Microinjected U snRNAs Are Imported to Oocyte Nuclei via the Nuclear Pore Complex by Three Distinguishable Targeting Pathways

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Abstract. The inhibitory effects of wheat germ agglutinin and mAb 414 on the nuclear import of all types of U snRNAs indicate that they cross the nuclear envelope through the nuclear pore complex. However, the import of different U snRNAs occurs by kinetically distinct targeting pathways that can be distinguished from one another by the competitive effects of free trimethylguanosine cap dinucleotide (m_3GpppG) and P(Lys)-BSA, an efficient synthetic karyophile based on the nuclear localization signal of SV40 large T antigen. The import of U snRNAs that contain 5′ m_3GpppN caps and are complexed by Sm proteins (U1, U2, U4, and U5) is competed by coinjection with free m_3GpppG, indicating a shared transport factor, but not by P(Lys)-BSA. The import of U6 snRNA, which lacks a m_3GpppN cap and is not complexed by the Sm proteins, is competed by P(Lys)-BSA but not by free m_3GpppG. Thus, by the criterion of kinetic competition, U6 snRNA import is identical to that of the karyophilic proteins P(Lys)-BSA and nucleoplasmin. Uniquely, the import of U3 snRNA, which contains a m_3GpppN cap but does not bind Sm proteins is not competed by either free m_3GpppG or P(Lys)-BSA. Thus, U3 snRNA appears to be imported by a novel third kinetic pathway.

THE active transport (Newmeyer and Forbes, 1988; Bataille et al., 1990) of macromolecules across the nuclear envelope occurs via the diaphragm-like central transporter assembly within the nuclear pore complex (NPC)[1] (Feldherr et al., 1984; Akey and Goldfarb, 1989; Akey, 1990). The energy-independent (Richardson et al., 1988; Newmeyer and Forbes, 1988) targeting of proteins to the NPC is probably mediated by primary cytoplasmic nuclear localization signal sequence (NLS) receptors (Goldfarb et al., 1986; Newmeyer and Forbes, 1990; Adam et al., 1990; Breeuwer and Goldfarb, 1990; Stochaj et al., 1991; Adam and Gerace, 1991). Discrete NLSs have been identified within the primary sequence of many nuclear proteins (Garcia-Bustos et al., 1991). These NLS sequences often resemble the SV40 large T-antigen signal, PKKKRKVEDP (Kalderon et al., 1984; Garcia-Bustos et al., 1991). Although it has not been shown directly, it is thought that T-antigen-like NLSs are the cell's predominant family of NLSs.

Two lines of evidence indicate that the NLSs of RNA polymerase II transcribed U snRNPs are distinct from T-antigen-like NLSs. First, the import of U1 snRNP requires both a 5′ trimethylguanosine cap (m_3GpppN) (Fischer and Luhrmann, 1990; Hamm et al., 1990) and the binding of Sm proteins (Mattaj and DeRobertis, 1985; Hamm et al., 1990). Second, the import of m_3GpppN-Sm protein-containing U snRNPs is kinetically noncompetitive with the import of karyophiles that contain T-antigen-like NLSs (Michaud and Goldfarb, 1991).

U1-U5 snRNA precursors are transcribed by RNA polymerase II (Dahlberg and Lund, 1988) and are exported from the nucleus by a 7-methylguanosine (m^7GpppN) cap–dependent process (Hamm and Mattaj, 1990). Once in the cytoplasm these U snRNAs (except U3) associate with a number of common Sm-proteins that bind to a consensus single stranded Sm-protein binding site (Mattaj, 1988). After RNP assembly, the m^7GpppN cap is hypermethylated to generate a 2,2,7-trimethylguanosine (m_3GpppN) cap (Mattaj, 1986; Reddy and Busch, 1988). While cap hypermethylation is dependent upon Sm protein binding (Mattaj, 1986), Sm proteins can bind microinjected m_3GpppN capped U snRNAs (De Robertis et al., 1982).

U6 snRNA, which is transcribed by RNA polymerase III, contains a posttranscriptionally added gamma-monomethylphosphoryl guanosine cap (Singh and Reddy, 1989), which is probably also found on other pol III transcripts (Shumyatsky et al., 1990). Although U6 lacks a consensus Sm-protein binding site, it does contain a single-stranded region that probably binds U6-specific proteins (Hamm and Mattaj, 1989; Groning et al., 1991). U4 and U6 snRNAs are normally found as a complex in the nucleus of somatic cells (Bringmann et al., 1984; Hashimoto and Steitz, 1984). However, in oocytes and early *Xenopus* embryos, U6 snRNA is present in excess over U4 and, even though it is probably not normally exported to the cytoplasm (Vankan et al.,

1. *Abbreviations used in this paper*: NLS, nuclear localization signal sequence; NPC, nuclear pore complex; WGA, wheat germ agglutinin.

1990), it can be imported as a separate particle after microinjection (Hamm and Mattaj, 1989). Because U6 snRNA import was competed by saturating concentrations of P(Lys)-BSA (Michaud and Goldfarb, 1991), we think its import is probably mediated by a protein(s) that contains a T-antigen-like NLS. Nucleolar U snRNAs, U3, U8, and U13 (Tyc and Steitz, 1989), are distinct in that they contain the characteristic pol II m_3GpppN caps but, like U6, they lack consensus Sm-protein binding sites (Luhrmann, 1988).

In the present study we show that free m_3GpppG cap dinucleotide reduces the import rate of m_3GpppN-Sm protein U snRNAs, but it has no effect on the import of either U3 or U6 snRNAs. The import of U6 snRNP is distinguished by its sensitivity to competition by P(Lys)-BSA (an efficient karyophile comprised of synthetic T-antigen NLS peptides conjugated to bovine serum albumin) (Goldfarb et al., 1986). The import of neither the m_3GpppN-Sm protein containing U snRNPs nor U3 snRNP are competed by P(Lys)-BSA. Thus, while the m_3GpppN cap of most U snRNAs appears to have a targeting function, the same structure on U3 snRNA is not required for nuclear localization. All U snRNAs tested are, however, sensitive to general inhibitors of NPC-mediated translocation.

Materials and Methods

Materials

Trimethylguanosine cap analogues, provided by S. Tahara (University of Southern California, Los Angeles, CA) (Stepinski et al., 1990), (m_3G-G, seco-m_3G-G and m_3GMP) were stored frozen at −20°C in 1 mM Tris-HCl (pH 7.0). Unmethylated G-G and m^7G-G were obtained from New England Biolabs (Boston, MA) and maintained as 10 mM stocks at −20°C in sterile H_2O. The U1 cDNA clone was provided by I. Mattaj (European Molecular Biology Laboratory, Heidelberg) (Hamm et al., 1987), and the U3 snRNA cDNA clone (Baserga et al., 1991) was provided before publication by S. Baserga (Yale University, New Haven, CT). Preparation and iodination of P(Lys)-BSA and nucleoplasmin are described in Michaud and Goldfarb (1991) except for the batch of P(Lys)-BSA used in the experiment shown in Fig. 7 C which was prepared with sulfo-MBS crosslinker (Pierce Chemical Co., Rockford, IL) dissolved in PBS instead of MBS dissolved in dimethylformamide. WGA (Sigma Chemical Co., St. Louis, MO) and concanavalin A (Sigma Chemical Co.) were dissolved in distilled water or intracellular medium (Bataillé et al., 1990) and used fresh.

