

Nuclear Export of Different Classes of RNA Is Mediated by Specific Factors

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Abstract. Various classes of RNA are exported from the nucleus to the cytoplasm, including transcripts of RNA polymerase I (large ribosomal RNAs), II (U-rich small nuclear RNAs [U snRNAs], mRNAs), and III (tRNAs, 5S RNA). Here, evidence is presented that some steps in the export of various classes of nuclear RNA are mediated by specific rather than common factors. Using microinjection into *Xenopus* oocytes, it is shown that a tRNA, a U snRNA, and an mRNA competitively inhibit their own export at concentra-

tions at which they have no effect on the export of heterologous RNAs. While the export of both U snRNAs and mRNAs is enhanced by their 7-methyl guanosine cap structures, factors recognizing this structure are found to be limiting in concentration only in the case of U snRNAs. In addition to the specific factors, evidence for steps in the export process that may be common to at least some classes of RNA are provided by experiments in which synthetic homopolymeric RNAs are used as inhibitors.

ANALYSIS of the mechanisms of transport of macromolecules from the cytoplasm to the nucleus is a field of very active study (reviewed by Garcia-Bustos et al., 1991; Silver, 1991; Dingwall and Laskey, 1992; Gerace, 1992; Newmeyer, 1993). In contrast, macromolecular export from the nucleus is much less well understood (Maquat, 1991; Nigg et al., 1991; Izaurralde and Mattaj, 1992). Since all cytosolic RNAs must be transported out of the nucleus this process is, not least in quantitative terms, an important cellular activity.

Much of the information available on RNA export has come from studies involving microinjection into *Xenopus laevis* oocytes. In this way it has been shown that tRNA, mRNA, and ribosome export from the nucleus are both energy requiring and saturable, and therefore carrier (or receptor)-mediated (Zaslhoff, 1983; Khanna-Gupta and Ware, 1989; Bataillé et al., 1990; Dargemont and Kühn, 1992). This has led to general acceptance of the idea that the export of RNAs from the nucleus involves their association with proteins. Indeed, RNA-protein complexes thought to be engaged in transport out of the nucleus can be visualized in the electron microscope in favorable cases (Stevens and Swift, 1966; Mehlin et al., 1992), but little is known about the identity of the proteins involved in RNA export.

Genetic studies have identified mutants of either tRNA (Zaslhoff et al., 1982; Tobian et al., 1985) or 5S RNA (Guddat et al., 1990) whose rate of export from the nucleus is considerably reduced. The 5S mutant whose export is most affected is incapable of interaction with either TFIIIA or

ribosomal protein L5, implicating these proteins as mediators of 5S export (Guddat et al., 1990). In the case of the tRNA mutants, a correlation between export and ability to bind to the metabolic enzyme glyceraldehyde-3-phosphate dehydrogenase has been established (Singh and Green, 1993), but no direct evidence for a role of this protein in tRNA export has as yet been published. In the case of ribosomes, nucleolar proteins that are associated with preribosomes but not mature ribosomes have been identified and proposed to function as mediators of preribosome exit from the nucleus (reviewed by Ware and Khanna-Gupta, 1991). Finally, the identification of temperature-sensitive yeast mutants that accumulate poly A in their nuclei at the restrictive temperature has led to the identification of genes whose products influence mRNA transport by mechanisms which are currently unclear (Amberg et al., 1992, 1993; Forrester et al., 1992; Kadowaki et al., 1992, 1993).

By far the bulk of the sequence complexity of exported RNA is represented by RNA polymerase II transcripts (U-rich small nuclear RNAs [U snRNAs] and mRNAs).¹ When in the nucleus, mRNAs and their precursors are found in association with a family of very abundant nuclear proteins as heterogeneous nuclear ribonucleoproteins (hnRNPs) (Dreyfuss et al., 1993). These proteins were long thought to be confined to the nucleus, but recent work has shown that a subset of the proteins moves between the nucleus and the cytoplasm (Piñol-Roma and Dreyfuss, 1991, 1992). In the cytoplasm,

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1. *Abbreviations used in this paper:* hnRNP, heterogeneous nuclear ribonucleoprotein; m⁷G, 7-methyl guanosine; U snRNA, U-rich small nuclear RNA.

as in the nucleus, the proteins are found in association with poly (A)⁺ RNA, leading to the suggestion that they may be transported along with mRNA and, in fact, may be involved in mediating mRNA nuclear export (Piñol-Roma and Dreyfuss, 1992).

Other work has implicated the termini of polymerase II transcripts in mediating their export. Eckner et al. (1991) provided good evidence that the RNA processing steps involved in generating 3' ends were, at least in the case of histone mRNAs, in some way coupled to the transport of these RNAs out of the nucleus. The mono-methyl guanosine (m⁷G) cap structure of pol II RNAs has also been shown to affect the rate of their export (Hamm and Mattaj, 1990). A nuclear cap-binding protein that may mediate this effect has been identified and purified (Izaurrealde et al., 1992; see also Ohno et al., 1990).

Thus, many different proteins have been implicated in the export of RNAs from the nucleus, but none has, as yet, been directly shown to be active in the transport process. It is a tacit assumption in many of the studies referred to above that export mediators will be specific, if not for an individual RNA, at least for a particular class of RNAs (e.g., polymerase II vs. polymerase III transcripts, U snRNAs vs. mRNAs, tRNAs vs. 5S RNA etc.). However, to our knowledge, the only experiment that directly addressed this question showed that export of a small amount of a microinjected, radioactively labeled, mRNA could be inhibited either by coinjection of an excess of unlabeled mRNA or of tRNA (Dargemont and Kühn, 1992), suggesting that common factors might mediate export of diverse RNAs. We therefore set out to systematically examine the substrate specificity of RNA export pathways. The results obtained show that in *Xenopus* oocytes, distinct essential factors mediate the export of tRNA, 5S RNA, U snRNA, and mRNA.

