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A new method for detecting sites of 2′-O-methylation in RNA molecules

YI-TAO YU, MEI-DI SHU, and JOAN A. STEITZ

Department of Molecular Biophysics and Biochemistry, Yale University School of Medicine, Howard Hughes Medical Institute, Boyer Center for Molecular Medicine, New Haven, Connecticut 06536-0812, USA

ABSTRACT

2′-O-methylation of eukaryotic ribosomal RNAs occurs in the cell nucleoli. At least 100 modification sites that are highly conserved among vertebrate rRNAs have been mapped. However, in part because of the insensitivity of current approaches, there are 2′-O-methylated sites that remain unidentified. We have developed an extremely sensitive method for detecting 2′-O-methylated residues that are predicted within a long RNA molecule. Utilizing RNase H cleavage directed by a 2′-O-methyl RNA–DNA chimeric oligonucleotide, this method has allowed identification of two methylated nucleotides, G1448 in *Xenopus* 18S rRNA and A394 in *Xenopus* 28S rRNA. The latter (A394 in 28S) had not been detected before. We have confirmed that the methylation at G1448 in 18S is dependent upon *Xenopus* U25 snoRNA and have demonstrated that the methylation at A394 in 28S requires U26 snoRNA. One advantage of this technique is that it can examine specific rRNA and precursor molecules. We show that about 30% of the 40S pre-rRNA has been methylated at these two sites and their methylation is complete at the stage of 20S (immediate precursor to 18S) and 32S (immediate precursor to 28S). We also show that methylation at these two sites is not essential for rRNA transport from the nucleus to the cytoplasm.

Keywords: nuclear export; 2′-O-methyl RNA–DNA chimeras; RNase H; rRNAs; U25 snoRNA; U26 snoRNA

INTRODUCTION

In eukaryotes, the 18S, 5.8S, and 28S ribosomal RNAs are cotranscribed by RNA polymerase I within the cell nucleoli. The precursor rRNA is processed subsequently through a series of endo- and exonucleolytic cleavages to create the mature rRNA species (Perry, 1976; Crouch, 1984; Sollner-Webb et al., 1995). Before being assembled into ribosomal subunits and exported to the cytoplasm, the rRNAs become modified extensively. The modifications include conversion of uridines to pseudouridines, base methylation, and 2′-O-methylation (Maden, 1990).

There are at least 100 2′-O-methylation sites within vertebrate rRNAs. Most occur very early in processing (at the ~40S pre-rRNA stage), whereas at least one site does not receive the methyl group until a late stage of maturation (Eladari et al., 1977). Strikingly, almost all 2′-O-methylation sites are conserved among vertebrate rRNAs (Maden, 1990), suggesting an important role in ribosome biogenesis or function.

Reprint requests to: Joan A. Steitz, Department of Molecular Biophysics and Biochemistry, Yale University School of Medicine, Howard Hughes Medical Institute, Boyer Center for Molecular Medicine, 295 Congress Avenue, New Haven, Connecticut 06536-0812, USA.

Recently, a number of new small nucleolar RNAs (snoRNAs) that are intron-encoded have been identified (Maxwell & Fournier, 1995; Tycowski et al., 1996a). Most contain two evolutionarily conserved sequence motifs named box C and box D. In cells, these snoRNAs exist as small nucleolar ribonucleoprotein particles that are immunoprecipitable by antibodies against fibrillarin, an abundant nucleolar protein whose binding requires an intact box C (Baserga et al., 1991). Almost all of these snoRNAs exhibit extensive complementarity (usually 12–17 contiguous base pairs) to conserved regions of rRNA (Steitz & Tycowski, 1995; Kiss-Laszlo et al., 1996; Nicoloso et al., 1996; Tycowski et al., 1996a). Within the potentially interacting rRNA region, a 2′-O-methylated nucleotide is found at a fixed distance of 5 nt from the conserved box D, or a box D-like motif (box D′) in the complementary snoRNA (Steitz & Tycowski, 1995; Kiss-Laszlo et al., 1996; Nicoloso et al., 1996; Tollervey, 1996; Tycowski et al., 1996a). The resulting hypothesis that these snoRNAs guide 2′-O-methylation at specific rRNA sites has been confirmed recently for both vertebrate (Tycowski et al., 1996b) and yeast (Kiss-Laszlo et al., 1996) cells. Alteration of the spacing between box D and the complementary region of a yeast snoRNA (U24) resulted in the predicted displacement of the methylated site (Kiss-Laszlo

et al., 1996). Accordingly, Cavaille et al. (1996) showed that expression in mammalian cells of an "artificial" snoRNA designed to base pair with an arbitrary rRNA sequence resulted in 2′-O-methylation at the predicted site within the rRNA sequence.