Antibody mAb 414 ascites was provided by L. Davis (Duke University, Durham, NC). mAb 414 was purified from ascites fluid by protein A-Sepharose chromatography. 10 ml of ascites fluid was brought to 3.0 M NaCl, 100 mM NaBorate (pH 8.9) and applied to 3 ml protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated in the same buffer. The column was washed with 10 column volumes of 3.0 M NaCl, 10 mM NaB (pH 8.9), then the antibody was eluted with 100 mM glycine (pH 3.0). 1-ml fractions were collected directly into 50 μl 1.0 M Tris-HCl (pH 8.0). The antibody was dialyzed into 150 mM NaCl, 10 mM Na_2HPO_4 (pH 7.5) and concentrated with centricon filtration units (Amicon Corp., Danvers, MA) to 25 mg/ml. Antibody concentration was determined by Bradford assay using bovine IgG (Bio-Rad Laboratories, Cambridge, MA) as the standard.

Isolation of HeLa RNAs

HeLa cells were grown and labeled with [^{32}P]orthophosphate essentially as described in Michaud and Goldfarb (1991), except the labeling period was 20–24 h. Labeled cells were pelleted then washed in cold PBS (0.9% NaCl, 10 mM Na_2HPO_4, pH 7.5) and lysed in 5 ml 2% SDS, 300 mM NaCl, 10 mM EDTA, 20 mM Tris-HCl (pH 7.5). The lysate was extracted twice with 5 ml phenol:chloroform:isoamyl alcohol (25:24:1). Nucleic acids were precipitated by addition of 2.5 vol cold ethanol to the aqueous phase. DNA was removed by spooling, and the remaining RNA was pelleted by centrifugation at 10,000 rpm at 4°C for 30 min in an SS-34 rotor (Dupont, Wilmington, DE). RNA pellets were washed with 70% ethanol and air dried. RNA either was resuspended in sterile H_2O and used for injection (see Fig. 6) or processed further. After resuspension of the RNA pellet in 3 ml 0.5 M NaCl, 1 ml 40% PEG 8000 was added to a final concentration of 10%. The mixture was incubated at room temperature for 30–60 min, and high MW RNA and DNA was pelleted by centrifugation at 10,000 rpm at 10–12°C for 10 min in an SS-34 rotor. Greater than 80% of the high MW RNA and 5.8S RNA, which remains hydrogen bonded to 28S rRNA, is removed by this step. Small MW RNAs that remain soluble in the supernatant were precipitated with 2.5 vol ethanol at −20°C for 30–60 min and collected by centrifugation at 10,000 rpm at 4°C for 45–60 min. The 70% ethanol-washed and air-dried RNA pellet either was resuspended in sterile water for injection (see Fig. 1) or resuspended in 100 μl formamide loading buffer (85% deionized formamide, 0.5× TBE, 0.1% SDS). [^{32}P]HeLa U2, U1, U6, and tRNAs were subsequently electrophoresed, gel purified, and prepared for injection as described in Michaud and Goldfarb (1991).

The total recovered radioactivity of gel-purified RNAs was determined by scintillation counting 1% of the measured, collected volume of eluate from each crushed gel slice. The recovered mass was calculated by measuring the absorbance of the remaining eluate at 260 nm. These measurements allowed us to calculate the specific activity of the gel-purified RNAs. The specific activity of gel-purified HeLa [^{32}P]RNAs typically ranged from 1000-1200 cpm/ng for U1 and U2, 400–600 cpm/ng for U6 and 1500-2500 cpm/ng for tRNA.

T7 Transcription of Xenopus U1 and Human U3 RNA

Wild-type *Xenopus* U1 (Hamm et al., 1987) or human U3 templates (Baserga et al., 1991) were linearized with BamHI or RsaI, respectively, and were transcribed with T7 RNA polymerase essentially as described in Hamm et al. (1987). The transcription reaction included 500 μM cap dinucleotide, either m_3G-G, m^7G-G, or G-G. Unlabeled GTP (50 μM) was present throughout the entire reaction. Typically, RNAs were transcribed at specific activities of 1–5 × 10^7 dpm/μg. Reactions were stopped by the addition of 90 μl 0.2% SDS. After phenol/chloroform extraction and ethanol precipitation in 2.5 M ammonium acetate, the transcript was gel purified from an 8% acrylamide, 8.3 M urea, TBE gel. Occasionally, U3 RNA was ethanol precipitated with 20 μg carrier yeast tRNA immediately after phenol/chloroform extraction and used for subsequent injection. The RNA was eluted from crushed gel slices in 0.3 M ammonium acetate, 1 mM EDTA, and 0.1% SDS, phenol/chloroform extracted, precipitated as above, and resuspended in distilled water.

Xenopus Oocyte Microinjection, Transport Assays, and Immunoprecipitation

Xenopus laevis oocytes were obtained and injected, and transport of either iodinated proteins or [^{32}P]-labeled RNAs analyzed as described in Michaud and Goldfarb (1991). Typically 50 nl of sample was injected and final intracellular concentrations are denoted in the figure legends in parentheses. Assembly of microinjected, Sm-binding site containing U snRNAs was assayed by immunoprecipitation with anti-Sm mouse mAb 7.13 provided by S. Hoch (The Agouron Institute, La Jolla, CA) (Billings et al., 1985) according to Michaud and Goldfarb (1991).

Results

U snRNA Import Occurs through the NPC

All evidence indicates that macromolecular nuclear transport into the nucleoplasm occurs through the NPC. A good criterion for signal-mediated import via the NPC is sensitivity to wheat germ agglutinin (WGA) or to antibodies directed against nucleoporins. The nucleoporins are a family of N-acetylglucosamine-containing proteins (Holt et al., 1987) that are localized to the cytoplasmic and nucleoplasmic faces of the central transporter assembly (Snow et al., 1987; Akey and Goldfarb, 1989). Although antinucleoporin antibodies and WGA do not inhibit the binding of karyophiles to the central transporter (Finlay et al., 1987; Newmeyer and Forbes, 1988; Akey and Goldfarb, 1989), translocation can be significantly reduced (Dabauvalle et al., 1988*a*,*b*; Featherstone et al., 1988, Bataille et al., 1990), perhaps due to cross-

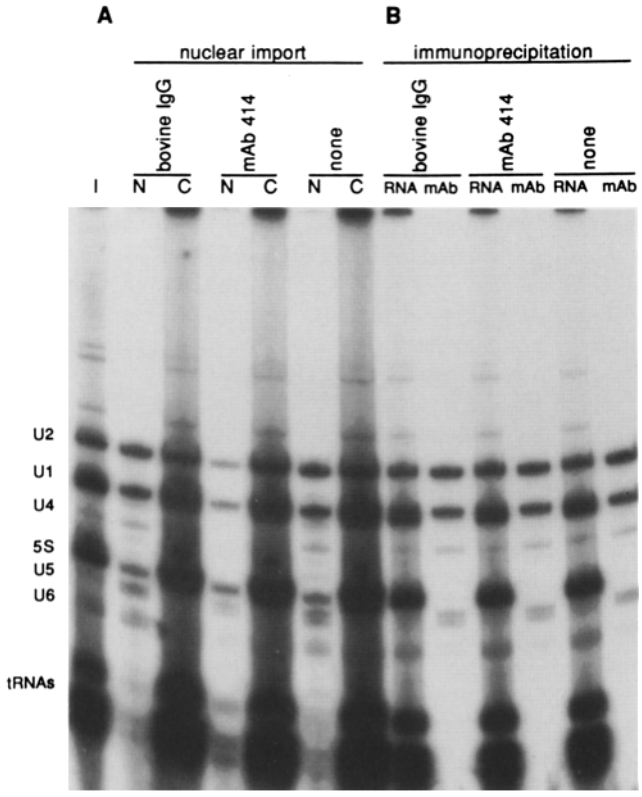


Figure 1. Effect of antinucleoporin antibody mAb 414 on snRNA transport and assembly in *Xenopus* oocytes. (*A*) *Xenopus* oocytes were injected with ~50 nl of 25 mg/ml mAb 414 or 25 mg/ml bovine IgG to a final concentration of ~2.5 mg/ml and incubated 2 h at room temperature before injection with 10% PEG soluble HeLa [^{32}P]RNA followed by a further 2-h incubation. Alternatively, PEG soluble HeLa [^{32}P]RNA was injected into oocytes that had not been previously injected. Nuclei (*N*) and cytoplasms (*C*) from groups of five oocytes were separated and RNA from each fraction purified. (*B*) Whole oocyte extracts were prepared from sibling oocytes and divided into two aliquots. RNA was prepared directly from one aliquot. Assembled snRNPs were immunoprecipitated from the other aliquot with anti-Sm mAb 7.13 (mAb), and RNA was prepared from the immunoprecipitate. RNAs from both aliquots were separated by denaturing 8% acrylamide gel electrophoresis.

linking transporter subunits (Featherstone et al., 1988; Akey, 1990).