Materials and Methods

Plasmids

Xenopus T7 UIΔSm (previously called ΔD), U2ΔSm, and U5ΔSm constructs have been described previously (Hamm et al., 1987; Scherly et al., 1990; Jarmolowski and Mattaj, 1993). The 77-nt long transcript was obtained by T3 transcription of EcoRI linearized plasmid UISII- as described (Izaurrealde et al., 1992).

The human tRNA^{met} and tRNA^{met} G57-U constructs used in this study were based on genes as described (Zasloff et al., 1982). *Xenopus* 5S wt and 5S M2 (Δ11-41) genes have been described previously (Guddat et al., 1990). The T7 promoter and restriction sites (DraI for 5S wt and 5S M2; BfaI for tRNA^{met} and tRNA^{met} G57-U) were introduced by PCR. The following sets of primers were used:

T7 5S wt:

5S/5'-CGGAATTCTAATACGACTCACTATAGGGCCTACGGCC
5S/3'-CGCGGATCCTTTAAAGCCTACGAC

T7 5S M2:

5S/mut 1-CGGAATTCTAATACGACTCCACTATAGGGCCTACG-
GCCTTCTCGGAAGC
5S/3'-as above

T7 tRNA^{met}:

tRNA/5'-CGGAATTCTAATACGACTCACTATAGGGAGCAG-
AGTGGCGC
tRNA/3'-GGCGGATCCTAGCAGAGGATGG

T7 tRNA^{met} G57-U:

tRNA/5'-as above
tRNA mut-GGCGGATCCTAGCAGAGGATGGTTTGTAGATCCATCG

Amplification was carried out in a volume of 100 μl in 20 mM Tris-HCl, pH 8.5, 1.5 mM MgCl₂, 50 mM KCl, 0.01% gelatin, 1 ng of DNA template, 100 ng of each primer, 2 U Ampliflag polymerase (Perkin-Elmer Corp., Norwalk, CT), and 0.2 mM dNTPs (Pharmacia Diagnostics Inc., Fairfield, NJ). 25 cycles were performed (94°C, 1 min; 52°C, 1.5 min; 72°C, 1.5 min) followed by 5-min final incubation at 72°C. Amplified fragments were digested with EcoRI and BamHI and cloned into pUC19. Plasmids were checked by sequencing.

The U1A cDNA (Sillekens et al., 1987) was cloned into the EcoRI site of the pGem 3zf(+) vector containing, between the BamHI and XbaI restriction sites, a DNA fragment coding for a 50-nucleotide long polyA stretch. For transcription, the plasmid was linearized with HindIII and transcribed with T7 RNA polymerase. Thus the 3' end of the RNA had the sequence:

A₅₀ UCUAGUGCAGGCAUGCAAGCA.

Preparation of RNA for Injection

³²P-labeled RNA was prepared as described previously (Jarmolowski and Mattaj, 1993) except 10 μCi [α ³²P]UTP (800 Ci/mmol) and 10 μCi [α ³²P]GTP (400 Ci/mmol) were used to obtain higher specific radioactivity of transcripts. To prepare nonradioactive RNA competitors, in vitro transcription was performed. 5–10 μg of linearized plasmid was transcribed in a volume of 100 μl in 40 mM Tris-HCl, pH 8.0, 8 mM MgCl₂, 10 mM DTT, 0.4 mg/ml BSA, 1.25 mM NTPs (Pharmacia), 10 U RNasin (Promega Corp., Madison, WI), 1.5 mM m⁷GpppG or ApppG dinucleotides (New England BioLabs, Beverly, MA) and 150–200 U T7 or T3 RNA polymerase (Stratagene Inc., La Jolla, CA). Trace amounts of [α ³²P]GTP (about 20,000–30,000 cpm) were added to enable determination of the concentration of synthesized RNA. After 3 h of incubation at 37°C the mixture was extracted with phenol/chloroform and unincorporated NTPs were eliminated by a Sephadex G-50 spin column. Ethanol-precipitated RNA was resuspended in 10 μl of water. Transcripts were checked on an 8% polyacrylamide gel containing 7 M urea, and concentration was determined using incorporation of [α ³²P]GTP into RNA. Homopolymeric polyribonucleotides (Sigma, St. Louis, MO) were sonicated to obtain a heterogeneous size distribution from ~2,000 to 100 nt. They were then extensively extracted with phenol/chloroform, precipitated with ethanol, and resuspended in H₂O.

Microinjection

Pieces of *Xenopus laevis* ovary were dissected, treated with collagenase, and individual oocytes prepared for microinjection (Hamm et al., 1989). In vitro transcribed RNAs were injected into the nucleus of oocytes and later extracted from dissected oocytes as described previously (Mattaj and De Robertis, 1985). To control nuclear injection, samples were mixed (1:1) with a 20-mg/ml solution of dextran blue (2,000,000 mol wt) (Serva Biochemica, Paramus, NJ) in water. This procedure slightly enhanced the rate of mRNA export, but did not affect the other RNAs. After dissection only oocytes with blue nuclei were used. For each sample five oocytes were pooled. In all experiments, 0.5 oocyte equivalents of RNA were loaded per lane on 8% polyacrylamide gels containing 7 M urea (for 5S, tRNA, and snRNAs) or 6% / 7 M urea polyacrylamide gels (for mRNA).

Quantitation of Export Data

Gels were quantified using the Molecular Dynamics (Sunnyvale, CA) Phosphorimager system with ImageQuant software, version 3.0.

