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Modification of human U4 RNA requires U6 RNA and multiple pseudouridine synthases

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ABSTRACT

Small nuclear RNAs (snRNA), cofactors in the splicing of pre-mRNA, are highly modified. In this report the modification of human U4 RNA was studied using cell extracts and *in vitro* synthesized, and therefore unmodified, U4 RNA. The formation of pseudouridine (Ψ) at positions 4, 72 and 79 in U4 RNA was dependent on an RNA-containing cofactor, since the activities in the extracts were micrococcal nuclease (MN) sensitive. Extracts were fractionated on glycerol gradients and there was a broad peak of reconstitution activity centered at 14 S. Reconstitution was not due to additional enzymatic activity, since the peak fraction was MN sensitive. Oligodeoxynucleotide-mediated RNase H digestion of U6 RNA in the extracts inhibited formation of Ψ in U4 RNA. From glycerol gradient analysis we determined that exogenously added U4 RNA that is associated with U6 RNA (sedimentation velocity 16 S) was significantly higher in Ψ content than U4 RNA not associated with U6 RNA (8 S). Competitive inhibitors of Ψ synthases, 5-fluorouridine-containing (5-FU) wild-type and mutant U4 RNAs, were used to investigate formation of Ψ in U4 RNA. Deletions and point mutations in these 5-FU-containing U4 RNAs affected their ability to inhibit Ψ synthase *in vitro*. With the aid of these potent inhibitors it was determined that at least two separate activities modify the uridines at these positions.

INTRODUCTION

Removal of introns from pre-mRNA and splicing of the remaining exons is an essential function in all eukaryotic cells. Splicing occurs in a large and dynamic structure termed the spliceosome, of which small nuclear ribonucleoprotein particles (snRNPs) are essential components (1). The spliceosomal snRNPs are composed of highly conserved small nuclear RNAs (snRNAs) U1, U2, U4, U5 and U6. Each snRNA binds a common core of proteins, the Sm proteins (excluding U6 snRNA), and proteins that are specific for the individual snRNAs (2,3). These snRNAs contain many modified bases, including methylated bases and pseudouridine (Ψ) (2,4). The U4 and U6 snRNAs are highly base paired (see Fig. 1) and form one snRNP which enters the spliceosome complexed with U5 in the form of a tri-snRNP. These interactions are essential for spliceosomal assembly and function (5–7).

Formation of Ψ in these snRNAs has been the subject of several reports. Recently it has been shown that base pairing with U4 RNA is a prerequisite for Ψ formation in U6 RNA (8). With the aid of 5-fluorouridine (5-FU)-containing RNAs, potent and specific inhibitors of Ψ formation, it was shown that there are multiple Ψ synthase activities that specifically recognize U1, U2 and U5 snRNAs (9,10).

The function of Ψ in snRNAs is unknown, but Ψ is found in regions of snRNAs that are necessary for snRNP function in splicing of pre-mRNA (11) and *in vitro* synthesized U2 RNA fails to complement a U2-deficient splicing extract whereas U2 RNA isolated from cells does (12). The function of Ψ in spliceosomal snRNAs in higher eukaryotes may be to strengthen snRNA–mRNA and snRNA–snRNA interactions or stabilize the secondary structure of the snRNAs (13). In addition, Ψ may serve to stabilize the tertiary structure of the RNA backbone of the spliceosome (13).

In this report we show that modification of U4 RNA requires U6 RNA and, with the aid of 5-FU-containing wild-type and mutant U4 RNAs, that formation of Ψ in U4 RNA *in vitro* requires at least two separate and specific Ψ synthase activities.

MATERIALS AND METHODS

Generation of point mutants and *in vitro* transcription

The point mutants mSP6-U4A4 and mSP6-U4A72 (T→A substitution at positions 4 and 72) were constructed by subcloning the 350 bp *Eco*RI–*Hin*dIII fragment from pSP6-U4 into the *Eco*RI and *Hin*dIII sites in M13mp19RF. Oligonucleotide-mediated site-directed mutagenesis was performed as previously described using the oligonucleotides 5′-CTATAGAAG-C**A**TTGCGCAGTG-3′ for mutagenesis of nucleotide 4 and 5′-TTGAAAACTT**A**TCCCAATACC-3′ for mutagenesis of nucleotide 72 (14). The mutations were confirmed by dideoxy sequencing the single-stranded phage DNA (15).

