Diversity in the Signals Required for Nuclear Accumulation of U snRNPs and Variety in the Pathways of Nuclear Transport

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Abstract. The requirements for nuclear targeting of a number of U snRNAs have been studied by analyzing the behavior of in vitro-generated transcripts after microinjection into the cytoplasm of *Xenopus* oocytes. Like the previously studied U1 snRNA, U2 snRNA is excluded from the nucleus when it does not have the 2,2,7mGpppN cap structure typical of the RNA polymerase II (pol II)-transcribed U snRNAs. Surprisingly, two other pol II-transcribed U snRNAs, U4 and U5, have a much less stringent requirement for the trimethyl cap structure. The γ-monomethyl triphosphate cap structure of the RNA polymerase III-transcribed U6 snRNA, on the other hand, is shown not to play a role in nuclear targeting. Wheat germ agglutinin, which is known to prevent the import of many proteins into the nucleus, inhibits nuclear uptake of U6, but not of U1 or U5 snRNAs. Conversely, a 2,2,7mGpppG dinucleotide analogue of the trimethyl cap structure inhibits transport of the pol II U snRNAs, but does not detectably affect the transport of either U6 snRNA or a karyophilic protein. From these results it can be deduced that U6 enters the nucleus by a pathway similar or identical to that used by karyophilic proteins. The composite nuclear localization signals of the trimethyl cap-containing U snRNPs, however, do not function in the same way as previously defined nuclear targeting signals.

THE vertebrate U-rich small nuclear RNAs (U snRNAs) can be divided into three categories: nucleoplasmic RNAs transcribed by RNA polymerase II, like U1, U2, U4, and U5 snRNAs; nucleoplasmic RNAs transcribed by RNA polymerase III, like U6 snRNA; and nucleolar RNAs transcribed by RNA polymerase II, like U3, U8, and U13. The major nucleoplasmic U snRNAs function in the processing of messenger RNA precursors (Steitz et al., 1988; Sharp, 1987; Maniatis and Reed, 1987), while, of the nucleolar RNAs, U3 has been shown to play a role in ribosomal RNA maturation (Tyc and Steitz, 1989; Kass et al., 1990).

The assembly of the RNA polymerase II class of nucleoplasmic U snRNAs with proteins, in the formation of U snRNPs, involves migration of the RNA components from the nucleus to the cytoplasm, assembly with proteins, and finally movement of the RNP back to the nucleus (for reviews see Mattaj, 1988 and Zieve and Sauterer, 1990). The binding of a group of common proteins to a region of the RNAs called the Sm binding site is of particular importance in this pathway. Mutant U snRNAs which cannot bind these proteins remain in the cytoplasm (Mattaj and De Robertis, 1985; Hamm et al., 1990). In addition, binding of the common proteins to U1 and U2 snRNAs has been shown to be required for the hypermethylation of their cap structures to the 2,2,7mGpppN structure characteristic of the U snRNAs (Mattaj, 1986; Hernandez and Weiner, 1986; Hamm et al., 1987). Recently, it has been demonstrated that the nuclear targeting signal of the U1 snRNP is bipartite. One component is the trimethylated cap structure and the other is thought to be a signal situated on the common U snRNP proteins (Hamm et al., 1990; Fischer and Lührmann, 1990). Both of these components are essential for efficient nuclear uptake of the U1 snRNP.

The intracellular movements of the RNA polymerase III-transcribed U6 snRNA have also been studied in *Xenopus* oocytes, albeit less extensively. After transcription, this RNA does not leave the nucleus (Vankan et al., 1990). However, if it is microinjected into the cytoplasm it will move from there to the nucleus (Hamm and Mattaj, 1989). Although this ability is apparently unnecessary in oocytes, it may well be important in dividing cells, where nuclear components have to reaccumulate after mitosis. A region of U6 snRNA (nucleotides 20–24) is required for nuclear migration. By analogy with the Sm binding site of the pol II U snRNAs, it has been proposed that this may be the binding site for a U6 snRNP protein (Hamm and Mattaj, 1989). Substitution of the γ-methyl triphosphate cap structure nor-

mally found on U6 snRNA (Singh and Reddy, 1989) with 2,2,7mGpppG allows nuclear import. However, if GpppG, 7mGpppG, or 2,7mGpppG is incorporated at the 5′ end of U6, the RNA remains in the cytoplasm (Hamm et al., 1990). This was interpreted as evidence for a role of the γ-methyl triphosphate cap in nuclear targeting.

Here, evidence is presented that different U snRNAs have diverse requirements for nuclear transport. First, it is shown that the γ-methyl triphosphate cap is not involved in intracellular transport of U6. Rather, the presence of a monomethyl (7mGpppG) cap structure on U6 is shown to inhibit nuclear transport by binding to a cytoplasmic component(s). Furthermore, wheat germ agglutinin, (WGA),[1] a general inhibitor of nuclear protein localization (Finlay et al., 1987; Dabauvalle et al., 1988), inhibits the transport of U6 snRNA, but does not prevent nuclear migration of the pol II class of U6 snRNAs. Conversely, a dinucleotide analogue of the trimethyl cap inhibits transport of the pol II U snRNAs, but does not affect transport of U6 snRNA or of the karyophilic Lamin L1 protein. These latter results confirm and extend the conclusions of a recent study (Michaud and Goldfarb, 1991) in which the existence of different pathways of nuclear transport was proposed.

Materials and Methods

Immunoprecipitation

Purified RNAs were precipitated directly. RNA was purified by homogenizing oocytes in homomedium (10 oocytes per ml; 50 mM Tris-HCl [pH 7.5], 5 mM EDTA, 1.5% SDS, 300 mM NaCl, 1.5 mg/ml proteinase K), extracting proteins with phenol equilibrated with 10 mM Tris-HCl (pH 8), 1 mM EDTA, and then with phenol-chloroform, and precipitating the RNA with 3 vol of ethanol. The purified RNAs were resuspended in 10 μl of H_2O per oocyte. Monoclonal anti-TMG cap antibodies (Bochnig et al., 1987) were used for each precipitation. Immunoprecipitation was performed as described previously (Mattaj and De Robertis, 1985), but at 4°C using buffer IPP_{50} (10 mM Tris-HCl [pH 8], 50 mM NaCl, 0.1% NP-40, 0.1% sodium azide).

U snRNA Genes

The U snRNA genes used for generation of the T7 U snRNA genes were a *Xenopus laevis* U1 gene (Zeller et al., 1984), an *X. laevis* U2 gene (Mattaj and Zeller, 1983), a chicken U4 gene (Hoffmann et al., 1986), an *X. laevis* U5 gene (Kazmaier et al., 1987), and an *X. tropicalis* U6 gene (Krol et al., 1987). The T7 promoter sequences and the restriction sites at the 3′ ends of the T7 genes (U1, BamHI; U2, DraI; U4, Eco47III; U5, Eco47III; U6, DraI) were introduced by site-directed mutagenesis (Kramer et al., 1984). The T7 constructs were generated by J. Hamm (see Hamm et al., 1987).

