Multiple cis-acting signals for export of pre-U1 snRNA from the nucleus

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We have identified cis-acting sequences that promote nuclear export of pre-U1 RNA injected into Xenopus oocyte nuclei. At least three elements, the 5’ m^G cap, the 3’-terminal stem–loop structure, and sequences in the 5’-terminal 124 nucleotides, contribute to efficient export of this RNA. Both the 5’ and 3’ export signals can function separately and do so independently of the cap structure. Experiments using hybrid RNAs indicate that the 5’ and 3’ export sequences of U1 RNA are sufficient to direct export of the heterologous, otherwise nonexportable, U6 RNA. The absence of comparable export signals in U6 RNA appears to be responsible for its retention in the nucleus. Stability of the pre-snRNAs in the nucleus depends on the presence of both a 5’ cap structure and a 3’ base-paired stem. The 5’ m^G cap is neither sufficient nor necessary for nuclear export. The m^G cap by itself did not promote export of U6 RNA or nonspecific small RNAs. Moreover, substitution of this cap with either an ApppG cap or γ-mpppG cap did not eliminate export of either full-length or a “minimal” U1 RNA (lacking most of the internal U1 RNA sequences), but it reduced the rate of export by about two to threefold. However, in the absence of the 3’ stem–loop, substitution of the m^G cap led to a greater decrease in export rate, underscoring the cooperative action of the three different export elements of pre-U1 RNA. The m^G cap analog, m^GpppG, selectively destabilized pre-U1 RNA within the nucleus. Thus, nuclear components that recognize the 5’ m^G cap may be important for both the stability and the export of pre-U1 RNA.

[Key Words: Export; RNA; U1 snRNA; nucleus; nucleo-cytoplasmic transport, Xenopus oocyte]

Received June 9, 1993; revised version accepted August 11, 1993.

Export of RNA from the nucleus to the cytoplasm is a highly selective, energy-requiring process that depends on specific cis-acting signals within the structure of the RNAs (Zasloff 1983; Guddat et al. 1990; Hamm and Mattaj 1990; Eckner et al. 1991; Dargemont and Kühn 1992; Sun et al. 1992; for review, see Maquat 1991; Izaurralde and Mattaj 1992). The transport of RNA through the large nuclear pore complexes (NPCs) (Dworetzky and Feldherr 1988; Meihlin et al. 1992) can be inhibited by general inhibitors of nuclear pore function, such as wheat germ agglutinin and anti-porcine protein antibodies (Featherstone et al. 1988; Bataille et al. 1990; Neuman de Veyvar and Dahlberg 1990; Dargemont and Kühn 1992, Michaud and Goldfarb 1992; for review, see Nigg et al. 1991, Forbes 1992).

The U small nuclear RNAs (snRNAs), which function in the processing of mRNAs and rRNAs (for review, see Steitz et al. 1988; Lührmann et al. 1990; Green 1991), offer unique model systems for studying the control of nuclear trafficking. This is because most snRNAs undergo bidirectional transport across the nuclear membrane, as they mature from precursor to functional forms.

Soon after synthesis by RNA polymerase II (RNAPII) (Dahlberg and Lund 1988), precursor U1 RNA is exported from the nucleus to the cytoplasm, where the 5’ m^G cap is hypermethylated, the 3’ end is partially shortened, and the RNA binds Sm proteins, common to many small nuclear ribonucleoprotein particles (snRNPs) (Parry et al. 1989; Lührmann et al. 1990). The assembled U1 snRNPs are then imported into the nucleus, where the final 3’-end maturation occurs (Yang et al. 1991). U1-specific proteins (called A, C, and 70K) are also components of the functional nuclear U1 snRNP, but it is unclear where in the cell and when in the life cycle of the RNA these proteins associate with the U1 snRNA (Feeney and Zieve 1990; Jantsch and Gall 1992; Kambach and Mattaj 1992, Terns et al. 1993). Import of U1 snRNPs depends on several structural features of U1 RNA, including the Sm protein-binding site, a 5’ trimethylated cap structure, and a proper 3’-end structure (Hamm et al. 1987, 1990; Fischer and Lührmann 1990; Neuman de Veyvar and Dahlberg 1990; Fischer et al. 1991, 1993; Michaud and Goldfarb 1992; this paper).