Many, but not all, vertebrate 2′-O-methylation sites predicted by the snoRNA guide model had been mapped previously (Maden, 1990). The classical approach for detection of 2′-O-methylated nucleotides involved in vivo labeling with [^{14}C]-methyl methionine or [^{32}P]orthophosphate followed by ribonuclease fingerprinting of newly synthesized rRNAs (Maden, 1986, 1988). More recently, methods based on reverse transcription or partial alkaline hydrolysis followed by reverse transcription (Maden et al., 1995; Kiss-Laszlo et al., 1996; Tycowski et al., 1996b) have facilitated detection (and mapping) of many rRNA 2′-O-methylation sites. However, in part because of the low sensitivity of these methods, some 2′-O-methylated sites have not been detected. Among these is the methylation site A394 in the *Xenopus* 28S rRNA, which is predicted to be directed by U26 snoRNA (Kiss-Laszlo et al., 1996; Nicoloso et al., 1996; Tycowski et al., 1996b).

Here, we have developed a highly sensitive new method for detection of 2′-O-methylated sites. Applying this method to *Xenopus* 18S rRNA, we first confirmed a 2′-O-methylated site, G1448, reported previously (Maden, 1986; Tycowski et al., 1996b) and the involvement of U25 in this modification (Tycowski et al., 1996b). Then, we detected a previously undetermined 2′-O-methylation at A394 in *Xenopus* 28S rRNA and demonstrated that it requires U26 snoRNA. An advantage of this method is that individual processing intermediates can be examined for modification: the 2′-O-methylation of G1448 in 18S rRNA and of A394 in 28S rRNA are both observed at very early times (the 40S pre-rRNA stage); they are complete at the stage of 20S (immediate precursor to 18S) and 32S (immediate precursor to 28S), respectively. Finally, we demonstrate that a lack of methylation at these two sites has no effect on rRNA export from the nucleus to the cytoplasm in the *Xenopus* oocyte.

RESULTS

Use of RNase H and chimeric oligonucleotides to detect 2′-O-methylation

RNase H nonspecifically cleaves the RNA strand of an RNA–DNA hybrid. However, it does not cleave any site where the 2′-O position of an RNA residue is methylated. Two groups (Inoue et al., 1987; Lapham & Crothers, 1996) have exploited this property of RNase H and reported that digestion of an RNA directed by a complementary 2′-O-methyl RNA–DNA chimeric oligonucleotide consisting of four deoxynucleotides flanked by 2′-O-methyl ribonucleotides is site-specific. Cleavage occurred at the phosphodiester bond 3′ to the ribonucleotide that is base paired with the 5′-most deoxynucleotide of the chimera (arrow A in Fig. 1A).

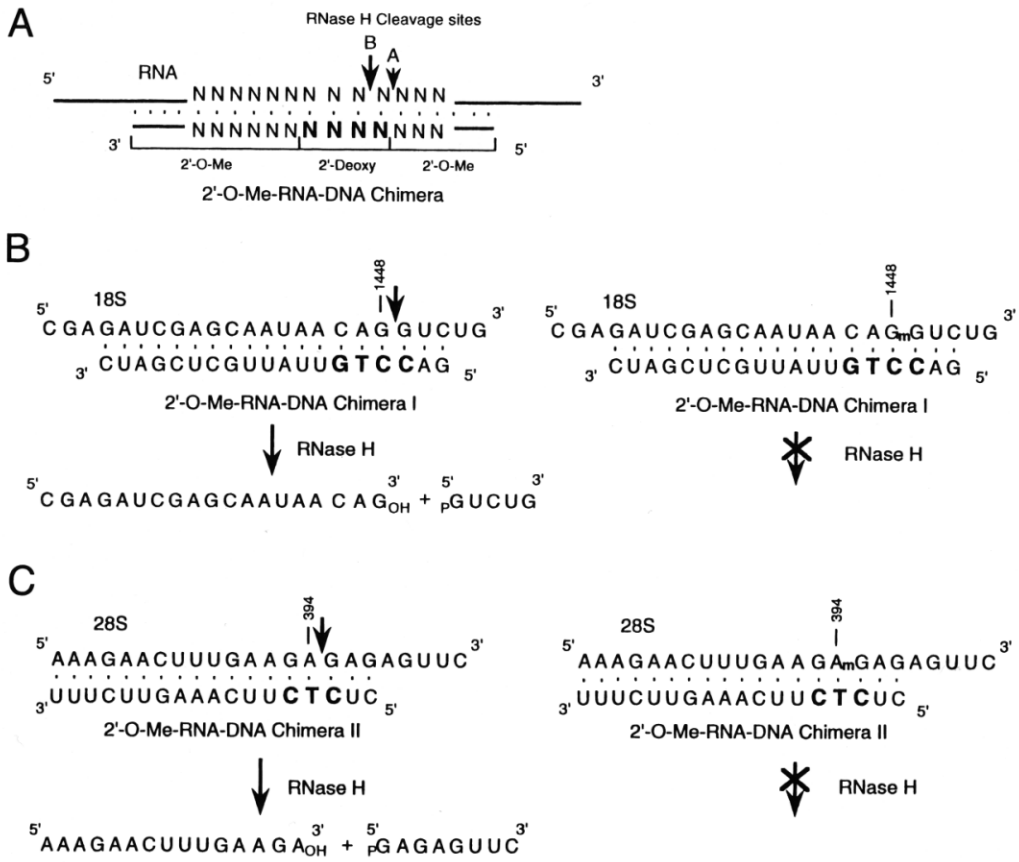
We reasoned that use of a 2′-O-methyl RNA–DNA chimera and RNase H digestion might therefore provide a direct assay for determining whether any particular nucleotide in a long RNA molecule carries a 2′-O-methyl group. If the 2′-O position of the targeted residue is methylated, RNase H cleavage should be blocked. If instead the 2′-O position is unmodified, RNase H should cut the RNA specifically at that site, yielding two RNA fragments (Fig. 1B,C).