T7 U snRNA Synthesis

The T7 U snRNA genes were linearized by cutting at the introduced restriction sites. 1 μg of linearized DNA was incubated in a 10-μl vol of 40 mM Tris-HCl (pH 8), 8 mM $MgCl_2$, 2 mM spermidine, 50 mM NaCl, 30 mM DTT, 0.04 mM ATP/GTP/CTP/UTP, 10 U of T7 RNA polymerase, 10 U of RNasin, 0.1 mM cap dinucleotide, 20 μCi [α^{32}P]UTP for 30 min at 37°C. Water was added to a final volume of 100 μl, proteins were extracted with phenol-chloroform, unincorporated nucleotides were removed with spun columns, and RNAs were precipitated with 3 vol of ethanol (including 3 mg of glycogen as a carrier). RNAs were suspended in 10 μl of H_2O. Dinucleotide cap analogues, synthesized as described (Darzynkiewicz et al., 1988, 1990) or obtained commercially were added to the reactions to a final concentration of 400 μM. γ-Methyl GTP (Darzynkiewicz et al., 1985) was added to similar transcription reactions to a final concentration of 1 mM.

1. *Abbreviation used in this paper*: WGA, wheat germ agglutinin.

Oocyte Injections

T7 U snRNAs were mixed in ratios to obtain similar intensities of radioactive signals on autoradiographs. Approximately 20 nl was injected into the vegetal half of oocytes. Oocytes were dissected manually after transfer into J buffer (70 mM NH_4Cl, 7 mM $MgCl_2$, 0.1 mM EDTA, 2.5 mM DTT, 20 mM Tris-HCl [pH 7.5], 10% glycerol), and RNA was extracted from the oocyte fractions with homomedium as described in the immunoprecipitations section. Extracted RNAs were analyzed on denaturing 8% polyacrylamide gels, and 0.5–1 oocyte equivalents of RNA were loaded on gels of 0.4 mm thickness. In the experiments where cap analogs were coinjected with the T7 U snRNAs, they were mixed with the RNA and injected at a final concentration of either 5 mM (most experiments) or 50 mM (Fig. 5 *B*). Because of variation in the rate and extent of accumulation observed between different experiments, internal controls were always included where appropriate, and each experiment was repeated at least three times.

Oocyte Injections of In Vitro Translated Protein L1

Injection and analysis of in vitro-translated protein was carried out as follows: 1–3 μg m7G-capped *Xenopus* Lamin L1 mRNA (transcribed from cDNA clone M8; Krohne et al., 1989) was added to 25 μl reticulocyte lysate (Promega Biotec, Madison, WI), 2.5 μl amino acid mix (1 mM) without methionine, 5 μl ^{35}S methionine (1,000 Ci/mmol), and 45 μl H_2O and incubated for 90 min at 30°C. The in vitro-translated protein was injected without further purification directly into the cytoplasm of oocytes and incubated in Barth's medium supplemented with 100 μg/ml cycloheximide.

Oocytes were dissected in 5:1 medium (83 mM KCl, 17 mM NaCl, and 10 mM Tris-HCl, pH 7.4). The isolated nuclei were fixed and pelleted in 95% ethanol. The cytoplasms were homogenized in ice-cold 5:1 medium, and the insoluble fraction was removed by centrifugation. The protein in the supernatant was precipitated with 5 vol acetone for 1 h at −80°C.

Precipitated proteins were dissolved in protein sample buffer and separated by a SDS-PAGE (Laemmli, 1970). The gels were subsequently fixed in 40% MeOH/10% acetic acid and rinsed for 30 min in amplify solution (Amersham International, Amersham, UK) before drying on a gel dryer. Exposure times varied between 1 and 3 d.

Results

The γ-Methyl Triphosphate Cap Structure Cannot Substitute for 2,2,7mGpppG on U1 snRNA

As mentioned in the introduction, U6 RNA with either a 5′ γ-methyl triphosphate (made in vivo) or a 2,2,7mGpppG cap structure (made in vitro) can migrate from the cytoplasm to the nucleus, while U6 with a 7mGpppG cap cannot. This suggested that the 2,2,7mGpppG and γ-methyl triphosphate cap structures might be interchangeable in nuclear targeting. To test this idea, U1 RNA with a γ-methyl triphosphate 5′ end was made. Chemically synthesized γ-methyl GTP (Darzynkiewicz et al., 1985) was used as a substrate in T7 RNA polymerase-catalyzed in vitro transcription reactions. Incorporation of γ-methyl GTP at the 5′ end of T7-transcribed U1 or U6 (T7U1, T7U6) RNAs was monitored both by direct analysis (data not shown) and by the fact that γ-methyl triphosphate-capped RNAs were stable upon injection into oocytes (Fig. 1 *A*), while the same RNAs with unmodified triphosphate 5′ ends were unstable (Hamm et al., 1990 and our unpublished data).

In the first experiment, T7U1 RNAs were made with γ-methyl triphosphate (γmTP) or 7mGpppG 5′ ends. They were coinjected with γ-methylated T7U6 RNA as an internal control. T7U1 RNA capped with 7mGpppG becomes trimethylated after injection, and moves to the nucleus (Hamm et al., 1990; Fischer and Lührmann, 1990). This is shown on the right of Fig. 1 *A*. The internal control, γ-methylated T7U6, also accumulates in the nucleus. T7U1 with a γ-methyl triphosphate 5′ end is, however, excluded (Fig. 1 *A*, *left*).

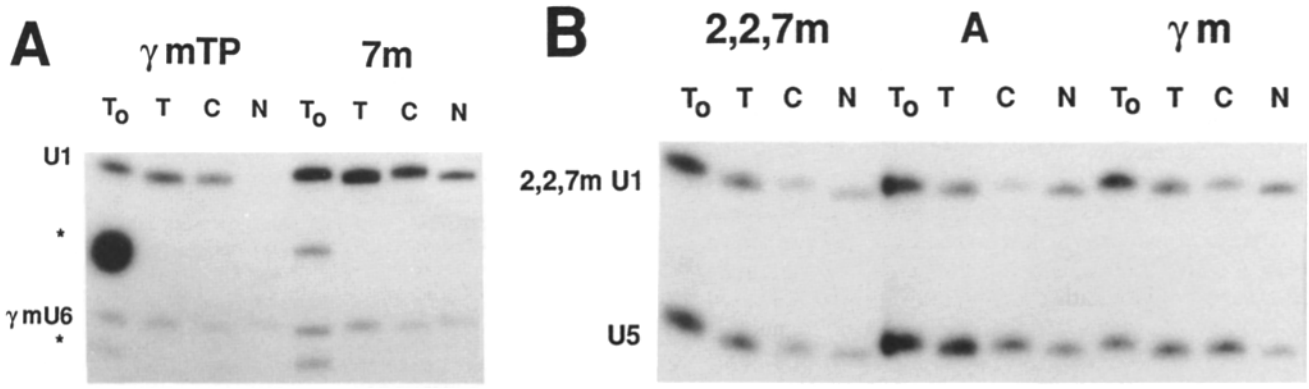


Figure 1. γ-Methyl triphosphate–capped U1 and U5 snRNAs. (*A*) T7U1 RNA with either a γ-methyl triphosphate (*left*) or a 7mGpppG (*right*) cap was coinjected into the cytoplasm of oocytes with γ-methyl triphosphate-capped T7U6 RNA. T_o, RNA extracted immediately after injection; T, C, N, RNA extracted from total oocytes or from cytoplasmic or nuclear fractions 16 h after injection. The RNA was analyzed on an 8% polyacrylamide denaturing gel. Asterisks mark transcripts of unknown structure generated in the T7U1 transcription reaction. The blob on the far left lane is an autoradiographic artefact. (*B*) T7U5 RNA with either a 2,2,7mGpppG, an ApppG, or a γ-methyl triphosphate cap was coinjected into the cytoplasm of oocytes with 2,2,7mGpppG-capped T7U1 RNA. RNA fractions were analyzed as in *A*.