In contrast to U1 RNA, U6 snRNA remains in the nucleus (Hamm and Mattaj 1989, this paper). Soon after synthesis by RNAPIII (Dahlberg and Lund 1988; Reddy and Singh 1992), the 5’ pppG of U6 RNA is converted
The mechanisms responsible for the selective and efficient export of pre-U1 RNA remain unclear. Export of pre-U1 snRNA is not strictly coupled to synthesis (Neumann de Veyvar and Dahlberg 1990), because precursor RNA injected into the nuclei of X. laevis oocytes is rapidly transported to the cytoplasm (Yang et al. 1992). The 5' m7G cap structure of pre-U1 RNA has been implicated as an important signal for U1 RNA export because reduced levels of pre-U1 RNA were detected in the cytoplasm when m7G cap analog was injected or when the U1 RNA was made with a 5' triphosphate end by transcription from an RNA polymerase III promoter (Hamm and Mattaj 1990; Izaurralde et al. 1992). However, it is unclear whether this reduction was solely the result of direct effects on export. Furthermore, these studies did not address the possibility that pre-U1 RNA might contain additional signals for export.

In this study we show that export of U1 RNA involves two domains present in the 5' and 3' regions of the RNA, each of which can act independently of the other to promote export. Also, we demonstrate that although the 5' m7G cap structure of precursor U1 RNA enhances the rate of U1 snRNA export significantly, it is neither sufficient nor essential for snRNA export. The localization of U6 RNA exclusively in the nucleus is most likely the result of the lack of export signals rather than the presence of active retention signals.

Results

We investigated the stability and intracellular localization of U snRNAs in X. laevis oocytes by microinjecting either snRNA genes [plus δ2P]GTP or δ2P-labeled precursor snRNAs. Individual oocytes were dissected at various times into nuclear and cytoplasmic fractions, and the intracellular distributions of the labeled RNAs were determined by polyacrylamide gel electrophoresis. Because the distribution of an RNA is a function of both its transport and stability, we also examined the stability of the RNAs within each cell compartment. Notably, we found that several RNAs were unstable in the nucleus although they were stable in the cytoplasm. The accuracy of nuclear injections and the quality of oocyte dissections were monitored by coinjection of U6 RNA, because this RNA is not exported from the nucleus (Hamm and Mattaj 1989) and is stable in both compartments of the oocyte (see below).

Figure 1. An m7G cap is not sufficient for export of U6 RNA from the nucleus. [A] Structure of templates used to express U6 RNA from a U1 promoter (RNAP II) or a U6 promoter (RNAP III). The U6-coding sequences (stippled region), the U1 3'-end signal (3' box), and the 5' and 3' ends of the primary U6 RNA transcripts are indicated. [B] Intracellular location of U6 RNAs synthesized by either RNAP II or III. Xenopus oocytes were coinjected with wild-type U1 genes (~4 ng of DNA/oocyte) and either the chimeric [RNAP II] or wild-type [RNAP III] U6 RNA genes (~4 and 1 ng of DNA/oocyte, respectively) plus 2H-GTP. After 5 hr of labeling, 1 oocyte equivalent of the δ3P-labeled RNA present in the nuclear (N) and cytoplasmic (C) fractions from 10 pooled oocytes was analyzed by electrophoresis in a denaturing 8% polyacrylamide gel. The autoradiographs of the U6 and U1 RNA regions of the gel are shown. [C] Immunoprecipitation analysis using antibodies against the 5' m7G cap structure. Precipitation was carried out with 3 oocyte equivalents of nuclear RNAs shown in the left panel of B, and both pellet (P) and supernatant (S) were analyzed by gel electrophoresis as in B.
of a coinjected U1 gene; Fig. 1B, top bands). Because m7G capped U6 RNA is stable following its injection into the cytoplasm [see Table 1 footnote, below; data not shown], it is unlikely that m7G capped U6 RNA was exported from the nucleus and degraded rapidly in the cytoplasm. We conclude that synthesis from a U1 promoter and possession of an m7G cap structure are not sufficient to specify snRNA export. In support of this conclusion, we have found that several comparably sized m7G-capped RNAs, such as antisense U4 RNA and artificial RNAs generated by transcription from pGEM vectors and DNA fragments containing SP6 and T7 RNA polymerase promoters, were either unstable or not exported from the nucleus [data not shown].

We tested directly whether the m7G cap was necessary for U1 RNA export by monitoring the transport of differently capped precursor U1 RNAs that were injected into oocyte nuclei [Fig. 2]. These precursor U1 RNAs, made in vitro using SP6 RNA polymerase, had either a nonphysiological ApppG or m7GpppG and γ-mpppG cap structures, which are normally present at the 5' ends of pre-U1 and U6 RNAs, respectively. To assay for export of U1 RNA in the absence of its import [Hamm and Mattaj 1990], in these and other experiments we used a variant U1 RNA, Ulsm- [Fig. 4A, below], which lacks the Sm-binding site needed for import [Hamm et al. 1987]. Control experiments indicated that the export rate and stability of Ulsm- RNA were similar to those of wild-type U1 RNA [data not shown], and so in the text below we refer to these RNAs synonymously unless indicated otherwise.

