The earliest time point, taken 30 min after injection, showed no incorporation of ^{32}P -UTP into pre-rRNA (data not shown). This is in contrast to untreated oocytes in which 40S was readily detected within 20 min (see Fig. 1B). After 7 h, there was still no significant incorporation of label into RNA, demonstrating a complete, rapid and long-lasting inhibition of transcription by AMD.

The effect of AMD on processing and turnover of an accumulated pool of labeled RNA was examined as follows. First, oocytes were injected with ^{32}P -UTP and incubated overnight to accumulate a pool of labeled RNA. The oocytes were then incubated in the presence of AMD and at various time points, nuclei were isolated and total RNA was extracted (see schematic above Fig. 5A). The results are shown in Figure 5A. After 1 h of AMD treatment, there was a significant decrease in the amount of full-length 40S pre-rRNA. Over time, this pre-rRNA was processed to yield mature rRNAs (Fig. 5A, lanes 2–4). By 2 h after AMD treatment, all the 20S precursor had been processed to

yield mature 18S rRNA (Fig. 5A, lane 3). By 4 h after AMD treatment, no 36S precursor and only a small amount of 32S precursor remained; the majority of both was converted to mature 28S (Fig. 5A, lane 4).

To examine whether the snoRNAs can facilitate processing of previously transcribed rRNA intermediates in the absence of new transcription, oocytes were injected with the antisense deoxyoligonucleotide specific for the snoRNA examined (U22 or U8) and ^{32}P -UTP. Oocytes were incubated for 12–18 h to allow an accumulation of the rRNA intermediates that appear in the absence of the snoRNA (see schematic above Fig. 5B). At $t = 0$, AMD was administered to the oocytes. One hour later, the corresponding snoRNA was microinjected into the cytoplasm of these AMD-treated (or –AMD control) oocytes and time points were taken as indicated. Figure 5B shows the results of the U22-snoRNA injection experiments. Lane 1 shows in vivo-labeled rRNA in untreated oocytes. Lane 2 contains RNA from an oocyte depleted of the endogenous U22 snoRNA; no mature 18S was detected. Lane 3 contains RNA from oocytes treated with AMD for less than 2 min (i.e., $t = 0$). Lanes 4–7 contain RNA from oocytes treated with AMD for 1 h before receiving a cytoplasmic injection of U22 snoRNA followed by incubation for the indicated length of time. The 20S precursor of mature 18S persisted for over 5 h after injection of U22 snoRNA. The –AMD samples in lanes 8–11 were treated similarly except that no AMD was added. In these samples the appearance of mature 18S rRNA was detected within the first hour after injection of U22 snoRNA.

DISCUSSION

In this work, the rate at which newly synthesized, mature rRNAs accumulate in *Xenopus* oocytes was examined. Microinjection of ^{32}P -UTP into *Xenopus* oocytes produced detectable levels of 40S pre-rRNA precursor within 20 min, demonstrating that full-length rRNA transcripts accumulate prior to processing. In *Xenopus* oocytes, trace amounts of mature 18S rRNA were detected as early as 20 min after injection, but were more abundant after 40–60 min. In contrast, mature 28S rRNA and 12S, the long-lived precursor to 5.8S rRNA, were not detected until 3–4 h after the UTP injection. Together, these data indicated that the processing events are the rate limiting step in the appearance of mature 18S and 28S rRNAs.

The rate at which mature rRNAs appear in *Xenopus* oocytes is slower than that previously reported for other species and other stages of *Xenopus* development (Shiokawa et al., 1987; Hadjiolova et al., 1993; Beltrame et al., 1994; Henry et al., 1994; Zanchin & Goldfarb, 1999). In general, a longer cell cycle appears to correspond with a longer time period required for rRNA biosynthesis. Another general feature of the process-

ing time course is that mature 18S rRNA typically appears prior to mature 25S/28S rRNA.