Results

Export of In Vitro Transcripts

In order to validate the experimental system chosen to study nuclear export, microinjection of in vitro T7 RNA polymerase transcripts into *Xenopus* oocyte nuclei, a number of preliminary experiments were carried out. Four RNAs were chosen; human initiator methionyl tRNA (tRNA^{met}), *Xenopus laevis* 5S RNA, *Xenopus laevis* UIΔSm RNA, a U1 snRNA mutant that is exported from the nucleus but, unlike wt U1, cannot re-enter the nucleus (Hamm and Mattaj, 1990), and the human mRNA encoding the U1 snRNP-

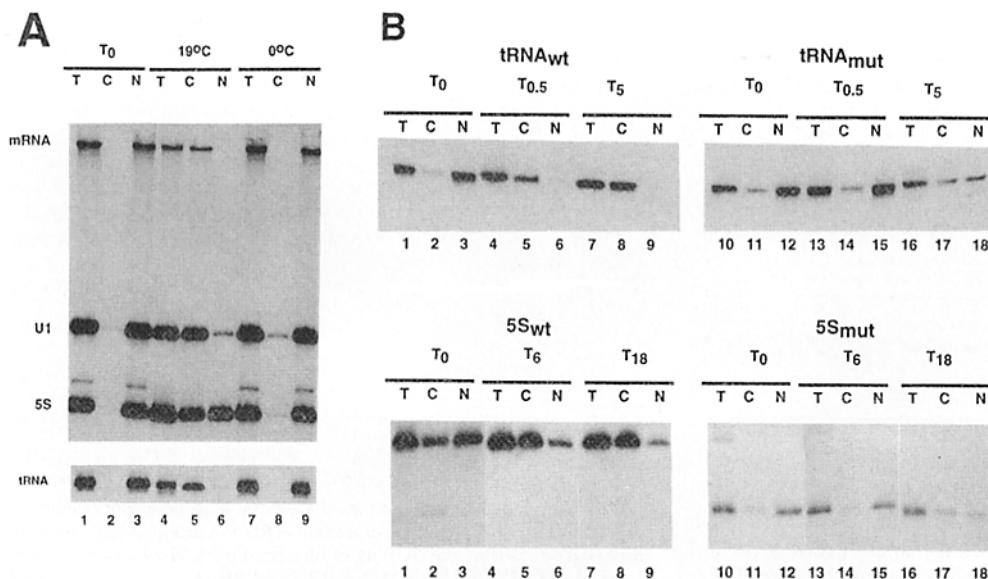


Figure 1. Export of T7 RNA polymerase-transcribed RNAs (A) RNA export is temperature dependent. A mixture of T7-transcribed UIA mRNA (0.001 pmol per oocyte), U1- Δ Sm RNA (0.01 pmol per oocyte), 5S RNA (0.01 pmol per oocyte), or T7-transcribed tRNA^{met} (0.025 pmol per oocyte) was microinjected into the nuclei of *Xenopus* oocytes. The oocytes were dissected either immediately (lanes 1-3) or after 360 min (top) or 60 min (bottom) of incubation at either 19°C (lanes 4-6) or on ice (lanes 7-9). RNAs extracted from either total oocytes (T) or from cytoplasmic (C) or nuclear (N) fractions were visualized by autoradiography after separation on

denaturing polyacrylamide gels. The transcript with a mobility intermediate between those of U1 Δ Sm and 5S was not identified. It is rapidly degraded at 19°C. (B) Mutant T7 RNA polymerase transcripts behave like *in vivo* transcripts. The rates of export of 0.025 pmol of T7-transcribed tRNA^{met} and a mutant derivative with a G₅₇ to U change (top) or of 0.01 pmol of wt 5S RNA and a mutant derivative M2 (bottom) were compared.

specific UIA protein (UIA mRNA). In pilot experiments, amounts of these RNAs that gave maximal export rates were determined (data not shown).

Export of purified, *in vivo* transcribed, tRNA^{met} has been shown to be temperature (i.e., energy) dependent (Zasloff, 1983). This was also the case for microinjected, T7-transcribed tRNA^{met} (Fig. 1 A, bottom). Similarly, cooling prevented the export of T7 transcripts of UIA mRNA, U1 Δ Sm RNA and 5S RNA (Fig. 1 A, top, compare lanes 4-6 with 7-9). The mRNA result is in agreement with previous studies which demonstrated that export of microinjected mRNA transcripts requires ATP (Dargemont and Kühn, 1992).

Further evidence for the physiological relevance of these experiments came from the study of mutant RNAs whose export rates are known to be reduced. These were mutants of tRNA^{met} (a G₅₇ to U mutation, Zasloff et al., 1982) and 5S RNA (mutant M2, a deletion of nts 11-41, Guddat et al., 1990). When T7 transcripts of these RNAs were tested by microinjection, their transport was considerably reduced compared to the wt RNAs (Fig. 1 B), although both did eventually reach the cytoplasm in agreement with the results of the earlier studies (lanes 16-18 of both panels). Experiments presented below demonstrate that export of T7-transcribed U1 Δ Sm RNA has the same cap dependence as does export of identical *in vivo* transcripts. In summary, these experiments all suggest that microinjected T7 transcripts of the RNAs studied are transported out of the nucleus in a manner similar to *in vivo* transcripts of the same RNAs.

tRNA Export

Previous work has shown that the export of microinjected tRNA (Zasloff, 1983) and mRNA (Dargemont and Kühn, 1992) from *Xenopus* oocyte nuclei are saturable processes. Different tRNAs (Zasloff et al., 1983) and different mRNAs

(Dargemont and Kühn, 1992) were shown to compete with one another for export, and, in addition, tRNA was shown to inhibit mRNA export when coinjected at high concentration (Dargemont and Kühn, 1992). These results raised the possibility that all RNAs would cross-compete for a limiting component of the export machinery. Initial experiments were designed to examine this question in more detail.

Radioactively labeled tRNA^{met} (0.025 pmol) was injected into oocyte nuclei either with or without increasing quantities of unlabeled tRNA^{met} (Fig. 2 A, top). As expected (Zasloff, 1982) the distribution of the tRNA after 45 min showed that, in the absence of competitor, essentially all had been exported (Fig. 2 A, top, first six lanes). Injection of competitor tRNA at between 0.1 and 5.0 pmol per oocyte resulted in a progressive inhibition of export, confirming that the process was saturable. When, instead of tRNA, similar amounts of either 5S RNA or U1 Δ Sm RNA were coinjected with the labeled tRNA, little or no effect was seen until the maximal amount of either competitor was injected (5 pmol, Fig. 2 A, middle and bottom). At this concentration both competitors inhibited export to some extent and, in addition, induced some degradation of the nuclear tRNA^{met}.