As shown in Figure 2A [lanes 2-5], U1 RNAs with the different 5' caps accumulated in the cytoplasm. These RNAs with alternative cap structures [either ApppG or γ-mpppG caps] were exported to the cytoplasm, although at a two- to threefold slower rate than m7G-capped U1 RNA [Figs. 2B and 3B, data not shown]. Again, export of ApppG-capped U1 RNA was independent of the presence of the Sm protein-binding site in the RNA [data not shown]. As expected, the coinjected control U6 RNA remained in the nucleus [lower panel]. In contrast to uncapped U1 RNA, which is highly unstable in both the nucleus and cytoplasm [data not shown; cf. Hamm and Mattaj 1990], the cap-substituted U1 RNAs were quite stable in both compartments. Thus, the m7G cap enhanced the rate of U1 RNA export, but it was not essential for export of U1 RNA.

The dinucleotide m7GpppG destabilizes pre-U1 RNAs in the nucleus

We examined possible functional interactions of the m7G cap of pre-U1 RNA with nuclear components by injecting an analog of the cap, the dinucleotide m7GpppG, which would be expected to compete for association with cap-binding proteins [Izaurralde et al. 1992]. To understand the effects of this compound on transport of pre-U1 RNA, it was first important to know whether it affected RNA stability. As shown in Figure 3, coinjection of the m7G cap analog significantly decreased the stability of m7G-capped U1 RNA precursors in the nucleus but not the cytoplasm [Fig. 3A, Ulsm- , lanes 2–9, summarized in Fig. 3B, left panels]. Surprisingly, this analog also destabilized U1 RNA, whose 5' cap was ApppG rather than m7GpppG [Fig. 3B, middle panels; see Discussion]. In contrast, variants of U1 RNA [see Fig. 4A] such as U1124 [lacking sequences at the 3' end] or U1Min [lacking sequences from the middle of U1 RNA] were not destabilized appreciably by the cap analog [Fig. 3B, right panels; data not shown]. As expected, m7GpppG did not affect the stability of U6 RNA or
Figure 3. The m7GpppG cap analog specifically destabilizes full-length pre-U1 RNAs in the nucleus. (A) Effect of the m7GpppG cap analog on the metabolism of several different types of RNAs. A mixture of m7G-capped Ulsm- RNA, γ-mpG-capped U6 RNA, and X. laevis mature tRNA Tyr (all labeled with 32P) was injected into oocyte nuclei [lanes 2-9] or cytoplasm [lanes 10-13] in the presence (+) or absence (-) of 50 mM m7GpppG cap analog. After 1 and 3 hr of incubation, the intracellular distributions of the RNAs were analyzed as in Fig. 2A; lane 1 shows the RNA prior to injection. (B) Quantitation of the effects of the m7GpppG cap analog on export and stabilities of m7G-capped Ulsm- RNA and m7G-capped Ul124 RNA. The results of experiments such as those shown in A were quantitated by scanning the autoradiographs; the bars show the percentage of the injected RNAs present in the nuclear [top] and cytoplasmic fractions [bottom] at different times after injections in the presence [shaded bars] or absence [solid bars] of the m7GpppG cap analog.

tRNA Tyr nor the transport of the tRNA [Fig. 3A, lanes 2-9].

The effect of the cap analog on U1 RNA export was difficult to evaluate because of the destabilization of the RNA. However, at early times after coinjection of the cap analog and pre-U1 RNA, before the RNA in the nucleus had been degraded, a significant level of export occurred [Fig. 3A, lanes 6, 7; see 1.3-hr time point in bottom left panel of Fig. 3B]. Moreover, the export rate of stable m7G-capped U1 RNA variants, such as Ul124 and UlMini, was inhibited only slightly [Fig. 3B, bottom right panel, data not shown]. Thus, it is very likely that the dinucleotide m7GpppG affects the stability of full-length precursor U1 RNA in the nucleus more than it affects the export of this RNA.

Sequences within U1 RNA that signal RNA export

We asked whether sequences within U1 RNA might direct its export. As noted above [Fig. 3B], two deletion variants of U1 RNA were stable and efficiently exported from the nucleus. These two U1 RNA derivatives were Ul124, which lacked the 3'-terminal stem–loop structure of U1 RNA [Fig. 4A, middle panel], and UlMini, which contained the first 27 nucleotides from the 5' end and the entire 3' stem–loop sequences but lacked the majority of internal U1 RNA sequences, including the Sm protein-binding site [Fig. 4A, right panel]. Both of these RNAs were exported from the nucleus at rates comparable with that of the full-length Ulsm- RNA [Fig. 4B]. Lacking functional Sm protein-binding sites, the deletion variants remained in the cytoplasm, where they were stable [data not shown]. The 5' 27 nucleotides of UlMini RNA are not essential for export, because comparable minimal U1 RNAs that lacked these 5' sequences, but contained the 3'-terminal stem–loop structure [Sumpter et al. 1992], were also very efficiently exported [data not shown]. These results suggest that U1 RNA contains two signals present in the 5' and 3' regions of the RNA, each of which can act independently of the other to promote export.