Thus, *Xenopus* oocytes appeared to be an ideal model system in which to examine the rate at which mature rRNA accumulates in a snoRNA-dependent manner. Here I present data examining the effect of exogenous U8 and U22 snoRNAs on the rate of pre-rRNA processing. U22 snoRNA is required for 18S accumulation (Tycowski et al., 1994) whereas U8 snoRNA is essential for accumulation of both 5.8S and 28S maturation (Peculis & Steitz, 1993). I have demonstrated that these snoRNAs, when microinjected into the cytoplasm of oocytes, could rescue processing of the pre-rRNA with kinetics that were slower than the rate of processing in an undisturbed oocyte.

In a U22-depleted oocyte subsequently injected with exogenous U22, mature 18S was first detected 40 min after snoRNA injection, as opposed to 20 min in an undisturbed oocyte. In the U8-depleted oocyte, exogenous U8 facilitated maturation of 28S and the 12S precursor such that both rRNAs were apparent within 4 h of snoRNA injection, rather than the 2–3-h time frame seen in undisturbed oocytes. The rate of mature rRNA appearance is slower than that seen in undisturbed oocytes. These data illustrate two key points. (1) The snoRNAs injected into the cytoplasm of oocytes can be imported into the nucleus. (2) Even for those snoRNAs that do not require a cytoplasmic maturation phase, there exists a mechanism that allows their import into the nucleus when injected into the cytoplasm. These data also raise two key questions. (1) Is the timing of the appearance of mature rRNA in an snoRNA-rescued oocyte consistent with the assembly of the microinjected exogenous snoRNA on a nascent pre-rRNA transcript? (2) Are the preexisting pre-rRNA templates a substrate for processing by the snoRNA? Each of these issues is discussed in turn below.

SnoRNAs can be transported from the cytoplasm into the nucleus

³²P-labeled snoRNAs injected into the cytoplasm can be seen to accumulate in the nuclear compartment over time

The snoRNAs examined here were radioactively labeled by *in vitro* transcription and injected into the cytoplasm of oocytes. At various time points, oocytes were dissected and nuclei and enucleated cytoplasms were collected. SnoRNAs were detected in the nucleus within 6 h of injection and continued to accumulate in the nucleus over the full 20-h time course.

Direct evidence for U3 nuclear import has previously been described. After microinjection of U3 into the cytoplasm of *Xenopus* oocytes, nuclear accumulation was assayed by microdissection (Baserga et al., 1992;

Michaud & Goldfarb, 1992; Murdoch & Allison, 1996). The results establish that U3 is imported via an active transport mechanism involving nuclear pore function and a targeting pathway independent of those used by karyophilic proteins or the spliceosomal U snRNAs (Michaud & Goldfarb, 1992). Use of separate import pathways could explain the slower import kinetics seen here for the snoRNAs versus U1 snRNA.

Although the literature includes many accounts of snoRNAs being both imported and exported from various types of cells under various conditions (Baserga et al., 1992; Glibetic et al., 1992; Michaud & Goldfarb, 1992; Peculis & Steitz, 1993, 1994; Azum-Gelade et al., 1994; Murdoch & Allison, 1996; Sienna et al., 1996), there are also contradicting published accounts wherein the authors fail to detect nuclear import of the snoRNAs examined (Speckmann et al., 1999).

These apparent differences might arise from differences in experimental procedures. Two variables that could be closely examined were the effects of coinjection of a large dye, blue dextran, with the snoRNA as previously described (Speckmann et al., 1999) and isolation of the nuclei in a different aqueous solution (J buffer versus 5:1 PO₄ plus 10 mM Mg²⁺). However, the results indicated that neither the injection nor the isolation conditions significantly affected the rate or efficiency of import of any of the small RNAs examined here. When import was not detected (Speckmann et al., 1999), the ovary had been collagenase treated, to chemically release individual oocytes. There are reports that this treatment can adversely affect oocyte metabolism (Smith, 1991; Terns & Goldfarb, 1998), and its effects can be variable, making this difficult to reproduce completely from laboratory to laboratory. The variation in viability observed for collagenase-treated oocytes prevented their use in the time course experiments described here. Thus, possible adverse effects of collagenase treatment were not tested, but might account for the differences in import observed by others.