SP6 transcription of *Dra*I-cut pSP6-U4, the point mutants mentioned above and U4 RNA mutants (see Fig. 1) pSP6-U4 ΔStem I (nt 56–63 deleted), pSP6-U4 ΔStem II (nt 1–16 deleted), pSP6-U4 Δ5′Stem–loop (nt 19–55 deleted) and *Xba*I-cut human U4 RNA mutant pSP6-U4 ΔSm (nt 91–145 deleted) were

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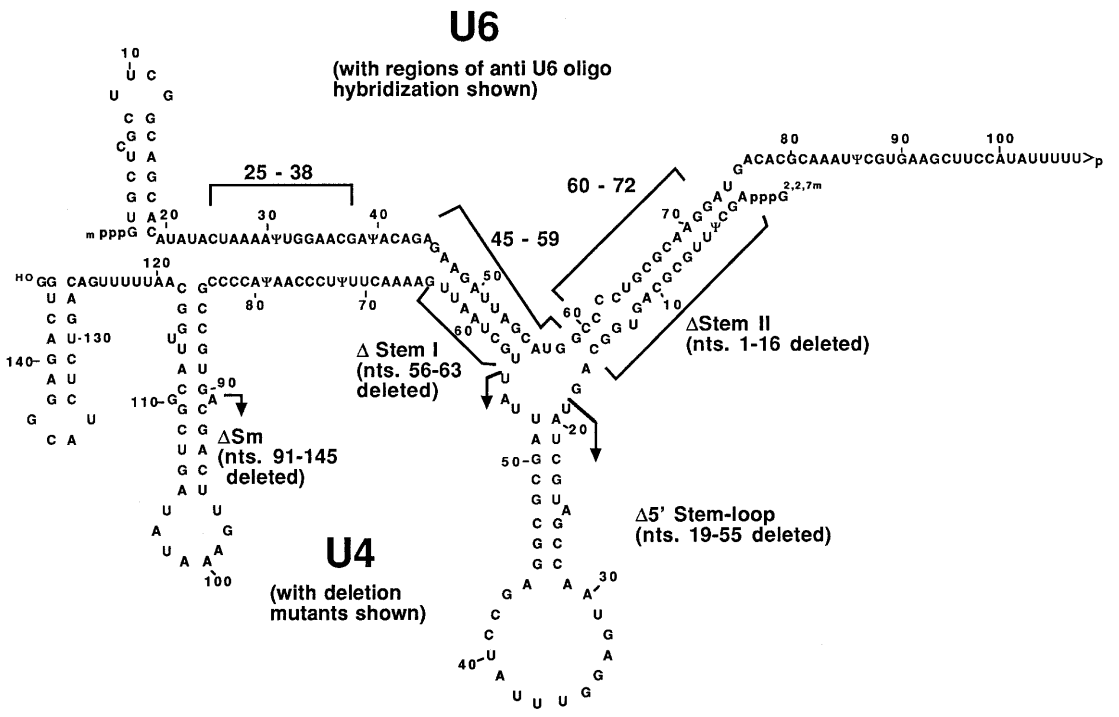

Figure 1. Interaction of U4 and U6 RNAs and diagram of U4 mutants and anti-U6 RNA oligodeoxynucleotides. The primary structure of U4 RNA with Ψ modifications and regions of intermolecular base pairing with U6 RNA are indicated. Regions of interaction, protein binding or secondary structure are denoted on the diagram (2 and 5–7). The U4 RNA deletion mutants used are indicated on the U4 RNA portion of the diagram. The regions of anti-U6 RNA deoxynucleotide hybridization are shown on the U6 portion of the diagram.

performed as described (9,10,16–18). The U4 deletion and wild-type clones were a generous gift from Albrecht Bindereif (Humbolt University, Germany) (5). Human U5 RNA was transcribed using SP6 RNA polymerase and *Bfa*I-cut pHU5a2 (16). The *in vitro* transcription reactions (25 µl) contained, depending upon the application, [α^{32}P]UTP (50 µCi, 800 Ci/mmol), [5-^{3}H]UTP (1 µCi, 17 Ci/mmol) or [5-^{3}H]UTP (50 µCi, 17 Ci/mmol), 50 µM GTP, 250 µM ATP and CTP and 1 mM m^{7}GpppG. To make ^{32}P-labeled and ^{3}H-labeled U4 RNA no UTP other than the label was added to the reaction. 5-FU-containing RNAs were made with 1 µCi [5-^{3}H]UTP, 1 mM 5-FUTP (Sierra Bioresearch, Tucson, AZ) and 250 µM CTP, GTP and ATP. All 5-FU containing RNAs were gel purified on a 10% polyacrylamide, 8.3 M urea gel prior to addition to HeLa extracts.