As with full-length Ulsm- RNA [Fig. 2], substitution of the m7G cap with an ApppG reduced the rate of export of UlMini RNA two to threefold [data not shown]. However, in the case of Ul124 RNA, the identical cap substitution resulted in a much greater reduction in the rate of export [approximately eightfold; data not shown]. This result indicates that the function of the 5' export signal is more dependent on the m7G cap structure than that of the 3' signal.

Export sequences of U1 RNA can function to direct export of heterologous RNA

To test whether the 5' and 3' U1 RNA domains were capable of directing export of another RNA, we fused these U1 RNA sequences to the U6 RNA [Fig. 5A]; this RNA is otherwise not exported from the nucleus even when it contains an m7G cap [Fig. 1]. When injected into nuclei, hybrid U6 RNAs containing either the 5' domain [nucleotides 1-124] or the 3' domain [nucleotides 130-164] of U1 RNA were exported. Thus, both domains contain signals that can promote export of a heterologous RNA [Fig. 5B, lanes 2-7]. However, the hybrid RNAs were not transported as efficiently as was full-length U1 RNA [Fig. 5B, cf. RNAs in lanes 4 and 5]. Because either domain of U1 RNA by itself can direct export of U6 RNA, two separate export signals apparently exist within U1 RNA.

Dual role for sequences within the 3' stem–loop of U1 RNA

We investigated the role of the 3'-end structure in U1
RNA export and stability by injecting several variant U1 RNAs containing either extensions or truncations at the 3’ ends (Fig. 6A). Unexpectedly, the m7G-capped U1 +40, U1 +80, and U1 +153 RNAs were degraded rapidly when injected into the cytoplasm (lanes 2–5); these same RNAs were stable for at least 24 hr when injected into the cytoplasm (lanes 6, 7; data not shown). Only the wild-type precursor U1 RNA, U1 +2 (with 2 extra nucleotides at the 3’ end), was stable and exported efficiently; a low amount of RNA in the U1 +8 preparation also was stable and exported, as evidenced by the appearance of shortened, mature forms back in the nucleus 3 hr later (lane 4). Thus, in addition to its role in export, the correct 3’-end structure of pre-U1 RNA contributes greatly to the stability of the RNA in the nucleus.

The roles of U6 RNA termini for stability and retention in the nucleus

The localization of newly made U6 RNA in the nucleus could result either from a lack of export signals in the RNA or from active retention. To distinguish between these two possibilities, we tested three sites within the U6 RNA for their abilities to keep the RNA in the nucleus (Fig. 7A). These were (1) the 5’ cap, (2) the single-stranded region (nucleotides 20–25), which is important for both 5’ capping (Singh et al. 1990) and import of U6 RNA from the cytoplasm to the nucleus (Hamm and Mattaj 1989), and (3) the 3’-terminal uridylate tract that serves as a binding site for the karyophillic La protein (Rinke and Steitz 1985; Reddy et al. 1987; Terns et al. 1992) and is subject to post-transcriptional modification (Lund and Dahlberg 1992; Tazi et al. 1993).

An example of these studies is shown in Figure 7B, in which γ-mpppG-capped U6 RNA lacking the 3’ uridylate stretch was injected into oocyte nuclei (lanes 2,3) or cytoplasm (lanes 4,5). Some of the 3’ truncated U6 RNA was elongated, apparently by nontemplated addition of a 3’ uridylate tract (Lund and Dahlberg 1992), in both cell compartments (lanes 2,5) to the point where it could bind La protein, as shown by its coprecipitation by anti-La antibodies (Fig. 8C). However, even the fraction of U6 RNA that was not elongated remained in the nucleus, making it unlikely that the nuclear localization of U6 RNA was the result of retention of the RNA via binding to the La protein.