To test the feasibility of this approach, we examined the U25-guided 2′-O-methylation site G1448 in the *Xenopus* 18S rRNA identified previously (Tycowski et al., 1996b) and the potential U26-directed methylation site A394 in the *Xenopus* 28S rRNA (Kiss-Laszlo et al., 1996; Nicoloso et al., 1996; Tycowski et al., 1996b). In preliminary experiments, we observed that the site of RNase H cleavage directed by a 2′-O-methyl RNA–DNA chimera cannot always be predicted accurately by the rules quoted above (Lapham & Crothers, 1996). Rather, many chimeric oligonucleotides direct RNase H cleavage one nucleotide upstream, 5′ to the ribonucleotide that base pairs to the 5′-most deoxynucleotide (arrow B in Fig. 1A; Y.-T. Yu, unpubl. data). This is the case for the 2′-O-methyl RNA–DNA chimeras (Chimera I and Chimera II), which we designed to direct RNase H cleavage at G1448 in the *Xenopus* 18S rRNA and at A394 in the *Xenopus* 28S rRNA (Fig. 1B,C; data not shown). For Chimera II, we found that a stretch of three deoxynucleotides directs specific RNase H cleavage more effectively than four deoxynucleotides (Fig. 1C,E).

To confirm that cleavage occurs only at an unmethylated but not at a methylated target site (G1448 in 18S rRNA or A394 in 28S rRNA), we designed and tested two pairs of chemically synthesized oligoribonucleotides (Fig. 1B,C). One pair corresponds to nt 1430–1453 of *Xenopus* 18S rRNA and the other pair corresponds to nt 380–402 of *Xenopus* 28S rRNA. One of the two RNAs in each pair contained a single 2′-O-methyl group at the target site (G1448 in 18S rRNA and A394 in 28S rRNA). The results of RNase H cleavage directed by 2′-O-methyl RNA–DNA Chimera I and Chimera II are shown in Figure 1D and E. The unmodified 3′ end ^{32}pCp-labeled RNA oligonucleotides were almost completely cleaved (lanes 4), whereas the RNA oligonucleotides carrying a 2′-O-methyl group (G1448 in 18S and A394 in 28S) were totally resistant to chimeric oligonucleotide-directed RNase H digestion (lanes 2).

Detection of 2′-O-methylation at G1448 in *Xenopus* 18S rRNA

Using this procedure, we next examined the 2′-O-methyl site G1448 in the endogenous 18S rRNA from



