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Nuclear Processing of the 3′-Terminal Nucleotides of Pre-U1 RNA in *Xenopus laevis* Oocytes

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U1 small nuclear RNA is synthesized as a precursor with several extra nucleotides at its 3′ end. We show that in *Xenopus laevis* oocytes, removal of the terminal two nucleotides occurs after the RNA has transited through the cytoplasm and returned to the nucleus. The activity is controlled by an inhibitor of processing, which we call TPI, for 3′-terminal processing inhibitor. This inhibitor is sensitive to both micrococcal nuclease and trypsin treatment, indicating that it is a nucleoprotein. TPI inhibits the 3′ processing of pre-U1 RNAs that have 5′ ends containing m^7G caps but not mature $m^{2,2,7}G$ caps; this finding suggests that TPI interacts directly or indirectly with the 5′ end of pre-U1 RNA. The inhibition of processing by TPI, almost complete at 19°C, is reversibly inactivated at slightly higher temperatures. TPI activity is solely in the soluble fraction of oocyte nuclear extracts, in contrast to the 3′-terminal processing activity, which is present in both the particulate and soluble fractions. We propose that the differential processing of the 3′-terminal nucleotides of pre-U1 RNA after its return from the cytoplasm, but not before its exit from the nucleus, may be due to the association of TPI with the m^7G cap on the newly synthesized pre-U1 RNA.

Small nuclear RNAs (snRNAs), which participate in the processing of precursors of mRNAs and rRNAs, must themselves undergo maturation before they are functional. Initially, pre-U1 RNAs contain monomethyl m^7G caps at their 5′ ends and short extensions at their 3′ ends (reviewed in reference 2). Much of the maturation of snRNAs occur in the cytoplasm, where the RNA associates with specific proteins to form ribonucleoprotein particles (snRNPs), the cap becomes hypermethylated to $m^{2,2,7}G$, and some of the extra nucleotides at the 3′ end are removed (see references 25 and 29 for recent reviews).

Relatively little is known about the trimming of the extra nucleotides from 3′ ends of pre-snRNAs. Recently, Neuman de Vegvar and Dahlberg (24) showed that human and frog pre-U1 RNA synthesized in the nucleus of *Xenopus laevis* oocytes probably is exported as the primary transcript (17). In the cytoplasm, pre-U1 RNAs are processed by 3′ exonucleases to yield molecules with only two extra 3′ nucleotides (24). It generally has been assumed that removal of the last two nucleotides of the U1 RNA precursor (the terminal processing) also occurred in the cytoplasm, prior to or concurrent with transport back into the nucleus (3, 19).

To address the questions of where and how the 3′-terminal processing of pre-U1 RNA occurs, we used *X. laevis* oocytes, taking advantage of their large and easily manipulable nuclei (germinal vesicles [GVs]). This system allowed us to isolate both nucleus-free cytoplasm (4, 7) and intact functioning nuclei (17, 18).

In this study, we demonstrate that removal of the last two nucleotides at the 3′ end of pre-U1 snRNA takes place within the nucleus after reimport. To characterize this process, we developed an accurate and efficient in vitro processing system derived from *X. laevis* GVs. We show that the GVs contain at least two activities related to the terminal processing of the 3′ end of U1 snRNA: one that removes the last one or two nucleotides of pre-U1 RNA, and another that inhibits this processing when the transcript contains an m^7G cap. We propose that this latter activity serves to protect primary transcripts from being processed until they have exited the nucleus and reentered it in the form of $m^{2,2,7}G$-capped snRNAs.

MATERIALS AND METHODS

Materials. The wild-type U1 template was the *X. laevis* XU1b1 gene (14, 16). The mutant U1 gene ΔD, lacking the Sm antigen binding site (11), was provided by I. W. Mattaj. The wild-type U2 (22), U4 (27), and U5 (13) templates were obtained from I. W. Mattaj. Anti-m^7G antibodies were generously supplied by T. W. Munns (23). Anti-$m^{2,2,7}G$ antibodies were a gift from R. Lührmann (1). The monoclonal anti-Sm (Y-12) antibodies were provided by J. A. Steitz (15), and the polyclonal anti-(U1) RNP antibodies were as described elsewhere (24). m^7(5′)GpppG(5′) and other cap analogs were from New England Biolabs (Beverly, Mass.); tobacco acid pyrophosphatase (TAP) and RNAsin were from Promega (Madison, Wis.). EDTA, EGTA, trypsin (type XIII), soybean trypsin inhibitor, and micrococcal nuclease (MN) were from Sigma Corp. (St. Louis, Mo.). Proteinase K was from Boehringer Mannheim (Indianapolis, Ind.).

Oocyte injection and RNA extraction and analysis. Stage VI oocytes were obtained from *X. laevis* frogs as described previously (14, 16). A solution (10 to 20 nl) containing 10 mM Tris-HCl (pH 7.6) and ^{32}P-labeled RNA from one to two GVs (see below) was injected into each oocyte. For analyses of in vivo-made snRNAs, DNA templates (~2 ng per oocyte) were coinjected with [α-^{32}P]GTP (~0.5 μCi per oocyte) (14). After incubation of the injected oocytes at 19°C for the desired times in MBS-H buffer [88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 10 mM *N*-hydroxyethylpiperazine-*N*′-ethanesulfonic acid (HEPES; pH 7.5), 0.8 mM $MgSO_4$, 0.3 mM $Ca(NO_3)_2$, 0.4 mM $CaCl_2$, 10 mg of penicillin per ml, 10 mg

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of streptomycin per ml (8)], the GVs and cytoplasms were isolated from individual oocytes by manual dissection under mineral oil (17, 18). Whole or dissected oocytes were homogenized in 80 μl of proteinase K solution (0.8 mg/ml in 50 mM Tris-HCl [pH 7.6]–10 mM EDTA–1% sodium dodecyl sulfate) (14). After digestion for 1 to 2 h at 37°C, RNAs were isolated by phenol-$CHCl_3$-isoamyl alcohol (24:24:1) extraction and ethanol precipitation. Purified RNAs were fractionated by electrophoresis in 8% (29:1) polyacrylamide gels (45 cm long by 0.4 mm thick) containing 7 M urea, 1× TEB buffer (89 mM Tris [pH 8.3], 88.9 mM boric acid, 2.8 mM EDTA) at 1,400 to 1,500 V for 5.5 h.