It was shown previously that karyophilic proteins enter the nucleus via the nuclear pore complex (Feldherr et al., 1984); however, the manner of U snRNP import is still unresolved. Although the export of U snRNAs is sensitive to WGA (Neuman De Vegvar and Dahlberg, 1990), Fischer et al. (1991) reported that U1 and U5 snRNA import is insensitive to WGA. We tested whether the import of U snRNPs was sensitive to the antinucleoporin mAb 414 (Davis and Blobel, 1986) and WGA. Fig. 1 *A* shows that the import of U snRNPs U1, U2, U4, U5, and U6 were significantly inhibited by mAb 414 but not by control bovine IgG. The import of U3 also was inhibited by preinjection of mAb 414 but to a lesser extent than U2 or U6 (Fig. 2). As shown in Fig. 1 *B*, the import and not the assembly of Sm protein–associated U snRNPs was affected by the antibody. mAb 414 also significantly reduced the import rates of P(Lys)-BSA and calf thymus histone H1; however, the diffusion of lysozyme into the nucleus was unaffected (M. Mangan and D. Goldfarb, unpublished observations). As shown in Fig. 2, preinjection of WGA reduced the import of U3, U2, and U6 snRNAs. The inhibitory effect of WGA was abolished by coinjection of 500 mM N-acetylglucosamine (Fig. 2). Preinjection of con A under similar conditions had no inhibitory effect (not shown). Association of Sm-antigen with U2 snRNA was not affected by WGA (not shown), indicating that RNP assembly was not inhibited.

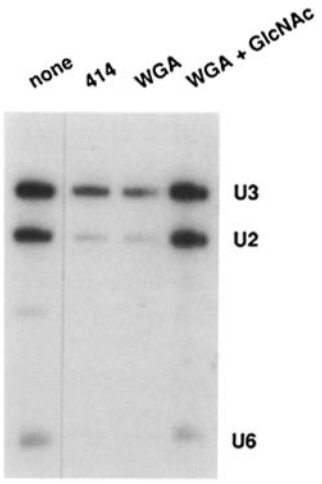


Figure 2. Effect of WGA and mAb 414 preinjection on U3, U2, and U6 snRNA import. Oocytes were preinjected with 50 nl 25 mg/ml mAb 414 (414), 10 mg/ml WGA (WGA), or 10 mg/ml WGA with 500 mM N-acetyl glucosamine (WGA + GlcNAc) to final concentrations of 2.5 mg/ml mAb 414, 1 mg/ml WGA, and 1 mg/ml WGA + 50 mM GlcNAc, respectively, then incubated for 2 h before injection of human [^{32}P]U3 (1 nM), transcribed and capped in vitro with m^{7}G-G, gel-purified HeLa [^{32}P]U2 (35 nM), and gel-purified HeLa [^{32}P]U6 (125 nM). Control oocytes (none) were not preinjected. RNA import was assayed at 1 h in groups of four oocytes. Only the nuclear fractions are shown. All cited concentrations in parentheses in this and all subsequent figure legends are final intracellular concentrations.

The inhibitory effect of preinjected WGA on HeLa U1, U2, and U6 import was dose dependent (Fig. 3) and, in the cases

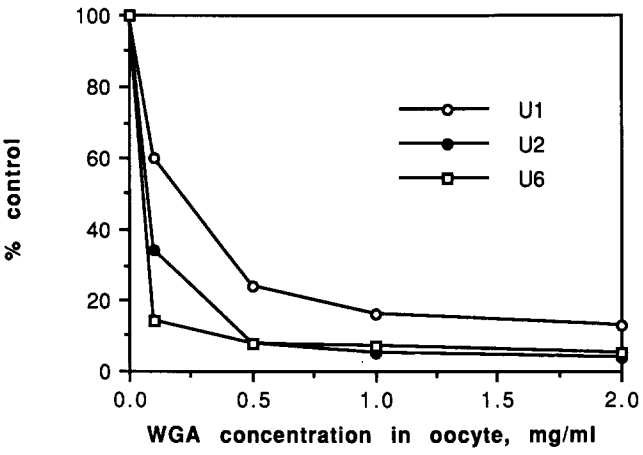


Figure 3. Dose-dependent inhibition of HeLa U1, U2, and U6 import by WGA. Oocytes were preinjected with various concentrations of WGA and incubated 2 h. The nuclear import of gel-purified HeLa [^{32}P]U1 (35 nM), HeLa [^{32}P]U2 (40 nM), and HeLa [^{32}P]U6 (245 nM) was assayed at 1 h after their injection into control (uninjected) and preinjected oocytes. RNA nuclear localization was measured in two groups of four to six oocytes. The final intracellular concentration of WGA is denoted on the abscissa. Transport in the presence of WGA is expressed as a percentage of the transport of each snRNA in control oocytes (% control). Transport in control oocytes was as follows: HeLa U1, 56.1 ± 2.3; HeLa U2, 54.8 ± 5.0; HeLa U6, 27.0 ± 14.4.

of U1, U2, and U3, apparent over an 8-h time course (Fig. 4, *a–c*). Interestingly, WGA differentially inhibits the import of different snRNAs (Fig. 3). Thus, U6 import is most drastically affected by WGA, with U2 then U1 import being less sensitive (Fig. 3). We conclude that the nuclear import of U1, U2, U3, U4, U5, and U6 snRNA is sensitive to general inhibitors of NPC-mediated transport.

Synthetic m_3G-G Specifically Competes the Import of U1, U2, U4, U5 snRNAs, but Not U6 or U3 snRNAs

The assembly and import of microinjected HeLa [^{32}P]U snRNAs into the nuclei of *Xenopus* oocytes can be monitored as a function of time (DeRobertis et al., 1982; Pan and Prives, 1988; Michaud and Goldfarb, 1991). We tested the effect of synthetic cap analogues on the import of various U snRNAs. Fig. 5 shows the dose-dependent effects of m_3GpppG (m_3G-G) and GpppG (G-G) dinucleotides on the import of [^{32}P]-labeled HeLa U1, U2, and U6 snRNAs, and in vitro transcribed *Xenopus* m_3GpppG-capped [^{32}P]U1 snRNA into oocyte nuclei. Qualitatively, the import of all U1 and U2 snRNA preparations was specifically inhibited by m_3G-G but not G-G. Quantitative differences between the response curves, such as the partial inhibition of HeLa U2 snRNA import by G-G (Fig. 5 *c*), were reproducible. By microinjecting total HeLa small [^{32}P]RNAs we also observed significant inhibition of U4 and U5 snRNA import by m_3G-G but not G-G or m^7GpppG (m^7G-G) (not shown). Although the import of the Sm protein-containing U snRNPs is competed by m_3GpppG, their association with the Sm proteins is unaffected (Fig. 6).

