A pre-export U1 snRNP in *Xenopus laevis* oocyte nuclei

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ABSTRACT

We demonstrate that precursors of U1 snRNA are associated with nuclear proteins prior to export to the cytoplasm. The ~15S complexes containing pre-U1 RNA, which we call pre-export U1 snRNPs, were identified in extracts of *Xenopus laevis* oocyte nuclei that were synthesizing U1 RNAs from injected U1 genes. The U1 snRNP-specific A protein was associated with nuclear pre-U1 RNA since both this protein and the RNA were co-precipitated by antibodies directed against either the m⁷G-cap of the precursor RNA or the U1-A protein. The interaction of the U1-A protein with pre-U1 RNA required sequences in the loop II region although this region of U1 RNA was not necessary for the association of U1 A protein with mature U1 snRNPs. The U1 A protein helps protect pre-U1 RNA against degradation in the nucleus.

INTRODUCTION

U1 small nuclear ribonucleoprotein particles (U1 snRNPs), which function in the removal of intron sequences from pre-mRNA, contain a single U1 RNA molecule (~164 ntds) complexed with at least 11 proteins (1, 2). Formation of U1 snRNPs involves a series of events in which a precursor of U1 RNA (pre-U1 RNA) is synthesized in the nucleus, undergoes maturation in the cytoplasm, and returns to the nucleus. Prior to export from the nucleus, precursor U1 RNA (pre-U1 RNA) has a 5' m⁷G cap and extra nucleotides at its 3' end (3, 4). In contrast, U1 RNA in mature U1 snRNPs contains a m^{2,2,7}G cap, lacks the extra 3' end sequences, and is bound by the Sm proteins (common to several snRNPs) (1, 5). At least two proteins that are specific to U1 snRNA, U1 A and U1 C proteins, can enter the nucleus independent of their interaction with U1 RNA (6–8) so it is unclear if these proteins become associated with U1 snRNA in the cytoplasm or the nucleus.

Efficient export of pre-U1 snRNA to the cytoplasm involves at least three cis-acting RNA elements, the 5' m⁷G-cap, the 3' terminal stem-loop structure and sequences in the 5' terminal 124 nucleotides (M. Terns, J.E. Dahlberg and E. Lund, *Genes and Development* in press; 9, 10). In this study we asked if the primary transcripts of U1 snRNA associate with nuclear proteins prior to export of the RNA from *Xenopus* oocyte nuclei. We

found that nuclear pre-U1 RNA is in a relatively large RNP complex. The U1 snRNP-specific protein, U1 A, interacts with precursor U1 snRNA and may help stabilize the RNA prior to its export. Analysis of variant U1 RNAs lacking the U1 A protein binding site, indicates that the initial interaction of the U1 A protein with nuclear pre-U1 RNA differs from the interaction of U1 A protein in mature U1 snRNPs.

MATERIALS AND METHODS

DNA and antibodies

U1_{wt}, the wild-type *Xenopus laevis* U1b1 gene was described previously (11). U1_{Sm-}, a *Xenopus laevis* U1 gene (U1ΔD) lacking a functional Sm protein binding site (12), was obtained from I. Mattaj. The U1_{A-} genes, in which sequences within the stem-loop II of the *Xenopus laevis* U1b2 gene (11) were deleted (L15) or substituted (L27) (13), were provided by A. Krol.

The antibodies against the monomethyl guanosine cap (m⁷G) (14) or trimethyl guanosine cap (m₃G) (15) were rabbit polyclonal antibodies provided by T. Munns and R. Lührmann, respectively. Monoclonal antibodies against Sm proteins (Y12) (16) or the U1 70k protein (2.73) (17) were furnished by J. Steitz and S. Hoch, respectively. U1 A protein antibodies were either from a patient serum (M54) (18) or a monoclonal antibody (9A9) (19) and were obtained from W. van Venrooij. Qualitatively similar results were obtained using the two sources of anti-U1 A protein antibodies; the experiments shown here were performed with the patient serum since it more efficiently immunoprecipitated both precursor and mature U1 RNAs from oocyte extracts.