The behavior of the differently capped T7U snRNAs in this and the following experiments is summarized in Table I.

U1 RNA is longer than U6, and has a very different predicted secondary structure, while U5 RNA resembles U6 more closely in both of these respects (Steitz et al., 1988). T7U5 RNA was therefore synthesized using γ-methyl GTP as a substrate, in order to test whether the modified triphosphate cap was functional in nuclear targeting when present on U5 RNA. γ-Methylated U5 RNA accumulated in the nucleus similarly to 2,2,7mGpppG-capped U5 (Fig. 1 *B, left and right panels*, trimethyl-capped T7U1 served as an internal control), suggesting that the γ-methyl triphosphate 5′ end might indeed function when placed on T7U5 RNA. However, as a further control of the specificity of this effect, T7U5 capped with ApppG, a dinucleotide whose incorporation prevents nuclear import of T7U1 RNA (Fischer and Lührmann, 1990; Hamm et al., 1990) was analyzed. To our surprise, this RNA also accumulated in the nucleus, to an extent similar to that of the trimethyl-capped RNA (Fig. 1 *B, middle*). In repeated experiments, ApppG-capped T7U5 RNA accumulation in the nucleus was always only slightly less efficient than that of trimethylated T7U5 when measured after overnight (16 h) incubation. The cytoplasmic/nuclear ratio of the ApppG-capped T7U5 RNA varied between 1.3 and 1.9 times greater than that of the 2,2,7mGpppG-capped RNA in different experiments (data not shown).

Table I. Nuclear Transport of U snRNAs by Cap Structures

	U1	U2	U4	U5	U6
2,2,7mGpppG	+	+	+	+	+
2,7mGpppG	+	+	+	+	−
7mGpppG	+	+	+	+	−
GpppG	+	+	+	+	−
γmpppG	−	NT	NT	±	+
βmppG	NT	NT	NT	NT	+
ApppG	−	−	±	±	+

NT, not tested. ± indicates that the cap allows nuclear transport, but with reduced efficiency. Note that 2,7mGpppG, 7mGpppG, and GpppG are converted to 2,2,7mGpppG upon microinjection when they are added to U1-U5 RNA but not to U6 RNA.

Differential Requirements for the Trimethyl Cap Structure

This result suggested that the previously characterized requirement for the 2,2,7mGpppG cap structure for nuclear migration might be confined to U1 snRNA. To investigate this possibility further, T7 transcripts of U1, U2, U4, and U5 RNAs with ApppG caps were generated. The U1, U2, and U4 RNAs were coinjected with 7mGpppG-capped U5 RNA as an internal control, while the U5 RNA was coinjected with 7mGpppG-capped U1 RNA. The results (Fig. 2 *A*) confirmed the fact that ApppG-capped U1 RNA is excluded from the nucleus while ApppG-capped U5 RNA is not. ApppG-capped U2 RNA, like U1, was excluded from the nucleus, while ApppG-capped U4 behaved in an intermediate way, showing reduced nuclear accumulation compared to 7mGpppG-capped U4 RNA (compare U4 in Fig. 2 *A* with the control 7mU4 of 2 *B*).

One possible trivial explanation for these results was that the (symmetrical) ApppG dinucleotide might for some reason be incorporated in the opposite orientation to that expected when incorporated into U4 and U5 RNA. If this were so, the G would be available for trimethylation. To exclude this possibility, mixtures of ApppG and 7mGpppG-capped RNAs were injected into oocytes and immunoprecipitated immediately upon injection or 16 h later. Immunoprecipitation was carried out with an antitrimethyl cap antibody under conditions where it exhibits weak affinity for 7mGpppG and strong affinity for 2,2,7mGpppG (see Materials and Methods). The first mixture (Fig. 2 *C*, lanes *1, 2, 5,* and *6*) contained ApppG-capped U2, U4, and U5 RNAs together with 7mGpppG-capped U1 RNA; the second (lanes *3, 4, 7,* and *8*) contained ApppG-capped U1 together with 7mGpppG-capped U5. The total RNAs from one oocyte equivalent at time zero (lanes *1* and *3*) or after 16 h (lanes *2* and *4*) are shown together with the immunoprecipitated RNAs from five oocytes (lanes 5–8). It is clear that the ApppG-capped RNAs are not immunoprecipitable, and, therefore, that they have cap structures in the expected orientation.

To confirm the unexpected differences in cap dependence, a competition experiment was carried out. It has been shown that coinjection of the dinucleotide cap analogue