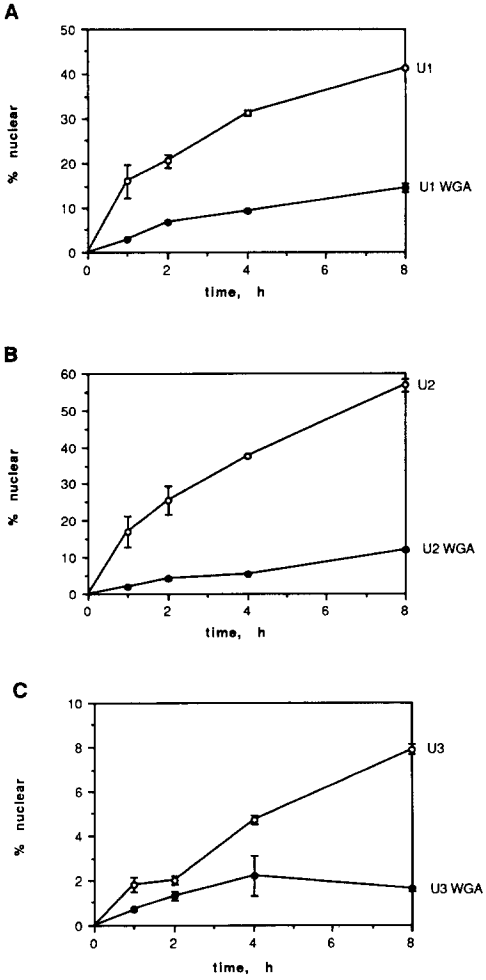


Figure 4. WGA inhibition of *Xenopus* U1, HeLa U2, and human U3 import as a function of time. Oocytes were preinjected with 50 nl of 15 mg/ml WGA to a final concentration of ~1.5 mg/ml and incubated 2 h. Preinjected or uninjected oocytes were injected with *Xenopus* [^{32}P]U1 (2 nM), transcribed and capped in vitro with m^7G-G, gel-purified HeLa [^{32}P]U2 (113 nM), and human [^{32}P]U3 (3 nM), transcribed and capped in vitro with m^7G-G, and incubated indicated times. Nuclear import of injected RNAs was assayed in two groups of six to eight oocytes at each time.

A key comparison in this set of experiments is between m_3GpppN-containing U snRNAs (Fig. 5, *a–c*) and U6 snRNA (Fig. 5 *d*). As expected, the import of U6 snRNA, which lacks a m_3GpppN cap and is competed by P(Lys)-BSA (Michaud and Goldfarb, 1991), is unaffected by either m_3G-G or G-G (Fig. 5 *d*).

U3 snRNA, because it contains a m_3GpppN cap but no Sm protein binding site, presents an interesting natural test case for competition by the m_3G-G dinucleotide and P(Lys)-BSA. Hamm et al. (1990) reported that the m_3GpppN cap of U1 snRNP functioned as a nuclear localization signal only when present together with an Sm protein binding site. As shown in Fig. 7 *a*, the import of U1 is efficiently competed by free m_3G-G but not by free m^7G-G or G-G. In contrast, the import of U3 is not effected by any of these cap analogues. In these experiments U3 snRNA was microinjected at a final concentration in the oocyte of between 2 and 5 nM. The import of U3 snRNA is not kinetically saturated at this concentration as the microinjection of 30 times (150 nM) this concentration did not diminish the rate of U3 snRNA import at 1, 8, and 19 h (not shown). The half-life of injected U3 both in the presence and absence of m_3G-G is ~18 h in oocytes (not shown). Thus by this criterion, the m_3GpppG cap of U3 snRNP is nonessential for nuclear import. These results are consistent with the results of S. Baserga and M. Gilmore-Hebert (Yale University, New Haven, CT) who have shown that U3 import occurs in the absence of a hypermethylated cap (personal communication). These effects can also be seen throughout a time course (Fig. 7 *b*). Here, the initial import rate of U1 snRNP is inhibited by 100 μM intracellular free m_3G-G but the import of U3 snRNP is unaffected.

Because U3 import was unaffected by free m_3G-G, we were especially curious to learn if, like U6, U3 import could be competed by saturating concentrations of P(Lys)-BSA. We found no significant inhibition of U3 snRNP import in the presence of 5 or 15 μM P(Lys)-BSA (Fig. 7 *c*). Competition experiments using higher P(Lys)-BSA concentrations (25 μM) were problematic due to nonspecific inhibitory effects where some batches of P(Lys)-BSA bound U snRNAs indiscriminately in vitro and inhibited U1 and U2 snRNP assembly in the oocyte. A separate experiment, where human [^{32}P]U3, HeLa [^{32}P]U2, and, [^{125}I]P(Lys)-BSA were injected alone or with 15 μM P(Lys)-BSA into four groups of five oocytes, showed that neither U3 nor U2 import was competed by P(Lys)-BSA at 1.5 h, even though [^{125}I]P(Lys)-BSA import was inhibited threefold (not shown). Two-tailed *t* tests indicated statistically significant differences in [^{125}I]P(Lys)-BSA import with and without competitor at the 99% confi-