Preparation of nuclear extracts. Dissections and homogenizations were done at room temperature. GVs were isolated under oil (17, 18) and transferred into a small nonsiliconized glass tube (made from a glass Pasteur pipet), using a 5-μl micropipet. Under a dissecting microscope, the GVs were manually homogenized for 1 to 2 min at 2.0 GV per μl in D buffer (250 mM sucrose, 50 mM Tris-HCl [pH 7.6], 25 mM KCl, 5 mM $MgCl_2$), using a pulled capillary tube (outer diameter, 0.63 mm; inner diameter, 0.20 mm; Drummond Scientific). After spinning at 4°C in an Eppendorf centrifuge at 14,000 × *g* for 2.5 min, the supernatant was used to make the nuclear soluble NS fraction; it was carefully collected and cleared by a second centrifugation (2.5 min). The pellet was used to make the nuclear particulate (NP) fraction by one to two washes with D buffer (2 μl per GV) and resuspension in D buffer at 2.0 GV per μl. All fractions were stored on ice until use.

In large-scale preparations of NS fraction, 100 oil-isolated GVs were placed into a 0.5-ml Eppendorf tube and spun for 10 s in a microfuge. The excess oil over the GV pellet was removed, and 50 μl of cold D buffer containing 3 mM dithiothreitol was added. The GVs were homogenized by drawing the solution up and down several times in a yellow pipet tip. The homogenate was spun at 10,000 rpm in a microfuge for 2.5 min. The NS supernatant fraction was removed, and RNAsin was added to a final concentration of 1 U/μl.

Immunoprecipitations. Anti-m^7G, anti-$m^{2,2,7}G$, anti-Sm, or anti-RNP antibodies (at ~20 mg of immunoglobulin G per ml) were coupled to protein A–Sepharose CL-4B beads (Sigma) at ~1.0 mg of immunoglobulin G per 23 mg of protein A-beads in IPP_H (500 mM NaCl, 10 mM Tris-Cl; [pH 7.6], 0.1% Nonidet P-40) according to Bringmann et al. (1). The antibody-Sepharose beads were washed five times with 1 ml of IPP_L (150 mM NaCl, 40 mM Tris-Cl [pH 7.6], 1 mM EDTA, 0.05% Nonidet P-40) before being mixed with the U1 RNA or U1 RNP preparation. The RNA-bead complexes or the RNP-bead complexes were washed five times with 1 ml of IPP_L and stored at 4°C. To release the bound RNAs, the RNA-beads were incubated in 150 μl of proteinase K solution for 0.5 to 2 h at 37°C with end-over-end rotation. RNAs were then extracted with phenol-$CHCl_3$-isoamyl alcohol, precipitated by ethanol, and resuspended in 10 mM Tris-HCl (pH 7.6) so that 1 μl of solution contained one GV-equivalent of RNA.

Preparation of ^{32}P-labeled processing reaction substrates. Pre-$U1_{+2}$ RNA was synthesized in vitro by using preinjected GVs (15) containing 1 to 2 ng of U1 template DNA per GV; oil-isolated GVs were labeled with 4 to 5 nl of [α-^{32}P]GTP (400 Ci/mmol) per GV (~0.5 μCi per GV) and incubated at 18 to 20°C for 1.5 to 2 h. After isolation of the RNA, m^7G-containing pre-U1 RNA was immunoprecipitated by anti-m^7G antibodies as described above.

m^7G-$U1_{+8/9}$ precursor was obtained by transcription with SP6 RNA polymerase (24), using a synthetic *X. laevis* U1 template extending to position +40 (15a); $U1_{+8/9}$ RNA was generated by incubation with a DNA antisense oligonucleotide and cleavage by RNase H (26).

$m^{2,2,7}G$-$U1_{+2}$ precursor and $m^{2,2,7}G$-$U1_{+2}$ snRNP were made by injecting ^{32}P-labeled pre-U1 RNA (see above) into cytoplasms of intact oocytes at 1 GV-equivalent of RNA per oocyte. After 5 to 7 h of incubation, oocytes were dissected under oil and the nucleus-free cytoplasm was homogenized in 140 mM NaCl–40 mM Tris (pH 7.6)–1.0 U of RNAsin per μl–3 mM dithiothreitol (80 μl of solution per oocyte). After centrifugation, the supernatant was divided into two aliquots. From one aliquot, the RNAs were extracted and subjected to immunoprecipitation with anti-$m^{2,2,7}G$-antibodies to make $m^{2,2,7}G$-$U1_{+2}$ RNA; from the other aliquot, $U1_{+2}$ snRNPs were immunoprecipitated directly with either anti-$m^{2,2,7}G$ or anti-Sm Sepharose-coupled antibodies to yield $m^{2,2,7}G$-containing snRNPs. Before being added to the in vitro processing reaction, the bead-bound snRNPs were washed two times with 0.5 ml of D buffer.

TAP treatment of pre-U1 RNA. Pre-U1 RNA (8 GV-equivalents) was incubated in 25 μl of 50 mM sodium acetate (pH 5.0)–1 mM EDTA–10 mM 2-mercaptoethanol with 9 U of tobacco acid pyrophosphatase (TAP) at 37°C for 30 min. RNAs were then phenol extracted and ethanol precipitated.

In vitro processing reaction and analysis. Nuclear extracts were prepared as described above and adjusted to 2 mM dithiothreitol and 1.0 U of RNAsin per μl. One GV-equivalent of substrates was added to 3 μl of the extracts, and reaction mixtures were incubated at the temperatures and times indicated in the text and figure legends. To stop processing, 80 μl of the proteinase K solution was added to each reaction mixture, and the samples were incubated at 37°C for 1 h. RNAs were extracted and analyzed on 8% polyacrylamide gels containing 7 M urea (see above).

Depletion of Sm antigen from NS fraction. The NS fraction (20 μl, or 40 GV-equivalents) was incubated with an excess of monoclonal Y-12 antibodies (15) coupled to protein A-Sepharose beads at 4°C for 8 h with occasional mixing. After centrifugation at 12,000 × *g* for 30 s, the supernatant (depleted NS) was collected and stored on ice until use. The extent of depletion of snRNPs was assayed by monitoring the level of U1 RNA by Northern (RNA) blot analysis; the depleted NS fraction did not contain any detectable U1 RNA (data not shown).

MN treatment of the NS fraction. Extracts were treated with 1 to 3 U of MN per μl in D buffer as described in the figure legends. MN was activated by the addition of 2 mM Ca^{2+} or inactivated by the addition of 5 mM EGTA (either before or after incubation, depending on the experiment). In control experiments, *Escherichia coli* 23S rRNA or tRNA (at a final concentrations of 50 to 500 μg/ml) was added to MN-treated NS fraction prior to substrate addition (see Fig. 4; also data not shown). These levels were chosen to be in excess of the endogeneous concentrations of RNA and DNA in GV extracts, estimated to be 20 to 30 μg/ml and 60 to 80 ng/ml, respectively (9).