In vitro modification and assays for Ψ formation

The *in vitro* modification reactions were carried out as previously described in 300 µl total volume using HeLa cytoplasmic (S100) and nuclear extract (NE) (8–10,16,19,20). Except where noted, the reaction mixture contained 60% HeLa extract (30% of each of the two extracts if a combination was used) by volume, 0.5 mM ATP, 20 mM creatine phosphate, 3.2 mM $MgCl_2$ and 2 mM dithiothreitol (DTT). RNAs were incubated in S100 for 30 min at 37°C, followed by addition of NE and further incubation for 2.5 h at 37°C unless otherwise noted in the text. Extracts to be micrococcal nuclease (MN) treated also contained 1 mM $CaCl_2$ and were treated with 1 U/µl MN for 30 min at 37°C. The MN was subsequently inhibited by adding EGTA to 10 mM and either poly(A)/poly(C) RNA or poly(U) RNA to a final concentration of 1 mg/ml, prior to addition of labeled U4 RNA (21). For the competitive inhibition experiments involving 5-FU-containing RNAs, the 5-FU RNAs at a 6-fold molar excess over substrate RNA were incubated in the extract/reaction mixture for 10 min at 37°C prior to addition of ^{32}P-labeled RNAs. ^{32}P-Labeled RNA was purified on a 10% polyacrylamide, 8.3 M urea gel after incubation in the reactions. To determine site-specific Ψ formation, the gel-purified, ^{32}P-labeled RNA was RNase T1 digested, electrophoresed and the fragments eluted from a 20% polyacrylamide, 8.3 M urea gel. The fragments were nuclease P1 digested and analyzed by thin layer chromatography (TLC) on cellulose plates in 2-propanol:concentrated HCl:water (70:15:15 v/v/v) (22). For total Ψ formation gel-purified RNA was subjected directly to nuclease P1 digestion and TLC. The ^{3}H release assay used to measure Ψ formation in ^{3}H-labeled U4 RNA is a modification (16) of the procedure described by Mullenbach *et al.* (23).

Glycerol gradient centrifugation

Extracts were fractionated on glycerol gradients (10–30%) prepared and centrifuged at 4°C in an SW41 rotor for 18 h at 40 000 r.p.m. (16). The buffer for the gradients contained 150 mM KCl, 20 mM Tris–HCl, pH 7.6, 1.5 mM $MgCl_2$, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 0.5 mM DTT and samples were diluted with 1 vol of this buffer prior to overlaying on the gradient. After centrifugation the gradients were fractionated from the bottom. Every two fractions were combined,

dialyzed against buffer D (20 mM HEPES, pH 7.9, 20% v/v glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT) and concentrated with Centricon 30 microconcentrators (30 kDa cut-off). Total HeLa extract RNA was made by proteinase K treating (100 µg/ml) S100 or nuclear extract at 37°C for 1.5 h. The resulting RNA was phenol/chloroform extracted, ethanol precipitated and resuspended to 1/10 of the starting volume.

To separate U4 RNA associated with U6 RNA from U4 RNA alone, *in vitro* assembly reactions with labeled U4 RNA were centrifuged on 10–30% glycerol gradients in a SW41 rotor for 15.5 h at 35 000 r.p.m. at 4°C. After centrifugation the gradients were fractionated from the bottom and the fractions counted (Cherenkov). Apoferritin and alcohol dehydrogenase were centrifuged on parallel gradients as markers. Fractions at 16S and 8S were combined, the RNA isolated and the U4 RNA from each region analyzed for Ψ content by TLC assay.

RNase H digestion

Oligodeoxynucleotide-mediated RNase H digestion was carried out as described (24). The digestion of U6 RNA was accomplished using individual or a mixture of oligodeoxynucleotides antisense to nt 25–38, 45–59 and 60–72 of human U6 RNA, each at a concentration of 0.6 µg oligo/µl extract. An antisense U1 oligodeoxynucleotide (nt 1–14 of human U1 RNA) was used as a control at the same 0.6 µg/µl concentration.