RNA synthesis

To synthesize U1 and U6 RNAs in vitro, templates were generated by PCR amplification of the RNA coding sequences of their genes using appropriate primer pairs with phage promoter sequences in the 5' primer as described (20; M. Terns, J.E. Dahlberg and E. Lund, *Genes and Development*, in press). In vitro transcription of SP6 or T7 PCR templates was performed essentially as described elsewhere (20). 5' capping of the RNAs was accomplished by lowering the GTP concentration from 500 to 50 μM and including 1mM of m⁷GpppG (New England BioLabs) or ApppG (Pharmacia) or 4 mM γ-mpppG (provided by R. Reddy). [α-³²P]-GTP (800 Ci/mmol, NEN Dupont) was

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used as source of label. The snRNA transcripts were purified by electrophoresis in 8% denaturing polyacrylamide gels prior to use in oocyte injections.

Oocyte microinjection and dissection and RNA analysis

Stage V and VI oocytes, obtained from *X. laevis* frogs (11), were injected either with U1 genes (~2ng/oocyte) or in vitro synthesized RNAs (~1 fmole of RNA/oocyte). For the RNA injection experiments, U6 RNA was co-injected with the test U1 RNA(s) to monitor the accuracy of the nuclear injection since this RNA normally is not exported to the cytoplasm and is stable (21; M.Terns, J.E.Dahlberg and E.Lund, Genes and Development, in press). Nuclei and cytoplasm were isolated from individual oocytes by manual dissection under mineral oil (22, 23) and RNAs within each compartment of at least four oocytes for each time point were purified and analyzed as previously described (20).

Oocyte extract preparation

Extracts containing ^{32}P -labeled RNAs or ^{35}S -methionine-labeled proteins were prepared from isolated GVs or cytoplasm and immunoprecipitations were performed as previously described (20). ^{35}S -methionine-labeling of oocytes was performed by incubating 100–150 oocytes in 1ml of MBS-H medium containing 1 mCi of ^{35}S -Methionine (~1000 Ci/mmol, NEN Dupont) for 40–45 hours at 18°C. To inhibit RNA export, wheat germ agglutinin (50 nl) was injected at 10–20 mg/ml into the cytoplasm of oocytes (3).

Figure 1. Identification of pre-export U1 snRNPs in *Xenopus* Oocyte nuclei. Glycerol gradient sedimentation analysis of precursor U1 RNA (top panel), pre-U1 snRNP (middle panel), or pre-U1 snRNP plus mature U1 snRNP (bottom panel, upper and lower brackets, respectively). *Xenopus laevis* U1 genes were injected into oocyte nuclei (GVs) and ^{32}P -labeled precursor or mature U1 RNAs were generated by labeling oil-isolated GVs or intact oocytes, respectively, with ^{32}P -[GTP]. GV extracts or free RNA from ten oocyte nuclei were fractionated in 5–20% linear glycerol gradients and the RNAs of each fraction were purified and analyzed by electrophoresis in 8% polyacrylamide 7M urea gels and autoradiography. Lanes P and M show the gel mobilities of precursor (Pre) and mature (Mat) U1 RNAs. The position of endogenous 5S ribosomal RNA (black dot) is indicated; 5S rRNA, which sediments as a ~7S RNP complex in extracts (30), serves as an internal control for sedimentation in different gradients. The direction of sedimentation is from left (top) to right (bottom of gradient).

Glycerol gradient sedimentation

Sedimentation analyses were performed essentially as described (24) using linear 4.3 ml glycerol gradients (5–20% vol/vol) containing 20 mM Hepes pH 7.9, 150 mM KCl, 1.5 mM MgCl_2 , 0.5 mM DTT, and 0.5 mM phenylmethylsulfonyl fluoride. Deproteinized ^{32}P -pre-U1 RNA or oocyte nuclear extracts containing ^{32}P -labeled precursor or mature U1 snRNA were layered onto the gradients and centrifuged in a Beckman SW60Ti rotor at 35,000 rpm for 20 hours at 4°C. Twenty equal-sized fractions were collected and RNAs from individual gradient fractions were purified and analyzed by electrophoresis in denaturing polyacrylamide gels and autoradiography.