As mentioned above, it has previously been shown that coinjection of tRNA inhibits mRNA export (Dargemont and Kühn, 1992). To investigate the converse situation, we coinjected unlabeled UIA mRNA competitor with labeled tRNA^{met} (Fig. 2 B). The length of the mRNA transcripts makes it technically impossible to achieve the same molar ratio as with the other competitor RNAs although, in terms of weight, more mRNA competitor is injected at the highest concentration than tRNA. The result obtained was similar to that observed with the 5S and U1 Δ Sm competitors. Only at the highest concentration of mRNA was an effect on transport seen, and this was accompanied by partial degradation of the nuclear tRNA.

These results indicate that a limiting factor in tRNA export

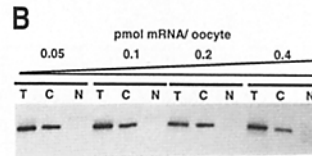
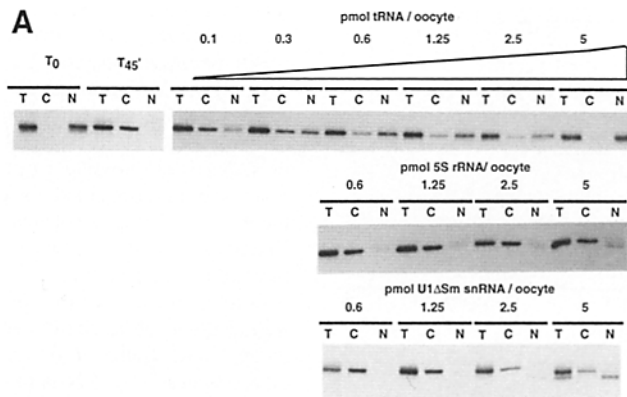


Figure 2. Saturability of tRNA^{met} transport. (A) The distribution between the nucleus and cytoplasm of 0.025 pmol of [³²P]labeled tRNA^{met} transcripts 45 min after their injection into the nucleus was determined either in the absence of co-injected RNA (top left) or in the presence of increasing amounts of unlabeled tRNA^{met} (top), 5S RNA (middle), or U1ΔSm RNA (bottom). (B) As in figure 2A, except that increasing amounts of unlabeled U1A mRNA, as indicated, were co-injected with 0.025 pmol of tRNA^{met}.

recognizes tRNA specifically in preference to other RNA polymerase III (5S) or II (U1ΔSm, mRNA) transcripts. The specificity of this recognition is underlined by the observation that the export-defective G₅₇ to U mutant did not compete tRNA^{met} export when 5 pmol of this RNA was co-injected with the labeled wt RNA (data not shown). In view of the demonstration that glyceraldehyde-3-phosphate dehydrogenase binds to wt tRNA^{met}, but not to transport defective mutants, and the suggestion that it might mediate tRNA export (Singh and Green, 1993) we attempted to overcome the saturation of tRNA export by coinjection of large quantities of glyceraldehyde-3-phosphate dehydrogenase, but without success (data not shown).

5S RNA Export

The concentration dependence of 5S RNA export was, unlike that of the other RNAs tested here, not a simple function. At the lowest concentrations tested the RNA was exported rather slowly compared to tRNA, but achieved a final cytoplasmic:nuclear distribution of ~3:1 (Fig. 3, lanes 1–9). At intermediate concentrations (0.04–0.15 pmol of injected RNA) the efficiency of export was reduced, as would be expected if a specific event in export were being saturated. However, when even more 5S RNA was injected (0.3 pmol or more, Fig. 3, lanes 19–21 and data not shown), a higher cytoplasmic:nuclear ratio was again achieved (2–3:1). Similar results were obtained in three independent experiments. These results suggest three possible interpretations. There may be more than one factor that can mediate the same step in 5S RNA export (see Guddat et al., 1990). If one export factor has a high affinity but a low capacity and a second has low affinity but high capacity one could expect the observed behavior. Alternatively, there may be a nuclear inhibitor of 5S RNA export with an affinity that is low relative to export factors and a limited capacity, such that its effect is only detected at intermediate 5S concentrations. Finally, it is known that 5S RNA can migrate to the nucleus after microinjection into the cytoplasm of *Xenopus* oocytes (De Robertis et al., 1982). The kinetics observed may be the result of differential affinities and saturability of the export and import processes.

Cross-competition experiments were carried out with tRNA^{met}, U1ΔSm RNA and U1A mRNA. Independent of the concentration of labeled 5S RNA co-injected, no inhibi-

tion of export was seen (data not shown). However, since up to 5 pmol of unlabeled 5S RNA did not reduce the export of labeled 5S RNA to a level below that seen in Fig. 3, lanes 19–21 (data not shown), saturation of the 5S export pathway could not be achieved. The cross-competition experiment is therefore only informative in light of the ability of the competitor RNAs to competitively inhibit their own export (see above and below).

U snRNA Export

Like tRNA, U1ΔSm export proved to be saturable (Fig. 4A, top) with export after 360 min being reduced to 50% in the presence of 0.6 pmol of unlabeled RNA and completely inhibited when 2.5 pmol or more competitor was co-injected. It has previously been shown that the m⁷GpppG cap structure of U1 snRNA is important for efficient nuclear export (Hamm and Mattaj, 1990; Izaurralde et al., 1992). To determine whether the cap structure was required for recognition by the factor limiting for U1ΔSm export, two additional competitors were tested. U1ΔSm capped with ApppG rather than m⁷GpppG was a very inefficient competitor of U1ΔSm export (Fig. 4A, middle). In contrast, a 77-nt long transcript unrelated to U1 snRNA in sequence, but capped with m⁷GpppG, inhibited U1ΔSm export ~50% as efficiently on a molar basis as U1ΔSm itself (Fig. 4A, bottom). In view of the recent suggestion that the effect of the cap structure on U1 snRNA export might be in large part due to its effect on stability of the RNA in the nucleus (Terns et al., 1993)

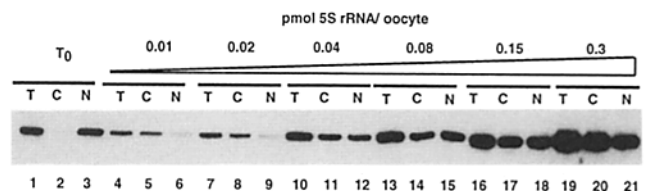


Figure 3. Saturability of 5S RNA export. Increasing amounts of ³²P-labeled 5S RNA, as indicated, were microinjected into the nuclei of *Xenopus laevis* oocytes. The oocytes were dissected 18 h later and RNA from the fractions analyzed. As a control for the accuracy of injection, oocytes were also dissected immediately after injection of 0.04 pmol RNA (lanes 1–3).