Functional analysis of pre-rRNA processing demonstrates nuclear import of cytoplasmic injected snoRNAs

We used the ability of snoRNAs to rescue pre-rRNA processing as a functional assay for nuclear import of these snoRNAs. With this functional assay as a method of detection for the snoRNAs, it appears that the snoRNAs were imported and assembled into active snoRNPs to facilitate pre-rRNA processing in less than 1 h for U22 snoRNA, and in less than 4 h for U8 snoRNA. A comparison of the time course for rescue of processing (Figs. 2 and 3) with that for nuclear import (Fig. 4B,C) reveals that functional processing is restored upon nuclear import of only a small fraction of the cytoplasmic-injected snoRNA. These amounts are less than can be detected directly by typical methods of RNA labeling

and autoradiography, and only a very small fraction of that routinely injected into oocytes. This suggests only a relatively small amount of snoRNA is required to restore processing. Because the *Xenopus* oocytes stockpile large amounts of the cellular machinery to be used later in development, the oocyte contains vastly more U8 or U22 snoRNA than is required for maintenance of oocyte rRNA processing. Import and nuclear accumulation of the snoRNAs throughout the time course suggests these processes may not be directly regulated by the demand for processing.

Can snoRNAs translocate across the nuclear envelope?

A decade or more ago it was believed that the snoRNAs, like the snRNAs, would require a transient cytoplasmic stage for 5' cap hypermethylation and RNP assembly. More recently it was demonstrated that when snoRNAs are injected into the nucleus, 3' end maturation (Cheng et al., 1995) and 5' cap hypermethylation (Peculis & Steitz, 1994; Terns & Dahlberg, 1994) could also occur in the nucleus. Some reports indicated that when U3 or U8 snoRNA was injected into the cytoplasm, it could not be subsequently detected in the nucleus (Speckmann et al., 1999). Thus, it was proposed that the snoRNAs did not have the ability to cross the nuclear envelope (Terns & Dahlberg, 1994; Terns et al., 1995). However, there was a large body of evidence that did not agree with this conclusion.

Previously, examples of normal, physiological processes involving U3 transport across the nuclear envelope have been described. One example is mitosis and nuclear envelope breakdown in higher eucaryotic cells, which is accompanied by cessation of rRNA synthesis and dispersal of U3 and U8 into the cytoplasm. Later in telophase, U3 and U8 assemble into prenucleolar bodies that ultimately associate with functional nucleoli (Azum-Gelade et al., 1994; Jimenez-Garcia et al., 1994; Matera et al., 1994; Weisenberger & Scheer, 1995).

A second example of snoRNA transport across the nuclear envelope has been observed in serum starvation of mouse fibroblasts (Sienna et al., 1996), which results in a relocation of ~40% of the U3 into a stable cytoplasmic pool. Within 5 h of subsequent serum stimulation, this cytoplasmic pool of U3 is relocated to the nucleus in a process sensitive to actinomycin D or cycloheximide. This process is specific to U3; U8 is retained in the nucleus. Finally, myoblast differentiation is accompanied by redistribution of U3 in a process that may also include the accumulation of a stored, cytoplasmic pool of U3 snoRNA (Glibetic et al., 1992).

The data presented here demonstrate at a functional level that the snoRNAs, when injected into the cytoplasm, are imported into the nucleus. This does not

alter or contradict the premise that in the normal maturation process, the snoRNAs need not cross the nuclear envelope. It does, however, demonstrate that cytoplasmic injected snoRNAs can ultimately assemble into stable functional particles that function in the nucleolus.

Is the appearance of the rRNA rescue consistent with assembly of the snoRNPs on nascent transcripts?

SnoRNA-depleted oocytes were microinjected with exogenous snoRNAs and time points were taken to identify the rate at which mature rRNAs were first detected. This time frame would represent the time required for assembly, import, and functional reconstitution of the ribosomal processing machinery. The data indicated that the introduction of exogenous snoRNA did result in the production of mature rRNA, but at a rate slower than that observed in an undisturbed oocyte.