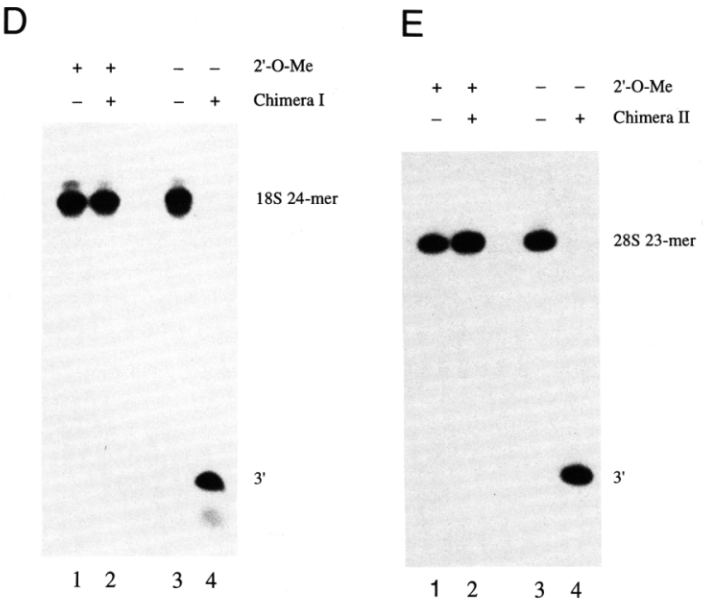


FIGURE 1. Site-specific RNase H cleavage directed by 2′-O-methyl RNA–DNA chimeric oligonucleotides. **A:** Schematic representation of RNase H cleavage sites. The top strand is an RNA sequence hybridized to a 2′-O-methyl RNA–DNA chimera, the bottom strand. In the chimera, deoxynucleotides are indicated as bold letters and 2′-O-methyl ribonucleotides as plain letters. Arrows A and B indicate the cleavage sites reported previously (Inoue et al., 1987; Lapham et al., 1996) and observed by us in many cases, respectively. **B:** RNase H cleavage of a pair of RNA oligonucleotides corresponding to nt 1430–1453 of *Xenopus* 18S rRNA. Bold letters and plain letters in Chimera I denote the deoxynucleotides and 2′-O-methyl ribonucleotides, respectively. Only the RNA oligonucleotide on the left is cleaved at the site indicated to yield two RNA fragments. **C:** RNase H cleavage of a pair of oligonucleotides corresponding to nt 380–402 of *Xenopus* 28S rRNA. Bold letters and plain letters in Chimera II represent deoxynucleotides and 2′-O-methyl ribonucleotides, respectively. Only the RNA oligonucleotide on the left is cleaved at the site indicated. **D:** Experimental data for B. 3′ End-^{32}P-pCp-labeled RNA oligonucleotides with (lanes 1 and 2) or without (lanes 3 and 4) a single 2′-O-methyl group at G1448 were subjected to Chimera I-directed RNase H digestion. Lanes 1 and 3 are controls where Chimera I was omitted. Positions of the original oligonucleotides and the cleaved 3′ fragment are indicated. **E:** Experimental data for C. 3′ End-^{32}P-pCp-labeled RNA oligonucleotides with (lanes 1 and 2) or without (lanes 3 and 4) a single 2′-O-methyl group at A394 were subjected to Chimera II-directed RNase H digestion. Lanes 1 and 3 are controls where Chimera II was omitted. Positions of the original oligonucleotides and the cleaved 3′ fragment are labeled.

Xenopus. By injecting an oligodeoxynucleotide complementary to U25 into the *Xenopus* oocyte, Tycowski et al. (1996b) showed previously that the U25 snoRNP can be depleted selectively. We used the same experimental paradigm to produce oocytes that were either depleted or mock depleted (as a control) of U25 and were labeled subsequently with [α^{32}P]UTP for approximately 16 h. Total nuclear RNAs were recovered, hybridized to 2′-O-methyl RNA–DNA Chimera I (which directs RNase H cleavage at position G1448 of 18S rRNA, see Fig. 1B), and then incubated with RNase H.

The results are shown in Figure 2. When the RNA from mock-depleted oocytes was analyzed, all precursor and mature rRNAs exhibited resistance to digestion; only the 40S pre-rRNA was partially cleaved (compare lane 2 with lane 1), suggesting that it is not completely methylated at G1448 within the 18S sequence. In contrast, the profile of RNA from the U25-depleted oocytes was altered dramatically by RNase H digestion. The 40S pre-rRNA, along with mature 18S rRNA and its immediate precursor 20S pre-rRNA, were cleaved completely, whereas mature 28S rRNA and its immediate precursor 32S pre-rRNA remained unchanged (lanes 3 and 4). Taken together, these results confirm the existence of a 2′-O-methyl group at G1448 in *Xenopus* 18S rRNA and show that the methylation requires the U25 snoRNA.

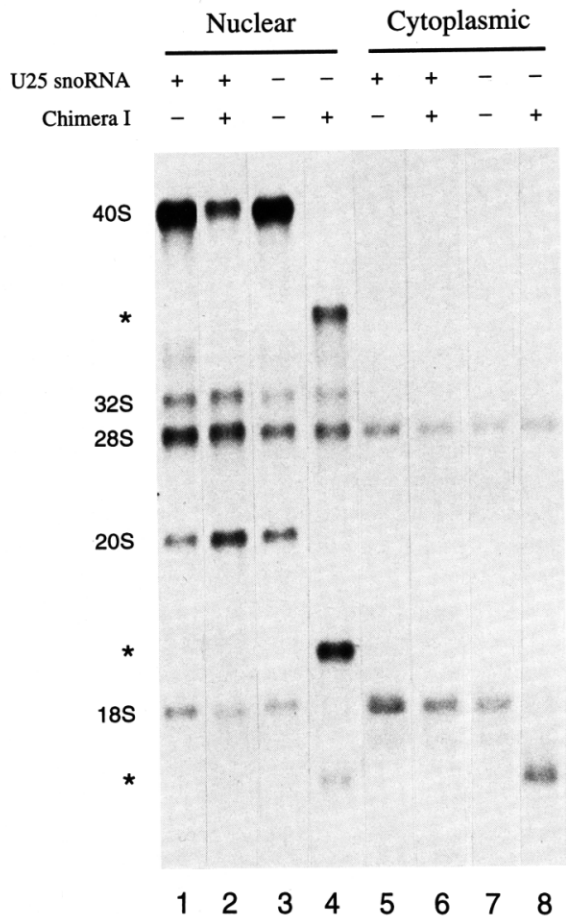


FIGURE 2. Chimera I-directed RNase H cleavage of *Xenopus* nuclear and cytoplsmic RNAs. [α-^{32}P]UTP labeled nuclear RNAs (lanes 1–4) and cytoplsmic RNAs (lanes 5–8) derived from mock-depleted (lanes 1, 2, 5, and 6) or U25-depleted (lanes 3, 4, 7, and 8) oocytes were subjected to RNase H digestion directed by Chimera I. Lanes 1, 3, 5, and 7 are controls where Chimera I was omitted. Positions of precursor and mature rRNAs are indicated, with bands generated by RNase H cleavage shown by asterisks (*).