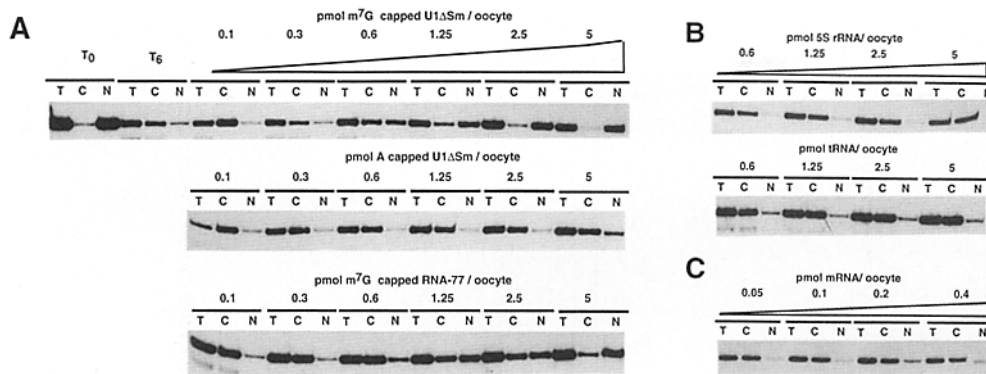


Figure 4. Saturability of U1 Δ Sm RNA export. (A) The distribution between the nucleus and cytoplasm of 0.01 pmol of ³²P-labeled U1 Δ Sm RNA transcripts 360 min after their injection into the nucleus was determined either in the absence of co-injected RNA (top left) or in the presence of increasing amounts of unlabeled m⁷GpppG-capped U1 Δ Sm RNA (top), ApppG-capped U1 Δ Sm RNA (middle panel), or an unrelated, 77 nt m⁷GpppG-capped transcript (bottom) as indicated. (B) As in Fig. 4 A except that unlabeled 5S rRNA (top) or tRNA^{phe} (bottom) were used as competitors. (C) As in Fig. 4 A except that unlabeled U1A mRNA was used as a competitor.

it is important to note that in these experiments injection of capped competitor RNAs has no detectable effect on U1 Δ Sm stability, but efficiently blocks its export from the nucleus.

These results provide strong evidence that interaction with a factor that recognizes the cap structure is limiting for U1 Δ Sm export. It was therefore perhaps not surprising that neither of the two RNA polymerase III transcripts (Fig. 4 B) nor U1A mRNA (Fig. 4 C), up to the maximal concentrations tested, inhibited U1 Δ Sm export under the conditions of this experiment. Note that although the U1A mRNA has a m⁷GpppG cap, the maximal amount injected (0.4 pmol) is below the level at which inhibition is seen with either U1 Δ Sm or RNA-77 at 360 min (Fig. 4 A). Comparable levels of inhibition of U1 Δ Sm export by 0.4 pmol of either U1A mRNA or U1 Δ Sm could be observed after shorter (120 min) incubation times (data not shown).

To determine whether the cap structure is generally involved in U snRNA export, the effect of U1 Δ Sm on U2 Δ Sm and U5 Δ Sm RNAs was compared to its autoinhibition. U1 Δ Sm was an efficient competitor of export of all three RNAs (Fig. 5). Interestingly, the three RNAs showed differences both in the rate of their export (U2 Δ Sm being the

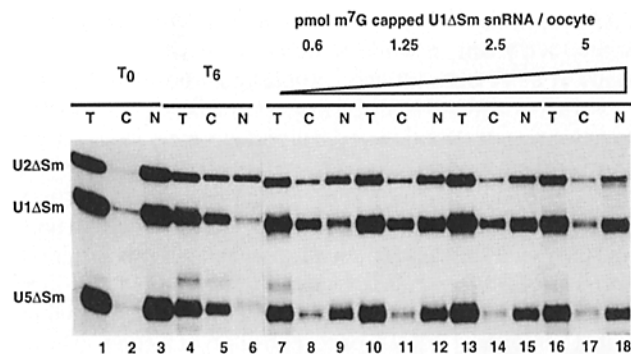


Figure 5. A common factor in U snRNA export. A mixture of 0.01 pmol each of U2 Δ Sm, U1 Δ Sm, and U5 Δ Sm RNAs were microinjected into the nuclei of *Xenopus* oocytes. The oocytes were either dissected immediately (lanes 1–3) or after 360 min (lanes 4–18). Export was monitored either without (lanes 4–6) or with increasing amounts of co-injected unlabeled U1 Δ Sm RNA as indicated (lanes 7–18).

slowest, Fig. 5, lanes 1–6) and in their susceptibility to inhibition. U5 Δ Sm export was inhibited most efficiently followed by U2 Δ Sm then U1 Δ Sm itself (Fig. 5, lanes 7–18), suggesting that the three RNAs have different affinities for a common nuclear export factor. ApppG-capped U1 Δ Sm RNA did not inhibit export of U2 Δ Sm or U5 Δ Sm, while m⁷GpppG-capped RNA-77 inhibited export of both RNAs roughly half as efficiently as did U1 Δ Sm (data not shown). Thus, the limiting factor in the export of all three U snRNAs is likely to be the same, and to require an m⁷G cap structure for U snRNA recognition.

mRNA Export

A U1A mRNA-coding sequence was constructed by joining the human U1A cDNA sequence (Sillekens et al., 1987) to a poly A stretch with, at its 3' end, 21 nt of polylinker sequence (see Materials and Methods). When radioactively labeled U1A mRNA was injected into the nucleus together with increasing amounts of unlabeled U1A mRNA, progressive inhibition of export was seen with 0.1 pmol or more of competitor (Fig. 6 A, upper panel). This molar amount of any RNA was not sufficient to inhibit export of U1, U2, or U5 snRNAs (see above). It therefore seemed possible that the limiting factor for U1A mRNA export might not be the same as for U snRNA export, and therefore might not depend upon the presence of a cap structure on the RNA. To test this, the ability of ApppG-capped U1A mRNA to inhibit export of m⁷G-capped labeled RNA was examined (Fig. 6 A, lower panel). In several experiments, there was no significant difference in the effectiveness of A or m⁷G-capped mRNAs as competitors, showing that association between U1A mRNA and the factor that becomes limiting for its export when mRNA concentration is increased does not require the m⁷G cap structure.