The stabilities and subcellular localization of this and other U6 RNA variants following injection into oocyte nuclei are summarized in Table 1. Maximal stability of U6 RNA in the nucleus was dependent on the presence of both the 5’ cap structure and the 3’-terminal uridylate tract (La-binding site). Deletion of the single-stranded region did not significantly affect the nuclear stability of U6 RNA containing wild-type termini. Variable effects on U6 RNA stability were observed when both the single-stranded region and the 5’ or 3’ termini were altered. The half-lives of all variants of U6 RNA were generally two- to fourfold longer in the cytoplasm than in the nucleus (not shown); it is therefore unlikely that export of U6 RNAs went undetected as a result of cytoplasmic degradation. Moreover, the U6 RNA variants probably did not undergo a rapid export/import cycle, because U6 RNAs lacking the single-stranded region needed for efficient import into the nucleus from the cytoplasm (Hamm and Mattaj 1989) did not accumulate in the cytoplasm. These data show that U6 RNA remains in the nucleus regardless of the type of 5’ cap that it has or whether it contains the single-stranded region and/or the 3’-terminal uridylate stretch. Thus, it is likely that U6 RNA lacks signal sequences necessary for RNA export.
Signals for export of pre-U1 snRNA

Export signals in pre-U1 RNA

The two sequence elements of pre-U1 RNA that promote export from the nucleus to the cytoplasm are located in the 3' stem-loop and within the 5' terminal, 124 nucleotides of this RNA, respectively. Variants of pre-U1 RNA lacking one or the other sequence element are exported (Fig. 4), indicating that neither element is essential and that the individual sequence elements can function independently of each other. When fused to U6 RNA, either element is able to direct export of this RNA (Fig. 5). It is likely that both sequence elements contribute to the efficient export of intact pre-U1 RNAs. The precise sequences responsible for export activity remain unknown, and the actual elements may be comprised of both sequence and structural information. Moreover, sequences that direct export of pre-U1 RNA overlap, at

Discussion

In analyzing cis-acting signals that participate in the export and stabilization of RNAs, we studied the precursors of two snRNAs, U1 and U6. Specific structures at the 5' and 3' ends of both RNAs are needed for their stability within the nucleus. Our results are consistent with the idea that export is not the default pathway for snRNAs. Two sequence domains within U1 RNA were identified that, in conjunction with the 5' m7G cap structure, direct efficient U1 RNA export. U6 RNA appears to lack such export signals, which may explain why this RNA remains in the nucleus.

Figure 6. A correct 3'-terminal stem-loop structure of U1 RNA is important for both nuclear stability and import from the cytoplasm. [A] Structure of wild-type pre-U1 RNA (U1s, U1s) and variant U1 RNAs that have truncated (U1s, U1s, U1s) 3' ends, all RNAs contained the Sm protein-binding site and therefore are subject to bidirectional nuclear transport. [B] Nucleocytoplasmic transport of 3'-truncated or -elongated forms of U1 RNA that were injected into oocyte nuclei [lanes 2-5] or cytoplasm [lanes 6-7]. The RNAs of individual oocytes were analyzed as in Fig. 2A, black dots indicate mature, m7G-capped U1s, o RNA forms that have been imported into the nucleus.
least partially, with sequences required to stabilize the RNA within the nucleus [Fig. 6].

An m^7G cap at the 5' end of an RNA cannot be a sufficient signal to direct export, as several small RNAs, including U6 RNA, remain in the nucleus even when they contain this cap [Fig. 1]. Nor is an m^7G cap necessary for generating such a signal, as shown by the transport of pre-U1 RNAs that have AppppG or γ-mpppG caps (Fig. 2). However, an m^7G cap contributes to the efficiency of export because substitution of the m^7GpppG cap with AppppG or γ-mpppG reduces the rate of export of full-length pre-U1 RNA two- to threefold. The influence of the m^7G cap on export is more pronounced with U1 RNAs that lack the 3' stem-loop structure, indicating that the export signals within pre-U1 RNA may act cooperatively. Without some type of cap, all U1-related RNAs are unstable.

Our results showing that an m^7G cap enhances the efficiency of export of pre-U1 RNA agree with the conclusions drawn from the results of others [Hamm and Mattaj 1990; Izaurralde et al. 1992]. An essential role for the 5' cap in export was suggested because a variant of pre-U1 RNA remained in the nucleus when the RNA was synthesized without a 5' cap, using RNAP III [Hamm and Mattaj 1990]. However, we have observed recently that such a pre-U1 RNA, in contrast to the m^7G capped precursor made by RNA polymerase II, was bound to the nuclear protein La (data not shown), which normally associates with the 3' ends of newly made RNA polymerase III transcripts [Rinke and Steitz 1982; Mathews and Francoeur 1984; Stefano 1984; Terns et al. 1992]. Thus, it is possible that retention of this uncapped pre-U1 RNA in the nucleus was the result of masking, by the bound La protein, of the export signal at the 3'-end structure rather than the result of the absence of a 5' m^7G cap.