RESULTS

Identification of a pre-export U1 snRNP

To study very early steps in U1 snRNP morphogenesis, we used *Xenopus laevis* oocytes that were actively synthesizing U1 snRNA from injected U1 genes. When extracts of isolated oocyte nuclei containing newly-made ^{32}P -labeled U1 RNAs were fractionated by centrifugation on glycerol gradients, pre-U1 RNA was found in an RNA/protein complex that sedimented at ~15S, much faster than protein-free pre-U1 RNA (Fig. 1A, compare top and middle panels), and slightly faster than ~12S mature U1 snRNPs (bottom panel). The complexes containing pre-U1 RNA, which we call the pre-export U1 snRNPs, were observed in both isolated

Figure 2. Association of the U1 A protein with precursor U1 RNA in the nucleus. **A)** Immunoprecipitation of U1 A protein by anti-m⁷G cap antibodies. Oocytes, which had been pre-labeled with ^{35}S -Methionine for forty hours, were (+) or were not (–) injected with *Xenopus laevis* U1 genes and one hour later all oocytes were injected with wheat germ agglutinin to inhibit pre-U1 RNA export. After an additional five hours of incubation, nuclear extracts were prepared from isolated nuclei and two oocyte equivalents were used for immunoprecipitation with anti-m⁷G-cap (lanes 1, 2), anti-Sm (lanes 3–5) or anti-U1 A protein antibodies (lane 6). The precipitated proteins were analyzed by electrophoresis in 15% polyacrylamide gels containing SDS. The positions of known snRNP proteins are indicated; lane 7 shows the total labeled proteins (T) present in 0.1 oocyte equivalents of the nuclear extract. **B)** Immunoprecipitation of pre-U1 RNA. Nuclear extract was prepared from oil-isolated nuclei that had been pre-injected with U1 genes and labeled with ^{32}P -GTP after isolation (22, 23). Nuclear RNAs from 1.25 oocyte equivalents were purified from total extract (T) and from the precipitate (P) and supernatant (S) fractions of the immunoprecipitations using antibodies against U1 A, U1 70k, and Sm proteins or m⁷G- and m³G-caps, as indicated. Polyacrylamide gel electrophoresis of the RNAs was as in Figure 1.

nuclei and nuclei from intact oocytes (not shown). Thus, pre-U1 RNA apparently associates with nuclear proteins prior to its export to the cytoplasm.

Association of U1 A protein with pre-U1 RNA within the nucleus

To identify nuclear proteins associated with pre-U1 RNA prior to export, we analyzed proteins that were co-precipitated with the RNA by m⁷G-cap antibodies (Fig. 2A). Nuclear extracts were prepared from ³⁵S-methionine labeled *Xenopus* oocytes that had been pre-injected with U1 RNA templates and treated with the lectin wheat germ agglutinin, to inhibit pre-U1 RNA export (3; E.Lund unpublished results). Immunoprecipitation of the newly made pre-U1 RNA by antibodies specific for the m⁷G-cap revealed the co-precipitation of a ³⁵S-labeled protein having the same electrophoretic mobility as the U1 snRNP A protein (Fig. 2A, lane 2).

The co-precipitation of this newly-made protein with anti-m⁷G-cap antibodies occurred only if oocytes were synthesizing pre-U1 snRNA from injected U1 genes; the protein was not precipitated when the U1 template was omitted (Fig. 2A, lane 1) or when a U4 RNA template was injected (data not shown). In contrast, the full array of snRNP proteins was co-precipitated by anti-Sm antibodies regardless of whether the oocytes had been injected with snRNA templates (lanes 3 and 4), indicating that these latter proteins were in mature, endogenous snRNPs that had been pre-labeled with ³⁵S methionine.