To determine more accurately the stage at which G1448 methylation occurs, we quantitated each band in the nuclear RNA on a phosphorimager. The results are summarized in Table 1. About 70% of the 40S pre-rRNA derived from the mock-depleted oocytes was cleaved. Because RNase H digestion directed by the 2′-O-methyl RNA–DNA chimera goes to completion under these conditions (see Fig. 2, lane 4; Fig. 1D, lane 4), we can conclude that about 30% of the molecules in the 40S pre-rRNA population have been 2′-O-methylated at position G1448. For mature 18S rRNA and its immediate precursor 20S pre-RNA, nearly 100% of the molecules were resistant, suggesting that, at these stages, the 2′-O-methylation at this position is complete.

We further asked whether 2′-O-methylation at G1448 has any effect on nuclear export of 18S rRNA. Because the new method examines only labeled (newly synthesized) RNAs, targeting cytoplasmic RNAs for RNase H digestion directed by chimeric oligonucleotides will establish whether transport of unmodified rRNA has occurred. Note that the absence of 40S, 32S, and 20S pre-rRNAs in Figure 2, lanes 5–8, demonstrates lack of nuclear contamination. We observed that cytoplasmic 18S and 28S rRNAs derived from mock-depleted oocytes were almost totally insensitive to digestion (Fig. 2, compare lane 6 with lane 5; Table 1). For cytoplasmic RNAs derived from U25-depleted oocytes, however, cleavage of 18S rRNA was nearly complete, whereas the 28S rRNA remained intact (Fig. 2, compare lane 8 with lane 7; Table 1). The lack of 2′-O-methylation at position G1448 in 18S rRNA recovered from the cytoplasm argues that 2′-O-methylation at this position is not essential for transport of 18S rRNA from the nucleus to the cytoplasm.

We noted that the sequence of U25 snoRNA that is complementary to nt 1445–1456 of the *Xenopus* 18S rRNA partially (7 of 12 nt) overlaps the sequence of the 2′-O-methyl RNA–DNA Chimera I that we designed to base pair with nt 1433–1451 of 18S rRNA. This raised the possibility that intact U25 present in the RNA preparation from mock-depleted oocytes may have had some inhibitory effect on the Chimera I-directed RNase H digestion. However, for several reasons, we believe this to be unlikely. First, the complementarity between Chimera I and the 18S rRNA sequence is longer (19 base pairs) than that between U25 and 18S (12 base pairs). Thus, Chimera I should compete successfully with U25 for hybridization to the

TABLE 1. Quantitation of RNase H cleavage of precursor and mature rRNAs directed by 2′-O-methyl RNA–DNA Chimera I.[a]

	Nuclear				Cytoplasmic			
	Mock-depleted		U25-depleted		Mock-depleted		U25-depleted	
Chimera I	+	−	+	−	+	−	+	−
40S	100	31	100	<1				
32S	100	99	100	99				
28S	100	97	100	102	100	95	100	105
20S	100	113	100	<1				
18S	100	90	100	<1	100	90	100	7

[a]Each RNA signal (40S, 32S, 28S, 20S, or 18S) observed after RNase H cleavage (shown in Fig. 2) was compared to that from control reaction where Chimera I was omitted. Relative ratios (in percentage) were determined by phosphorimager. The experiment was repeated several times with comparable results.

18S rRNA sequence. Second, 2′-O-methyl RNA–DNA Chimera I was present in vast excess (~1,000-fold) compared to U25. Third, if U25 were interfering with RNase H digestion, equivalent effects on 40S pre-rRNA, 20S pre-rRNA, and 18S rRNA should have been observed. Instead, only the 40S pre-rRNA was cleaved partially.

To completely eliminate the possibility of U25 snoRNA interference, we pre-treated nuclear RNAs from mock-depleted oocytes with RNase H and the oligodeoxynucleotide that had been used to deplete U25 from oocytes. We confirmed that digestion of U25 was complete by primer-extension analysis (data not shown), and then used this RNA preparation for 2′-O-methyl RNA–DNA Chimera I-directed RNase H cleavage. The resulting RNA profile (data not shown) was no different from that presented in Figure 2, lanes 1 and 2, indicating that the U25 in the mock-depleted RNA preparation did not have any inhibitory effect on RNase H cleavage directed by 2′-O-methyl RNA–DNA Chimera I.

U26 snoRNA-dependent 2′-O-methylation of A394 in *Xenopus* 28S rRNA

Xenopus U26 snoRNA exhibits extensive complementarity (13 base pairs) to 28S rRNA nt 391–403 (see Fig. 1C). Based on the guide RNA model (Kiss-Laszlo et al., 1996; Nicoloso et al., 1996; Tycowski et al., 1996), A394 of 28S rRNA should therefore be 2′-O-methylated. This methylation site has never been reported (Maden, 1990; Kiss-Laszlo et al., 1996; Nicoloso et al., 1996), although Maden (1988) predicted a methylation site G-A_m-G somewhere in the first 800 nt of 28S rRNA.