It has previously been shown that the export from the nucleus of an adenovirus-derived mRNA was significantly slower when the RNA had a tri-methyl guanosine cap structure than when it was capped with a mono-methyl guanosine (Hamm and Mattaj, 1990). To determine whether this effect was specific for the tri-methyl cap structure we compared the kinetics of export of m⁷GpppG and ApppG capped U1A mRNAs. The nonphysiological ApppG cap caused a reproducible decrease in the rate of export (Fig. 7), the $t_{1/2}$ for

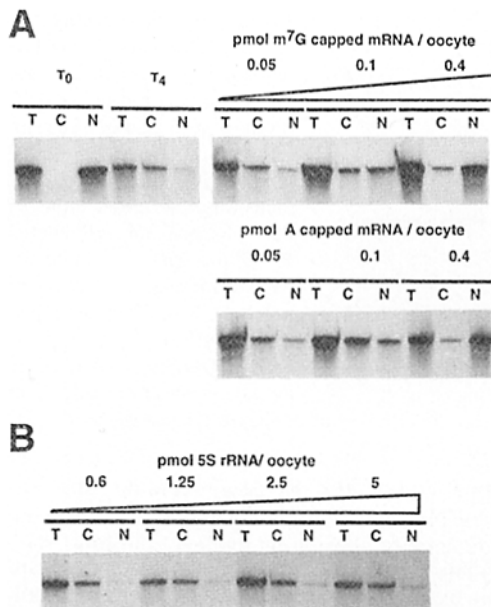


Figure 6. Saturability of U1A mRNA export. (A) The distribution between the nucleus and cytoplasm of 0.001 pmol of ^{32}P -labeled U1A mRNA transcripts 240 min after their injection into the nucleus was determined either in the absence of coinjected RNA (top left, compare T_0 and T_4) or in the presence of increasing amounts of unlabeled m⁷GpppG-capped U1A mRNA (top right) or ApppG-capped U1A mRNA (bottom) as indicated. (B) As in Figure 6 A, but with increasing amounts of unlabeled 5S rRNA coinjected with the ^{32}P -labeled U1A mRNA.

export being increased by approximately a factor of 2. Similarly, coinjection of unlabeled m⁷GpppG-capped U1ΔSm RNA reduced the rate of export of U1A mRNA, but did not prevent it (data not shown). Thus, the presence of a monomethyl cap structure on an mRNA enhances the rate of its export. This is in agreement with the observation that the m⁷GpppG dinucleotide inhibited mRNA export in an experiment in which both were coinjected into *Xenopus* oocyte nuclei (Dargemont and Kühn, 1992).

In order to test the specificity of inhibition of U1A mRNA export we coinjected the two RNA polymerase III transcripts used previously. 5S rRNA had no detectable effect on export when up to 5 pmol per oocyte was injected (Fig. 6 B). tRNA^{met}, consistent with an earlier report (Dargemont and Kühn, 1992), inhibited U1A mRNA export, but only partially and at the highest level of competitor tested (5 pmol, data not shown).

It has previously been suggested that the poly A tail may play a role in mRNA export (Wickens and Gurdon, 1983; Eckner et al., 1991). To test this we compared the export rate of U1A mRNA with that of a truncated version of the RNA from which the poly A stretch had been removed. The rate of export of the poly A⁻ mRNA was slightly (30–50% in different experiments) slower than that of the poly A⁺ (data not shown). A similar effect was seen when a genuine poly A tail of ~200 nt in length was added to U1A mRNA in vitro using poly A polymerase (data not shown). This effect was relatively small, but was reproducibly observed in several independent experiments, suggesting that the pres-

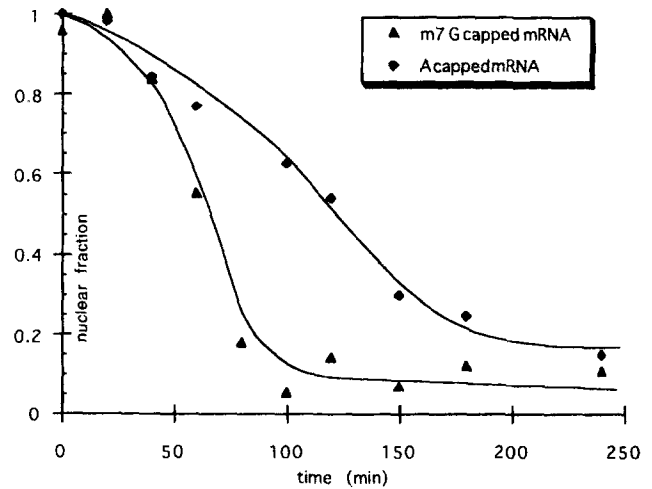


Figure 7. The effect of the cap structure on mRNA export. 0.001 pmol of either m⁷GpppG-capped or ApppG-capped U1A mRNA was microinjected into the nuclei of *Xenopus* oocytes. At various times thereafter, oocytes were dissected and the amount of intact mRNA present in the nuclear and cytoplasmic fractions was determined by phosphorimager analysis. In the graph, export is expressed as the remaining fraction of RNA in the nuclear compartment.

ence of poly A does have a direct effect on transport rate. However, neither the presence of the cap structure nor of poly A is essential for U1A mRNA export.