To confirm the association of U1 A protein with pre-U1 RNA we used a polyclonal antibody that specifically immunoprecipitated the U1 A protein (Fig. 2A, lane 6). This anti-U1 A antibody co-precipitated labeled pre-U1 RNAs (Fig 2B, lane 2), as did anti-U1 A monoclonal antibodies (not shown). Pre-U1 RNA was not co-precipitated by antibodies against U1 70k or Sm proteins (Fig. 2B, lanes 4–7), showing that this RNA

Figure 3. Binding of U1 A protein to pre-U1 but not pre-U1_{A-} RNA. U1 genes encoding wild-type (U1_{wt}) or a mutant RNA (U1_{A-}) which lacks stem-loop II sequences of the U1 A protein binding site, were co-injected into oocyte nuclei together with ³²P-GTP. After five hours of labeling, oocytes were dissected into nuclear and cytoplasmic fractions and extracts were prepared for immunoprecipitation with antibodies against U1 A, U1 70k and Sm proteins, as indicated. The ³²P-labeled U1 RNAs in total extracts (N and C) and in the precipitate (P) and supernatant (S) fractions of nuclear (lanes 1–7) and cytoplasmic (lanes 9–14) extracts were purified and analyzed as in Figure 1. The near complete immunoprecipitation of U1 snRNPs with anti-Sm protein antibodies, but not with U1 70k or U1 A protein antibodies, probably reflects differences in the stability of these RNA/protein interactions under the conditions of immunoprecipitation used here (see also ref. 12). Precursor U1 RNA (Pre) contains two extra nucleotides at its 3' end, one or both of which are absent in nuclear mature (Mat) U1 RNA (3). Due to the lower level of cytoplasmic pre-U1 RNA (compare N and C, lanes 7 and 8), the autoradiogram shown in lanes 9–14 was exposed about four times longer than that of lanes 1–6.

was not in mature U1 snRNPs. Furthermore, the RNA was precipitable by antibodies specific for m⁷G but not m^{2,2,7}G caps (lanes 8–11) confirming that it was indeed pre-U1 RNA.

Association of U1 A protein with U1 RNAs lacking the U1 A protein binding site

Sequences in loop II of U1 RNA are essential for binding of the U1 A protein to free U1 RNA (18, 25–27). To test if this region is also necessary for the binding of pre-U1 RNA to U1 A protein in oocyte nuclei, we attempted to co-precipitate precursor and mature forms of U1 RNA variants (U1_{A-}) mutated in loop II, using antibodies to the U1 A protein. Mature-sized U1 RNAs were precipitated regardless of whether or not they had the wild-type loop II region (Fig. 3, lanes 1, 2). However, the only pre-U1 RNA precipitated by the anti-U1 A antibody was the one with the wild type loop II (denoted by the dot in lane 1). The same pattern of U1 A protein binding was observed using a U1_{A-} RNA mutant in which sequences of loop II were substituted with

Figure 4. Instability of pre-U1 RNA lacking a U1 A protein binding site and a 5' m⁷G-cap structure. **A)** Stabilities of ApppG-capped pre-U1 RNAs with or without a U1 A protein binding site. ApppG-capped U1 RNAs, lacking either the Sm protein binding site (lanes 1–3) or the U1 A protein binding site (lanes 4–8) were made in vitro using SP6 RNA polymerase and injected directly into oocyte nuclei. At the indicated times, individual oocytes were dissected into nuclear (N) and cytoplasmic (C) fractions and the ³²P-labeled U1 RNAs within each fraction were analyzed by polyacrylamide gel electrophoresis as in Figure 1. In lanes 4–8, m⁷G-capped U1_{Sm-} RNA was co-injected with the ApppG-capped U1_{A-} RNA as a positive control for export, stability and recovery of RNA from the oocytes. U1_{Sm-} RNA, which lacks the Sm protein binding site needed for nuclear import (12), behaves like U1_{wt} RNA with respect to nuclear export and stability (M.Terns, J. E.Dahlberg and E.Lund, submitted). **B)** Rapid degradation of pre-U1 RNAs lacking the U1 A protein binding site in the presence of m⁷GpppG dinucleotide cap analogue. A mixture of m⁷G-capped wildtype (U1_{wt}) and mutant U1 RNA (U1_{A-}) was injected directly into oocyte nuclei (lanes 2–9) or cytoplasm (lanes 10–13) in the absence (lanes 2–5, 10 and 11) or presence (lanes 6–9, 12 and 13) of 50 mM m⁷GpppG cap analogue. At the times indicated, RNAs present in the nuclear (N) and cytoplasmic (C) fractions of individual oocytes were analyzed as in (A). Lane 1 (M) shows the RNA prior to injection. U1 RNA which has been imported back into the nucleus following cytoplasmic maturation, were distinguished from pre-U1 RNA which had not been exported by the presence of m³G caps and shortened 3' ends. (3, 4); greater than 80% of the injected U1 RNA was re-imported at 5 hours (e.g., compare mobility of nuclear RNAs in lanes 1 and 4; anti-m³G cap antibody immunoprecipitations not shown).