RESULTS

Requirement of an RNP cofactor for Ψ formation in U4 RNA

When extracts were treated with micrococcal nuclease (MN) prior to addition of U4 RNA, Ψ formation in U4 RNA was inhibited (Table 1). The RNA assayed for Ψ was full-length, gel-purified ^{32}P-labeled U4 RNA. This result suggests that an RNA cofactor is required for Ψ formation in U4 RNA. The percent of theoretical is a way of expressing the amount of Ψ formed in U4 RNA during the reaction. Human U4 RNA has three Ψ at positions 4, 72 and 79 (Fig. 1), therefore 100% of theoretical would be equivalent to 3 mol Ψ/mol U4 RNA. The percent Ψ for U4 is 7.32%, since there are three Ψ out of 41 uridine plus Ψ residues. So, in untreated or mock-treated extracts between 63 and 74% of the theoretical amount of Ψ that could have been formed was detected in this exogenously added U4 RNA.

A sample of combined nuclear and S100 extracts was subjected to sedimentation velocity centrifugation on 10–30% glycerol gradients in order to determine the approximate size of this cofactor. Fractions from the gradients were collected from the bottom and every two fractions were combined, dialyzed and concentrated. The glycerol gradient fractions were then added to MN-treated, combined extracts, where the MN had been inactivated by addition of EGTA and poly(U) RNA. Then ^{3}H-labeled U4 RNA was added and incubated for 30 min (see Materials and Methods). The extent of Ψ formation was determined using the ^{3}H release method (8,23). Since the U4 substrate RNA was synthesized with [5-^{3}H]UTP, when Ψ is formed a ^{3}H ion is released to solvent, which forms the basis of the assay. The assays were carried out in triplicate for all samples and the results are shown in Figure 2. Partial activity in a broad peak is restored with the addition of glycerol gradient fractions having an average sedimentation velocity of 14 S. This experiment was repeated using a different gradient fractionation of combined extracts and ^{32}P-labeled U4 RNA as substrate with TLC as the assay. The incubation time was much longer (3 h) but the results were basically the same (data not shown). This peak fraction might contain Ψ synthase but the activity would have a molecular mass of ~400 kDa, much larger than any known Ψ synthase (25–29).

Table 1. The effect of micrococcal nuclease digestion on Ψ formation in U4 RNA

Treatment	Percent Ψ (± SD)[a]	Percent of theoretical (± SD)[b]
None	4.6 (0.06)	63 (0.8)
MN-treated	0.2 (0.01)	3 (0.1)
Mock-treated (EGTA added before MN)	5.4 (0.05)	74 (0.7)

[a]To obtain percent Ψ the TLC plate was exposed to film and the autoradiograph used to identify the uridine and Ψ spots. These spots were scraped from the TLC plates, counted in scintillant for 1 h, corrected for background and the counts used to obtain a ratio of Ψ counts to the total counts in uridine and Ψ spots. In addition, a 'no extract' control value for percent Ψ (0.53%) was subtracted from the values for RNAs incubated in extracts.
[b]Percent of theoretical is obtained by comparing the observed percent Ψ and the theoretical percent of Ψ ([Ψ/U +Ψ] × 100) expected from the known sequence. The theoretical percent for the entire sequence of U4 RNA is 7.32%.

Table 2. Micrococcal nuclease sensitivity of the peak glycerol gradient fraction

Sample	Mean c.p.m. ^{3}H released (± SD)[a]
Mock, no MN added to extracts	922 (35)
MN-treated extracts, no fraction added	101 (14)
MN-treated extracts and peak fraction	380 (13)
MN-treated extracts and MN-treated peak fraction	73 (23)

[a]Counts from three separate assays were corrected for background by a 'no extract' control and the mean is reported. In this experiment 19 c.p.m. ^{3}H was subtracted from the counts released by the RNAs incubated in the extracts.