To determine whether A394 of 28S rRNA is methylated in *Xenopus*, we again used RNase H digestion directed by a 2′-O-methyl RNA–DNA chimeric oligonucleotide. Chimera II, designed to direct cleavage at this site (see Fig. 1C,E), was incubated with (α^{32}P)UTP-labeled nuclear RNAs from normal oocytes. With the exception of 40S pre-rRNA, all precursor and mature rRNAs were insensitive to Chimera II-directed RNase H digestion (Fig. 3, lanes 1 and 2). The 40S pre-rRNA was cleaved partially (~70%) (Table 2), again suggesting that methylation starts at very early times (~30% complete at the 40S stage) and ends before processing is complete.

Because methylation of A394 in 28S rRNA is believed to be guided by U26, we injected two different antisense U26 oligodeoxynucleotides (complementary to nt 12–26 or 19–35 of U26) separately into *Xenopus* oocytes. Each of these directed complete degradation of U26 by endogenous RNase H, as judged by primer-extension analysis (data not shown). After [α-^{32}P]UTP-labeling for approximately 16 h, nuclear RNAs were recovered and subjected to RNase H digestion directed by Chimera II. The 40S pre-rRNA, mature 28S rRNA, and its immediate precursor 32S pre-rRNA were all cleaved nearly to completion, whereas 18S rRNA and its immediate precursor 20S pre-rRNA remained uncleaved (Fig. 3, lanes 3–6; Table 2). The band immediately below the 28S band (indicated by an asterisk) is a degradation product. Upon further electrophoresis, this band can be separated more clearly from the 28S band (data not shown). These results demonstrate that 2′-O-methylation of A394 in the *Xenopus* 28S rRNA requires U26 snoRNA.

Again, we controlled for the possibility that U26 present in normal (mock-depleted) nuclear RNAs might block RNase H cleavage by competing with 2′-O-methyl RNA–DNA Chimera II for hybridization to precursor and mature 28S rRNAs. We pre-treated total nuclear RNAs with RNase H and an oligodeoxynucleotide designed to target the U26 region, which has potential to compete with Chimera II prior to the RNase H digestion directed by Chimera II (data not shown). This pre-treatment did not alter RNA pattern after RNase H treatment in the presence of Chimera II (data not shown).

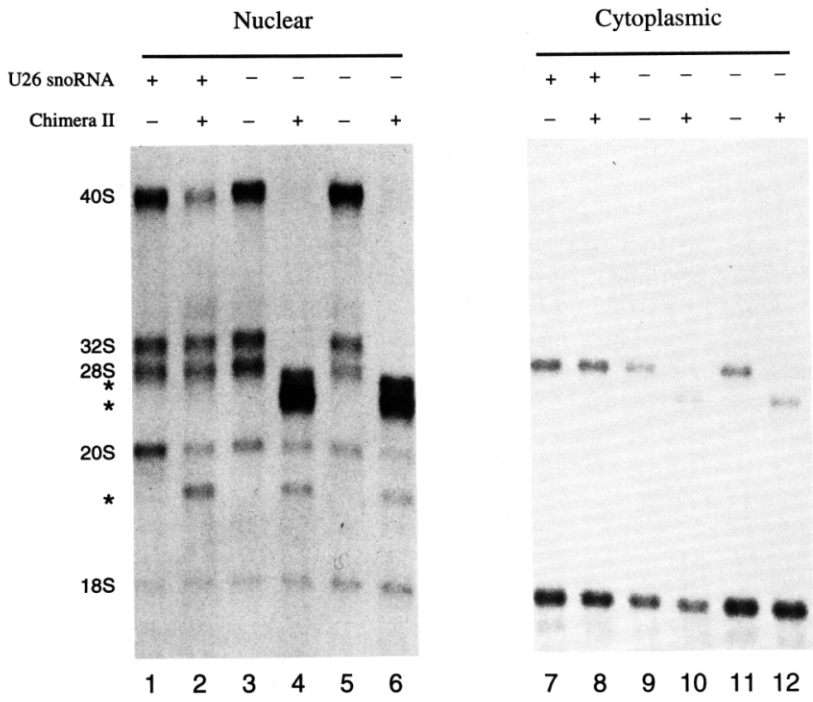


FIGURE 3. Chimera II-directed RNase H cleavage of *Xenopus* nuclear and cytoplasmic RNAs. [α-^{32}P]UTP labeled nuclear RNAs (lanes 1–6) and cytoplsmic RNAs (lanes 7–12) derived from mock-depleted (lanes 1, 2, 7, and 8) or U26-depleted (lanes 3–6, 9–12) oocytes were subjected to RNase H digestion directed by Chimera II. U26 was depleted by an oligodeoxynucleotide complementary to nt 12–26 of U26 in lanes 3, 4, 9, and 10, or by one complementary to nt 19–35 of U26 in lanes 5, 6, 11, and 12. Lanes 1, 3, 5, 7, 9, and 11 are controls where Chimera II was omitted. Positions of precursor and mature rRNAs are indicated, with bands generated by RNase H cleavage indicated by *. Nuclear 20S pre-rRNA appears slightly low compared to controls. This was probably caused by nonspecific RNase H digestion.