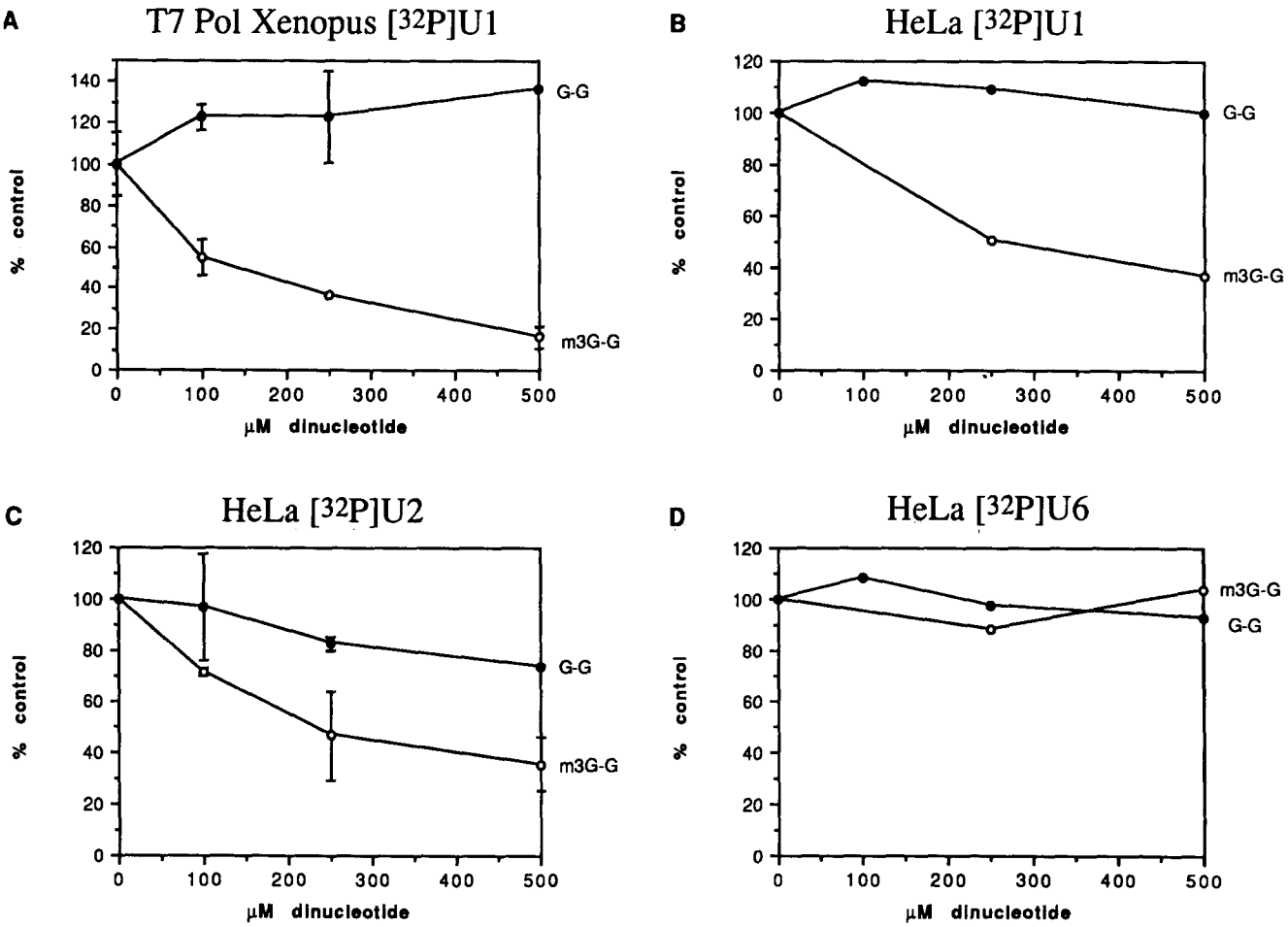


Figure 5. Effect of m_3G-G on the import of in vitro transcribed *Xenopus* U1, HeLa U1, HeLa U2, and HeLa U6 snRNP. Each gel-purified [^{32}P]snRNA was mixed with increasing concentrations of m_3G-G (*open circles*) or G-G (*closed circles*) cap dinucleotide and injected into the cytoplasm of *Xenopus* oocytes. Nuclear transport was assayed at 1 h. The intracellular concentration of each dinucleotide is denoted on the abscissa. Transport in the presence of either dinucleotide was normalized to transport in the absence of dinucleotide. (*A*) *Xenopus* [^{32}P]U1 RNA (7 nM), transcribed and capped with m_3G-G in vitro by T7 RNA Polymerase, was injected into two groups of five oocytes. (*B*) HeLa [^{32}P]U1 RNA (50 nM) was injected into 10 oocytes. (*C*) HeLa [^{32}P]U2 (50 nM) was injected into two groups of 8–12 oocytes. (*D*) HeLa [^{32}P]U6 (200 nM) was coinjected with HeLa [^{32}P]U1 into 10 oocytes. HeLa U1 import is shown in *B*.

dence level. In contrast, there were no statistical differences in U3 or U2 import with and without P(Lys)-BSA competitor even at the 80% confidence level. U3 snRNA is, therefore, unique in being imported by a mechanism that is not inhibited by either m_3G-G or P(Lys)-BSA.

Chemical Specificity of Cap Analogue Inhibition

We tested the ability of different cap analogues to inhibit HeLa U2 snRNA import. We asked which structural moieties of the m_3G-G cap dinucleotide were responsible for inhibiting U2 snRNA import. Similar studies have been performed on the inhibition of protein synthesis by m^7G analogues (Carberry et al., 1990). The analogue m_3Gp (m_3GMP) lacks a dinucleotide linkage, m^7G-G lacks two methyl groups, G-G lacks all three methyl groups, and seco-m_3G-G contains a disrupted ribose ring on the terminal guanosine (Stepinski et al., 1990). The effect of 500 μM of each of these analogues is shown in Table I. Only m_3G-G significantly inhibited U2 snRNA import. Therefore, the factor that complexes the m_3G-G cap in situ recognizes trimethylation, an inverted dinucleotide motif, and an intact terminal ribose. Consistent with the seco-m_3G-G results, Fischer and Luhrmann (1990) showed that the alkaline hydrolysis of the purine rings in m_3G-G dinucleotide destroyed its ability to compete U1 snRNA import.

Free m_3G-G Has No Affect on the Import of P(Lys)-BSA or Nucleoplasmin

Saturating concentrations of P(Lys)-BSA compete the import of nucleoplasmin and U6 snRNA but not the m_3GpppN containing U snRNAs (Michaud and Goldfarb, 1991). As shown, m_3G-G competed the import of m_3GpppN-Sm containing U snRNAs but not U3 or U6 snRNA import. If the import pathway of m_3GpppN-Sm containing U snRNAs is, in fact, distinct from the pathway taken by T-antigen-like NLS containing karyophiles, then m_3G-G should not compete the import of either P(Lys)-BSA or nucleoplasmin. Figs. 8 *a* and 9 *a* show that over a wide concentration range m_3G-G and G-G had no effect on the import of [^{125}I]P(Lys)-BSA and [^{125}I]nucleoplasmin at 1 h after microinjection. Figs. 8 *b* and 9 *b* show the time courses of import for these

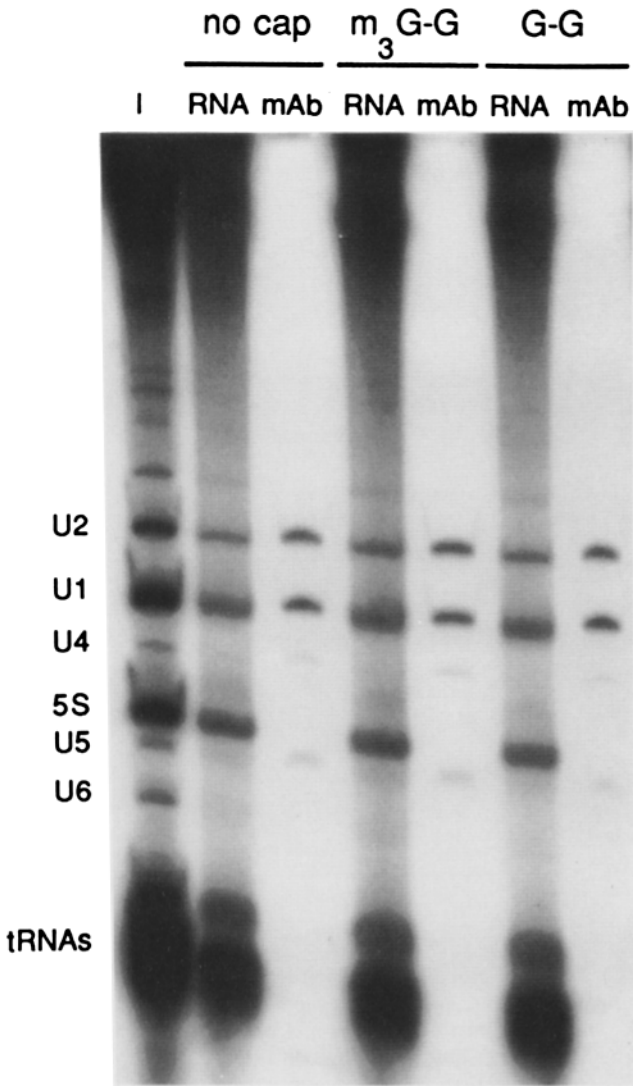


Figure 6. U snRNP assembly in the presence of cap dinucleotides. Total HeLa [^{32}P]RNA was injected alone, with m_3G-G (500 μM), or with G-G (500 μM) into groups of 10 oocytes. After 1 h incubation, extracts from whole oocytes were prepared. RNA (RNA) was purified directly from one-half the extract. The other half was incubated with anti-Sm mAb (mAb) and RNA was prepared from the immunoprecipitate. RNAs were separated on 8% denaturing acrylamide gels.

karyophiles in the presence of either 500 μM m_3G-G or G-G. No cap-specific inhibition of P(Lys)-BSA or nucleoplasmin import was observed.