RESULTS

To study nucleocytoplasmic transport and processing of U1 snRNA, we microinjected ^{32}P-labeled *X. laevis* pre-$U1_{+2}$ RNA into nuclei or cytoplasm of *X. laevis* oocytes. At various times after RNA injection, the oocytes were dissected into nuclear and cytoplasmic fractions and the content of ^{32}P-labeled RNAs was analyzed by electrophoresis in

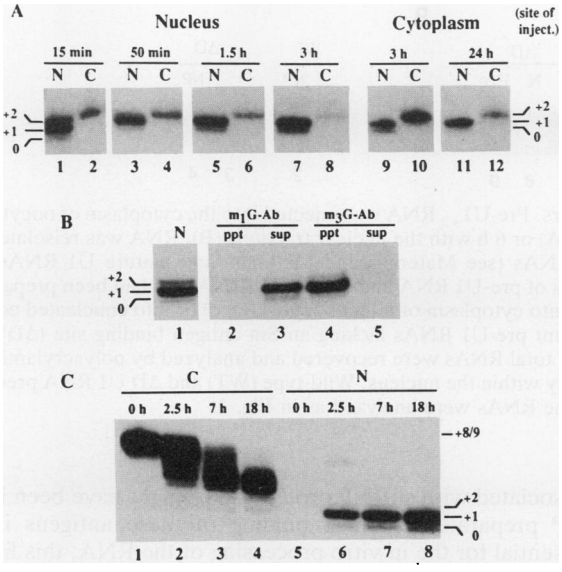

FIG. 1. Nuclear transport and 3′-end processing of U1 RNA in *X. laevis* oocytes. In this and other figures, +8/9, +2, +1, and 0 indicate the number of extra nucleotides at the U1 RNA 3′ end (24). (A) Nucleocytoplasmic distribution after microinjection into nuclei or cytoplasms. ^{32}P-labeled pre-U1$_{+2}$ RNA was injected into the oocyte nucleus (lanes 1 to 8) or into oocyte cytoplasm (lanes 9 to 12). RNAs were then isolated from both nucleus (N) and cytoplasm (C) of individual oocytes after increasing incubation periods as indicated and were analyzed by electrophoresis in an 8% polyacrylamide gel containing 7 M urea. (B) Cap structure of U1 RNAs after nuclear transport. Total nuclear U1 RNAs (N) were isolated 1 h after injection of m^{7}G-capped preU1$_{+2}$ RNA into the nucleus (lane 1) and were analyzed for their cap structures by immunoprecipitation with Sepharose-bound m^{7}G (lanes 2 and 3) or m^{2,2,7}G antibodies (lanes 4 and 5). RNAs from both precipitates (ppt) and supernatants (sup) were isolated and analyzed as in panel A. (C) Cytoplasmic 3′-end processing of longer U1 RNA precursors. ^{32}P-labeled pre-U1$_{+8/9}$ RNA was injected into the oocyte cytoplasm, and the extent of cytoplasmic 3′-end trimming (lanes 1 to 4) and nuclear transport (lanes 5 to 8) was monitored as a function of time. RNAs were isolated from pooled nuclei or cytoplasms, and one oocyte equivalent was analyzed as in panel A.

denaturing polyacrylamide gels. We specify the various forms of U1 snRNA and its precursors as U1$_0$, U1$_{+1}$ and U1$_{+2}$, with the subscript number referring to the number of extra 3′ nucleotides relative to the 164-nucleotide-long mature U1 RNA (24).

Location of processed U1 RNAs solely in the nucleus. When m^{7}G-capped pre-U1$_{+2}$ RNA was injected into the nuclei of oocytes, it appeared in the cytoplasm after a brief delay, still as the +2 form (Fig. 1A, lanes 1 and 2). In the cytoplasm, these RNAs did not change in size (lanes 4, 6, and 8) and, like the injected pre-U1$_{+2}$ RNA (lane 10), migrated as a single band. Longer heterogeneous m^{7}G cap-containing ^{32}P-labeled RNAs that were present in the pre-U1$_{+2}$ RNA preparations (presumably partial degradation products of mRNAs that were also precipitated by the m^{7}G antibodies) served as an internal control for the accuracy of injection into the nucleus and for leakage of injected RNAs out of the nucleus. These mRNA fragments remained in the nucleus, confirming that the pre-U1 RNA molecules were indeed injected into the nucleus and suggesting that the export of pre-U1 RNA is a selective process (not shown). With time, increasing amounts of the injected U1 RNA reappeared in the nucleus, now exclusively in the form of U1$_{+1}$ and U1$_0$ RNAs (Fig. 1A, lanes 3, 5, and 7). Since these processed nuclear U1$_{+1}$ and U1$_0$ RNAs were no longer precipitable with m^{7}G antibodies (Fig. 1B, lanes 2 and 3) and they all contained m^{2,2,7}G caps (lanes 4 and 5), they must have transited through the cytoplasm (24).

Comparable results were obtained when the pre-U1 RNA was injected into the cytoplasm rather than the nucleus (Fig. 1A). Again, all of the cytoplasmic RNAs were U1$_{+2}$ and the nuclear RNAs were U1$_{+1}$ or U1$_0$ RNA (compare lanes 10 and 12 with lanes 9 and 11, respectively). Paradoxically, the appearance of U1$_{+1}$ and U1$_0$ RNA in the nucleus was significantly slower when U1$_{+2}$ RNA was injected directly into the cytoplasm rather than into the nucleus (compare lanes 9 and 10 with lanes 7 and 8); the reason for this difference in import kinetics is unclear, but it may reflect differences in the factors associated with the RNA or in the local concentrations of U1 RNA in the region immediately adjacent to the nucleus.

Inspection of the cytoplasmic and nuclear RNAs in Fig. 1A shows that the cytoplasmic pre-U1 RNAs seem to accumulate as U1$_{+2}$ and then appear as U1$_{+1}$ or U$_0$ RNA in the nucleus. Moreover, as previously reported (24), longer U1 RNA precursors with more than two extra nucleotides at their 3′ ends must be trimmed by a cytoplasmic exonuclease to U1$_{+2}$ before entry into the nucleus can occur (Fig. 1C; compare lanes 1 to 4 with lanes 5 to 8).

A nuclear processing activity for U1$_{+2}$ RNA. The results shown in Fig. 1 indicate that the nucleus plays a role in terminal processing of pre-U1 RNA. To test directly whether the processing activity that converts U1$_{+2}$ to U1$_{+1}$ (or U1$_0$) is nuclear, we injected pre-U1 RNA into enucleated oocytes or, as a control, into the cytoplasm of intact oocytes (Fig. 2A). Twelve hours after injection, about a third of the U1 RNA in whole oocytes was processed (lane 3), but no U1 RNA was processed in enucleated oocytes (lane 4). Thus, the processing activity requires the nucleus.

