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Reverse 5′ caps in RNAs made in vitro by phage RNA polymerases

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ABSTRACT

We show that about one-third of the RNAs produced in vitro by viral RNA polymerases in the presence of m^7GpppG dinucleotides have unusual 5′ caps. In these RNAs, the initiating dinucleotide is incorporated in an orientation opposite to that expected so that the 7-methyl guanine (m^7G) nucleotide is adjacent to the body of the RNA, making a "reverse" cap. The doubly methylated dinucleotide, m^7GpppG$_m$, containing a 2′ *O*-methylated guanine (G$_m$) is incorporated only in the reverse orientation. Precursors of U1 snRNAs containing reverse caps are recognized by antibodies specific for the m^7G cap structure. When injected into *Xenopus laevis* oocyte nuclei, reverse-capped pre-U1 RNAs are exported considerably more slowly than normal. Furthermore, U1 RNAs with reverse caps exhibit a striking defect in nuclear import that can be attributed to the failure of reverse caps to be hypermethylated to $m^{2,2,7}$G caps. Thus, the presence of reverse-capped RNAs in RNA preparations may affect conclusions about the efficiency and extent of certain m^7G cap-dependent processes.

Keywords: in vitro transcription; m^7G caps; RNA transport; SP6, T7, T3 viral RNA polymerases; *Xenopus laevis* oocytes

INTRODUCTION

The 5′ ends of RNA polymerase II transcripts contain 5′–5′ triphosphate-linked guanine caps and ribose 2′ *O*-methylated nucleotides (Wei & Moss, 1975; Shatkin, 1976; Banerjee, 1980). Although a functional role for the ribose methylations is yet to be determined, the m^7G cap structure of mRNAs and snRNAs influences several cellular events, such as their stability (Furuichi et al., 1977; Shimotohno et al., 1977), splicing (Konarska et al., 1984; Edery & Sonenberg, 1985; Ohno et al., 1987; Patzelt et al., 1987; Inoue et al., 1989), translation (Muthukrishnan et al., 1975; Adams et al., 1978; Shatkin, 1985), and nuclear export (Hamm & Mattaj, 1990; Izaurralde et al., 1992, 1995; Terns et al., 1993a).

The m^7G cap structure of precursor U1 small nuclear RNA is essential for maturation of this molecule into a functional RNP complex (U1 snRNP) capable of participating in splicing. Following synthesis by RNA polymerase II (Dahlberg & Lund, 1988), pre-U1 RNA is incorporated into a relatively large RNA–protein complex termed the pre-export U1 snRNP (Terns et al., 1993b). Efficient export of pre-U1 RNA requires both the 5′ m^7G cap, which is recognized by a nuclear cap-binding complex (CBC) (Izaurralde et al., 1992, 1995), and structural features within the RNA itself (Terns et al., 1993a). Once in the cytoplasm, the RNA associates with the Sm-core proteins (Parry et al., 1989; Lührmann et al., 1990), a prerequisite for hypermethylation of the m^7G cap to $m^{2,2,7}$G (Mattaj, 1986). This latter structure is required for import of U1 and several other snRNPs into nuclei (Fischer et al., 1991; Marshallsay & Lührmann, 1994). Finally, the snRNP is imported back into the nucleus (Mattaj, 1988) where additional protein binding (Feeney & Zieve, 1990) and RNA 3′ end trimming (Yang et al., 1992) occur to produce the mature U1 snRNP. Thus, the 5′ cap structure plays a key role in both the export and import of snRNAs.

Study of the role of m^7G caps in RNA function and processing requires the efficient production of RNAs containing the m^7G cap structure. Typically, this is done by synthesizing transcripts in vitro using viral RNA polymerases (SP6, T3, T7) (Melton et al., 1984; Yisraeli & Melton, 1989) in the presence of dinucleotide cap analogues like m^7GpppG and m^7GpppG$_m$. Although these polymerases efficiently use the m^7GpppG

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dinucleotide to initiate RNA synthesis (Konarska et al., 1984), the precise structure of the 5′ ends of the product RNAs have not been analyzed carefully.

In this study, we show that m^7GpppG and m^7GpppG_m dinucleotides can be incorporated into RNA in the "reverse" orientation (i.e., $Gpppm^7G$- and G_mpppm^7G-, respectively). In fact, we never observe m^7GpppG_m incorporated into RNA in the normal orientation. We also show that RNAs containing reverse caps are recognized efficiently by antibodies specific for the m^7G cap structure, but apparently not by some cellular proteins that interact with caps. Consequently, reverse-capped pre-U1 RNAs injected into nuclei of *Xenopus* oocytes are exported 2–3-fold more slowly than are RNAs containing normal caps. Moreover, once in the cytoplasm, pre-U1 RNAs with reverse caps are not matured and, hence, are not imported back into the nucleus.

RESULTS

RNA polymerases of phages SP6, T7, and T3 are often used to synthesize RNAs with 5′ m^7G cap structures similar to those of mRNAs and precursors of small nuclear RNAs (pre-snRNAs). Although these enzymes have a strict requirement for GTP as the initiating nucleotide (Melton et al., 1984), this GTP can be replaced by a dinucleotide like NpppG, in which the γ-phosphate position of the GTP is derivatized. If the derivatizing group is m^7G, an RNA can result that has a cap resembling the caps at the 5′ ends of RNA polymerase II transcripts (e.g., $m^7GpppGpNp$ . . .). For clarity, we refer to the nucleotides that are linked to the body of the RNA through a 3′–5′-phosphodiester or through a triphosphate bridge as being in the α- or γ-positions of the cap ($G^{\gamma}ppp^{\alpha}G$-RNA), respectively.

Because of the quasi-symmetric nature of m^7GpppG and because both of its bases can pair with dC in a template, this dinucleotide presumably could be used in either orientation by the polymerases. Consequently, the resulting RNA would have either m^7G or G at the γ-position of its 5′ terminus (Table 1). To test if that occurred, we analyzed the 5′ caps of RNAs made in vitro using m^7GpppG as the initiating nucleotide.

5′ cap structures of RNAs synthesized in vitro using viral polymerases plus cap analogue dinucleotides

The orientations of the 5′ caps of RNAs made in vitro were determined by digestion of ^{32}P-labeled transcripts with various nucleases and analysis of the 5′ products by TLC or PAGE. Consistent with previous reports (Konarska et al., 1984), RNase T2 digestion of RNAs made by SP6 RNA polymerase in the presence of GpppG or m^7GpppG cap dinucleotides showed that the RNAs were initiated almost exclusively with the dinucleotides, rather than with GTP (Fig. 1A,C). However, RNA initiated with m^7GpppG had two forms of 5′ cap, as revealed by prior digestion with tobacco acid pyrophosphatase (TAP), which cleaved the 5′–5′ triphosphate bridge of the cap structure (compare Fig. 1B and D). These two 5′ end products were identified as pGp and pm^7Gp (see legend to Fig. 1), showing that in vitro-synthesized SP6 transcripts were initiated with caps in both the normal (m^7GpppG-) and reverse ($Gpppm^7G$-) orientations (Table 1). Comparable results were obtained using T3 (Fig. 1E, F) and T7 (data not shown) viral RNA polymerases to transcribe their respective templates in the presence of the m^7GpppG dinucleotide.