Retention of U6 RNA in the nucleus

We cannot exclude the possibility that U6 RNA is actively retained in the nucleus through binding to an unidentified nuclear component via RNA sequences that were not tested in this study. However, our experiments indicate that the retention of U6 RNA in the nucleus results from the absence of export signals rather than from the presence of a retention element. For example, the sequences of U6 RNA are exported when they are linked to either of the export signals of pre-U1 RNA [Fig. 8].
mTG cap of pre-U1 RNA, either independently or as part of other proteins such as the 80-kD cap-binding protein (CBP) (Ohno and Mattaj 1990; Izaurralde et al. 1992). The larger than mature U1 snRNPs, as assayed by glycerol gradient centrifugation of oocyte nuclear extracts (Terns et al. 1992). Taken together, these results indicate that U6 RNA is not normally retained within the nucleus through binding of nuclear proteins that specifically recognize U6 RNA sequences.

**Nuclear metabolism and export of pre-U1 snRNA**

It is likely that the export signals in pre-U1 RNA function through interactions with nuclear proteins. We have shown recently that pre-U1 RNA is in an RNP complex larger than mature U1 snRNPs, as assayed by glycerol gradient centrifugation of oocyte nuclear extracts (Terns et al. 1993). Two nuclear factors that interact with pre-U1 RNA are TPI, a nucleoprotein that recognizes both the 5' cap and the 3' end (Yang et al. 1992). In addition, a role for the U1–A protein in stabilization is indicated by our recent finding that pre-U1 RNAs that lack both the binding site for this protein and the mG cap are highly unstable in the nucleus (Terns et al. 1993).

We propose (Fig. 8) that pre-U1 RNA is in a specific complex, which we call a pre-export snRNP, and that such a complex both stabilizes the RNA against attack by nuclear RNases and directs the RNA to nuclear pore complexes for export to the cytoplasm. It is possible that additional proteins, acting as receptor molecules, bind transiently to bring the pre-export snRNP complex to the nuclear pore. Furthermore, there may be components at the pores that interact specifically with the pre-export snRNP–receptor complex to facilitate efficient export of the pre-snRNA.

The role of the mG cap structure in pre-U1 RNA export has been probed by testing the effects of a dinucleotide cap analog on cytoplasmic accumulation of the RNA. In our experiments, injection of mGpppG into oocyte nuclei destabilized both mG- and ApppG-capped, full-length pre-U1 RNA (Fig. 3, data not shown). This destabilization indicates that the RNA is normally stabilized by a cap-binding protein. Moreover, the fact that both mGpppG- and ApppG-capped RNAs are similarly affected shows that the factor responsible (perhaps TPI or CBP) does not discriminate well between the two types of 5' cap structures. Because of the destabilizing effect of the mGpppG analog, we were unable to monitor its long-term effects on the export of injected pre-U1 RNA. However, a significant level of export occurred at early times, before the pre-U1 RNA was degraded (Fig. 3). Thus, the effects of the analog appear to be more on the stability than on the transport of pre-U1 snRNAs.

In contrast to full-length pre-U1 RNA, certain deletion variants of pre-U1 RNA were not destabilized in the presence of the cap analog (Fig. 3B). This indicates either that the variants do not require a cap-binding protein for stability (i.e., are inherently stable in the nucleus) or that their binding to this factor is relatively resistant to the dinucleotide competitor. The export of these stable variants was largely unaffected by injection of the cap analog (Fig. 3B). However, both the deletion variants and full-length pre-U1 RNA were exported more efficiently when they contained an mG cap rather than an ApppG cap. Taken together, these results suggest that a cap-binding protein (or the mG cap structure itself) participates in the export of pre-U1 RNA and that the mGpppG cap analog is not an effective competitor of the export of injected RNAs.

Our results contrast with previously published results of others who reported that this analog directly inhibited export of pre-U1 RNA synthesized in the nucleus (Hamm and Mattaj 1990; Izaurralde et al. 1992). The reason for this discrepancy is unclear, but the mGpppG cap analog may act at several levels to affect both the stability and export of U1 RNA under different experimental conditions.

### Table 1. Nuclear localization and stability of U6 RNA

| U6 RNA structure | Behavior following nuclear injection |
|------------------|-------------------------------------|
|                  | 3-uridylate stretch | Single-stranded region | Half-life in nucleus [hr] | Location in oocyte |
| 5' end           |                      |                      |                          |                    |
| pppG             | +                    | +                    | 3-4                     | N                   |
|                  | +                    | -                    | 6-8                     | N                   |
|                  | -                    | -                    | <0.5 (unstable)         | N                   |
| γ-mpppG          | +                    | +                    | >24                     | N                   |
|                  | +                    | -                    | >24                     | N                   |
|                  | -                    | -                    | 8-12                    | N                   |
|                  | -                    | -                    | 3-4                     | N                   |
| mGpppG           | +                    | +                    | 8-12                    | N                   |
|                  | +                    | -                    | 3-4                     | N                   |
|                  | -                    | +                    | 8-12                    | N                   |