To examine whether the 3′-terminal processing activity might be on the cytoplasmic side of the nuclear membrane (i.e., accessible to RNAs that are still cytoplasmic), either wild-type or a mutant pre-U1 RNA (ΔD) lacking the Sm antigen binding site (11) was injected into the cytoplasm of intact oocytes (Fig. 2A, lanes 5 to 9). The latter RNA cannot be transported into the nucleus because it fails to associate with Sm proteins (21). Lanes 7 to 9 show that 12.5 h after injection, the mutant RNA was unchanged in size and remained in the cytoplasm.

A crude particulate fraction of isolated nuclei (NP) was able to process the 3′ end of U1$_{+2}$ RNA (Fig. 2B, lanes 1 and 2). Moreover, ΔD U1$_{+2}$ RNA was processed by the NP fraction (lanes 3 and 4), demonstrating that the failure of this mutant RNA to be processed after injection into cytoplasm of intact oocytes was not due to its inability to serve as a substrate of the processing activity. These observations, taken together with our findings that U1$_{+1}$ and U1$_0$ RNAs are not observed in the cytoplasm (Fig. 1), led us to conclude that processing of U1$_{+2}$ takes place within the nucleus or as the RNA is transported through the nuclear envelope.

The activity in the NP fraction responsible for the 3′ processing was probed by treatment with various agents prior to assay (Fig. 3A). The component(s) was sensitive to treatment by heat (lane 2) or trypsin (lane 3) but was resistant to treatment with MN (lanes 4 and 5), indicating that this nuclear factor is a protein. The 3′ trimming activity requires Mg^{2+} ions, is unaffected by K^+ ions over a wide range, and is accelerated at elevated temperatures. The pH

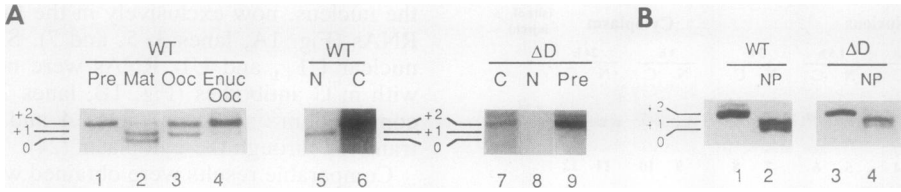

FIG. 2. Location of activities capable of processing of U1 RNA precursors. Pre-$U1_{+2}$ RNA was injected into the cytoplasm of oocytes (A) or incubated with the NP fraction (B). After 12 h of incubation in oocytes (A) or 6 h with the nuclear fractions (B), RNA was reisolated and analyzed by electrophoresis as in Fig. 1. Pre and Mat refer to pre-U1 RNAs (see Materials and Methods) and mature U1 RNAs (24), respectively; (A) Requirement for nuclear transport. Lanes 1 and 2, markers of pre-U1 RNA and mature U1 RNA that had been prepared as for Fig. 1A, lane 7. In lanes 3 and 4, wild-type (WT) pre-U1 RNA injected into cytoplasm of intact oocytes (Ooc) or into enucleated oocytes (Enuc Ooc). In another experiment (lanes 5 to 9), wild-type (WT) or mutant pre-U1 RNAs lacking an Sm antigen binding site (ΔD) were injected into the cytoplasms of intact oocytes. Twelve hours after injection, total RNAs were recovered and analyzed by polyacrylamide gel electrophoresis as in Fig. 1. N, nucleus; C, cytoplasm. (B) Processing activity within the nucleus. Wild-type (WT) and ΔD U1 RNA precursor and transcripts were incubated with the NP fraction at 19°C for 6 h, and the RNAs were analyzed as in Fig. 1.

optimum is broad, ranging from pH 6.4 to 8.3 (data not shown).

The products of the in vitro processing reaction were analyzed by immunoprecipitation with antibodies specific for m^7G caps, $m^{2,2,7}G$ caps, Sm antigens, and U1-specific RNP proteins (Fig. 3B, m_1G, m_3G, Sm, and RNP, respectively). The processed U1 RNA still had the m^7G cap at its 5′ end (lanes 3 and 4), showing that the trimming must have occurred at the 3′ end. This conclusion was confirmed by RNase T_1 fingerprinting, which showed that $U1_0$ RNA had the 3′-terminal oligonucleotide of mature U1 RNA (not shown). The processed $U1_{+1}$ and $U1_0$ RNA was not precipitable by antibodies directed against Sm or RNP antigens (lanes 7 to 10), demonstrating that the pre-U1 RNA had not

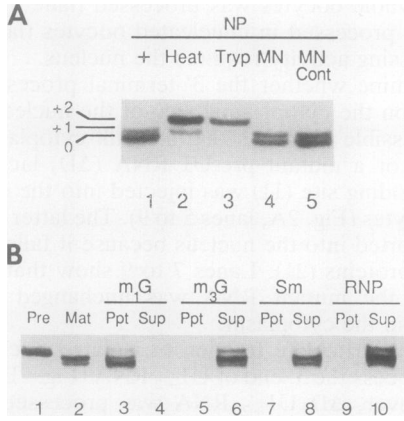

FIG. 3. Characteristics of the 3′ processing activity in the NP fraction. (A) Pretreatments of the NP fraction. Pre-U1 RNAs were incubated at 19°C for 4.5 h with untreated NP (lane 1) or with pretreated NP (lanes 2 to 5). In lane 2, NP was heated at 65°C for 3 min and cleared by centrifugation; in lane 3, NP was treated with trypsin (1 mg/ml) at 37°C for 30 min, and then soybean trypsin inhibitor (1.6 mg/ml) was added; in lanes 4 and 5, NP was treated with MN (3 U/μl) either in the presence of 2 mM $CaCl_2$ at 37°C for 30 min, followed by the addition of 5 mM EGTA (lane 4), or in the presence of both 5 mM EGTA and 2 mM $CaCl_2$ at 37°C for 30 min (lane 5). RNAs were analyzed as in Fig. 1. (B) 5′ caps and proteins associated with the RNA products. Wild-type pre-U1 RNA was incubated with NP at 19°C for 6 h and subjected to immunoprecipitation with anti-m^7G, anti-$m^{2,2,7}G$, anti-Sm, and anti-RNP antibodies coupled to Sepharose beads (m_1G, m_3G, Sm, and RNP, respectively). RNAs were then isolated both from precipitates (Ppt) and from supernatants (Sup) for analysis.

associated with snRNP proteins that might have been in the NP preparations. Thus, binding of these antigens is not essential for the in vitro processing of the RNA; this finding is in agreement with the fact that the ΔD RNA was processed well (Fig. 2B, lane 4).