We also assessed the possible effect of 2′-O-methylation at position A394 in 28S rRNA on nuclear export. When cytoplasmic mature rRNAs derived from U26-depleted oocytes were targeted by 2′-O-methyl RNA–DNA Chimera II, we observed complete RNase H cleavage of 28S rRNA, whereas the 18S rRNA was unchanged (Fig. 3, lanes 9–12; Table 2). In contrast, cytoplasmic mature 28S rRNA from the mock-depleted oocytes was not cleaved (Fig. 3, lanes 7 and 8; Table 2). These results argue that 2′-O-methylation at position A394 in 28S rRNA, like position G1448 in 18S, has no effect on transport of rRNA from the nucleus to the cytoplasm.

DISCUSSION

We have developed a new method for detecting 2′-O-methylation of an RNA that involves RNase H digestion directed by 2′-O-methyl RNA–DNA chimeras. For several reasons, we believe that this technique has advantages over methods used previously. First, it is highly sensitive. Because the *Xenopus* pre-rRNAs and rRNAs can be ^{32}P-labeled readily in vivo, we simply monitored the radioactive RNA species after RNase H digestion and thereby detected two 2′-O-methylation sites in the *Xenopus* rRNAs, one of which (A394 in 28S) had not been determined and mapped (Maden, 1990;

TABLE 2. Quantitation of RNase H cleavage of precursor and mature rRNAs directed by 2′-O-methyl RNA-DNA Chimera II.[a]

	Nuclear						Cytoplasmic					
	Mock-depleted		U26-depleted[b]		U26-depleted[c]		Mock-depleted		U26-depleted[b]		U26-depleted[c]	
Chimera I	+	−	+	−	+	−	+	−	+	−	+	−
40S	100	33	100	3	100	3						
32S	100	95	100	<1	100	<1						
28S	100	95	100	<1	100	<1	100	100	100	3	100	3
20S	100	60	100	85	100	87						
18S	100	110	100	110	100	107	100	97	100	90	100	97

[a]Data from Figure 3 were treated as in Table 1.
[b]The oligodeoxynucleotide, 5′-CAGTAAGAGAGAGTTCA-3′, was used to deplete U26 snoRNA from oocytes (see Materials and Methods).
[c]The oligodeoxynucleotide, 5′-AGAGTTCATAGGTAT-3′, was used to deplete U26 snoRNA from oocytes (see Materials and Methods).

Kiss-Laszlo et al., 1996; Nicoloso et al., 1996). This method is therefore easily applicable to low abundance RNAs, such as yeast snRNAs or snRNAs from the human AT-AC spliceosome (where limiting material has made other approaches impossible). Second, because our method involves only one RNase H digestion step, it is much easier and simpler to use than other methods. Finally, because the RNase H cleavage simultaneously reveals the 2′-O-methylation status for both precursor and mature RNA molecules present in the mixture, the method allows assessment of the stage at which the methylation takes place. About 30% of the 40S pre-rRNA is 2′-O-methylated at G1448 in 18S and at A394 in 28S (Tables 1, 2), whereas methylation at these two sites is complete at the stage of 20S (immediate precursor to 18S) and 32S (immediate precursor to 28S). These data suggest that methylation may start on nascent rRNA transcripts and that the rate of methylation is slower than that of rRNA transcription. However, because the methylation at these two sites is complete in the processing intermediates (20S and 32S), the methylation rate is apparently faster than the processing rate.

Recently, it has been proposed, and in some cases demonstrated, that certain snoRNAs direct 2′-O-methylation at specific sites within rRNA sequences with which they potentially base pair. U25 and U26 snoRNAs contain sequences complementary to conserved regions of 18S and 28S rRNAs, respectively (Kiss-Laszlo et al., 1996; Nicoloso et al., 1996; Tycowski et al., 1996b). In agreement with a previous study (Tycowski et al., 1996b), we confirmed U25 dependent 2′-O-methylation at G1448 in the *Xenopus* 18S rRNA. We also proposed and verified the U26-dependent 2′-O-methylation site at A394 of 28S rRNA. Depletion of U26 prevented the 2′-O-methylation (Fig. 3); injection of synthetic U26 into oocytes restored the methylation (data not shown). With the emergence of a rapidly increasing number of new snoRNAs with potential guide functions, the method presented here will be well suited for detection of the corresponding 2′-O-methylation sites in rRNAs.

By analyzing cytoplasmic rRNAs, we have shown that 2′-O-methylations at G1448 in 18S and at A394 in 28S are not essential for transport. Tycowski et al. (1996b) observed previously that nuclear 18S rRNAs derived from U25-depleted *Xenopus* oocytes lacked a 2′-O-methyl group at position G1448, whereas this deficiency could not be detected in cytoplasmic RNA. The method they used, alkaline hydrolysis followed by primer extension, had the limitation that the total population of cytoplasmic RNAs—including mature rRNAs generated before depletion of U25—were examined. Therefore, no conclusions regarding the effect of 2′-O-methylation on rRNA transport could be reached (Tycowski et al., 1996b). In the studies presented here, only RNAs labeled after U25 depletion in *Xenopus* oocytes were examined. Thus, the lack of 2′-O-methylation at G1448 in cytoplasmic 18S rRNA (Fig. 2; Table 1) and at A394 in cytoplasmic 28S rRNA (Fig. 3; Table 2) demonstrated unequivocally that 2′-O-methylation at these sites is not required for transport of rRNAs from the nucleus to the cytoplasm.