non-U1 sequences (data not shown). Thus, U1 A protein requires the loop II region for its interaction with pre-U1 RNA. In contrast, the U1 A protein may use additional ways to associate with mature U1 snRNPs, most likely protein-protein interactions (26, 28).

In agreement with the results in Figure 2, neither U1 70k or Sm proteins bind to pre-U1 RNA (lanes 3–6). As expected, binding of the U1 70k and Sm proteins (and hence co-precipitation of U1 RNA) was not affected by alteration of loop II (12, 18, 26). Deletion or substitution of loop II did not appear to affect the stability, transport, or 3' end processing of U1 RNA.

Upon export to the cytoplasm, pre-U1 RNA associates with the U1 A, U170k and Sm proteins regardless of whether the RNA has the wildtype or mutant loop II region (lanes 9–14). This observation, and the finding that pre-U1 RNA injected directly into oocyte cytoplasm is efficiently co-precipitated by antibodies against the U1 A protein (not shown), shows that the association of U1 RNA with U1 A protein can occur in either the oocyte nucleus or cytoplasm.

Importance of the U1 A protein in pre-U1 RNA nuclear stability

The stability of pre-U1 RNA in the nucleus appears to require binding sites in the RNA for either U1 A protein or a cap binding protein. As shown in Figure 3, deletion of the binding site for U1 A protein did not destabilize m⁷G-capped pre-U1 RNA. Likewise, pre-U1 RNA containing a 5' ApppG-cap instead of the m⁷G-cap, when injected into the oocyte nucleus, was stable for several hours and is exported to the cytoplasm (Fig. 4A, lanes 2 and 3; M.Terns, J.E.Dahlberg and E.Lund, *Genes and Development*, in press). In contrast, ApppG-capped U1 RNA lacking the binding site of U1 A protein, was rapidly degraded in the nucleus, with little or no RNA being detected three hours after injection (lower bands, lanes 5–8). In this experiment, a normally capped RNA (upper bands, m⁷G-U1_{Sm-}) was co-injected with the ApppG-capped U1_{A-} RNA as a control to demonstrate stability and recovery of m⁷G-capped RNA in the same oocytes.

To test more directly if a cap binding protein is required for pre-U1 RNA stability in the absence of bound U1 A protein, we saturated cap binding activities in oocyte nuclei by injecting the cap analogue, m⁷GpppG. As we have shown elsewhere, this cap analogue destabilizes wild-type pre-U1 RNA (Fig. 4B, compare the levels of U1_{wt} in lanes 2 and 6; M.Terns, J.E.Dahlberg and E.Lund, *Genes and Development*, in press); however this effect is much more extreme when the RNA lacks loop II (compare U1_{wt} and U1_{A-} in lanes 6). Essentially the same results were obtained when these two RNAs were synthesized in the nuclei, rather than introduced by injection of in vitro-made RNAs (not shown). As expected, both RNAs were stable in the nucleus in the absence of the cap analogue (lanes 2 and 4). We conclude that the binding of either U1 A protein or an m⁷G-cap binding protein is needed to stabilize pre-U1 RNA in the nucleus.

Both the wild-type and mutant pre-U1 RNAs were stable in the cytoplasm regardless of whether the analogue was present (lanes 10 and 12). Furthermore, the stability of mature U1 snRNPs that were imported into the nucleus from the cytoplasm was unaffected by the analogue (lanes 11 and 13). Thus, the destabilization induced by the excess m⁷G cap analogue, is a nuclear event specific to the precursor RNA.