To quantitate the proportion of RNAs made by SP6 RNA polymerase that have reverse caps, pre-U1 RNA (made in the presence of $\alpha[^{32}P]$-ATP and -UTP) was digested with RNase T1 and analyzed by PAGE (Fig. 2). The encoded sequence at the 5′ end of this transcript is (5′) cap-GpApApUpApCpUpUpApCpCpUpGp (Table 1), so if the first nucleotide were a nonmethylated G, RNase T1 would cleave after the first and 13th nucleotides to produce a unique 12-mer oligonucleotide. However, if the G in the α-position of the cap contained a methyl group at the N-7 of the guanine (m^7G) or at the 2′ O of the ribose (G_m), it would be resistant to cleavage by RNase T1 and a longer oligonucleotide

TABLE 1. Expected 5′ end products of pre-U1 RNA generated by enzymatic and chemical treatments.[a]

Initiating dinucleotide	Possible pre-U1 RNA 5′ end sequences	Observed yield (%)	Expected ^{32}P-labeled 5′ end digestion products: T2	TAP → T2	T1	TAP → T1	Ox/β → T2
m^7GpppG	$m^7GpppGp^*Ap^*(Np)_{10}Gp$	~60	$m^7GpppGp^*$	pGp^*	$m^7GpppGp^* + Ap^*(Np)_{10}Gp$	$pGp^* + Ap^*(Np)_{10}Gp$	$pppGp^*$
	$Gpppm^7Gp^*Ap^*(Np)_{10}Gp$	~35	$Gpppm^7Gp^*$	pm^7Gp^*	$Gpppm^7Gp^*Ap^*(Np)_{10}Gp$	$pm^7Gp^*Ap^*(Np)_{10}Gp$	$pppm^7Gp^*$
m^7GpppG_m	$m^7GpppG_mp^*Ap^*(Np)_{10}Gp$	<1	$m^7GpppG_mp^*Ap^*$	$pG_mp^*Ap^*$	$m^7GpppG_mp^*Ap^*(Np)_{10}Gp$	$pG_mp^*Ap^*(Np)_{10}Gp$	$pppG_mp^*Ap^*$
	$_mGpppm^7Gp^*Ap^*(Np)_{10}Gp$	~80	$_mGpppm^7Gp^*$	pm^7Gp^*	$_mGpppm^7Gp^*Ap^*(Np)_{10}Gp$	$pm^7Gp^*Ap^*(Np)_{10}Gp$	$_mGpppm^7Gp^*$
GpppG	$GpppGp^*Ap^*(Np)_{10}Gp$	~95	$GpppGp^*$	pGp^*	$GpppGp^* + Ap^*(Np)_{10}Gp$	$pGp^* + Ap^*(Np)_{10}Gp$	$pppGp^*$

[a] Relevant phosphates labeled by $\alpha[^{32}P]$-ATP and $\alpha[^{32}P]$-UTP are indicated by the asterisks (*). [b] The observed 5′ structures of the in vitro capped RNAs were quantitated as percentages (%) of the total RNA products as in Figure 2; T1 or T2, RNase T1 or RNase T2, respectively; TAP, tobacco acid pyrophosphatase; Ox/β, periodate oxidation of 2′, 3′ *cis* diols followed by β-elimination.

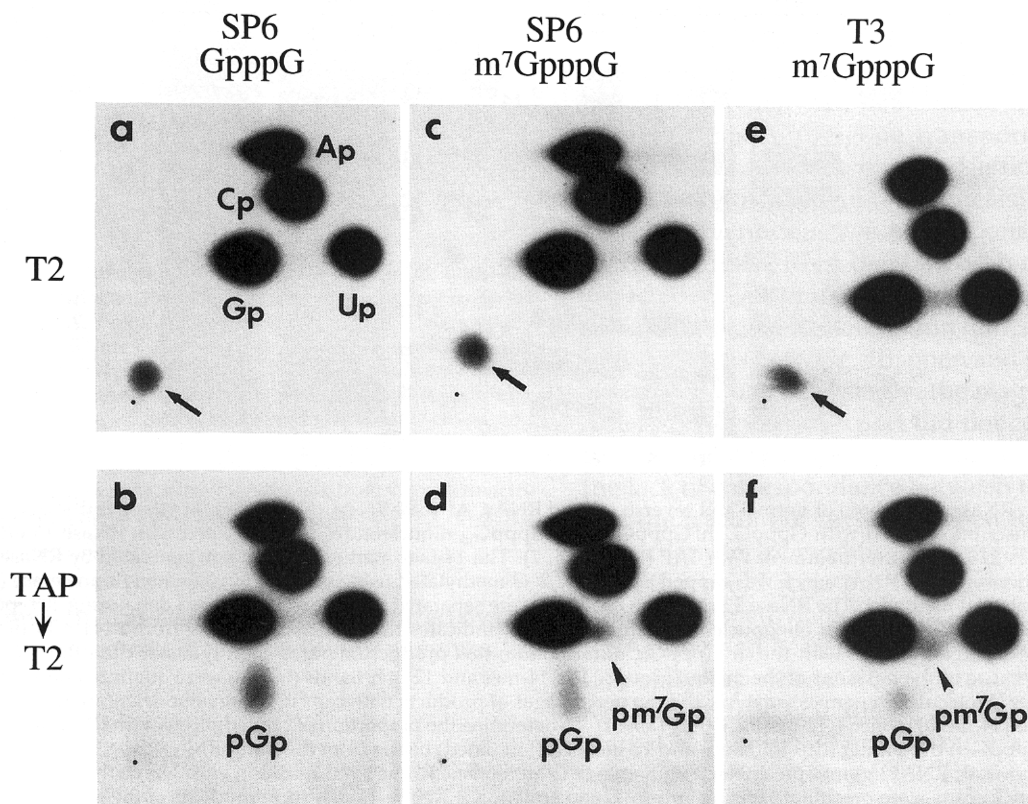

FIGURE 1. 5′ end structures of capped RNAs transcribed in vitro. RNAs were made in vitro with SP6 (**A–D**), or T3 (**E**, **F**) RNA polymerases in the presence of α[^{32}P]-ATP plus α[^{32}P]-UTP (SP6) or α[^{32}P]-GTP (T3) and with GpppG (**A**, **B**) or m^{7}GpppG (**C–F**) as initiating nucleotides. RNAs were digested with RNase T2 either alone (**A**, **C**, **E**) or after treatment with TAP (**B**, **D**, **F**). Digestion products were separated by two-dimensional TLC using an acidic second-dimension solvent. Dinucleotide 5′ end products are indicated with arrows and a dot indicates each origin. Assignment of pm^{7}Gp (Nishimura, 1972) was confirmed by TAP plus alkaline phosphatase digestion of the RNA made in the presence of m^{7}GpppG prior to treatment with RNase T2 (data not shown).