Homoribopolymers as Export Competitors

Thus far, the competition experiments presented all led to the conclusion that different specific factors were limiting in concentration for the export of different classes of RNA. Indeed, the lack of cross-competition except at very high levels of competitor RNAs suggested that common steps on the pathway of export of different classes of RNA, if they exist, were not easily saturable. To try to find evidence for such common steps the effects of synthetic homopolymeric RNAs on export of the different RNA substrates was tested.

The result of one such experiment, involving coinjection of U1ΔSm, 5S, and tRNA, is shown in the lower panel of Fig. 8. After 6 h, as expected, U1ΔSm and tRNA^{met} are almost entirely cytoplasmic, while 5S rRNA is distributed approximately equally between the cytoplasmic and nuclear fractions (Fig. 8, lanes 1–6). Coinjection of 50 ng (due to the extensive size distribution of the homopolymers, no accurate value for their molarity could be obtained) of either poly A, poly C, or poly U had little or no effect on export of the three RNAs (lanes 7–9 and 13–18). On the other hand, both poly I and poly G at the same concentration had strong inhibitory effects on U1ΔSm and 5S rRNA export (lanes 10–12, 19–21). Note that poly G and poly I were the only RNAs found thus far that inhibited 5S rRNA export strongly. In other experiments it was established that U1A mRNA and U1ΔSm responded identically to the homoribopolymers at this time point, i.e., U1A mRNA export after 360 min was inhibited efficiently by poly G and, to a lesser extent, by poly I (data not shown). In the upper panel of Fig. 8, the results of inhibition of U1A mRNA export by the ribohomopolymers at an earlier time point, 120 min, is shown. Unlike U1ΔSm or 5S,

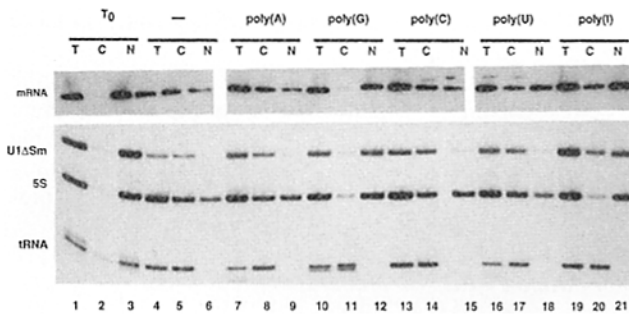


Figure 8. Inhibition of export by homopolymeric RNAs. (*Top*) 0.001 pmol of UIA mRNA was microinjected into the nuclei of *Xenopus* oocytes, and re-extracted from total or fractionated oocytes either immediately (lanes 1–3) or 120 min later (lanes 4–21). The RNA was injected either alone (lanes 4–6) or with 50 ng of one of 5 synthetic RNA homopolymers as indicated (lanes 7–21). (*bottom*) A mixture of 0.01 pmol U1ΔSm RNA, 0.01 pmol 5S RNA, and 0.05 pmol tRNA^{met} was microinjected into the nuclei of *Xenopus* oocytes, and re-extracted from total or fractionated oocytes either immediately (lanes 1–3) or 360 min later (lanes 4–21). The RNAs were injected either alone (lanes 4–6) or together with 50 ng of one of 5 synthetic RNA homopolymers as indicated (lanes 7–21).

at this time point UIA mRNA export is also inhibited to some extent by poly U (lanes 16–18). While the inhibition by poly G and poly I persisted until 360 min, that of poly U was no longer detected at the later time point (data not shown).

In contrast to these three RNAs (U1ΔSm, 5S, and UIA mRNA) no effect was seen on tRNA^{met} export (Fig. 8, lanes 1–21). The lack of inhibition of export of this RNA was confirmed in kinetic experiments at early time points (data not shown). These data provide evidence that there may be common components involved in the export of at least some different classes of nuclear RNA.

Discussion

Specific Steps in RNA Export

Transport between the nucleus and cytoplasm of diverse *in vitro*-transcribed RNAs microinjected into *Xenopus laevis* oocytes was studied. Control experiments suggest that this experimental protocol faithfully reflects several aspects of the *in vivo* export pathway including temperature (energy) dependence, response to mutation, in the case of two RNA polymerase III transcripts, and cap dependence, in the case of RNA polymerase II transcripts.

The purpose of the study was to determine whether factors involved in mediating the export of different RNAs, or classes of RNA, were specific or common. By injecting increasing concentrations of different RNAs, it was possible to specifically saturate the export of tRNA, U snRNA, and mRNA, providing evidence that in each of these cases at least one specific, titratable factor is essential for export. The export of 5S RNA as a function of concentration showed complex behavior, and even high amounts of 5S RNA did not saturate transport. Evidence has been presented that two proteins, TFIIIA and ribosomal protein L5, may both mediate the export of 5S RNA from oocyte nuclei (Guddat et al.,

1990), arguing that specific export factors exist also in this case.

Previous studies have demonstrated a role for the m⁷G cap structure of RNA polymerase II transcripts in facilitating their export from the nucleus (Hamm and Mattaj, 1990; Dargemont and Kühn, 1992; Izaurralde and Mattaj, 1992). The results presented here confirm and extend these conclusions. In the case of U snRNAs, the export factor that is most readily titratable by injecting increasing amounts of competitor RNA is only affected by RNAs with a cap structure. In a recent study (Terns et al., 1993) it was suggested that the effect of the cap structure on U1 snRNA export might be related to its effect on the nuclear stability of U1 RNA. Previous data (see Fig. 3 in Hamm and Mattaj, 1990 and Fig. 2 in Izaurralde et al., 1992) had, however, indicated that these two effects could be separated. In this study, we show that m⁷GpppG-capped competitor RNAs, including one unrelated to U1 snRNA, can completely block export of the RNA without detectably altering its nuclear stability (Figs. 4 and 5), confirming that U1 stability in the nucleus and U1 export can be affected independently by competitors that presumably act by preventing cap-binding proteins from associating with the RNA. The fact that the same two m⁷GpppG-capped RNAs could efficiently block export of not only U1ΔSm, but also of U2ΔSm and U5ΔSm RNAs (Fig. 5) speaks against the general existence of specific structural elements or sequences in U snRNAs that have been proposed, in the case of U1, to direct export independently of the cap structure (Terns et al., 1993).