A 3′-terminal processing inhibitor in nuclei. The location of the processing activity within the nucleus led us to question why newly made transcripts (24) and pre-$U1_{+2}$ RNAs injected into the nucleus (Fig. 1A) are not processed before they are exported to the cytoplasm. We tested whether the lack of processing in this instance was due to the presence of an inhibitor in nuclei that would prevent the snRNA(s) from being processed prematurely. As shown in Fig. 4A (lanes 2 and 3), the NS fraction inhibited the processing activity present in the NP fraction. Thus, the NS fraction contains an inhibitor activity, which we call TPI (for 3′-terminal processing inhibitor).

Inhibition of the trimming of the 3′ end of the pre-U1 RNA by TPI requires recognition of the 5′ m^7G cap structure of the precursor. The presence of a large amount of free m^7GpppG cap competitor overcame the inhibition by TPI (Fig. 4A, lane 4), as did addition of anti-m^7G cap antibody (lane 5). Moreover, TPI did not inhibit 3′ trimming of RNAs lacking an m^7G cap (lanes 6 and 7) or bearing an $m^{2,2,7}G$ cap (lanes 8 and 9).

The ability of free m^7GpppG caps to overcome the inhibitory effect of TPI (Fig. 4, lane 4) is likely the result of a competition for TPI binding between the free and RNA-associated m^7G caps, as illustrated by the titration experiment shown in Fig. 4B (lanes 2 to 4). Furthermore, TPI does not appear to recognize the unmethylated form of the cap, GpppG (lanes 5 and 6); other experiments (not shown) demonstrate that the nucleoside m^7G also is not recognized by the factor. Normally, the m^7G cap at the 5′ end is converted to $m^{2,2,7}G$ once pre-U1 RNA is in the cytoplasm (20); thus, the specificity of TPI for RNAs with m^7GpppG caps could explain why precursor snRNA transcripts are not processed until they have transited the cytoplasm.

The nucleoprotein character of TPI. The nature of the processing inhibitor in the NS fraction was studied by adding pretreated NS fractions to processing reactions. In this assay, processing of pre-U1 RNA would be an indication that TPI had been inactivated. The results (Fig. 5A) show that TPI is inactivated by heating and digestion with trypsin (compare lane 4 with lanes 5 and 9), indicating that TPI is proteinaceous in nature. Unexpectedly, we found that pretreatment of the NS fraction with MN also destroyed the

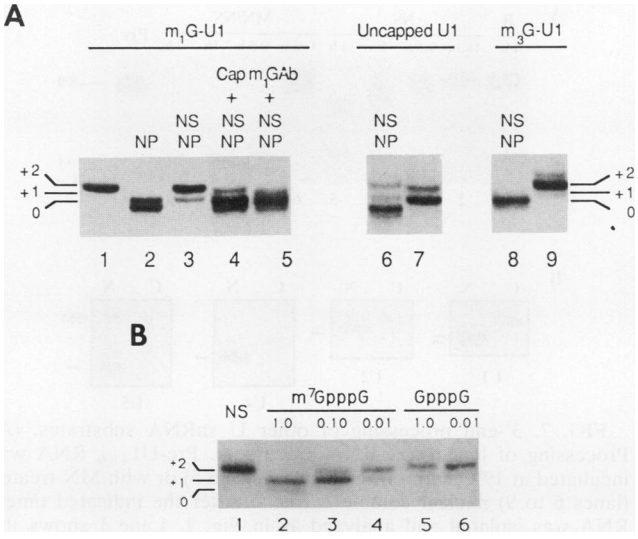

FIG. 4. Inhibition of pre-U1 RNA processing by TPI. (A) An inhibitor of 3′ processing in the NS fraction. Pre-$U1_{+2}$ RNA (m_1G-U1) was incubated with NP alone (lane 2) or with NP plus NS (lanes 3 to 5); the reaction mixtures also contained either 0.4 mM m^7(5′)GpppG(5′) cap analog (lane 4) or anti-m^7G antibody (m_1GAb) that bound the cap on the pre-U1 RNA (lane 5). Pre-U1 RNAs lacking a 5′ m^7G cap (uncapped U1) as a result of treatment with TAP (lanes 6 and 7) or having an $m^{2,2,7}$G cap (m_3G-U1; lanes 8 and 9) were also incubated in the presence of both NP and NS (19°C, 5.5 h; lanes 6 and 8). RNA products were analyzed as in Fig. 1. (B) Competition of cap analogs for TPI. Pre-$U1_{+2}$ RNA containing an m^7G cap was incubated with the NS fraction NS prepared by the large-scale procedure. Incubations were in the presence of no cap analog (lane 1) or m^7GpppG at final concentrations of 1.0, 0.1, and 0.01 mM (lanes 2 to 4, respectively) or with GpppG at final concentrations of 1 and 0.01 mM (lanes 5 and 6).

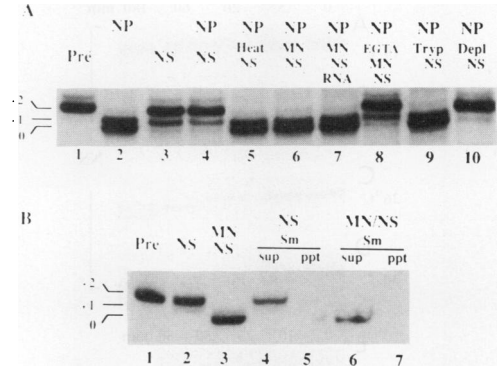

FIG. 5. Characteristics of TPI. (A) Partial characterization of the TPI factor(s). Untreated NS (lanes 3 and 4) or NS pretreated in various ways (lanes 5 to 10) was included in processing reactions in the presence of (lanes 4 to 10) or absence (lane 3) of NP. Lane 5, NS heated at 65°C for 3 min. In lanes 6 and 7, NS was treated with MN (37°C, 30 min) in the presence of 2 mM $CaCl_2$; then the reaction was stopped by addition of 5 mM EGTA and assayed in the absence (lane 6) or presence of 60 μg of *E. coli* 23S rRNA per ml (lane 7). Lane 8, NS treated with MN (37°C, 30 min) in the presence of both 2 mM $CaCl_2$ and 5 mM EGTA, which prevents MN digestion. Lane 9, NS treated by trypsin (0.1 mg/ml) at 37°C for 30 min, followed by inactivation with soybean trypsin inhibitor (1.0 mg/ml). Lane 10, NS depleted by anti-Sm antibodies (see Materials and Methods). Lane 2 is the processing reaction in the presence of NP but absence of NS. (B) Immunoprecipitation by anti-Sm antibodies of pre-U1 RNAs incubated with untreated or MN-treated nuclear soluble fraction. Untreated (NS) or MN-treated (MN/NS) NS fraction was incubated for 4 h at 19°C with pre-$U1_{+2}$ RNA. The NS and MN/NS fractions were then mixed with Sepharose beads (lanes 2 and 3) or subjected to immunoprecipitations at 4°C with anti-Sm antibodies coupled to Sepharose beads (lanes 4 to 7). RNAs in both the immunoprecipitates (ppt) and supernatants (Sup) were analyzed as in Fig. 1.