would be generated. As illustrated in Figure 2A, a slowly migrating band (corresponding to a 14-mer) was produced by RNase T1 digestion of pre-U1 RNA made with m^{7}GpppG (lane 4), but not with GpppG (lane 2). The 14-mer band was not observed when the RNA was treated with TAP prior to RNase T1 digestion (lane 5); instead, a 13-mer was generated that co-migrated with a 13-mer from elsewhere in the molecule. Thus, the 14-mer originated from the 5′ ends of transcripts whose caps were in the reverse orientation (Table 1). Quantitation of the 14-mer and the 12-mer bands (lane 4) showed that about one-third of the transcripts that were initiated with the m^{7}GpppG dinucleotide had reverse caps.

Given that the cap dinucleotide can be used in either orientation, we asked why the two orientations were not equally represented in the products. One explanation is that the pK_a of the N-1 position of m^{7}G is about 7.5 (Rhoads et al., 1983), so a significant fraction of this nucleotide would not be protonated under the conditions of RNA synthesis and, thus, would not be able to pair with dC in the DNA template (Hendler et al., 1970). To test that model, transcription reactions were performed at various pHs and the fractions of RNAs with normal or reverse caps were determined by quantitation of 5′ terminal 12-mer and 14-mer RNase T1 oligonucleotides, as in Figure 2A. As predicted, the percentage of RNA transcripts with reverse caps increased to close to 50% as the pH of the reaction decreased (Table 2). Because the m^{7}GpppG dinucleotide can be incorporated in the reverse orientation only when the m^{7}G is protonated, the ratios of transcripts with reverse or normal caps can be used to calculate the apparent pK_a of this group at each of the different pHs tested (see footnote to Table 2). Surprisingly, the calculated pK_a values varied with the pHs of the transcription reactions, perhaps as a result of local conditions induced by the RNA polymerase. Additionally, the positive charge of the protonated m^{7}G could affect the ability of the enzyme to bind the dinucleotide.

Phage RNA polymerases normally cannot incorporate 2′ *O*-methylated nucleoside triphosphates (pppN$_m$)

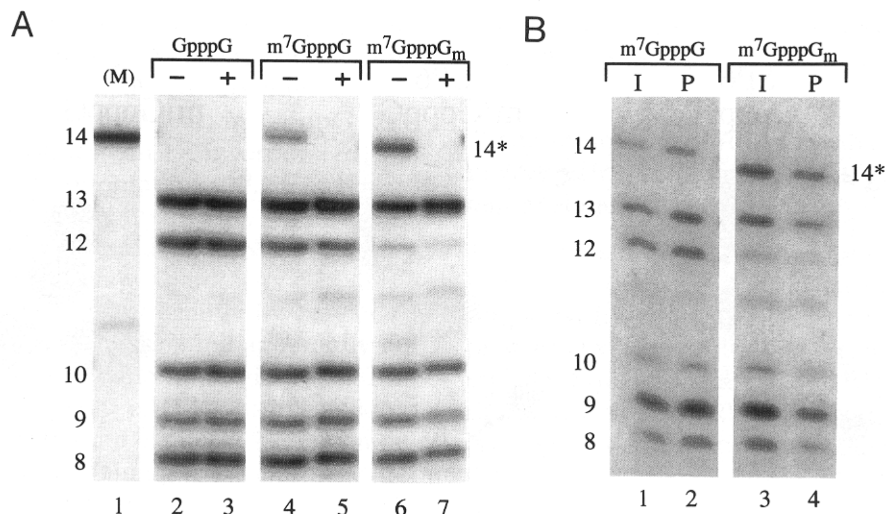

FIGURE 2. RNase T1 analysis of normal and reverse-capped RNAs. **A:** Total RNAs. ^{32}P-labeled, in vitro synthesized pre-U1$_{Sm-}$ transcripts initiated with GpppG, m^{7}GpppG, or m^{7}GpppG$_m$ dinucleotides were digested with RNase T1 either alone (lanes 2, 4, 6) or after treatment with TAP (lanes 3, 5, 7). The 14-mer marker (lane 1) was generated by RNase T1 digestion of pre-U1$_{Sm-}$ RNA, which was capped and ribose 2′ *O*-methylated using vaccinia virus guanylyltransferase (see the Materials and methods). The RNase T1 digestion products were separated by electrophoresis in a denaturing 20% polyacrylamide gel and the relevant oligonucleotides are shown. 14* indicates the 5′ 14-mer resulting from RNase T1 digestion of pre-U1 RNA capped with the m^{7}GpppG$_m$ dinucleotide; this product migrates slightly faster than the other 5′ 14-mers because of the positions of the methyl groups. The 14-mer and 12-mer bands (lane 4) were quantitated by PhosphorImager (Molecular Dynamics) analysis and the percentages of products with caps in the reverse orientation were determined as [14-mer]/[14-mer + 12-mer] × 100% (Table 1). To determine the proportion of capped products in RNAs initiated with m^{7}GpppG$_m$, the 14-mer (cap-initiated) and 12-mer (GTP-initiated) bands from the input RNA (lane 6) were quantitated as above. **B:** RNAs immunoprecipitated with anti-m^{7}G antibodies. RNAs synthesized in vitro with either m^{7}GpppG or m^{7}GpppG$_m$ caps were precipitated using anti-m^{7}G cap antibodies. RNase T1 digestion products of the input (I, lanes 1, 3) and precipitate (P, lanes 2, 4) fractions were separated by gel electrophoresis as in A. The absence of the 12-mer in the precipitate RNA in lane 4 indicates that this oligomer was generated from uncapped (pppG-initiated) molecules.

near the 5′ ends of transcripts (Conrad et al., 1995). Therefore, the doubly methylated cap analogue, m^{7}GpppG$_m$, should be incorporated only in the reverse orientation (denoted G$_m$pppm^{7}G-). Analysis of the 5′ end products generated by RNase T1 (Fig. 2A, lane 6) digestion showed that only about 80% of the transcripts made in the presence of this dinucleotide contained any cap. As expected, the m^{7}GpppG$_m$ dinucleotide was incorporated only in the reverse orientation (i.e., G$_m$pppm^{7}G-), because RNase T2 digestion produced G$_m$pppm^{7}Gp, but not m^{7}GpppG$_m$pAp, and TAP plus RNase T2 digestion produced pm^{7}Gp, but not pGmpAp (Fig. 3A,B, respectively, and data not shown). Consistent with these results, RNAs made with m^{7}GpppG$_m$, unlike those made with m^{7}GpppG, were resistant to oxidation/β-elimination of their 5′ cap nucleoside (Fig. 3C and data not shown; Table 1).