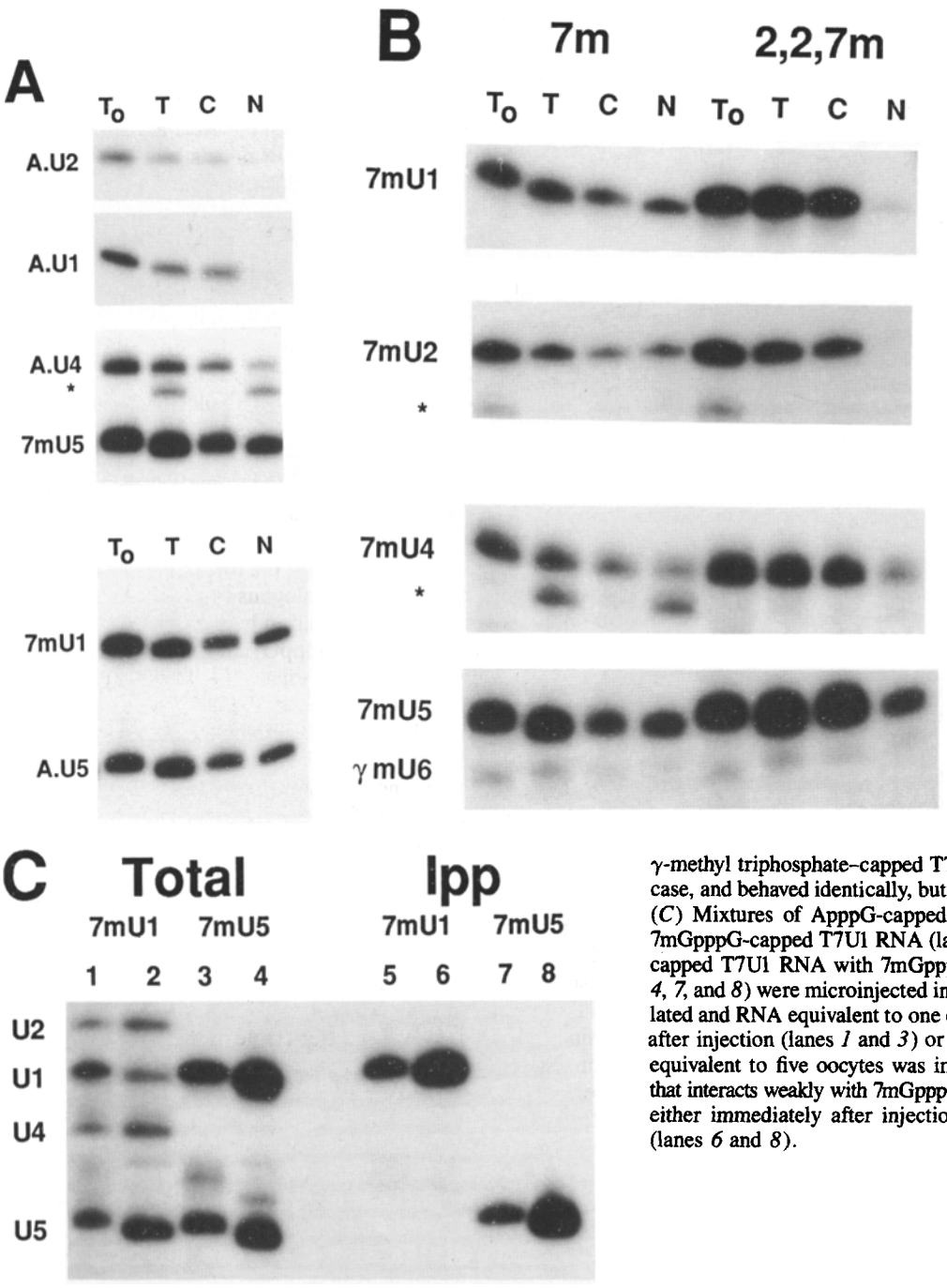


Figure 2. Differential requirements for the trimethyl cap structure. (*A*) T7-transcribed U2, U1, and U4 RNAs with an ApppG cap structure were coinjected with T7U5 RNA capped with 7mGpppG. The three RNAs were coinjected with T7U5 separately. The behavior of the T7U5 was identical in all three cases, but is only shown for the T7U4 experiment. In the lower part of the figure, a mixture of ApppG-capped T7U5 and 7mGpppG-capped U1 were coinjected. RNA fractions were analyzed as in Fig. 1 *A*. The asterisk marks a degradation product of T7U4 produced after microinjection. (*B*) T7U1, U2, U4, and U5 RNAs capped with 7mGpppG were coinjected into the cytoplasm of oocytes with γ-methyl triphosphate-capped T7U6 RNA and either 7mGpppG or 2,2,7mGpppG dinucleotide cap analogue (5 mM). RNA fractions were analyzed as in Fig. 1 *A*. Asterisks mark transcripts generated from T7U2 during transcription or from T7U4 after injection. The internal control γ-methyl triphosphate-capped T7U6 RNA was coinjected in each case, and behaved identically, but is only shown in the T7U5 panel. (*C*) Mixtures of ApppG-capped T7U2, U4, and U5 RNA with 7mGpppG-capped T7U1 RNA (lanes *1*, *2*, *5*, and *6*) or of ApppG-capped T7U1 RNA with 7mGpppG-capped T7U5 RNA (lanes *3*, *4*, *7*, and *8*) were microinjected into oocytes. Total RNAs were isolated and RNA equivalent to one oocyte was analyzed immediately after injection (lanes *1* and *3*) or 16 h later (lanes *2* and *4*). RNA equivalent to five oocytes was immunoprecipitated with an mAb that interacts weakly with 7mGpppG and strongly with 2,2,7mGpppG either immediately after injection (lanes *5* and *7*) or 16 h later (lanes *6* and *8*).

2,2,7mGpppG can inhibit nuclear migration of U1 RNA (Fischer and Lührmann, 1990). The inhibition was specific since similar concentrations of 7mGpppG had no effect. These two dinucleotides were therefore coinjected with 7mGpppG-capped U1, U2, U4, and U5 RNAs. γ-Methyl triphosphate-capped U6 was coinjected with each RNA as an internal control. The trimethylated dinucleotide had a large inhibitory effect on the nuclear accumulation of U1 and U2 RNAs. However, while the nucleo-cytoplasmic ratios of U4 and U5 RNA were altered when compared with samples coinjected with monomethylated analogue, the inhibition of U4 and U5 transport by the trimethylated dinucleotide was clearly less than for U1 or U2 (Fig. 2 *B*). Thus, the trimethyl cap structure did indeed have a differential effect on the nuclear uptake of different U snRNAs.

The Trimethyl Cap Structure Has a Kinetic Effect on U5 snRNP Nuclear Migration

In all the above experiments the nucleo-cytoplasmic ratio of the RNAs was determined after 16 h of incubation. To determine whether the cap structure had any effect at all on U5 snRNP migration, a kinetic experiment was performed. The rates of nuclear accumulation of 2,2,7mGpppG and ApppG-

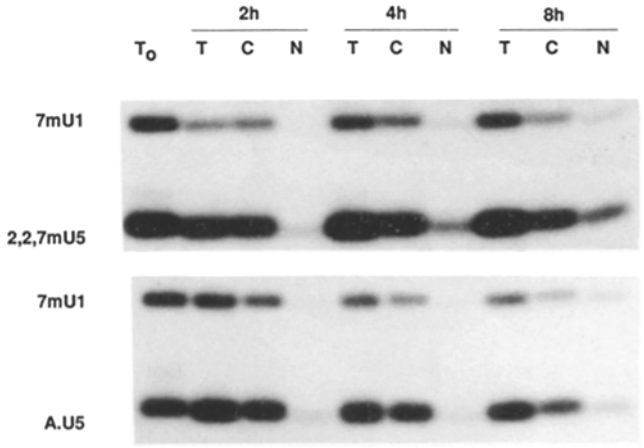


Figure 3. Kinetics of T7U5 accumulation. T7U5 RNA with either a 2,2,7,mGpppG (*top*) or an ApppG can (*bottom*) was coinjected into the cytoplasm of oocytes with 7mGpppG-capped T7U1 RNA. RNA was extracted and analyzed either from total oocytes or from cytoplasmic and nuclear fractions 0, 2, 4, and 8 h later.

capped U5 RNAs were compared, with 7mGpppG T7U1 as an internal control. The result (Fig. 3) showed that the trimethyl cap did have an effect on the kinetics of nuclear accumulation. When the cytoplasmic/nuclear ratio of ApppG or 2,2,7mGpppG T7U5 RNA was quantified by densitometry the C/N ratio of ApppG-capped T7U5 was >20:1 after 4 h and 4.2:1 after 8 h. The corresponding ratios for the trimethylated T7U5 RNA were 10:1 and 2.5:1, respectively. Similar results were obtained in three separate experiments. As mentioned above, the cytoplasmic/nuclear ratio observed with the ApppG-capped T7U5 RNA was also consistently higher than that of the trimethylated T7U5 RNA when measured after 16 h. Additionally, the inhibition of transport by the 2,2,7mGpppG dinucleotide seen in Fig. 2 *B*, where the cytoplasmic/nuclear ratio is increased from 0.9:1 to 3.5:1 by the inhibitor, further confirms that the trimethyl cap has a role in the nuclear transport of U5 RNA. Thus, although the trimethyl cap structure is not essential for nuclear uptake in the case of the U5 snRNP, its presence does affect the rate of import.