One interpretation of these data is that the delay in appearance of mature rRNAs (20 min for U22-dependent processing of 18S and 1–2 h for U8-dependent processing of 12S and 28S rRNAs) could reflect solely the rate of assembly, import, and functional reconstitution of these snoRNA. In this scenario, the rate of import is initially limiting for processing. However, once imported, the functional snoRNPs may process either or both the preexisting intermediates and nascent precursor rRNAs.

An alternative interpretation would be that the snoRNAs are present and functional in the nucleolus, but need to co-assemble with the nascent pre-rRNAs. In this scenario, the snoRNA would not effectively process the previously transcribed (but unprocessed) pre-rRNAs that accumulated in the absence of the snoRNA. Thus one might expect a lag or delay in the accumulation of mature rRNAs, during which nascent transcripts would initiate transcription and upon which the snoRNA would assemble cotranscriptionally to facilitate processing.

Are the preexisting pre-rRNA templates a substrate for processing by the snoRNA?

To examine the transcriptional state of the pre-rRNAs that are processed after introduction of the snoRNA, we examined the ability of the snoRNAs to facilitate processing in an oocyte where polymerase I transcription had been inhibited. A pool of in vivo-labeled pre-rRNA precursors was generated by transcription of the endogenous rDNA genes in the presence of ³²P-UTP, and in the absence of one specific snoRNA. New transcription was then inhibited by the addition of AMD. Following microinjection of the snoRNA, pre-rRNA was examined for evidence of processing of the precursors in a transcriptional-independent manner. The data for

the AMD experiments would support a cotranscriptional requirement for the snoRNA.

In the presence of AMD in an undisturbed oocyte, new transcription is halted and the previously accumulated rRNA precursors are processed to mature 18 and 28S rRNAs. However, in the absence of either U8 or U22, there is no significant accumulation of mature 28S or 18S, respectively. As is shown in Figure 5B, there is a very weak band comigrating at the position of mature 18S rRNA for the U22 samples. However, there is no increase in the intensity of this band over time, nor is there a disappearance of the 20S rRNA, the precursor from which 18S could form. At this point, the identity of this weak band is not known. Three possibilities are (1) a fortuitously comigrating band, (2) misfolded/misassembled authentic 18S that cannot be exported to the cytoplasm, or (3) a trace amount of 18S from a transcript not completely inhibited by AMD that was cleaved, in a U22-dependent manner, but not exported to the cytoplasm.

The AMD experiments are faulted by a lack of knowledge about the extent to which other cellular processes may be disrupted by these treatments (i.e., nuclear transport, polymerase II activity or, translation). Because it is possible that other cellular functions essential for pre-rRNA processing may have been inhibited by AMD, the negative result from the attempted rescue in the absence of transcription is difficult to interpret. Although the results are entirely consistent with a cotranscriptional requirement for U22, these data fall short of confirming this conclusion.

The data presented here demonstrate at an observational as well as a functional level that snoRNAs injected into the cytoplasm can be imported into the nucleus of *Xenopus* oocytes. The two snoRNAs examined here, U8 and U22, rapidly import into the nucleus, assemble, and assist in the processing of the mature rRNAs. In rescued oocytes, where an snoRNA has been depleted and exogenous snoRNA introduced, the mature rRNAs accumulate with slightly slower kinetics than in undisturbed oocytes. These results are consistent with a cotranscriptional requirement for the snoRNAs, which would also allow them to play the previously proposed role of rRNA chaperones (Steitz & Tycowski, 1995; Peculis, 1997b) to assist in folding as well as cleavage of the pre-rRNA.

MATERIAL AND METHODS

RNAs

Endogenous U8 snoRNA was depleted via microinjection of the previously described U8-specific deoxyoligonucleotide, U8-40 (Peculis & Steitz, 1993). U8 RNA was generated by T7 runoff of the pspU8-2 construct, after digestion with *Bst*BI as previously described (Peculis & Steitz, 1993).