TABLE 2. Effect of pH during in vitro transcription on the percentages of products with reverse caps.[a]

Observed pH	% RNAs with reverse caps	Calculated pK_a
6.6	48%	8.0
7.3	43%	8.0
7.5	35%	7.9
8.4	28%	8.5

[a] SP6-transcription reactions of pre-U1$_{Sm-}$ RNAs using m^{7}GpppG as initiating dinucleotide were performed at the indicated pHs (see the Materials and methods). Products were digested with RNase T1 and analyzed as in Figure 2 (data not shown). pK_a values were calculated by assuming that the acid form (A) of m^{7}GpppG could be incorporated equally well in either the normal or reverse orientations and the base form (B) could be incorporated only in the normal orientation. Thus, the fraction of the reverse capped RNAs (R) is given by: $R = 1/2(A)/(A + B)$. This equation can be written as $B = A(1 - 2R)/2R$, so the Henderson–Hasselbalch equation becomes: $\mathrm{pH} = \mathrm{p}K_a + \log(1 - 2R)/2R$.

Effect of position of the m^{7}G structure in RNA on protein recognition

In normal caps, the m^{7}G nucleotide is in the γ-position, separated from the body of the RNA by a triphosphate bridge. To learn if the m^{7}G nucleotide in the α-position is accessible to protein recognition, we used polyclonal antibodies specific for the m^{7}G cap (Munns et al., 1982) to immunoprecipitate RNAs that contained normal or reverse caps and analyzed the resulting RNAs by RNase T1 digestion. Both forms of the RNA were precipitated (Fig. 2B, lanes 2, 4), showing that the m^{7}G nucleotide in a reverse cap is accessible to proteins. However, these results do not indicate

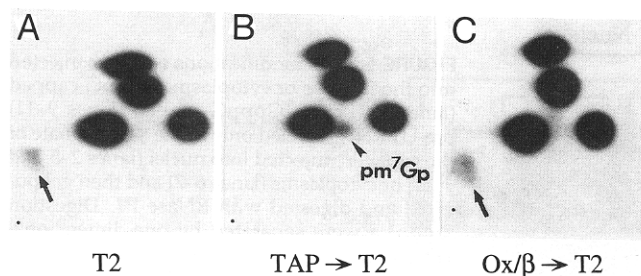

FIGURE 3. 5′ End structure of RNA made in vitro with the m^7GpppG_m cap analogue. Pre-$U1_{Sm^-}$ RNA synthesized in vitro in the presence of $\alpha[^{32}P]$-ATP plus $\alpha[^{32}P]$-UTP and m^7GpppG_m dinucleotide was digested with RNase T2 either alone (**A**), or after treatment with TAP (**B**) or $NaIO_4$ plus lysine (Ox/β) (**C**). Digestion products were analyzed as in Figure 1; the 5′ dinucleotide G_mpppm^7Gp end products are indicated with arrows, pm^7Gp is indicated by an arrowhead, and a dot indicates each origin. The G_mpppm^7Gp assignment in A was verified by TLC analysis using a different second-dimension solvent (neutral pH), which separates this dinucleotide from the potential trinucleotide product m^7GpppG_mpAp (data not shown).

whether reverse caps also would be recognized by cellular cap-binding proteins or modifying enzymes.

Effect of the reverse cap structure on export of pre-U1 RNA.

In earlier studies on the export of snRNAs injected into nuclei of *Xenopus laevis* oocytes, we had noticed a difference in export kinetics between RNAs that had been made in vivo or in vitro. When compared directly in the same batch of oocytes, pre-$U1_{Sm^-}$ RNA made in vitro by SP6 polymerase was exported consistently about one-third as fast as RNA made in vivo (Fig. 4A, solid squares and triangles, respectively). Moreover, pre-U1 RNA that had been made in vitro and subsequently had undergone transport (see legend to Fig. 4A) still was not exported efficiently when reinjected into the nucleus (solid circles). Thus, prior residence in the nucleus was not responsible for the rapid export of pre-U1 RNA that had been made in vivo. As expected, G_mpppm^7G-capped RNAs (100% reverse) were exported more slowly than RNAs with m^7GpppG caps (35% reverse) (Fig. 4B, open and solid squares, respectively) but, surprisingly, the magnitude of the decrease in the rate of export did not correlate with the proportion of RNAs with reverse caps. The reason for the lack of correspondence between the rate of export and the fraction of reverse-capped RNAs in unclear.

To characterize modifications of the 5′ cap of pre-U1 RNA that could occur prior to export from the nucleus, we used unmethylated GpppG- or m^7GpppG-capped RNAs that had been labeled in the γ-phosphate position of the cap structure using guanylyltransferase and $\alpha[^{32}P]$-GTP. The G in the γ-position was fully methylated at N-7 within 30 min of nuclear injection (Fig. 5, lanes 1–3), but most of the caps were not methylated in their ribose 2′ *O*-positions. This lack of 2′ *O*-methylation was independent of the presence of the normal m^7G-methylated cap structure on the injected RNA (compare lanes 3, 9). Furthermore, neither N-7