Nuclear Migration of U6 snRNA

The results presented in Fig. 1 showed that the γ-methyl triphosphate cap structure could not substitute for a trimethyl cap structure in nuclear import of U1 snRNA. We therefore wished to examine its role in U6 RNA migration in more detail. Previous experiments (Hamm et al., 1990) had indirectly suggested that γ-methylation might be important for nuclear migration. T7U6 RNA with a triphosphate 5′ end is very unstable when injected into oocytes (our unpublished data). T7U6 with either a 7mGpppG or 2,2,7mGpppG cap structure is stable. While the 7mGpppG-capped U6 remains in the cytoplasm after microinjection, the 2,2,7mGpppG-capped RNA moves to the nucleus (Fig. 4 *A*; see also Hamm et al., 1990). T7U6 with a γ-methyl triphosphate 5′ end also migrates to the nucleus with an efficiency similar to that of the internal U1 RNA control (Fig. 4 *A*). These results would support the possibility that the γ-methyl cap has an active role in transport and that it can be functionally replaced by the trimethyl cap structure.

However, two further cap structures which would not be expected to have an active function in transport, ApppG and β-methyl diphosphate, both allowed nuclear accumulation of U6 RNA (Fig. 4 *A*, *right*). These results cast doubt on the role of the γ-methyl cap, and so further experiments were undertaken to clarify this issue.

First, it was shown that none of the cap structures allows nuclear accumulation of U6 RNA in an unspecific way. It has previously been shown that mutation of nucleotides 20–25 of the U6 sequence drastically decreases its entry into the nucleus after cytoplasmic injection (Hamm et al., 1990). This U6 mutant, U6ΔD, was made in vitro with four different cap structures. None of these RNAs was able to accumulate in the nucleus (Fig. 4 *B*). The same was true of β-methyl diphosphate-capped U6ΔD transcripts (data not shown). Thus, the migration of the ApppG and β-methyl diphosphate–capped RNAs was not due to some fortuitous positive effect of these structures on transport.

A second approach to defining the role of the γ-methyl cap structure in U6 migration, analogous to Fig. 2 *B* above, was to attempt to inhibit U6 migration by the coinjection of cap analogs. γ-methyl GTP, 7mGpppG, and 2,2,7mGpppG were therefore coinjected with γ-methyl triphosphate–capped T7U6 RNA and trimethyl-capped T7U1 RNA as an internal control. While, as before, the trimethylated dinucleotide inhibited U1 transport (Fig. 5 *A*, *right*), neither the monomethyl dinucleotide nor the γ-methyl triphosphate affected U1 transport, and none of the cap analogues affect the transport of γ-methylated U6 RNA (Fig. 5 *A*).

This was strong evidence against the idea that the γ-methyl triphosphate and 2,2,7mGpppG cap structures had a functionally equivalent, or interchangeable, role in transport since, if they had, the 2,2,7mGpppG dinucleotide should also have inhibited U6 migration and an effect of γ-methyl GTP on U1 transport might have been expected. To determine whether any evidence for a role of the γ-methyl structure in transport could be obtained a kinetic experiment was performed. γ-Methyl triphosphate and ApppG-capped U6 RNAs were coinjected into the cytoplasm with 7mGpppG-capped U1 snRNA. At various times after injection oocytes were dissected and the nucleo-cytoplasmic distribution of the RNAs was determined. The results (Fig. 6) showed that there was no difference in the rate of accumulation between the ApppG and the γ-methyl triphosphate–capped U6 snRNAs. This strongly implied that the γ-methyl triphosphate cap has no role in nuclear import.

This left unexplained the result which had first suggested to us that the γ-methylated structure might have a function in transport. Why did 7mGpppG-capped U6 snRNA not migrate to the nucleus when every other cap structure tested allowed migration to take place? A possible reason was that cytoplasmic cap–binding proteins (Shatkin, 1985) might bind to the RNA and anchor it in the cytoplasm. To test this possibility, 7mGpppG-capped U6 RNA was coinjected into the cytoplasm together with the 7mGpppG dinucleotide, which can bind to at least some cytoplasmic cap–binding proteins (Darzynkiewicz et al., 1988 and references therein). GTP was coinjected in control oocytes. GTP, as expected, had no effect on 7mGpppG-capped T7U6 RNA mi-