Nuclear retention of U6 RNAs. U6 RNA variants with and without nucleotides 20–25 [the single-stranded region (nucleotides 20–25) required for nuclear import of U6 RNA (Hamm and Mattaj 1989)] and/or the 3' terminal uridylate stretch (the binding site for the nuclear protein La; see Fig. 7A) were synthesized in vitro without [pppG] or with different 5' caps (γ-mpppG, mGpppG, and ApppG). The 32P-labeled RNAs were injected into oocyte nuclei or cytoplasm, and their intracellular distribution was determined as a function of time, as in Fig. 2A. The nuclear half-lives were estimated from the autoradiographs. All U6 RNA variants tested were two- to fourfold more stable in the cytoplasm than in the nucleus [data not shown].
The precise roles of the sequence-encoded export signals and the 5' m7G cap in stabilization and export of pre-U1 RNA remain to be defined. Clearly, these elements and structures interact with specific proteins that stabilize the RNA and promote its export either directly or through their association with components of the nuclear pore complex (Fig. 8). Identification and functional analysis of these proteins will help to clarify the mechanism of pre-snRNA nucleocytoplasmic transport. It remains to be determined whether mRNAs and snRNAs utilize common pathways for their transport from the nucleus.

Materials and methods

DNA constructs

The wild-type *X. laevis* U1 gene used in this study was U1bl (Krol et al. 1985). The mutant U1 gene (U1AD) containing a substitution of nucleotides 124–128 of the Sm protein-binding site (Hamm et al. 1987) was provided by I. Mattaj, EMBL, Heidelberg; a unique *XhoI* site within the substituted sequence was used in generating several of the constructs described below. The wild-type *Xenopus tropicalis* U6 gene (Krol et al. 1987) was obtained from A. Krol, and a mutant U6 gene (As, lacking U6 nucleotides 20–25; Hamm and Mattaj 1989) was provided by I. Mattaj.

Plasmid constructions involved standard (Innis et al. 1990) and recombinant (Higuchi 1990) polymerase chain reaction (PCR) techniques and general DNA cloning procedures (Maniatis et al. 1989). Oligonucleotides used in PCR reactions were synthesized from Promega (Madison,WI) or University of Wisconsin Biotechnology Center. pGEM3ZF+ (Promega) was used as the vector for all constructs except pU1/U6, for which pUC19 was used. All DNA constructs were confirmed by DNA sequencing using the dideoxynucleotide chain-termination method with modified T7 DNA polymerase ( Sequenase, U.S. Biochemical).

Plasmids

pU1sm contains the 5’-flanking sequences (promoter) of the U1bl gene and the coding and 3’-flanking sequences of the U1AD gene. pU1smM contains the U1AD gene. pU1sm was derived from pU1sm* by deletion of coding sequences from nucleotides +27 to +124 [between *BclI* and *XhoI* cleavage sites] and replacement of these sequences with a *BglII* linker (CAGATCTG). pU1/U6 contains the 5’-flanking sequences (promoter) of the U1bl gene, coding region of the U6 gene, and the 3’-end box of the U1bl gene. The construct was designed such that transcription starts at G+1 of U6 and ends in a run of thymidylates generating U6 RNA containing an m7G cap and four to seven 3’-terminal uridylate residues. pU1(U1/U6) contains the 5’-flanking sequences (promoter) of the U1bl gene and the coding sequences of U1AD (nucleotides 1–124), fused to U6â€Â±ss sequences (nucleotides 14–107). The 3’-flanking sequences are from the U1bl gene as in pU1(U6), pU6(U6/U1) contains the 5’-flanking sequences (promoter) of the U6 gene and the coding sequences of U6â€Â±ss [nucleotides 1–98] fused to U1AD sequences (beginning at nucleotide 124 of the coding region and including the 3’-flanking region). The deletion of the 3’-terminal thymidylate residues of the U6 RNA-coding region (within nucleotides 99–107) ensured that transcription termination occurred only at the thymidylate stretch present immediately downstream of the U1AD-coding sequences (cf. Hamm and Matta 1990).

In vitro RNA synthesis

Templates used for in vitro synthesis of U snRNAs were either linearized plasmid DNAs (containing SP6 promoter sequences) or DNA fragments generated by PCR amplification of the RNA-coding regions of the plasmids described above, using appropriate 5’ and 3’ primer pairs. The 5’ primer used in the PCR reactions contained SP6 (all RNAs with 5’ U1 RNA sequences) or T7 (all RNAs with 5’ U6 sequences) phage RNA polymerase promoter sequences. The transcripts generated from these templates have the same sequence as the *Xenopus pre-U1* and pre-U6 RNAs except for two additional residues at their 5’ ends: GA for U1 RNA and GG for U6 RNA. The presence of the extra nucleotides at the 5’ end does not appear to affect the metabolism or transport of the RNAs (Terns et al. 1992; data not shown). The 3’-end primers were designed to give RNAs with 3’ ends identical to those observed in RNAs made in vivo (Neuman de Vegvar and Dahlberg 1990, Terns et al. 1992).