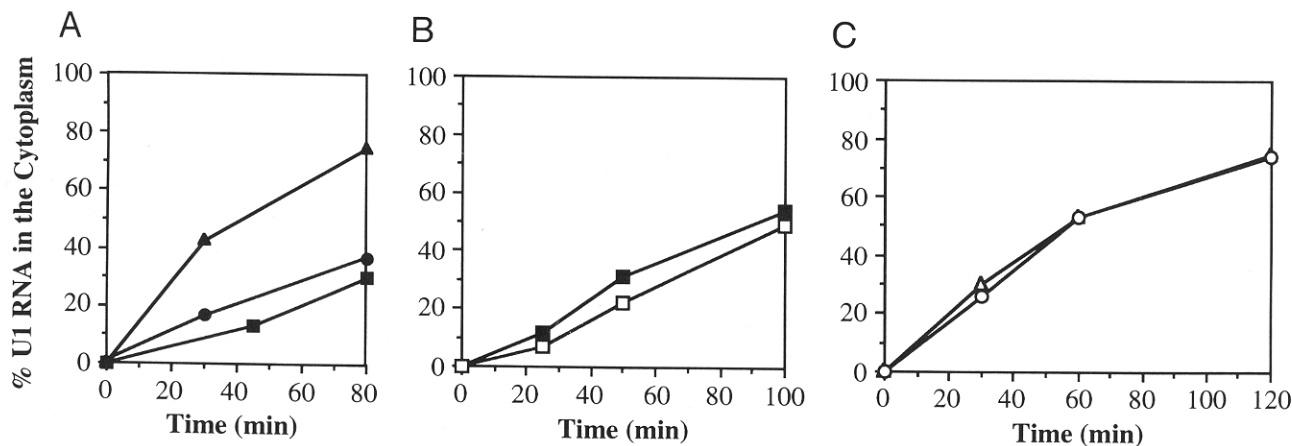

FIGURE 4. Export kinetics of pre-U1 RNAs with normal and reverse caps. **A:** Export of U1 RNAs made in vivo and in vitro. The export substrate, pre-$U1_{Sm^-}$ RNA (which remains in the cytoplasm after export from the nucleus [Hamm et al., 1987]), was made in isolated oocyte nuclei (solid triangles), or by SP6-transcription in the presence of m^7GpppG (solid squares), or by SP6-transcription in the presence of m^7GpppG followed by injection into nuclei and isolation from cytoplasms after export (solid circles). Prior to use in the transport assays, all RNAs were precipitated with anti-m^7G antibodies. The three types of U1 RNAs plus control U3 RNA were injected into oocyte nuclei and, at the indicated times, the labeled RNAs present in the nuclear and cytoplasmic fractions were isolated and analyzed by electrophoresis in a denaturing 8% polyacrylamide gel (data not shown). Nucleocytoplasmic distributions of the injected $U1_{Sm^-}$ RNAs were quantitated by PhosphorImager (Molecular Dynamics) analysis of this gel and the percent U1 RNA in the cytoplasm was calculated as $[C]/[C + N] \times 100\%$. **B:** Export of RNAs containing different percentages of reverse caps. Pre-$U1_{Sm^-}$ RNAs transcribed in vitro in the presence of m^7GpppG, 35% reverse (solid squares), or m^7GpppG_m, 100% reverse (open squares) were injected into oocyte nuclei and their export was analyzed as in A. **C:** Export of RNAs containing m^7GpppG- (open triangles) or GpppG- (open circles) caps. Pre-$U1_{Sm^-}$ RNAs were injected into oocyte nuclei and their export was analyzed as in A.

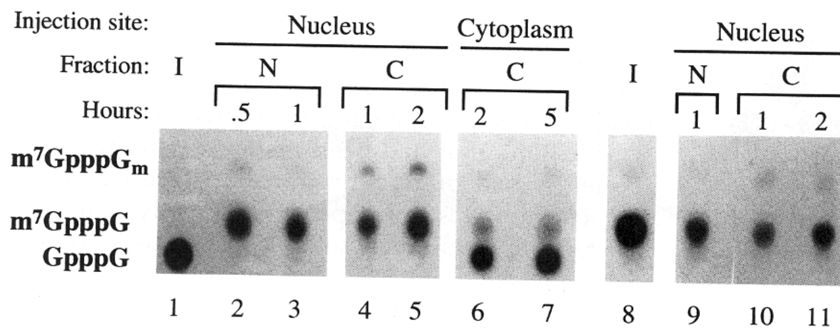

FIGURE 5. Cap modifications of RNAs injected into the nucleus or cytoplasm. GpppG-capped (lanes 2–7) or m^7GpppG-capped (lanes 9–11) pre-U1 RNAs labeled only in the γ-phosphate of the cap were injected into nuclei (lanes 2–5 and 9–11) or cytoplasms (lanes 6–7) and then gel purified and digested with RNase P1. Digestion products were separated by one-dimensional TLC; labeled cap products are indicated. Lanes 1 and 8 show the input (I) RNAs.

methylation (lanes 6, 7) nor 2′ *O*-methylation (lanes 4, 5 and lanes 10, 11) was efficient in the cytoplasm.

The rate at which N-7 methylation of GpppG-capped RNAs occurred indicated that this step would not retard export of the RNA. Indeed, this RNA was exported as fast as pre-U1 RNA containing fully normal caps (Fig. 4C). Because the rapidly exported RNAs with normal caps had very few 2′ *O*-methyl groups in their caps (Fig. 5, lanes 9–11), that modification cannot be required for efficient export. Therefore, the slow kinetics of export of pre-U1 RNAs containing reverse caps most likely is due to the presence of m^7G in the α-position.

Effect of reverse caps on nuclear import of U1 RNA

Upon export from the nucleus, wild-type pre-U1 RNA associates with stored cytoplasmic Sm-core proteins (Parry et al., 1989; Lührmann et al., 1990), and the γ-m^7G of its cap is trimethylated to $m^{2,2,7}$G (Mattaj, 1986); in *X. laevis* oocytes, this modification is required as a signal for subsequent import of U1 snRNPs back into the nucleus (Fischer et al., 1991; Marshallsay & Lührmann, 1994). We asked if pre-U1 RNAs with reverse caps also could be hypermethylated and imported. Preparations of wild-type pre-U1 RNA with 0%, 35%, or 100% reverse caps were injected into oocyte cytoplasms and the nucleocytoplasmic distributions of the RNAs were examined (Fig. 6A). Although RNAs with normal caps were efficiently imported into the nucleus (lanes 2–5), virtually none of the molecules with reverse caps were (lanes 11–14). Moreover, in a mixed pool of U1 RNAs with reverse and normal caps (lanes 7–10), only the molecules with normal caps entered the nucleus, as determined by two-dimensional RNase T1 fingerprinting of the U1 RNAs from cytoplasmic and nuclear fractions (data not shown). Reverse-capped RNA that had been injected into the nucleus, to allow for possible N-7 methylation of the γ-G, also remained in the cytoplasm after export (Fig. 6B, lanes 4, 5). We conclude that U1 RNAs with the m^7G in the α-position are inappropriate substrates for the snRNP import machinery regardless of whether the RNAs were injected directly into the cytoplasm or were exported from the nucleus.

We probed the reverse-capped RNAs that had been exported to the cytoplasm in order to determine why they were not imported. These RNAs were efficiently co-immunoprecipitated by anti-Sm antibodies (Fig. 6C, lanes 3, 4), showing that they were able to associate with Sm proteins. In contrast, the RNAs were not precipitable by anti-$m^{2,2,7}$G antibodies (lanes 1, 2), but remained precipitable by anti-m^7G antibodies (lanes 5, 6), indicating that hypermethylation of the cap had not occurred. We therefore attribute the lack of import to the inability of cells to modify the reverse cap into an $m^{2,2,7}$G-containing cap structure.