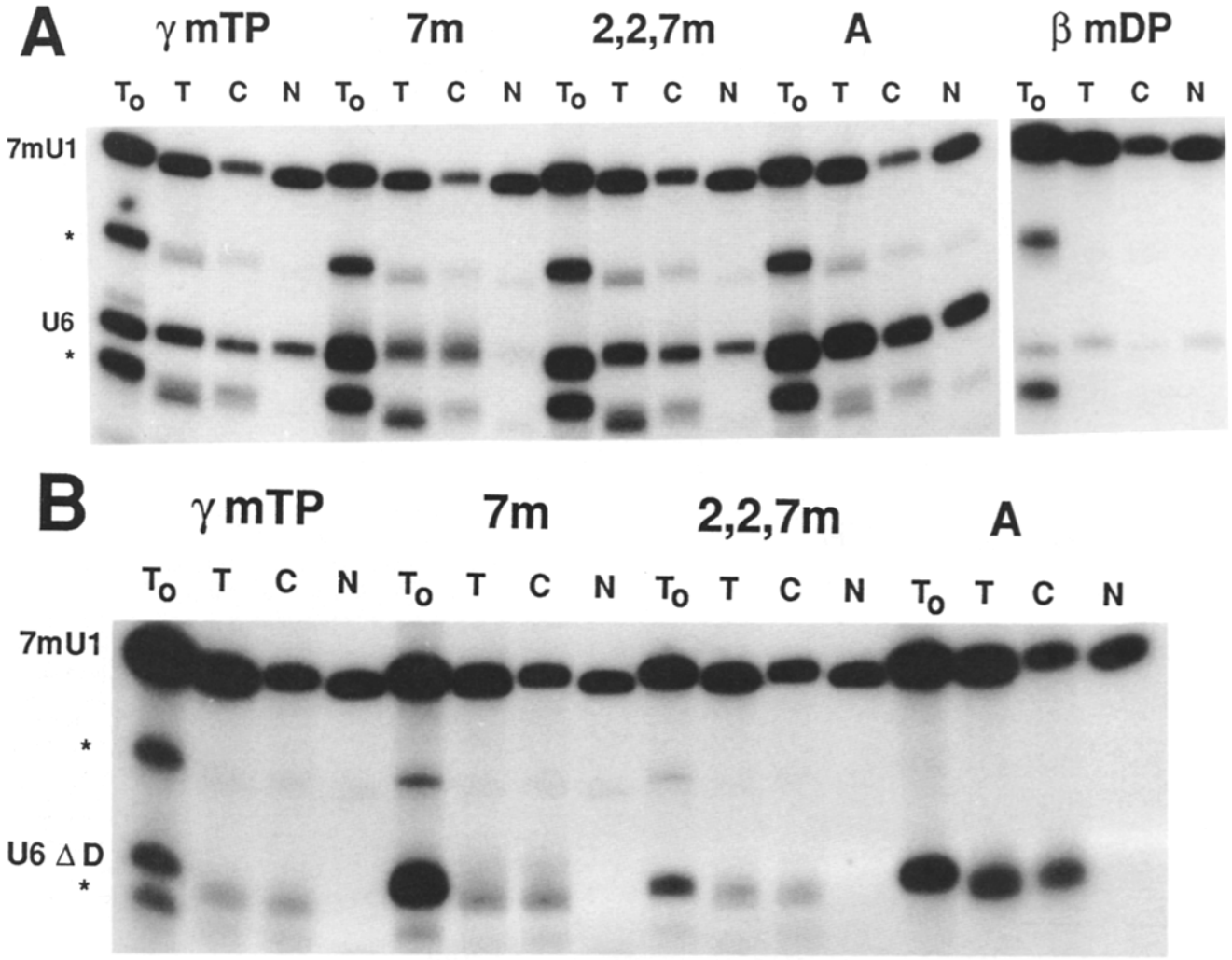


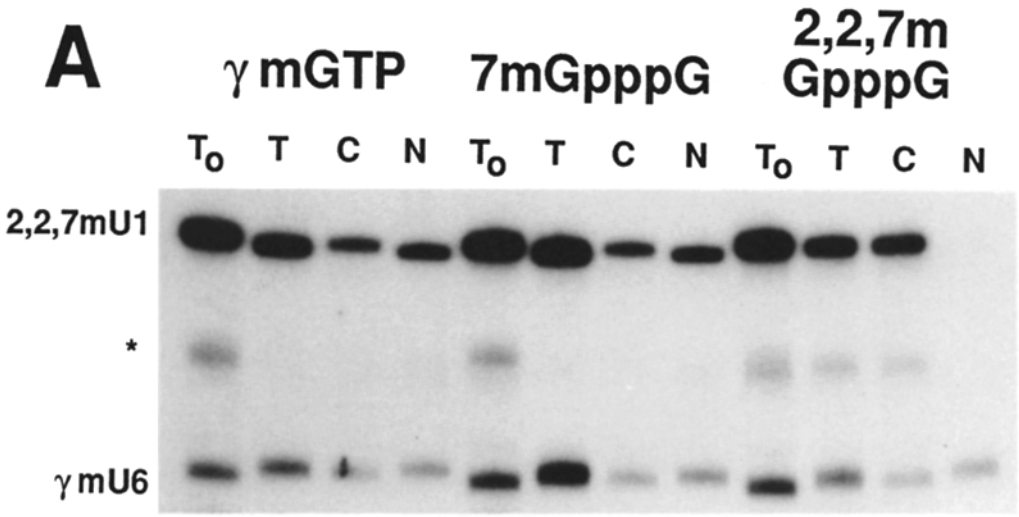
Figure 4. Cap requirements for U6 transport. (*A*) T7U6 RNA with either a γ-methyl triphosphate, a 7mGpppG, a 2,2,7mGpppG, an ApppG, or a β-methyl diphosphate cap structure was coinjected into the cytoplasm of oocytes together with 7mGpppG-capped T7U1 RNA. RNA was extracted and analyzed as in Fig. 1 *A*. Asterisks mark truncated products of unknown structure generated during T7U1 transcription. (*B*) T7U6ΔD is a mutant form of U6 in which nucleotides 20–25 are changed from UAUACU to CUCGAG. T7U6 RNA with the four indicated cap structures was coinjected with 7mGpppG-capped U1 RNA, and analyzed as in *A*.

gration (Fig. 5 *B*, *left*), while the monomethyl cap analogue allowed the 7mGpppG-capped RNA to accumulate in the nucleus to a level similar to U1 RNA (compare Fig. 5 *B*, *right* with Fig. 5 *A*, *left*). This result strongly suggests that the 7mGpppG-capped U6 snRNA is retained in the cytoplasm because it binds to a monomethyl cap-binding protein. Further experiments (not shown) with other cap analogues showing different affinities for cap-binding proteins are fully consistent with this proposal. The significance of this observation for the intracellular migration of the pol II U snRNAs is discussed below.

Differential Inhibition of Nuclear Import

We wished to obtain further, more direct, evidence for the role of protein-based signals in U snRNP transport, and for this purpose made use of transport inhibitors. In a first experiment it was shown that the nuclear accumulation of microinjected, in vitro made, *Xenopus* Lamin L1 protein (Krohne et al., 1989) was not affected by coinjection of the 2,2,7mGpppG dinucleotide (Fig. 7, *A* and *B*). As expected, coinjection of wheat germ agglutinin (WGA)[1] did inhibit L1 migration (Fig. 7 *C*).

Recently Michaud and Goldfarb (1991) have shown that injection of high concentrations of a specific nuclear localization signal (a peptide from SV40 T antigen) coupled to BSA inhibited nuclear uptake of a karyophilic protein. The same inhibitor also reduced U6 snRNA migration, but had no effect on the uptake of U2 snRNA. WGA represents a more efficient and possibly a more general (Finlay et al., 1987; Dabauvalle et al., 1988) inhibitor of the nuclear uptake of proteins. We therefore tested the effect of WGA on the transport of trimethyl-capped T7U1 and T7U5 snRNAs. Under identical conditions to those in which Lamin L1 transport was quantitatively inhibited, WGA allowed U1 or U5 snRNP transport to proceed, although the rate appeared to be somewhat reduced (Fig. 8). The kinetics of T7U1 transport are slower than those of L1 transport (compare Figs. 3 and 7), raising the possibility that the lack of inhibition might be due to WGA having a limited half-life. However, T7U1 transport was seen even after repeated WGA injection (data not



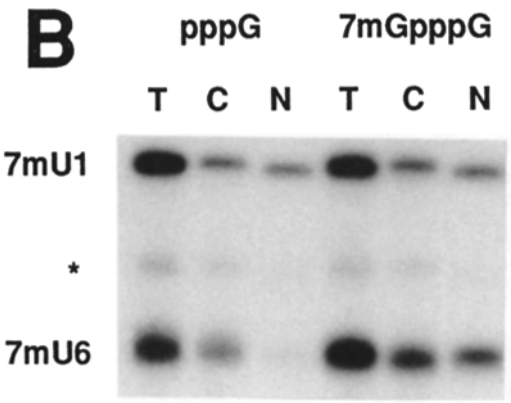


Figure 5. Cytoplasmic retention of U6. (*A*) γ-Methyl triphosphate-capped T7U6 RNA was coinjected with 2,2,7mGpppG-capped T7U1 RNA and either γ-methyl guanosine triphosphate, 7mGpppG dinucleotide, or 2,2,7mGpppG dinucleotide (all three at 5 mM). RNA was analyzed as in Fig. 1 *A*. The asterisk marks a truncated product generated during T7U1 transcription. (*B*) 7mGpppG-capped T7U6 and T7U1 snRNAs were coinjected into the cytoplasm of oocytes together with GTP or 7mGpppG dinucleotide (both at 50 mM). RNA was analyzed as in Fig. 1 *A*.