Discussion

U snRNP Import Occurs through the NPC

A central assumption in our studies is the essential role of the NPC in the translocation of karyophilic macromolecules across the nuclear envelope. This is supported by various lines of evidence (Garcia-Bustos et al., 1991), including the inhibition of both import and export by antinucleoporin antibody (Featherstone et al., 1989) and WGA (Finlay et al., 1987; Dabauvalle et al., 1988*b*; Bataille et al., 1990; Neuman De Vegvar and Dahlberg, 1990). Recently, we have shown that kinetically distinguishable classes of karyophiles exist (Michaud and Goldfarb, 1991) and others have shown that a member of one of these distinct classes, U1 snRNA, contains a novel NLS (Fischer and Luhrmann, 1990; Hamm and Mattaj, 1990). Although we favored the idea that the two karyophile classes were targeted to the pore complex by different cytoplasmic receptors and then transported into the nucleus by the same pore complex–mediated mechanism, it remained a possibility that U1 snRNA, for example, used an alternative translocation mechanism. The criteria we used to establish that the U snRNAs entered the nucleus via the pore complex was sensitivity to WGA and mAb 414. Although neither of these reagents exhibits completely monospecific binding properties, thus admitting the possibility of artifactual inhibition, our results strongly suggest that the U snRNAs are imported by the pore complex much in the same manner as P(Lys)-BSA and nucleoplasmin. Recently Fischer et al. (1991) reported that of several U snRNAs tested only U6 import was sensitive to WGA. We have gone to some length to try and reconcile our data with those of Fischer et al. (1991) who looked only at relatively long times after U snRNA microinjection. However, all of our experiments with WGA and mAb 414 show significant inhibition of U snRNAs at both early (1 h) and late (8 h) times (Figs. 1–4). We think that the WGA inhibition is a specific phenomenon because the effect was abolished by coinjection with N-acetylglucosamine. It is relevant to note that the phenomenon of transitory WGA inhibition was observed in the case of nucleoplasmin import in cultured cells (Dabauvalle et al., 1988*b*), and E. Lund (University of Wisconsin, Madison, WI) also sees specific, early inhibition of U1 snRNA import by WGA in oocytes (personal communication).

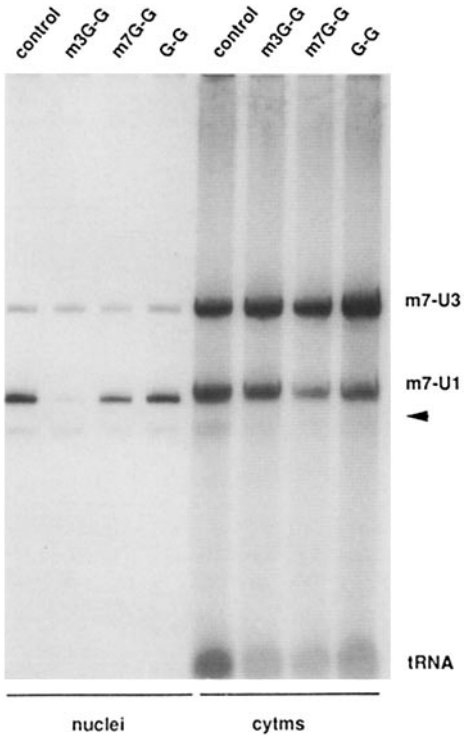
WGA and mAb 414 did, however, inhibit the import of the various karyophiles to different extents (also see m_3G-G effects below). Akey and Goldfarb (1990) have shown that nucleoplasmin and mAb 414 bind to adjacent, if not overlapping, sites on the transporter. Thus the differential inhibitory effects of WGA and mAb 414 on karyophile import may reflect something as simple as binding differences due to steric hindrance by WGA and mAb 414. Importantly, these quantitative differences, in contrast to qualitative differences between karyophiles in competition assays (Michaud and Goldfarb, 1991), do not necessarily indicate the use of different receptors either in the cytoplasm or at the transporter. For example, U6 snRNA import was more sensitive to mAb 414 than U1 snRNA import, and H1 import was more sensitive than P(Lys)-BSA import. We have shown that U1 and U6 snRNAs belong to noncompeting import classes, while H1 and P(Lys)-BSA belong to the same kinetic class (Michaud and Goldfarb, 1991; Mangan, M., and D. Goldfarb, unpublished results). Thus, significant differential inhibition of import by WGA or mAb 414 can occur between and within competing and noncompeting karyophile classes. As these differences are large and reproducible, they are probably significant; yet their significance must await further study into the mechanism of inhibition.

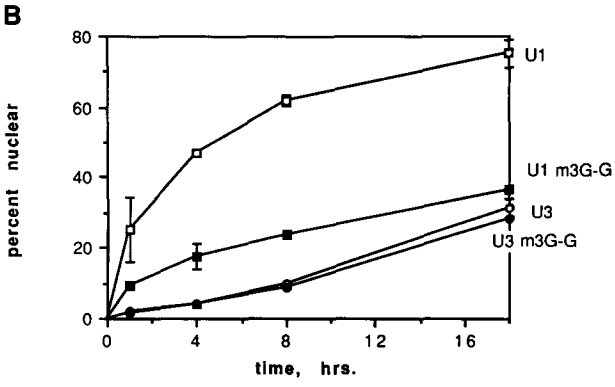
Competitive Inhibition of Nuclear Import

Synthetic peptide NLS-mediated nuclear import is almost as well studied as the transport of endogenous karyophiles and

A Human U3 Nuclear Import is not Inhibited by Cap Dinucleotides

	% nuclear import	
cap analogue	U3	U1
control	7.0 + 0.8 (100)	34.1+ 2.4 (100)
m_3G-G	6.4 + 0.5 (91)	4.8 + 0.1 (14)
m^7G-G	6.3 + 0.4 (90)	37.8 + 1.7 (111)
G-G	6.0 + 0.2 (86)	37.7 + 1.0 (111)





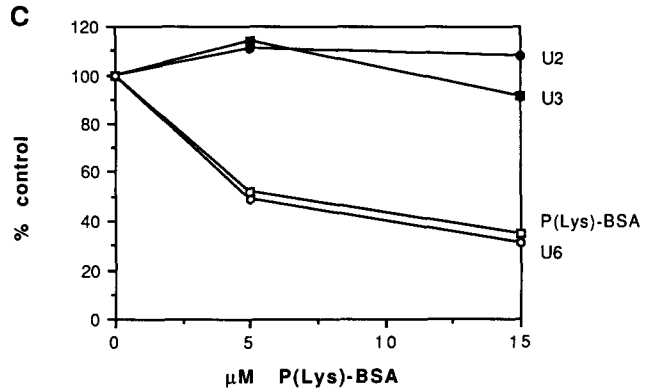


Figure 7. U3 import in the presence of m_3G-G and P(Lys)-BSA. (*A*) Human [^{32}P]U3 (5 nM), transcribed and capped with m_3G-G in vitro, *Xenopus* [^{32}P]U1 (5 nM), transcribed and capped with m_3G-G in vitro, and gel excised HeLa [^{32}P]tRNA (75 nM) were injected alone (control) or with m_3G-G (500 μM), m^7G-G (500 μM), or G-G (500 μM) and incubated for 6 h. Transport was assayed in groups of five oocytes. Transport was quantitated by scintillation counting of excised bands corresponding to U3 and U1. The number within parenthesis is percent control. The tabulated results are from three separate experiments. (*B*) Human [^{32}P]U3 (5 nM), transcribed and capped with m_3G-G in vitro, and *Xenopus* [^{32}P]U1 (8 nM), transcribed and capped with m_3G-G in vitro, were injected alone (U3, *open circles*; U1, *open squares*) or with m_3G-G (100 μM; U3, *closed circles*; U1, *closed squares*). Injected oocytes were incubated for the indicated times and U3 and U1 transport was assayed in groups of four to six oocytes. (*C*) Human [^{32}P]U3 (5 nM, *closed squares*), transcribed and capped with m^7G-G in vitro, gel-purified HeLa [^{32}P]U2 (62 nM, *closed circles*), gel-purified HeLa [^{32}P]U6 (105 nM, *open circles*), and [^{125}I]P(Lys)-BSA (50 nM, *open squares*) were mixed with either 0.1 M NH_4HCO_3 pH 8.0 (control), or P(Lys)-BSA (5 or 15 μM) in 0.1 M NH_4HCO_3 pH 8.0 then injected into oocytes. The oocytes were incubated for 1.5 h, then transport was assayed in two groups of four to seven oocytes. Transport in the presence of unlabeled P(Lys)-BSA is expressed as a percentage of transport in control oocytes uninjected with unlabeled P(Lys)-BSA. Percent nuclear localization in control oocytes was as follows: Human U3, 2.2 + 0.1; HeLa U2, 13.0 + 0.1; HeLa U6, 6.1 + 0.4; [^{125}I]P(Lys)-BSA, 27.5 + 2.3.