What role does the 2′-O-methylation of rRNA play in the cell? The experimental data are fragmentary and contradictory. Early experiments employing a methylation inhibitor in mammalian cells suggested that methylation of pre-rRNA is critical for its processing (Swann et al., 1975; Wolf & Schlessinger, 1977). In contrast, genetic depletion of one known methylase in yeast indicated that methylation is dispensable for pre-rRNA processing (Tollervey et al., 1993). In another report, 2′-O-methylation at a conserved site in yeast mitochondrial 21S rRNA appeared to be required for the assembly of functional large ribosomal subunits (Sirum-Connolly & Mason, 1993). Most recently, Kiss-Laszlo et al. (1996) have shown that inhibition of 2′-O-methylation at several sites in the yeast 25S rRNA does not affect growth. Surely a more detailed understanding of the sites of 2′-O-methylation of rRNA molecules will facilitate insights into the cellular functions of this class of RNA modifications.

MATERIALS AND METHODS

Depletion of U25 and U26 snoRNPs from *Xenopus* oocytes and RNA preparation

The procedure is essentially as described previously (Tycowski et al., 1994). Briefly, 32 nL of 2 mg/mL oligodeoxynucleotides complementary to either U25 (5′-ACAGGTCTG TGAT-3′, nt 18–31) or U26 (5′-CAGTAAGAGAGAGTTCA-3′, nt 19–35; or 5′-AGAGTTCATAGGTAT-3′, nt 12–26) were injected into the cytoplasm of the *Xenopus* oocytes. For mock depletion, a nonspecific oligodeoxynucleotide (5′-ATCTGGAA TCTACCTGCC-3′) was injected. After about 22 h at 18 °C, oocytes were reinjected with 150 nCi of [α^{32}P]UTP and incubated for another 16 h. Oocytes were then dissected into GVs (germinal vesicles) and cytoplasms. Nuclear RNAs and cytoplasmic RNAs were isolated subsequently (Tycowski et al., 1994) and used for 2′-O-methyl RNA–DNA chimera-directed RNase H cleavage (see below). To monitor depletion of U25 and U26 snoRNAs, a fraction (~six GVs) of nuclear RNA was subjected to primer extension analysis. The primer used for detecting U25 was as published (Tycowski et al., 1996b) and the primer for U26 was an oligodeoxynucleotide complementary to nt 53–70 of U26 (5′-ACTCAGATTATAAAGTCT-3′). Usually, depletion was nearly complete; no intact U25 and U26 were detected.

2′-O-methyl RNA–DNA chimera-directed RNase H cleavage

A 5-μL mixture containing 0.1 ng of 3′ end ^{32}P-pCp-labeled (England & Uhlenbeck, 1978) chemically synthesized RNA oligonucleotides (nt 1430–1453 of *Xenopus* 18S rRNA, 5′-CGAGAUCGAGCAAUAACAGGUCUG-3′ and 5′-CGAGA

UCGAGCAAUAACAG$_{2'\text{-O-m}}$ GUCUG-3′; nt 380–402 of *Xenopus* 28S rRNA, 5′-AAAGAACUUUGAAGAGAGAGUUC-3′ and 5′-AAAGAACUUUGAAGA$_{2'\text{-O-m}}$ GAGAGUUC-3′) and 2.5 ng of 2′-O-methyl RNA–DNA chimeric oligonucleotide (Chimera I for 18S, 5′-GAdCdCdTdGUUAUUGCUCGAUC-3′; or Chimera II for 28S, 5′-CUdCdTdCUUCAAAGUUC UUUC-3′) was heated to 95 °C for 3 min and transferred to 37 °C for 10 min. The annealed mixture then received 1 unit RNase H (Boehringer Mannheim) and 2 units of RNase inhibitor (Boehringer Mannheim) in 5 μL buffer containing 40 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 200 mM KCl, 0.2 mM DTT, 10% (w/v) sucrose. The RNase H cleavage reaction was performed at 37 °C for 30 min. RNAs were then extracted with PCA and precipitated with ethanol in the presence of 5 μg of carrier RNA (yeast RNAs). Recovered RNAs were resolved on 10% polyacrylamide–8 M urea gels and subjected to autoradiography.

For *Xenopus* RNAs, the 2′-O-methyl RNA–DNA chimera-directed RNase H cleavage was performed essentially as above except that each reaction contained 10 ng of Chimera I or Chimera II and nuclear RNAs collected from four GVs or cytoplasmic RNAs derived from two oocytes. After RNase H digestion, RNAs were resolved on horizontal 1% agarose-formaldehyde gels and transferred to Zeta-Probe GT membranes (Bio-Rad) for autoradiography.

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