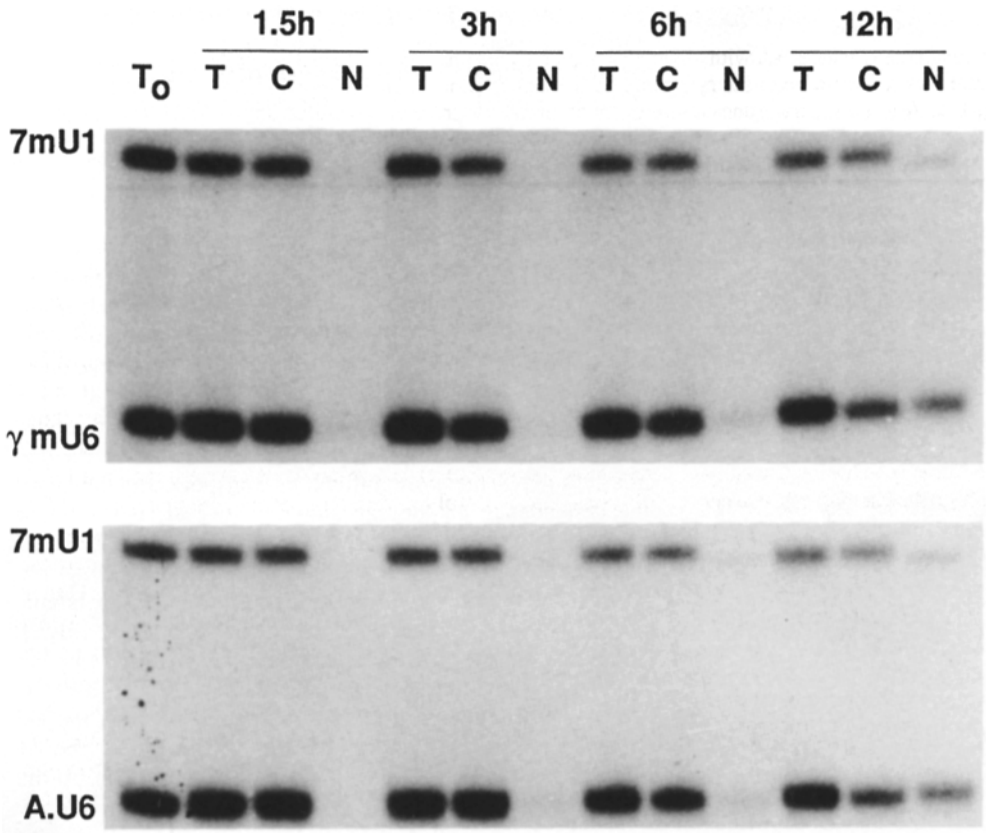


Figure 6. Kinetics of U6 RNA accumulation. T7U6 RNA with either a γ-methyl triphosphate (*top*) or an ApppG (*bottom*) cap structure was coinjected into the cytoplasm of oocytes together with 7mGpppG-capped T7U1 RNA. RNA was extracted and analyzed either from total oocytes or from cytoplasmic and nuclear fractions 0, 1.5, 3, 6, and 12 h later.

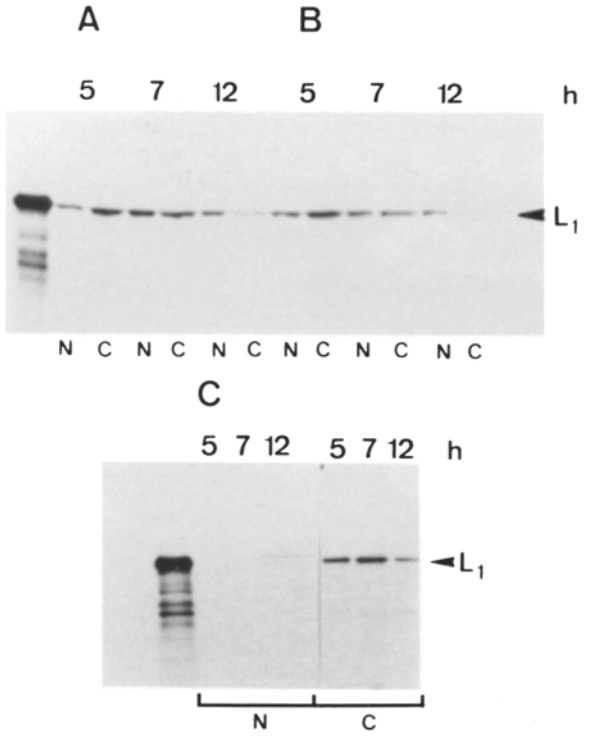


Figure 7. Protein transport is inhibited by WGA but not by m2,2,7GpppG cap analogue. In vitro-translated Lamin L1 was injected alone (*A*), with 5 mM m3GpppG (*B*), or with a WGA solution saturated in PBS, pH 7.4 (*C*) into the cytoplasm of oocytes. Proteins from the nuclear (*N*) and cytoplasmic (*C*) fraction were analyzed 5, 7, and 12 h later.

shown), and WGA is able to inhibit transport of U6 RNA over a period of at least 12 h (see below).

Having established that 2,2,7mGpppG and WGA were differential inhibitors of nuclear transport, it was possible to use them to investigate transport of U6. As shown previously (Fig. 5 *A*), the trimethylated cap analogue does not inhibit U6 transport. WGA, on the other hand, completely prevented nuclear uptake of U6. This can be seen by comparing the 12-h time points in the control panel (Fig. 9 *A*) with those in the WGA-injected panel (Fig. 9 *B*).

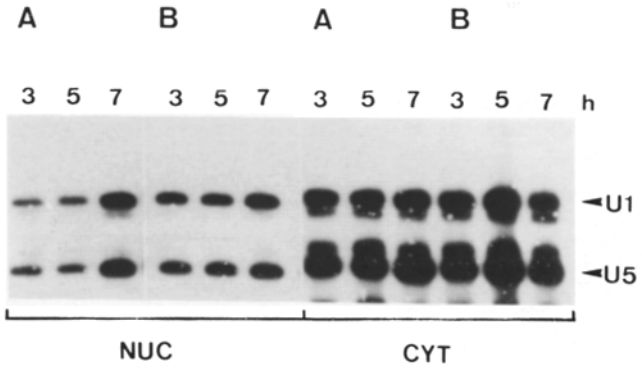


Figure 8. WGA does not inhibit nuclear migration of pol II U snRNAs. m2,2,7GpppG-capped U1 and U5 snRNAs were injected either alone (*A*) or with a saturated WGA solution (*B*), dissected into nuclear (*N*) and cytoplasmic (*C*) fractions, and analyzed at the indicated timepoints as described in Fig. 1 *A*.