DISCUSSION

In this study we have demonstrated that prior to its export from the nucleus, newly-made pre-U1 RNA is in a relatively large RNA/protein complex, which we call the pre-export U1 snRNP. We also showed that the U1 A protein, previously identified as a component of the fully mature U1-snRNP, binds to the pre-U1 RNA indicating that this protein is a component of the pre-export U1 snRNP. To our knowledge, this is the first report of a protein that binds to a precursor form of a spliceosomal snRNA prior to its export from the nucleus.

The U1 A protein interacted differently with U1 RNA before and after export from the nucleus (Fig. 2). Prior to export, binding of U1 A protein was dependent on RNA sequences in the stem-loop II of U1 RNA. In contrast, once the RNA was exported it was associated with the U1 A protein regardless of whether the RNA contained the binding site for this protein. Apparently, U1 A protein can interact with U1 RNA through protein/protein interactions involving the newly-bound 70k or Sm proteins. These results are consistent with previous in vitro studies using extracts (26) or purified U1 A protein (27). Thus, the interaction between the U1 A protein and the U1 RNA appears to be dynamic, involving both RNA/protein and protein/protein contacts with neighbouring molecules of the maturing U1 snRNP.

Endogenous U1 A protein is present both in the nucleus and cytoplasm of *Xenopus* oocytes (Figures 2 and 3). This finding is in agreement with earlier reports demonstrating that a population of unbound U1 A protein is normally present in nuclei of mammalian cells (6) and that purified U1 A protein shuttles between the nucleus and cytoplasm when injected into *Xenopus* oocytes (7). Because of the presence of U1 A protein in both cellular compartments, it is unclear whether the U1 A protein in the final mature U1 snRNP is the same molecule that bound initially to the nuclear pre-U1 RNA or whether U1 snRNP assembly involves the association and dissociation of different molecules of U1 A protein during nucleo-cytoplasmic RNA transport. Likewise it is unclear if the U1 A protein on pre-U1 RNA comes from a pool of free protein in the nucleus or from mature U1 snRNPs.

Deletion or substitution of the U1 A protein binding site appeared not to affect the stability or the transport of mutant U1 snRNA provided the RNA contained an 5' m⁷G-cap structure. Since deletion of the U1 A protein binding site did not prevent the export of pre-U1 RNA, it is unlikely that U1 A protein is essential for U1 RNA export.

Interestingly, pre-U1 RNA was very unstable in the nucleus when it lacked the binding sites for both a cap binding protein (i.e. the m⁷G cap) and the U1 A protein (stem-loop II) (Figure 4A). However, the RNA was stable when one or the other site was present, implying that the binding of either protein was sufficient to stabilize pre-U1 RNA. In contrast, a single compound, the cap analogue m⁷GpppG, destabilized both the wild-type and mutant (U1_{A-}) forms of RNA suggesting that the analogue may titrate both the cap-binding protein and the U1 A protein. While titration of the m⁷G-cap binding protein was expected, the loss of protection by the U1 A protein was a surprise. The cap analogue might prevent association of the U1 A protein with the RNA either directly or indirectly, via interactions that are mediated by a m⁷G-cap binding protein.

Good candidates for cap-binding factors of the pre-export U1 snRNP are TPI, a nucleoprotein that recognizes the 5' m⁷G cap of pre-U1 RNA and protects against premature nuclear 3' end

trimming (4), and a recently characterized nuclear 80 kDa m⁷G cap-binding protein (10, 29).

Because the U1 A protein interacts with both the precursor and mature forms of U1-RNAs in the nucleus, the levels or activities of the U1 A protein may affect the amounts of functional U1 snRNPs. For example, if only a limited number of U1 A proteins are available for U1-RNA binding, an excessive level of mature U1 RNAs could deplete the pool of U1 A protein available to form pre-export U1 snRNP complexes leading to increased turnover of pre-U1 RNA and decreased U1 RNA accumulation. Thus, the U1 A protein is potentially a regulatory protein impacting both on U1 snRNA assembly and function.

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