DISCUSSION

We have shown that a significant proportion of the m^7G-capped RNA made in vitro by viral RNA polymerases has an unusual cap. In these RNAs, the initiating m^7GpppG dinucleotide is incorporated with the methylated G proximal to the body of the RNA (reverse cap). The reverse cap has dramatic effects upon the transport of RNA: pre-U1 RNAs containing reverse caps are exported significantly more slowly than RNAs with normal caps and, more strikingly, such RNAs are not imported back into the nucleus.

Production of reverse cap structures in RNAs made in vitro

The incorporation of the dinucleotide in both the normal and reverse orientations is consistent with the fact that both G and m^7G can base pair with dC. However, the products did not contain equimolar mixtures of molecules with caps in both orientations, with only about one-third of them having reverse caps. The preference for incorporation of this dinucleotide in the normal orientation can be explained by the pK_a of m^7G. Around pH 7.5, a significant proportion this nucleotide cannot form a hydrogen bond with dC in the template as a result of dissociation of a proton from its N-1 position (Hendler et al., 1970). Assuming that the reverse-

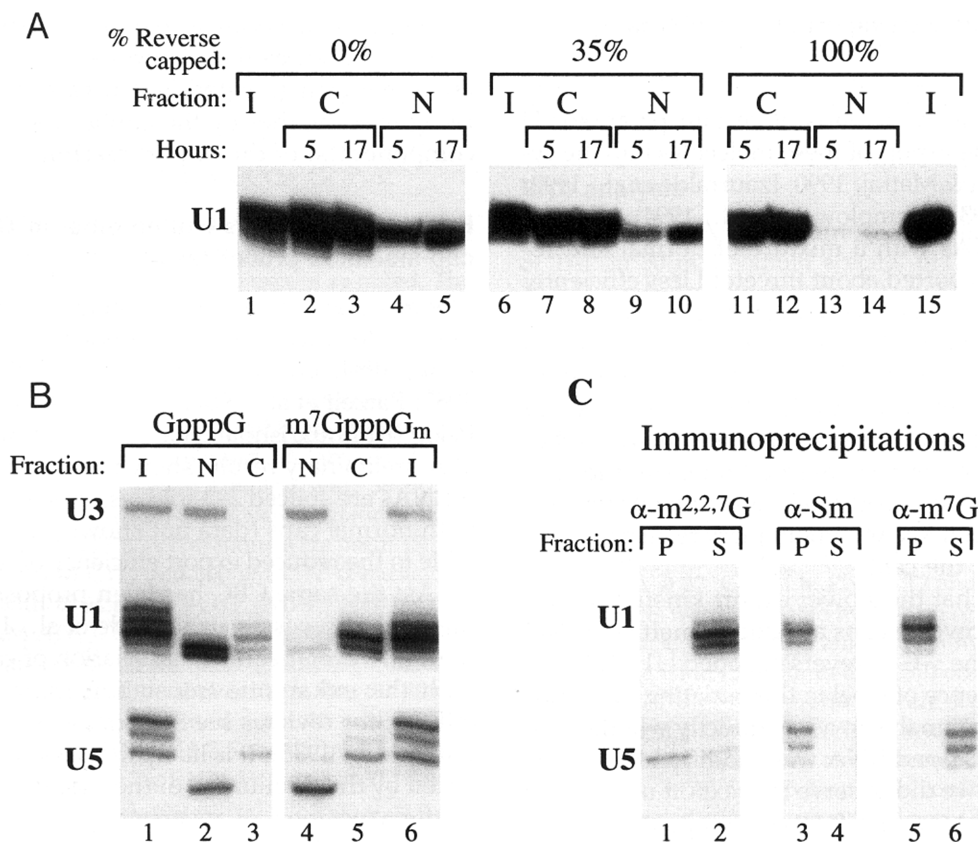

FIGURE 6. Nuclear import and cap-hypermethylation of U1 RNAs with normal and reverse caps. **A:** Transport of U1 RNAs injected into the cytoplasm. Wild-type pre-U1 RNAs (Sm+) with different percentages of reverse caps, 0% (lanes 2–5), 35% (lanes 7–10), and 100% (lanes 11–14), were injected into oocyte cytoplasms and the nucleocytoplasmic distributions (N and C) were analyzed with time by electrophoresis in a denaturing 8% polyacrylamide gel. Lanes 1, 6, and 15 show the input (I) RNAs. **B:** Transport of U1 RNAs injected into the nucleus. ^{32}P-labeled, pre-U1 RNAs synthesized in vitro in the presence of GpppG or m^{7}GpppG$_m$ dinucleotides were injected into oocyte nuclei along with control U3 and U5 RNAs. RNAs were isolated after 2.5 h and analyzed as in A. Lanes 1 and 6 show the input (I) RNAs. **C:** Immuno-precipitation analyses. Samples of RNAs shown in lane 5 of B (i.e., isolated from the cytoplasm 2.5 h after nuclear injection) were immunoprecipitated with anti-m^{2,2,7}G antibodies (lanes 1, 2). RNAs from a similar experiment, but isolated 6 h after nuclear injection, were immunoprecipitated with anti-m^{7}G antibodies (lanes 5, 6); extracts from this time point also were used for co-immunoprecipitation of the RNAs with anti-Sm antibodies (lanes 3, 4). Precipitate (P) and supernatant (S) fractions were analyzed by electrophoresis in denaturing 8% polyacrylamide gels.

capped products result only from incorporation of the protonated form of m^{7}G, a decrease in the fraction of molecules with reverse caps at higher pHs would indicate that the dinucleotide is exposed to the reaction environment at the time of nucleotide discrimination. When the reaction pH was increased, this fraction did decrease (Table 2). However, the apparent pK_a of m^{7}G calculated from the fraction of reverse-capped RNAs made at each pH showed that the enzyme did influence the extent of protonation of the bound nucleotide, at least at the highest pH tested (8.4). That could occur by direct interaction between the positively charged nucleotide and amino acids of the enzyme, or by stabilization of the protonated form through enzyme-promoted base pairing with the template.

Consistent with previous reports that viral RNA polymerases do not utilize 2′ *O*-methylated nucleoside triphosphates efficiently (Conrad et al., 1995), we find that the m^{7}GpppG$_m$ cap analogue is incorporated only in the reverse orientation (i.e., G$_m$pppm^{7}G-) (Fig. 3). Also, in the presence of this doubly methylated dinucleotide, the fraction of transcripts initiated with a cap is reduced to less than 80% (Fig. 2), compared to greater than 95% capped RNA made when using GpppG or m^{7}GpppG (Fig. 1). Presumably, this decrease in capping, even in the presence of a 10-fold molar excess of dinucleotide over GTP, is due to a twofold or greater reduction in the concentration of utilizable dinucleotide. The viral RNA polymerases incorporate 2′ *O*-methylated NTPs, but only in the presence of Mn^{+2} and not near 5′ ends of the RNA transcripts (Conrad et al., 1995). Therefore, it is unlikely that use of m^{7}GpppG$_m$ dinucleotide could result in the production of any m^{7}GpppG$_m$-capped RNA.