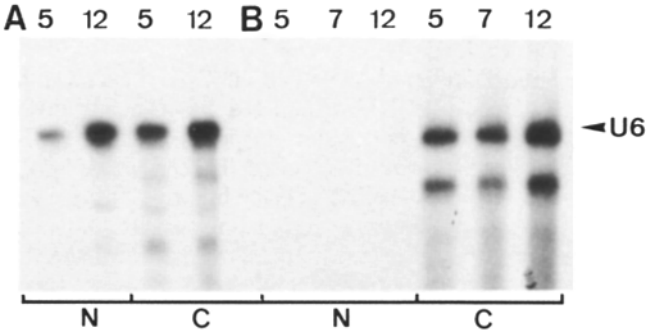


Figure 9. Nuclear migration of U6 RNA is inhibited by WGA. T7 U6RNA with a γ-methyl triphosphate cap structure was injected either alone (*A*) or with a saturated WGA solution (*B*) into the cytoplasm of oocytes. The oocytes were dissected at the indicated timepoints into nuclear and cytoplasmic fractions and analyzed as described in Fig. 1 *A*.

Discussion

A series of experiments that illustrate the diversity of the requirements of different U snRNAs/snRNPs for nuclear migration have been presented. The pol II-transcribed, 2,2,7mGpppG-capped RNAs clearly fall into a different class than the pol III-transcribed, γ methyl-phosphate containing U6 RNA. However, diversity was uncovered even within the pol II class, with U1 and U2 snRNAs showing a much stronger dependence on the trimethyl cap structure for nuclear migration than did the shorter U4 and U5 snRNAs.

This latter difference was unexpected. However, even in the case of U5, where the effect of the trimethyl cap structure was least apparent, it was possible to demonstrate that the trimethyl cap had an effect on the kinetics of nuclear accumulation. This suggests a unified model for the signaling of nuclear localization among the pol II-U snRNPs in which the cap functions as an accessory signal to increase the efficiency of nuclear transport. In the absence of the cap, the presence of an Sm binding site, whose function is discussed below in more detail, would be sufficient to allow nuclear accumulation of the U5 snRNP, but not of the U1 or U2 snRNPs. What might be the reason for this difference?

One possible clue may lie in the apparent inverse correlation between RNA size and transport efficiency in the absence of the cap. U2 and U1, the longer RNAs, are excluded from the nucleus if they have an ApppG cap, and their transport is completely inhibited by the dinucleotide 2,2,7mGpppG analogue (Fig. 2). U5, the smallest RNA, is least affected by these treatments and U4, which lies between U1 and U5 in size, shows an intermediate behavior with respect to inhibition of its nuclear transport. It may, therefore, be that the bigger the snRNA the more difficult its transport, and the larger the dependence on the cap signal. However, it should be borne in mind that it is not the naked snRNA, but rather the (at least partially) assembled snRNP, that moves to the nucleus (Mattaj, 1988; Zieve and Sauterer, 1990). It is currently unknown whether only the common, or core, snRNP proteins associate with the RNAs before migration or whether the proteins that are specific to particular snRNPs also assemble before transport. If the latter were the case, the U5 snRNP would actually be more massive than either the U1 or U2 snRNPs (Lührmann, 1988; Bach et al., 1989). At the present state of knowledge, it is therefore too early to draw

any firm conclusions about the molecular basis of the observed variability.

Compared to the relatively small differences between the individual pol II U snRNPs, there is a more obvious divergence between them as a class and the pol III-transcribed U6. Contrary to our previous hypothesis (Hamm et al., 1990), the cap structure of this RNA, a γ-methyl triphosphate, was shown here not to be involved in signaling nuclear migration. The experiments that demonstrated this revealed, however, that the trimethyl cap plays a dual role in transport. First, the cap must interact directly with some component of the transport machinery, since injection of a 2,2,7mGpppG cap analogue prevents transport, presumably by competing for binding to the transport component. Second, the trimethyl cap prevents interaction with some 7mGpppG cap-binding component that anchors RNA in the cytoplasm. This was shown by the activation of transport of 7mGpppG-capped U6 RNA by coinjection of the 7mGpppG dinucleotide analogue. Again the likely mechanism is competition for binding to a (this time monomethyl) cap binding protein. The best-characterized cytoplasmic cap binding proteins are involved in the initiation of translation (Shatkin, 1985). This binding activity may well also anchor mRNAs in the cytoplasm, since mRNA has never been reported to show anything other than unidirectional export from the nucleus to the cytoplasm. The pol II U snRNAs escape this inhibition since their caps are rapidly trimethylated in the cytoplasm (Mattaj, 1988; Zieve and Sauterer, 1990).

While transport of U6 is not affected by the 2,2,7mGpppG cap analogue, it is inhibited by coinjection of WGA. It has recently been reported that BSA-coupled peptides corresponding to the nuclear localization signal of SV40 T antigen, which act as inhibitors of protein uptake into the nucleus, also reduce U6 import while having no effect on the nuclear accumulation of U2 RNA (Michaud and Goldfarb, 1991). This was interpreted as evidence for the existence of two pathways of migration into the nucleus. Our results confirm and extend this hypothesis by showing that WGA, a general inhibitor of protein import into the nucleus (Finlay et al., 1987; Dabauvalle et al., 1988), prevents U6 accumulation while it has only a minor effect on the movement of pol II U snRNPs. The trimethylated cap dinucleotide acts in the inverse way, inhibiting pol II U snRNP transport, but not that of U6 snRNA or a karyophilic protein.

Several points arise from these observations. The first concerns U6 migration. It would appear from these and earlier results that U6 transport closely resembles protein migration in that it is inhibited by WGA, reduced by BSA-nuclear localization signal conjugates, and cap independent. Thus, the signal for migration of the U6 snRNP is likely to be similar to the better-characterized signals on karyophilic proteins (for review see Dingwall and Laskey, 1986). On the other hand, the signals on the pol II U snRNPs may have nothing in common with this class. The evidence that proteins are involved in signaling migration of these RNPs rests on the fact that mutation of the binding site (on the RNA) for the common set of U snRNP proteins prevents nuclear migration (Mattaj and De Robertis, 1985; Hamm et al., 1990). This is clearly an indirect argument. More direct experimental identification of the "non-cap" part of the bipartite targeting signal of these U snRNPs remains an elusive goal.

Thanks are due to Georg Krohne for providing us with the *Xenopus* Lamin L1 cDNA clone and for advice concerning the use of in vitro-translated L1 protein for transport studies. We wish to thank Elisa Izaurralde, Christian Kambach, and Angus Lamond for their comments on the manuscript, and Heide Seifert and Helen Fry for help in its preparation.

E. Darzynkiewicz was supported by the following grants: G-MEN-104/90, CPBR3.13, and CPBP/01.06, S. M. Tahara by National Institutes of Health grant GM38512 and BRSG award 2507-RR05356, and R. Lührmann by a grant from the Deutsche Forschungsgemeinschaft. U. Fischer was supported by a fellowship from the graduate student program for cell and tumor biology, Marburg.

Received for publication 27 November 1990 and in revised form 16 January 1991.

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