is indistinguishable by many basic criteria. Synthetic peptide NLS-BSA conjugates can contain as many as 20 peptides per monomer and, owing to the phenomenon of multivalency in nuclear transport (Roberts et al., 1987; Lanford et al., 1988; Dworetsky et al., 1988; Goldfarb, 1989; Lanford et al., 1990), this type of artificial karyophile exhibits high affinity for the transport apparatus. As a result, the import of P(Lys)-BSA is saturable with a micromolar K_m apparent in oocytes (Goldfarb et al., 1986). While the import rates of native karyophiles such as nucleoplasmin have not been shown directly to be saturable, their import can be competed by saturating concentrations of P(Lys)-BSA and thus indirectly shown to be receptor mediated (Michaud and Goldfarb, 1991). The competition of one karyophile's import by saturating concentrations of a second karyophile indicates that they share at least one common intermediate along their respective nuclear import pathways.

Initially, we recognized that the saturable component of P(Lys)-BSA import could be either a cytoplasmic receptor or a component of the NPC (Goldfarb et al., 1986). Subse-

Table I. Effect of Cap Analogues on HeLa U2 snRNA Nuclear Import

Cap analogue	Nuclear import
	%
control	28.0 ± 0.1 (100)
G-G	27.3 ± 0.9 (97.5)
m^7G-G	20.3 ± 0.3 (72.5)
m_3G-G	7.0 ± 0.4 (25.0)
seco m_3G-G	23.3 ± 3.1 (83.2)
m_3GMP	25.8 ± 0.3 (92.1)

HeLa [^{32}P]U2 (25 nM) was coinjected with various cap analogues (500 μM) or alone (control) into *Xenopus* oocytes. U2 nuclear import was assayed after 1 h in two groups of six oocytes. The number within parentheses represents (% control).

quent competition studies have indicated that the saturable component is probably a primary NLS receptor(s) located in the cytoplasm (Breeuwer and Goldfarb, 1990; Michaud and Goldfarb, 1991). This is supported by biochemical studies indicating a role for cytoplasmic signal binding factors in import (Silver, 1991; Garcia-Bustos et al., 1991), and the recent demonstration that a cytoplasmic signal binding protein is required for transport in permeabilized cells (Adam and Gerace, 1991). Although our competition studies do not directly identify cytoplasmic receptors, they indicate the existence of biochemical intermediates that plausibly consist of karyophile-cytoplasmic receptor complexes. The main argument is as follows. In competition studies between biochemically distinct karyophiles, the import of U2 snRNP is unaffected by 25 μM intracellular P(Lys)-BSA, a concentration capable of completely saturating P(Lys)-BSA and nucleoplasmin import (Michaud and Goldfarb, 1991). Here, we report that the import of other U snRNAs, including U1, U3, U4, and U5 import also was unaffected by competition with P(Lys)-BSA (not shown). Thus, under conditions where P(Lys)-BSA import is saturated, the NPC translocation channel remains unsaturated and available for the translocation of m_3GpppN-Sm protein-containing U snRNPs. Thus, the saturable step in P(Lys)-BSA import must occur before occupation of the translocation channels, which also can reasonably be assumed to be saturable, although this has yet

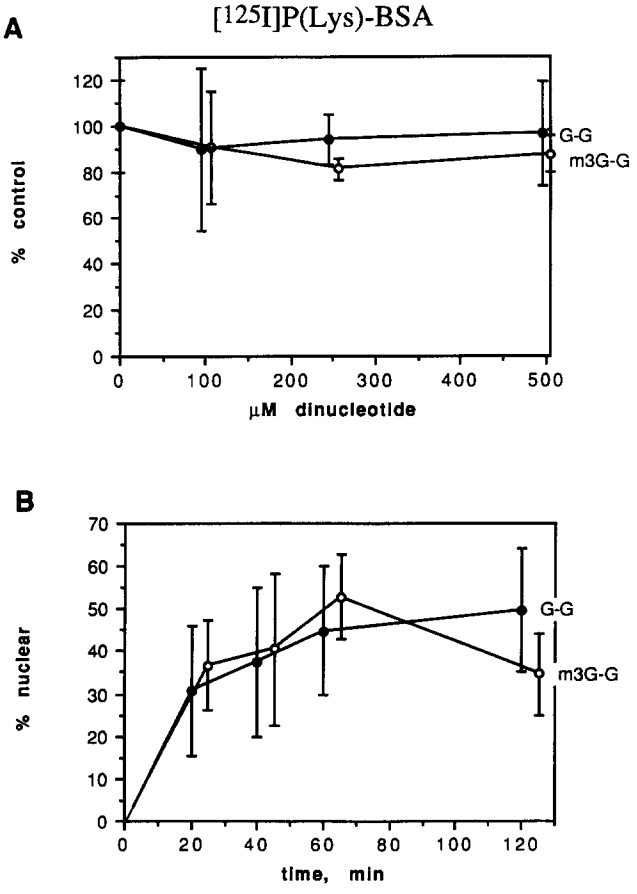


Figure 8. [^{125}I]P(Lys)-BSA import in the presence of m_3G-G. (*A*) [^{125}I]P(Lys)-BSA (15 nM) was coinjected with increasing concentrations of m_3G-G (*open circles*) or G-G (*closed circles*), and its import was compared with [^{125}I]P(Lys)-BSA import alone. Three groups of four to six oocytes were injected and transport was assayed after 1 h in pooled nuclei and cytoplasms by gamma counting. [^{125}I]P(Lys)-BSA import in control oocytes was 37.3%. (*B*) [^{125}I]-P(Lys)-BSA (200 nM) was injected with m_3G-G (500 μM, *open circles*) or G-G (500 μM, *closed circles*) into 12–15 oocytes. At each time, oocytes were fixed in 20% TCA and individual nuclei and cytoplasms were counted.

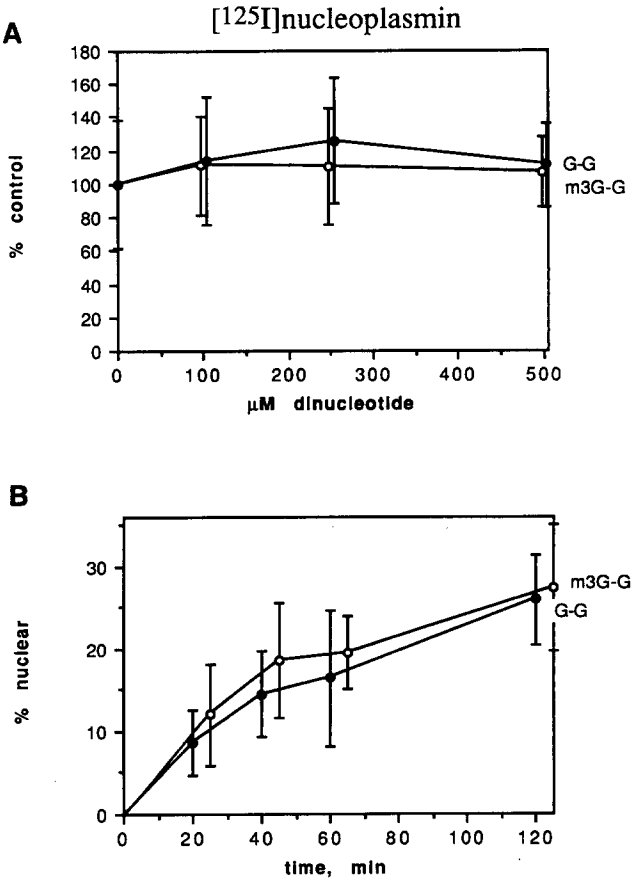


Figure 9. [^{125}I]nucleoplasmin import in the presence of m_3G-G. (*A*) [^{125}I]nucleoplasmin (80 nM) was injected with increasing concentrations of m_3G-G (*open circles*) or G-G (*closed circles*) and its import was compared with [^{125}I]nucleoplasmin import alone. Groups of 12 oocytes were injected and transport assayed after 1 h. [^{125}I]nucleoplasmin was 15.7% nuclear in control oocytes. (*B*) [^{125}I]nucleoplasmin (80 nM) was injected with m_3G-G (500 μM, *open circles*) or G-G (500 μM, *closed circles*) into 12 oocytes and transport was assayed at indicated times.

to be shown. Cytoplasmic or transiently pore complex-associated signal receptors are ideal candidates for the saturable component. Other models, such as karyophile-specific pore complex binding sites, have been discussed (Michaud and Goldfarb, 1991).