Nucleocytoplasmic transport of U1 snRNAs with reverse caps

The m^7G caps at the 5′ ends of certain precursor U snRNAs promote export of these molecules into the cytoplasm (Hamm & Mattaj, 1990; Izaurralde et al., 1992; Terns et al., 1993a; Jarmolowski et al., 1994). We find that pre-U1 RNAs with a mixture of normal and reverse caps are exported about threefold less efficiently than RNAs with exclusively normal caps (Fig. 4A). Surprisingly, the export rates for samples of U1 RNAs containing 100% or only 35% reverse caps were similar (Fig. 4B). It is unclear whether the reverse-capped U1 RNAs interfere with the export of normally capped U1 RNAs or whether the observed rates are the sum of two independent export rates (affecting normal versus reverse-capped RNAs). Preliminary results indicate that the latter is the case.

It is possible that the slower export kinetics of pre-U1 RNAs with reverse caps are due to inefficient N-7 methylation of the γ-G in reverse-capped U1 RNA. Because of the absence of label in the initiating cap dinucleotide, we were unable to assay directly whether the γ-G in reverse-capped RNA was modified before export. However, we did observe that export of U1 RNA preparations containing 35% reverse caps was not accelerated by prior participation in nucleocytoplasmic transport (Fig. 4A). The caps of U1 RNAs made in vivo are fully 2′ *O*-methylated, but U1 RNAs made in vitro with normal m^7G caps are exported efficiently (Fig. 5C) without ribose modifications (Fig. 6, lanes 9–11). Thus, it is unlikely that the lack of 2′ *O*-methylation of U1 RNAs with reverse caps is responsible for the slow export of these RNAs.

Another possibility is that the N-7 methylation is efficient on reverse-capped RNA, but the resulting m^7Gpppm^7G-RNA does not interact correctly with the export machinery. The pre-export U1 snRNP is a large complex (Terns et al., 1993b), whose assembly may be affected by whether the RNA cap is in the normal or reverse orientation. If the cap-binding protein complex (CBC), which facilitates export of m^7G-capped U1 snRNAs (Izaurralde et al., 1992, 1995), binds poorly to molecules with reverse caps, RNA export could be retarded. Alternatively, if the CBC has a greater affinity for RNAs with caps in the reverse orientation, the resulting tight RNP complex could reduce other protein interactions necessary for efficient transport of U1 RNA out of the nucleus.

The inability of reverse-capped U1 snRNA to enter the nucleus, despite its association with Sm-core proteins (Fig. 6), emphasizes the importance of the 5′ cap structure for maturation of this RNA. In *Xenopus* oocytes, hypermethylation of the m^7G cap to the $m^{2,2,7}G$ structure is obligatory for import of U1 snRNA (Fischer et al., 1991; Marshallsay & Lührmann, 1994). Presumably, U1 RNAs with reverse caps fail to become hypermethylated because they are not precipitated by anti-$m^{2,2,7}G$ antibodies. However, it is possible that an abnormal trimethylated 5′ structure is created that is recognized neither by the antibodies nor by a crucial component(s) of the import machinery.

Effect of the reverse cap on other m^7G cap-mediated processes

The m^7G cap influences the metabolism and function of mRNAs, including their proper splicing (Konarska et al., 1984; Edery & Sonenberg, 1985; Ohno et al., 1987; Patzelt et al., 1987; Inoue et al., 1989) and translation (Muthukrishnan et al., 1975; Adams et al., 1978). Our preliminary results show that reverse-capped pre-mRNAs are spliced 2–3-fold less efficiently than RNAs with normal caps (data not shown), an effect comparable to the reduced export efficiency of reverse-capped RNAs; the same CBC has been proposed to mediate these two processes (Izaurralde et al., 1994). Translation, however, requires association of capped mRNA with the eukaryotic translation initiation factor 4E (eIF-4E; for reviews see Shatkin, 1985; Rhoads, 1988; Sonenberg, 1988). It is likely that this complex is influenced by the orientation of the cap; the unmodified G in the γ-position of reverse-capped RNAs not only lacks the N-7 methyl group, but also is not ionized under physiological pH conditions and both of these properties appear to be critical for eIF-4E recognition of capped mRNAs (Rhoads et al., 1983; Darzynkiewicz et al., 1988). We propose that the 35% of RNAs with reverse caps account for the 35% of RNAs made in vitro that are not recognized by the Protein A–eIF-4E fusion protein (Edery et al., 1995). Moreover, a failure of eIF-4E to bind reverse-capped RNAs would explain the poor translation of mRNAs made in vitro in the presence of the m^7GpppG_m cap analogue (Drummond et al., 1985). Thus, the presence of reverse caps in RNA preparations could greatly influence interpretations about several important steps in gene expression. With an awareness of these effects, reverse-capped RNAs could be used as novel tools for examining the dynamic RNA–protein interactions moderated by the m^7G cap structure.

MATERIALS AND METHODS

In vitro RNA synthesis

Templates for in vitro RNA synthesis were DNA fragments generated by PCR amplification of the RNA-coding regions of various snRNA genes. 5′ primers contained either SP6 or T7 RNA polymerase promoter sequences; SP6-$U1_{wt}$ and SP6-$U1_{Sm^-}$ and SP6-U3 (Terns et al., 1993a, 1995) have been described previously. The T7-$U1_{S7}$ (Sturchler et al., 1992) was provided by A. Krol. The template containing the T3 promoter was the Δ3′ss template of BSAd1 (Hamm & Mattaj, 1990). The SP6-$U5_{wt}$ template was produced using the

X. laevis U5.11H gene (Kazmaier et al., 1987) and the following primer pairs (C. Grimm, pers. comm.):

5′-GGA ATT CGA TTT AGG TGA CAC
TAT AGA ATA CTC TGG TTT CT-3′

5′-AGT ACC TGG TGT GAA CCA GGC-3′.