A nuclear cap-binding protein that is a candidate mediator of U snRNA export has been identified and partially characterized (Izaurralde and Mattaj, 1992). On the other hand, the same factor is not likely to be what is titrated first by increasing concentrations of mRNA substrate, since mRNAs with either m⁷GpppG or AppppG at their 5' ends are equally good competitors of mRNA export, and the molar amount of mRNA required to inhibit export is below the level at which titration of the cap-binding factor is observed in the case of the U snRNAs. Kinetic experiments showed, however, that in the presence of an m⁷G cap structure, mRNA is exported at a higher rate (see also Hamm and Mattaj, 1990). It is important to note that there is no contradiction between these two results, since the export factor that is most easily titratable (i.e., limiting in concentration) for any RNA is not necessarily the same as the factor(s) that determines the rate of export of that RNA. Indeed, the fact that the presence of both an m⁷G cap structure and of poly A affect the rate of mRNA export, while neither is necessary to titrate the factor that is most limiting in concentration in a titration experiment, introduces the important concept that several factors may recognize, and affect the export of, any RNA substrate. It may either be that more than one factor is essential for the export of a given class of RNA, or that factors recognizing different determinants on the same RNA substrate have redundant functions in export. One earlier study of mRNA export has been published in which similar methods to those employed here were applied (Dargemont and Kühn, 1992). For the most part, our results are in good agreement with the conclusions of that study since we also find that mRNA export is temperature (energy) dependent, saturable, positively influenced by the m⁷G cap structure, and partially inhibited by coinjection of a large quantity of tRNA. The one differ-

ence between the two studies concerns the kinetics of export. While Dargemont and Kühn (1992) reported that the two mRNAs they tested were exported very rapidly, with a half-time in the nucleus of ~ 2.5 min, we consistently observed much slower kinetics with a lag time of ~ 20 – 25 min during which export was not detected, possibly reflecting the assembly of an export-competent RNP, followed by export with a half-time of ~ 30 min. Since similar quantities of mRNA were used in each study, and since the levels of mRNA required to saturate export were also similar, it is not obvious what caused this difference. It is possible that the different quantitation methods employed (we measured the export of only full-length mRNA while Dargemont and Kühn measured the total radioactivity in cytoplasmic or nuclear fractions) could affect the result obtained. A second possibility, that the identity of the mRNA utilized affects the export kinetics, was investigated by using *in vitro* transcribed dihydrofolate reductase mRNA. This mRNA exhibited kinetics indistinguishable from those of U1A mRNA (unpublished data).

Common Steps in Export

RNA, probably in the form of RNP, is exported to the cytoplasm through nuclear pore complexes (Stevens and Swift, 1966; Dworetzky and Feldherr, 1988; Mehlin et al., 1992). A monoclonal antibody that recognizes a subset of nuclear pore glycoproteins has been shown to reduce 5S and tRNA export to some extent (Featherstone et al., 1988) while the lectin wheat germ agglutinin, probably through interactions with a number of pore glycoproteins, strongly reduces export of U1 snRNA, tRNA, and ribosomal subunits (Bataillé et al., 1990; Neuman de Vegvar and Dahlberg, 1990). These results suggest the existence of common steps on the pathway of export of different RNPs, probably situated at a late stage in the process, and possibly involving either recognition of the transport substrates by pore complex components or actual movement through the pores.

It is at present unclear whether recognition events that occur at the pore complex will involve contacts with the RNA or the protein components of the exported RNPs. Since only minor cross competition was seen between the different physiological RNAs studied, an attempt was made to identify common steps in the export of the diverse RNAs using synthetic homopolymeric RNAs. Strikingly, 5S RNA, mRNA, and U snRNAs showed similar patterns of inhibition with these competitors. Poly G and poly I inhibited export of the three classes of RNA, but poly A, C, or U had no effect on 5S RNA or U snRNA at the concentrations tested. Poly U did have a minor inhibitory effect on mRNA export. The results obtained with poly G and poly I suggest that there may be a common factor involved in the export of these three RNAs, although it is not possible to exclude the possibility that three different factors with similar recognition specificity are involved. It is unlikely that the inhibition of export by the RNA homopolymers reflects blockage of pore complexes since the same competitors had no effect on tRNA export. The hnRNP proteins are attractive candidate proteins that may be involved in either specific or, particularly, common steps in export since they are both very abundant and known to move between the nucleus and cytoplasm in somatic cells (reviewed by Piñol-Roma and Dreyfuss, 1993). Unfortunately, little is known about either the abundance or

state (RNA bound vs. free, nuclear vs. cytoplasmic) of these proteins in *Xenopus* oocytes.

Thus, our data provide evidence that there are specific RNA-recognizing factors involved in the export of different classes of RNA as well as common factors that influence the transport of diverse RNAs. The different RNA-binding specificities of these factors will hopefully provide a biochemical tool with which to identify and purify them.

Comparison with Nuclear Import

The observation that different RNAs require specific saturable components for their export is reminiscent of earlier studies on the import of macromolecules to the nucleus. The initial demonstration that the import pathway was saturable utilized a peptide encoding the nuclear localization signal of SV-40 T antigen coupled to a carrier protein. This conjugate was actively imported to the nucleus, and served as an efficient competitive inhibitor of the transport of other karyophilic proteins (Goldfarb et al., 1986). Extension of this experimental approach to other karyophiles has more recently provided evidence for saturable components that are specifically involved in the nuclear transport of either karyophilic proteins or a subset of U snRNPs, respectively (Fischer and Lüthmann, 1990; Fischer et al., 1991, 1993; Michaud and Goldfarb, 1991). Preliminary evidence for a third saturable transport factor specifically required for U3 snRNP import has also been obtained (Michaud and Goldfarb, 1992). It is to be hoped that these import studies and the export studies presented here will lead to the identification of some of the mediators of macromolecular transport between the nucleus and cytoplasm.

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