ability of TPI to inhibit processing (lane 6). Addition of excess carrier RNA (e.g., *E. coli* tRNA or 23S rRNA) prior to addition of the pre-U1 RNA substrate (see Materials and Methods) did not reverse this effect of MN treatment (lane 7), suggesting that it was not due simply to release of nonspecific RNA-binding proteins. Pretreatment with MN in the presence of EGTA (lane 8) had no effect on TPI activity, showing that MN digestion is required for TPI inactivation. To control for the possibility that the effect of the MN might be mediated through the release of competitors such as free m^7G caps from endogenous mRNAs, equal amounts of digested and undigested extracts were mixed and assayed; under these conditions, no processing was observed, indicating that the TPI in the untreated extract was still active (not shown). Taken together, these results suggest that TPI is a nucleoprotein. However, the activity of TPI was unaffected by prior depletion of snRNPs from the NS fraction as a result of preincubation with Sepharose-bound anti-Sm antibodies (lane 10), indicating that TPI probably does not contain Sm antigens such as those present in the major snRNPs.

The ability to destroy TPI with MN allowed us to examine whether the NS fraction itself contained processing activity. The assay shown in lane 3 of Fig. 5B demonstrates that MN-treated NS was indeed a rich source of 3′-terminal processing activity; however, this activity cannot be detected (lane 2) if TPI has not first been destroyed by treatments such as MN digestion. To investigate whether snRNP-specific proteins released by MN treatment of the abundant endogenous snRNPs in the NS fraction (6, 28) could bind to U1 RNA under these conditions (and thereby limit access of TPI to the substrate), pre-$U1_{+2}$ RNA was incubated with NS or MN-treated NS and then assayed for its association with snRNP proteins. As shown in Fig. 5B, in neither case were the treated U1 RNAs precipitable by anti-Sm antibodies (lanes 4 to 7); likewise, we detected no difference in precipitation with either anti-70K or anti-RNP antibodies (data not shown). Thus, it is unlikely that the inactivation of TPI function by MN is mediated indirectly through the release of endogenous snRNP proteins, which could bind to and alter the RNA substrate.

Temperature sensitivity of TPI. The activity of TPI in untreated NS is remarkably sensitive to temperature, as illustrated by the kinetics of 3′-terminal processing at various temperatures over the range from 19 to 30°C (Fig. 6). Whereas little or no conversion of $U1_{+2}$ to $U1_{+1}$ (or $U1_0$) RNA occurred at 19°C during 3 h of incubation with untreated NS (Fig. 6A), the conversion was essentially complete within 20 min at 30°C (Fig. 6D). Intermediate temperatures resulted in intermediate kinetics. Inactivation of TPI at the higher temperature was reversible, since processing was prevented at 19°C even if the NS had been preincubated at 30°C for 0.5 h; the processing enzymes in such a preparation were not inactivated by this treatment (data not shown).

The effect of the temperature of incubation was due primarily to inactivation of TPI rather than stimulation of the processing enzymes. That was shown comparing the kinetics of processing at 30°C (Fig. 6D) with those observed at 19°C,

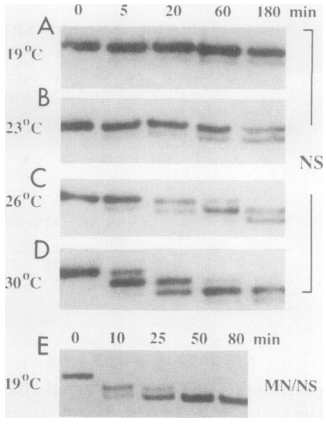

FIG. 6. Processing of pre-U1 RNA in the NS fraction as a function of temperature. (A to D) A large-scale preparation of the NS fraction was divided into four aliquots, each of which was preincubated at the desired temperature for 5 min; the reaction was initiated by the addition of pre-$U1_{+2}$ RNA, and at the indicated times, a portion was removed into proteinase K solution containing carrier tRNA. (E) The NS fraction was pretreated with MN in D buffer for 30 min at 37°C in the presence of 2 mM Ca^{2+}, and the reaction was stopped by addition of 5 mM EGTA; the processing reaction, assayed at 19°C, was initiated by addition of pre-$U1_{+2}$ RNA; at the indicated times, the RNA was isolated and analyzed as in Fig. 1.

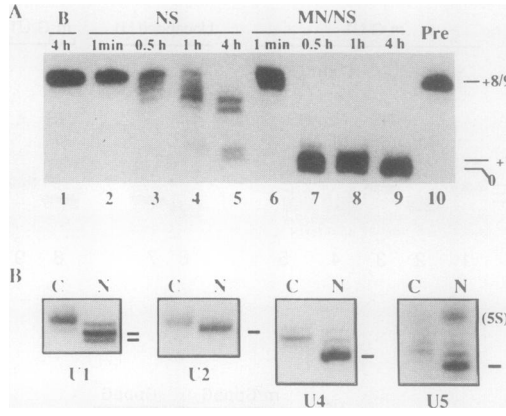

FIG. 7. 3′-end processing of other U snRNA substrates. (A) Processing of longer U1 RNA precursors. Pre-$U1_{+8/9}$ RNA was incubated at 19°C with untreated (lanes 2 to 5) or with MN-treated (lanes 6 to 9) nuclear soluble extracts. After the indicated times, RNA was isolated and analyzed as in Fig. 1. Lane 1 shows the substrate RNA after incubation with buffer (B) alone. (B) Terminal processing of other U snRNAs in the nucleus. U1, U2, U4, and U5 DNA templates were injected into oocyte nuclei together with [α-^{32}P]GTP. After 3 h of labeling, the newly made RNAs in the nuclei (N) and cytoplasms (C) were isolated and analyzed as in Fig. 1. Dashes indicate the mobilities of mature, nuclear forms of the snRNAs; the slower-migrating, minor bands in lanes N represent m^7G-capped precursors of the snRNAs (15a).

using an NS extract that had been pretreated with MN to destroy TPI (Fig. 6E). Under the conditions used here, processing was about as efficient at 19°C as at 30°C.