3’-Altered U1 RNAs were generated by cleaving wild-type SP6–U1 templates at appropriate restriction sites, U115A (HpaI) and U1-40 (EcoRI), or by oligonucleotide-directed RNase H cleavage of U11-40 RNA to generate U1 +40 RNAs (Yang et al. 1992). U124 was generated by cleaving the SP6–U1sm- DNA template with *XhoI*. 3’-truncated U6â€Â±ss RNA was generated by incorporating an *NcoI* cleavage site into a T7–U6 DNA fragment using a primer that is complementary to the 3’ end of U6 gene but contains two additional G residues between positions 101 and 102.

In vitro transcription of T7 or SP6 DNA templates was performed essentially as described previously (Terns et al. 1992), except that 5’ capping of the RNAs was accomplished by lowering the GTP concentration from 500 to 50 µM and including 0.5 or 1 mM of m7GpppG (NEB) or ApppG [Pharmacia] or 4 mM of γ-mpppG [kindly provided by R. Reddy, Baylor College of Medicine, Houston, TX]. [α-32P]GTP (NEN Dupont) was used as source of label. To obtain RNAs with defined 3’ ends [confirmed by two-dimensional RNase T1 fingerprinting analysis], all transcripts were purified by electrophoresis in high-resolution 8% denaturing polyacrylamide gels.

Oocyte microinjection analysis of RNA transport

Stage V and VI oocytes, obtained from adult female *X. laevis* (Krol et al. 1985), were injected into the nuclei or cytoplasts with 12 nl of solution containing up to 1 fmole of each 32P-labeled snRNA. The results were unaffected by a 10-fold increase or decrease in the amount of RNA injected [data not shown]. In experiments where the RNAs were co-injected with the dinucleotide cap analog, the solution contained 50 mM m7GpppG [New England Biolabs]. For nuclear injections [which may result in partial nuclear “hits” and cytoplasmic “mishits”], at least four individual oocytes were analyzed per time point, for cytoplasmic injections [which easily avoid nuclear hits], pools of three oocytes were used. In either case, the nuclei and cytoplasts were isolated from individual oocytes by manual dissection under mineral oil [Lund and Dahlberg 1989; Lund and Paine 1990], and after proteinase K digestion, RNAs within each compartment were purified by phenol extraction and ethanol precipitation (Terns et al. 1992). The 0.5 to 1 oocyte equivalents of RNA from selected oocytes were then analyzed by electrophoresis for 3–5 hr at ~40 V/cm in 8% [29 : 1] polyacrylamide gels [40 cm long and 0.4 mm thick] containing 7 M urea and 0.5 × TBE buffer [45 mM Tris-borate at pH 8.3, 1.15 mM EDTA]. Autoradiography was for 12–48 hr with, or 1–10 days without, an intensifying screen. Quantitation of the autoradiographs was done using AppleScan and Scan Analysis [BioSoft] software programs.
To monitor the accuracy of the nuclear injections, U6 RNA was co-injected with the test RNAs as this RNA remains within the nucleus and is stable in both the nucleus and cytoplasm. When variant U6 RNAs were tested, γ-mpGG-capped U1/U6 hybrid RNA (which is exported very slowly from the nucleus, data not shown) was used instead. In all cases, the transport properties of injected RNAs were comparable with those of RNAs made in vivo from injected plasmid DNAs.

**Immunoprecipitation**

Extract preparation from isolated nuclei and cytoplasm and immunoprecipitations were performed as described previously (Terns et al. 1992). The antibodies used in these experiments include a monoclonal [Y12] anti-Sm antibody [Lerner et al. 1981] [from J. Steitz, Yale University, New Haven, CT], rabbit polyclonal antibodies against the mG cap [Bringmann et al. 1983] [from R. Lührmann] or mG cap antibodies [Munns et al. 1982] [from T. Munns], and anti-La patient sera (A-114 and A-306; described in Terns et al. 1992] [from D. Kenan and J. Keene, Duke University, Durham, NC].

**Acknowledgments**

We thank A. Krol and I. Mattaj for supplying U1 and U6 genes, R. Reddy for furnishing the γ-mpGG cap analog, and J. Steitz, R. Lührmann, T. Munns, D. Kenan, and J. Keene for providing antibodies used in this study. This work was supported by a grant from the National Institutes of Health (NIH) [GM30220] to J.E.D. and an NIH postdoctoral fellowship [GM14704] to M.P.T.

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*Genes Dev.* 1993, 7:
Access the most recent version at doi:10.1101/gad.7.10.1898

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