In vitro transcription using viral RNA polymerases (Melton et al., 1984) was performed according to the enzyme manufacturer's conditions (Promega), except as noted. The standard 20-μL reactions included: 40–80 ng of DNA template; 1× transcription buffer (40 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 10 mM NaCl, 2 mM spermidine); 2 μg bovine serum albumin (BSA); 10 mM DTT; 40 U RNasin (Promega); 20 U SP6 or T7 or T3 RNA polymerases; 1 mM GpppG or 1 mM m^7GpppG (both from New England Biolabs) or 1 mM m^7GpppG$_m$ (Pharmacia). When labeling with 30 μCi of α[^{32}P]-ATP and 20 μCi of α[^{32}P]-UTP (New England Nuclear [NEN]), the following NTP concentrations were used: 150 μM ATP and UTP, 300 μM CTP, and 100 μM GTP (Pharmacia). When labeling with 20 μCi α[^{32}P]-GTP (NEN), NTP concentrations of 500 μM ATP, UTP, and CTP and 50 μM GTP were used. The 1× transcription buffers for synthesis at different pHs (Table 2) were the same as above except that the 40 mM Tris-HCl, pH 7.5, was substituted with 40 mM MOPS, pH 6.8, or 40 mM Tris-HCl, pH 7.6, or 40 mM Tris-HCl, pH 8.5. The actual pHs during RNA synthesis at 37 °C were measured as 6.6, 7.3, and 8.4, respectively. It should be noted that transcription reactions performed at pHs different from the optimal pH (7.5) resulted in decreased total yields of RNA products.

In vitro capping of unlabeled transcripts with α[^{32}P]-GTP using guanylyltransferase from vaccinia virus (Gibco, BRL) was done as described previously (Terns et al., 1995) in the presence or absence of 50 μM *S*-adenosyl methionine (SAM) to create the m^7GpppG or GpppG cap structures, respectively. The guanylyltransferase preparation also contained ribose 2′ *O*-methyltransferase activity, so that a small fraction of the products were m^7GpppG$_m$-capped (Fig. 5B, lane 8). For preparation of fully ribose 2′ *O*-methylated RNA (Fig. 2, lane 1), 100 fmol of m^7GpppG cap-labeled pre-U1$_{Sm^-}$ RNA (see above) were incubated in a 10-μL reaction volume containing 50 μM SAM, 50 mM Tris-HCl, pH 7.8, 1.25 mM $MgCl_2$, 6 mM KCl, 2.5 mM DTT, 1 μg BSA, 20 U RNasin and 4 U vaccinia virus guanylyltransferase (Gibco, BRL) for 2 h at 37 °C. The RNA was phenol extracted and ethanol precipitated before use.

In vivo RNA synthesis

To produce RNA in vivo that was identical in sequence to U1 RNA made in vitro by SP6 RNA polymerase, two nucleotides (GpAp) were inserted immediately upstream of the normal +1 transcription start site of a *X. laevis* U1$_{Sm^-}$ gene (Terns et al., 1993a) using the Transformer™ Site-Directed Mutagenesis Kit (Clontech). The sequence of this U1$_{Sm^-}$ (+GA) template was confirmed by dideoxynucleotide sequencing (Sanger et al., 1977) using Sequenase (US Biochemicals). The U1 RNA transcribed from the pU1$_{Sm^-}$ (+GA) gene after injection into oocyte nuclei behaved like U1$_{Sm^-}$ RNA that lacked the two-base insert with respect to cap modification and nucleocytoplasmic transport (data not shown).

For injection into oocyte nuclei, the pre-U1 RNAs made in vivo were produced in oil-isolated germinal vesicles (GVs) of stage V and VI oocytes of *X. laevis* as described previously (Yang et al., 1992).

RNA analysis

Digestion with TAP (Epicentre Technologies) was with 5 U of TAP in 50 mM sodium acetate, pH 5.0, 1 mM EDTA, 10 mM 2-mercaptoethanol plus 10 μg cRNA at 37 °C for 1 h. TAP-treated RNAs were phenol extracted, ethanol precipitated, and gel purified before additional RNase digestion. Oxidation plus β-elimination were done using 25 mM sodium periodate and 1 mM lysine, pH 8.4, (Lund & Dahlberg, 1992) and the treated RNAs were ethanol precipitated prior to further digestion. RNase T2 digestion was with 5 U RNase T2 (Calbiochem) in 0.2 M sodium acetate, pH 4.6, plus 10 μg carrier RNA (cRNA) at 37 °C for 1 h. RNAse P1 digestion was with 1 U RNase P1 (Calbiochem) in 50 mM MES, pH 6.5, plus 10 μg cRNA at 37 °C for 1 h. RNase T2 and RNAse P1 digestion products were separated by TLC on cellulose plates with isobutyric acid, water, and ammonium hydroxide (66:33:1) in one dimension (bottom to top) (Silberklang et al., 1979); the RNase T2 digestion products were separated further in a second dimension (left to right) with acidic solvent containing isopropanol, water, and concentrated HCl (70:15:15) (Nishimura, 1972) or with neutral solvent containing isopropanol, saturated ammonium sulfate, and 1 M sodium acetate, pH 7.0 (2:80:18) (Konarska et al., 1984). RNAse T1 digestion was with 5 U RNase T1 (Sankyo Company, Limited) in 10 mM Tris, pH 8.5, 1 mM EDTA plus 10 μg cRNA for 1.5 h at 37 °C and the digestion products were separated in denaturing 20% (19:1) polyacrylamide gels containing 7 M urea. PhosphorImager (Molecular Dynamics) analysis was used to quantitate the RNase T1 digestion products.

Immunoprecipitation

Deproteinized RNAs were immunoprecipitated with rabbit polyclonal antibodies specific for the $m^{2,2,7}$G cap (Bringmann et al., 1983) (from R. Lührmann) or the m^7G cap (Munns et al., 1982) (from T. Munns) and RNAs from oocyte extracts were co-immunoprecipitated with mouse monoclonal antibodies (mAb Y12) specific for Sm proteins (Lerner et al., 1981) (from J. Steitz) as described previously (Neuman de Vegvar & Dahlberg, 1990).

RNA transport

Stage V and VI oocytes were obtained from *X. laevis* frogs as described previously (Krol et al., 1985). Nuclei were injected with U1 RNAs (~1 fmol/oocyte) synthesized either in vivo or in vitro. Co-injection of U3 RNA (made in vitro), which is not exported to the cytoplasm (Terns & Dahlberg, 1994), and blue dextran (Jarmolowski et al., 1994) served as controls for accuracy of the nuclear injections and dissections; wild-type U5 RNA was included as a control for normal nucleocytoplasmic transport. For the import assays, oocyte cytoplasms were injected with in vitro synthesized U1 RNAs (10 fmol/oocyte). At different times after injections, nuclei and cytoplasms were isolated from individual oocytes (2–4 per time point) by man-

ual dissection under mineral oil (Lund & Paine, 1990). After Proteinase K (1 mg/mL) digestion for 1–2 h at 37 °C, the RNAs were phenol extracted, ethanol precipitated, and 0.5 oocyte equivalents were analyzed by electrophoresis in 8% (30:0.8) polyacrylamide gels containing 7 M urea. Quantitation of the U1 RNA in the nuclear or cytoplasmic fractions was conducted using PhosphorImager analysis.

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