A logical next experiment would be to saturate U snRNP import and determine whether or not this condition affects the import of P(Lys)-BSA. To this end, the studies of Fischer and Luhrmann (1990) and Hamm et al. (1990) indicate that free m_3G-G might serve as a suitable competitor of m_3GpppN-Sm protein containing U snRNP import. In the present study we show that m_3G-G inhibits the import of m_3GpppN-containing U snRNPs (see Fischer et al., 1991), but not the import of P(Lys)-BSA, nucleoplasmin, U3, or U6 snRNA. Thus, kinetically at least, m_3G-G does behave much like P(Lys)-BSA in its ability to compete the import of its cognate NLS-containing karyophile class. This result supports the hypothesis that m_3G-G competes for a limiting component of the m_3GpppN-Sm protein containing snRNP nuclear import apparatus, perhaps a cytoplasmic receptor.

The competition analysis of the import of different U snRNAs indicates additional complexity in targeting pathways. Thus, we can organize the major U snRNAs into three import classes (Table II). One class contains the U snRNAs that have a m_3GpppN cap and bind Sm proteins, a second class contains karyophiles which, by various criteria, contain T-antigen-like NLSs, and a third class is comprised of U3 snRNA, which is excluded from the other two classes by lack of any competition. The behavior of U4 and U5 snRNAs is more complex than U1 and U2 snRNAs because they can be imported without a m_3GpppN cap, albeit less efficiently (Fischer et al., 1991).

Mechanism of m_3G-G Inhibition of m_3GpppN-capped U snRNP Nuclear Import

The use of m_3G-G as a transport competitor was suggested by experiments that showed that the m_3GpppN cap is essential for U1 snRNP import and might have a role in signalling (Hamm et al., 1990; Fischer and Luhrmann, 1990). The implication of these results is that the m_3GpppN cap is complexed by signal receptors of the nuclear transport apparatus. There is, however, no direct evidence that the m_3GpppN cap acts as a classic targeting signal which is complexed by a transport receptor. In fact, U3 snRNA, which naturally contains a m_3GpppN cap, is imported by a cap-independent pathway (S. Baserga, personal communication). Because of the unusual modular nature of the U1 snRNP signal (Hamm et al., 1990), the m_3GpppN cap has been shown to be essential but not sufficient for U1 snRNP import. The Sm proteins are also required. One possible explanation is that the transport receptor simultaneously complexes both the m_3GpppN cap and a motif on one of the Sm proteins. Thus, U3 snRNP would not be a substrate for binding by this receptor, because it does not bind Sm proteins. Alternatively, the cap might be complexed by a non-transport factor that is required for the "karyophilic activation" of the snRNP but is not itself recognized by a transport receptor. The relationship between the Sm binding site and Sm proteins is such an example. By this analogy, large amounts of an oligonucleotide that contains the Sm-binding site sequence might compete with comicroinjected [^{32}P]U snRNA for Sm proteins and by this indirect mechanism inhibit the assembly and subsequent import of the [^{32}P]U snRNA. Free m_3G-G dinucleotide could also function in this fashion to compete the binding of a cap binding protein. Experiments showing that a m_3GpppN cap is essential for import do not distinguish between these alternatives.

Table II. Competition of snRNP Nuclear Import

		snRNP elements		Transport competitor	
	RNA Pol	m_3G cap	Sm	T-antigen NLS	m_3G-G
U1	II	+	+	−	+
U2	II	+	+	−	+
U4	II	+	+	−	+
U5	II	+	+	−	+
U3	II	+	−	−	−
U6	III	−	−	+	−

The key to resolving this issue will be the biochemical identification and functional characterization of the m_3GpppN cap binding factor. Although m^7GpppN cap binding proteins have been identified (Rhoads, 1988; Ohno et al., 1990), a cytoplasmic m_3GpppN cap binding factor has not been found. In fact, indirect evidence argues against a stable m_3GpppN cap-protein complex. A cap binding protein that remains associated with the U snRNP might be expected to mask the cap, making it inaccessible to antibodies. However, in some cases 80–90% of nuclear U snRNPs could be purified by immunoaffinity chromatography using anti-m_3GpppN cap antibodies (Krainer, 1988). Furthermore, nuclear snRNPs can be localized in tissue culture nuclei by immunofluorescence using anti-cap antibodies (Reuter et al., 1984). Thus, the cap appears to be mostly accessible in the nucleus. These observations support the hypothesis that the m_3GpppN cap is transiently complexed by a transport factor and is not bound by a permanently associated snRNP protein.

Why would U3 snRNA contain a m_3GpppN cap but not be recruited onto the same import pathway as the m_3GpppN-Sm protein containing U snRNAs? Interestingly, U3 snRNA in plants is transcribed by pol III and therefore lacks the m_3GpppN cap found on animal and lower eukaryotic U3 snRNAs (Kiss et al., 1991). Kiss et al. (1991) propose that the promoter conversion from pol II to pol III in plants occurred relatively recently in evolution. When plant U3 snRNA is artificially transcribed by a pol II promoter it has an m_3GpppN cap and is imported as an snRNP into nuclei. If one assumes that the function of U3 snRNP is conserved between plants and animals, even though the cap is not conserved, one might conclude that the cap does not play an essential role in import. This conclusion is supported by our competition results and the unpublished results of S. Baserga, M. Gilmore-Hebert, and X. W. Yang showing that U3 import in *Xenopus* oocytes is m_3GpppN cap independent. The promoter conversion postulated by Kiss et al. (1991) would not, therefore, have initially precluded the nuclear import of U3 snRNP in plants. In contrast, such a conversion would play havoc with the import of m_3GpppN-Sm-containing U snRNPs because they depend on the m_3GpppN cap for import.

Darzynkiewicz et al. (1988) and Kleinschmidt and Pederson (1990) postulated that the hypermethylation of m^7G caps serves to prevent the association of translation factors

with snRNAs. Recently, Hamm et al. (1990) have shown that when U6 snRNA is artificially transcribed by pol II instead of pol III it receives a m^{7}GpppN cap and is exported to the cytoplasm where it remains unhypermethylated. This m^{7}GpppN-capped U6 snRNA is probably anchored to cytoplasmic cap binding proteins in as much as it can be chased into the nucleus by microinjecting large amounts of free m^{7}G-G cap dinucleotide (Fischer et al., 1991). Thus, in animals and lower eukaryotes the hypermethylation of the U3 snRNA pol II cap, which must be mediated by proteins other than the Sm proteins, may function to prevent cytoplasmic anchoring. Alternatively, it may serve a functional role in the nucleus. For example, artificially m$_3$GpppN-capped plant U3 snRNP, although imported, is unable to assemble onto preribosomal particles (Kiss et al., 1991).

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