Processing of other snRNA substrates. In vivo, nuclear U1 RNA precursors often have more than two extra nucleotides at their 3′ ends (3, 19, 24). We have found that the nuclear processing of these longer precursors is also inhibited by TPI. As illustrated in Fig. 7A, incubation of pre-$U1_{+8/9}$ RNAs with untreated NS resulted primarily in a limited exonucleolytic degradation (lanes 2 to 4), with only a slow accumulation of the mature forms (lane 5). In contrast, incubation with MN-treated NS led to very rapid conversion the pre-$U1_{+8/9}$ RNAs to $U1_{+1}$ and $U1_0$ RNAs (lanes 6 and 7). Thus, the inhibition of processing of extended U1 precursors, like that of shorter pre-U1 RNAs, was relieved by the destruction of TPI activity.

As shown in Fig. 2, terminal processing of pre-U1 RNA occurs only after the RNA has returned to the nucleus. To investigate whether terminal processing at the 3′ ends of other snRNAs also occurred only after they returned to the nucleus, we analyzed the mobilities of newly synthesized U2, U4, and U5 RNAs present in either the cytoplasm or the nucleus of intact oocytes. In all cases, longer precursors but no mature forms were observed in the cytoplasm (Fig. 7B). Thus, it is likely that events similar to those described above for pre-U1 RNA also operate on the other pre-snRNAs. It remains to be established whether the same TPI factor participates in the processing of these RNAs.

DISCUSSION

The primary transcript of the U1 snRNA gene is exported from the nucleus to the cytoplasm, where it is trimmed at the 3′ end to a size that is two nucleotides longer than the mature form (24; this study). At that point it can be transported back to the nucleus, where final 3′-end trimming occurs. While it is unclear what limits the extent of processing in the cytoplasm to $U1_{+2}$, our data suggest that a factor, called TPI, prevents 3′ processing of the RNA in the nucleus prior to export. By recognizing the m^7G cap at the 5′ end of the pre-U1 RNA, this factor interferes with processing at the 3′ end (Fig. 4). Because nuclear snRNA precursors contain m^7G caps, TPI would inhibit their processing prior to export to the cytoplasm. In contrast, because hypermethylation of the cap to $m^{2,2,7}G$ is required for reentry of U1 snRNPs into the nucleus (5, 10), snRNAs would no longer be recognized by TPI as a target for inhibition after they return to the nucleus.

TPI may have multiple roles in snRNA metabolism. For example, in addition to controlling premature processing of pre-snRNAs, TPI might facilitate the efficient export of these RNAs from the nucleus. In this regard, it is notable that export of pre-snRNAs has been reported to require a 5′ m^7G cap (12). Moreover, part of this export complex might remain associated with the snRNA, to facilitate its reimportation back into the nucleus. Such a shuttling "chaperonin" activity would help to explain the accelerated kinetics of import of snRNAs injected into the nucleus relative to those of cytoplasmically injected RNAs (Fig. 1).

As illustrated in Fig. 7, U2, U4, and U5 RNAs also have cytoplasmic forms that are longer than their nuclear forms. Again, no mature-length RNA is observed in the cytoplasm, indicating that these RNAs also are subject to terminal 3′-end processing once they have reentered the nucleus. Moreover, recent experiments in our laboratory have demonstrated that when nuclear export of pre-snRNAs is inhibited, longer, m^7G-capped precursor forms of U2, U4, and U5 (as well as U1) RNAs accumulate in the nucleus (17a). Thus, it is likely to be a general phenomenon that 3′-terminal processing in the nucleus is prevented by the presence of m^7G caps and occurs only after $m^{2,2,7}G$-capped snRNAs have returned to the nucleus.

It is unclear what function(s) the extra nucleotides at the 3′

end might have in snRNA metabolism and why their controlled removal might be important. As noted above, the extra nucleotides at the 3′ end of the primary transcript might be part of a cytoplasmic localization signal that would help usher the newly synthesized pre-U1 RNAs from the nucleus immediately after transcription. If so, part of the retention of mature snRNPs in the nucleus might be due to the lack of these extra nucleotides at the 3′ end. However, transport from the nucleus cannot be obligatorily coupled to the presence of extra sequences at the 3′ end since m^7G-capped $U1_0$ RNAs injected into nucleus are exported, albeit at a slower rate (27a).

The precise length of the 3′ extension clearly affects transport from the cytoplasm to the nucleus. Pre-U1 RNAs with long 3′ extensions are not imported efficiently into the nucleus even when they are associated with Sm and RNP antigens and contain $m^{2,2,7}G$ caps (24). Instead, such molecules are substrates for a 3′ exonuclease activity in the cytoplasm that digests the RNAs to a limit of only two nucleotides longer than the mature RNA; at that point, the pre-snRNAs are rapidly imported into the nucleus (Fig. 1C). Furthermore, import of U1 RNAs lacking one or two nucleotides from the mature 3′ end (i.e., $U1_{-1}$ or $U1_{-2}$) also appears to be blocked (24). It is unclear what enzymes are responsible for the cytoplasmic shortening and what factors limit the extent of the processing.

In this work, we have equated the 3′-terminal processing to the $U1_{+1}$ and $U1_0$ forms because they seem to occur under the same conditions, in a stepwise fashion (e.g., Fig. 6). We have no direct evidence that the same enzyme is responsible for both steps, nor can we say that the 3′ processing activity in the NP fraction is the same as that in the NS fraction, which is revealed by inactivation of TPI (Fig. 4 and 5).

We have also tested the ability of nuclear extracts to process $m^{2,2,7}G$-capped $U1_{+2}$ RNA present in snRNPs, rather than as free RNAs. As expected, the $m^{2,2,7}G$-capped $U1_{+2}$ RNA in snRNPs was processed by a mixture of the NP and NS fractions, and it was not necessary to treat the NS fraction with MN to obtain processing, since this pre-RNA has the hypermethylated cap and thus is resistant to inhibition by TPI (data not shown). Unexpectedly, in this case both NP and NS fractions appear to be required for processing.

The nature of TPI and its mode of action are intriguing. The sharp but reversible inactivation of this activity over the range from 19 to 29°C (Fig. 6), along with its sensitivity to treatment with MN, makes it possible that the function of this inhibitor requires an easily denaturable RNA-RNA or RNA-protein interaction. Thus, TPI could be an RNP, part of which recognizes a m^7G cap and the other part of which interacts directly with an RNA sequence, perhaps the 3′ end of $U1_{+2}$ RNA. It will be interesting to isolate this unusual factor and to determine the mechanism of how it protects $U1_{+2}$ RNA from being processed and whether TPI can also affect the processing (and possibly transport) of other snRNAs in the cell nucleus that bear m^7G caps.