Endogenous U22 snoRNA was depleted following microinjection of the previously described U22-specific deoxyoligo-

nucleotide, X-U22-3' (Tycowski et al., 1994). U22 RNA was generated by T7 runoff transcription of the X-U22 template, after digestion with *Pst*II. XU22 was generated via PCR using *Xenopus* genomic DNA as template and the following deoxyoligonucleotides complementary to *Xenopus* U22 as previously published (Tycowski et al., 1994). XU22-5'RI 5'-AAA CCGAATTCCCCCAATGATGAAACCTTC-3' contained an *Eco*RI site followed by 19 nt identical to the 5' end of U22. XU22-3'Pst 5'-TCCTCCTCTGCAGCCCCCTCAGACAGTTC CAGC-3' was complementary to 20 nt at the 3' end of U22 RNA followed by a 3' *Pst*I site. This PCR product was digested with *Eco*RI and *Pst*I and cloned into the *Eco*RI and *Pst*I sites of pGem3z (Promega). A T7 runoff transcript from the vector-encoded promoter produced a sense strand U22 RNA containing extra (polylinker) sequence at both the 5' and 3' ends. U1 snRNA was generated by T7 runoff transcription of pU1-T7 (Baserga et al., 1991), after digestion with *Eco*RI. Pre-tRNA^{Phe} was generated by T7 runoff transcription of T7pre^{Phe} (Haselbeck & Greer, 1993) after digestion with *Bst*NI. U3 RNA was generated by T7 transcription of U3.3A (Baserga et al., 1991) after digestion with *Rsa*I. Transcription protocols were as previously described (Peculis & Steitz, 1993) using GpppG cap analog (Pharmacia) when indicated.

Oocyte injections

Xenopus oocytes were manually dissected from ovary tissue and maintained in MBS (Peng, 1991) continuously. The stage 5/6 oocytes were injected as indicated schematically in the figure or described in the text. ³²P-UTP injections consisted of 23 nL of ³²P-UTP 3,000 Ci/mmol (Amersham) injected into the cytoplasm of each oocyte. RNA injections consisted of 23 nL volume delivered to the cytoplasm at the equator of the oocyte, directed toward the vegetal pole. The RNA (or pooled RNAs) were each present in the stock solution at a concentration of 25 ng/μL. For pre-tRNA controls, the nuclear injection consisted of 9.2 nL of pre-tRNA^{Phe} of a stock RNA at 20 ng/μL. For cytoplasmic RNA injections containing blue dextran, the RNA was at 25 ng/μL and the final concentration of blue dextran (Sigma) was 20 mg/mL.

Nuclear isolations

Except in those situations described in the text, all nuclei were manually dissected in 5:1 PO₄ buffer plus 10 mM Mg²⁺ as previously described (Gall et al., 1991; Peculis & Steitz, 1993). For isolations in J buffer (Birkenmeier et al., 1978), oocytes were transferred into J Buffer and the nuclei isolated immediately. Total RNA was isolated and resolved on denaturing agarose or polyacrylamide gels as previously described (Peculis & Steitz, 1993, 1994).

Transcriptional inhibitors

Oocytes were manually dissected and maintained in MSB. The oocytes were injected as indicated schematically in the figure or described in the text. ³²P-UTP injections consisted of 23 nL of ³²P-UTP 3,000 Ci/mmol (Amersham) injected into the cytoplasm of each oocyte. For snoRNA depletions, the corresponding deoxyoligonucleotide was injected into the cytoplasm. Actinomycin D (Sigma) was administered inter-

nally (injection of 23 nL of a 1 mg/mL stock into the cytoplasm) or externally (oocytes soaked in MBS + AMD at a final concentration of 1 mg/mL). The overall results were identical, although the time of transcriptional inhibition differed slightly with internal versus external application of AMD. RNA injections consisted of 23 nL volume of a 25 ng/ μ L RNA stock delivered to the cytoplasm at the equator of the oocyte, directed toward the vegetal pole.

Quantitation

The amount of snoRNA imported into the nucleus was determined via Fuji Phosphorimager, and calculated as amount imported/(amount imported + amount remaining in cytoplasm). This calculation was performed on the pre-tRNA in each lane and the percent of cytoplasmic contamination determined. This percentage was subtracted from the percentage import of each snoRNA and the reported values represent the adjusted amount.

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