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REFERENCES

1. **Bringmann, P., J. Rinke, B. Appel, R. Reuter, and R. Lührmann.** 1983. Purification of snRNPs U1, U2, U4, U5 and U6 with 2,2,7-trimethylguanosine-specific antibody and definition of their constituent proteins reacting with anti-Sm and anti-(U1) RNP antisera. EMBO J. **2**:1129–1135.
2. **Dahlberg, J. E., and E. Lund.** 1988. The genes and transcription of major small nuclear RNAs, p. 38–70. *In* M. L. Birnstiel (ed.), Structure and function of major and minor small nuclear ribonucleoprotein particles. Springer-Verlag, Berlin.
3. **Eliceiri, G. L.** 1974. Short-lived, small RNAs in the cytoplasm of Hela cells. Cell **3**:11–14.
4. **Feldherr, C. M., and P. A. Richmond.** 1978. Manual enucleation of Xenopus oocytes. Methods Cell Biol. **17**:75–79.
5. **Fischer, U., and R. Lührmann.** 1990. An essential signaling role for the m^3G cap in the transport of U1 snRNP to the nucleus. Science **249**:786–790.
6. **Forbes, D. J., T. B. Kornberg, and M. W. Kirschner.** 1983. Small nuclear RNA transcription and ribonucleoprotein assembly in early Xenopus development. J. Cell Biol. **97**:62–72.
7. **Ford, C. C., and J. B. Gurdon.** 1977. A method for enucleating oocytes of *Xenopus laevis*. J. Embryol. Exp. Morphol. **37**:203–209.
8. **Gurdon, J. B.** 1974. Control of gene expression in animal development. Harvard University Press, Cambridge, Mass.
9. **Gurdon, J. B., and M. P. Wickens.** 1983. The use of *Xenopus* oocytes for the expression of cloned genes. Methods Enzymol. **101**:370–386.
10. **Hamm, J., E. Darzynkiewicz, S. M. Tahara, and I. W. Mattaj.** 1990. The trimethylguanosine cap structure of U1 snRNA is a component of a bipartite nuclear targeting signal. Cell **62**:569–577.
11. **Hamm, J., M. Kazmaier, and I. W. Mattaj.** 1987. *In vitro* assembly of U1 snRNPs. EMBO J. **6**:3479–3485.
12. **Hamm, J., and I. W. Mattaj.** 1990. Monomethylated cap structures facilitate RNA export from the nucleus. Cell **63**:109–118.
13. **Kazmaier, M., G. Tebb, and I. W. Mattaj.** 1987. Functional characterization of *X. laevis* U5 snRNA genes. EMBO J. **6**:3071–3078.
14. **Krol, A., E. Lund, and J. E. Dahlberg.** 1985. The two embryonic U1 RNA genes of *Xenopus laevis* have both common and gene-specific transcription signals. EMBO J. **4**:1529–1535.
15. **Lerner, E. A., M. R. Lerner, C. A. Janeway, Jr., and J. A. Steitz.** 1981. Monoclonal antibodies to nucleic acid-containing cellular constituents: probes for molecular biology and autoimmune disease. Proc. Natl. Acad. Sci. USA **78**:2737–2741.

15a. **Lund, E.** Unpublished data.

16. **Lund, E., C. J. Bostock, and J. E. Dahlberg.** 1987. The transcription of *Xenopus laevis* embryonic U1 snRNA genes changes when oocytes mature into eggs. Genes Dev. **1**:47–56.
17. **Lund, E., and J. E. Dahlberg.** 1989. *In vitro* synthesis of vertebrate U1 snRNA. EMBO J. **8**:287–292.

17a. **Lund, E., and J. E. Dahlberg.** Unpublished data.

18. **Lund, E., and P. L. Paine.** 1990. Nonaqueous isolation of transcriptionally active nuclei from Xenopus oocytes. Methods Enzymol. **181**:36–42.
19. **Madore, S. J., E. D. Wieben, and T. Pederson.** 1984. Intracellular site of U1 small nuclear RNA processing and ribonucleoprotein assembly. J. Cell Biol. **98**:188–192.
20. **Mattaj, I. W.** 1986. Cap trimethylation of U snRNA is cytoplasmic and dependent on U snRNP protein binding. Cell **46**:905–911.
21. **Mattaj, I. W., and E. M. DeRobertis.** 1985. Nuclear segregation of U2 snRNA requires binding of specific snRNP proteins. Cell **40**:111–118.
22. **Mattaj, I. W., and R. Zeller.** 1983. *Xenopus laevis* U2 snRNA genes: tandemly repeated transcription units sharing 5′ and 3′ flanking homology with other RNA polymerase II transcribed genes. EMBO J. **2**:1883–1891.
23. **Munns, T. W., M. K. Liszewski, J. T. Tellam, H. F. Sims, and R. E. Rhoads.** 1982. Antibody-nucleic acid complexes. Immu-

nospecific retention of globin messenger ribonucleic acid with antibodies specific for 7-methylguanosine. Biochemistry **21:** 2922–2928.

24. **Neuman de Vegvar, H. E., and J. E. Dahlberg.** 1990. Nucleocytoplasmic transport and processing of small nuclear RNA precursors. Mol. Cell. Biol. **10:**3365–3375.
25. **Parry, H. D., D. Scherly, and I. W. Mattaj.** 1989. "Snurpogenesis": the transcription and assembly of U snRNP components. Trends Biochem. Sci. **14:**15–19.
26. **Patton, J. R., R. J. Patterson, and T. Pederson.** 1987. Reconstitution of the U1 small nuclear ribonucleoprotein particle. Mol. Cell Biol. **7:**4030–4037.
27. **Vankan, P., C. McGuigan, and I. W. Mattaj.** 1990. Domains of U4 and U6 snRNAs required for snRNP assembly and splicing complementation in *Xenopus* oocytes. EMBO J. **9:**3397–3404.

27a. **Yang, H.** Unpublished data.

28. **Zeller, R., T. Nyffenegger, and E. M. De Robertis.** 1983. Nucleocytoplasmic distribution of snRNPs and stockpiled snRNA-binding proteins during oogenesis and early development in *Xenopus laevis*. Cell **32:**425–434.
29. **Zieve, G., and R. Sauterer.** 1990. Cell biology of the snRNP particles. Crit. Rev. Biochem. Mol. Biol. **25:**1–46.