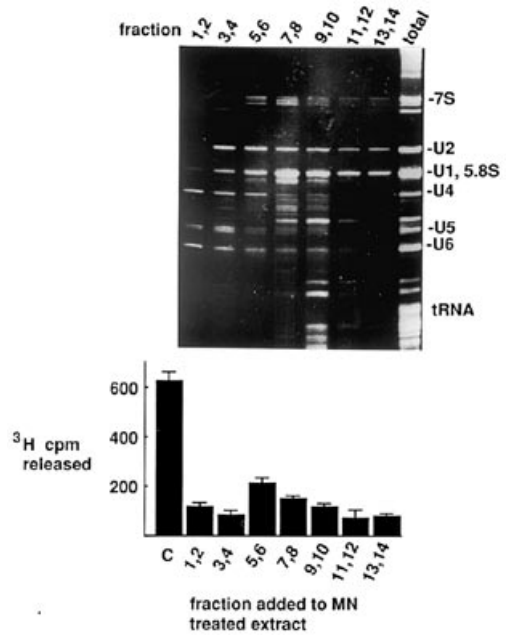

Figure 2. Fractionation of complementation activity by glycerol gradient centrifugation of cellular extracts. Glycerol gradient fractions from sedimentation velocity gradient centrifugation on 10–30% gradients in a SW41 rotor at 40 000 r.p.m. at 4°C for 18 h. The S values were calculated by running standards with known S values in parallel on identical glycerol gradients. Every two fractions were combined, dialyzed and used in a complementation assay that involved adding aliquots of the fractions to MN-treated extracts, incubation for 30 min and ^{3}H release assay. Counts from three separate assays were corrected for background by a 'no extract' control and the mean and standard deviation between the assays is reported. In this experiment 73 c.p.m. ^{3}H were subtracted from the counts released by the RNA incubated in the extracts for the 'no extract' control. C is mock-treated extract with no fraction added.

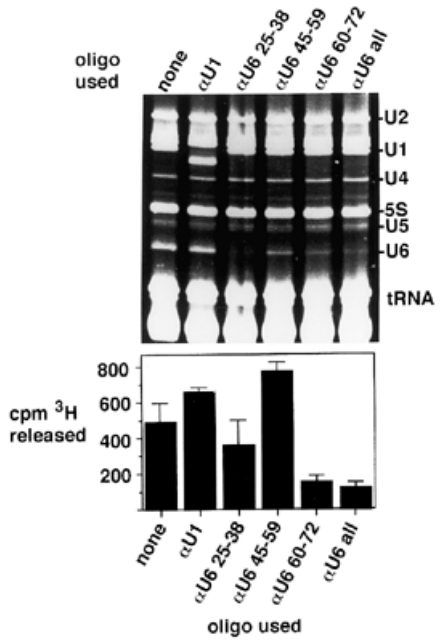

Figure 3. Oligodeoxynucleotide-mediated digestion of U6 RNA inhibits formation of Ψ in U4 RNA. Extracts were incubated with the oligodeoxynucleotides (shown in Fig. 1) indicated at the top and bottom of the figure prior to addition of the ^{3}H-labeled U4 RNA. The amount of ^{3}H released (three aliquots) during incubation was measured as described in Materials and Methods. RNA from an aliquot of the reaction was isolated and electrophoresed on a 10% polyacrylamide, 8.3 M urea gel and stained with ethidium bromide. A picture of a portion of the gel with UV illumination is shown with the prominent small RNA bands noted to the right of the figure. Fluorography of the gel was used to confirm that the ^{3}H-labeled U4 RNA was still intact (data not shown).

The activity found in the peak fraction is MN sensitive, as shown in Table 2. Extracts composed of a combination of S100 and NE were treated with MN and shown to be sensitive to the nuclease (see the first two rows in Table 2). The low residual activity is most likely due to the use of this assay on crude extracts rather than significant amounts of the RNA cofactor remaining undigested, since the residual levels are much lower in Table 1, where the TLC assay was used. When a portion of the peak fraction was added to the MN-treated extracts there was a partial restoration of activity, but when that fraction was itself treated with MN the activity was lost (compare the last two rows in Table 2). These data show that the activity found in the peak fraction is due to an RNA-containing cofactor and not due to significant additional enzymatic activity in the fraction. It is possible that the cofactor is an RNA-containing enzyme, but the cofactor will need to be isolated in order to make that determination.

U6 RNA is required for Ψ formation on U4 RNA

The most likely candidate for the RNA cofactor would be U6 RNA, since the two RNAs, U4 and U6 RNA, form a single RNP. Pseudouridine formation on U6 RNA depends on its interaction with U4 RNA (8). In order to determine if U6 RNA is the cofactor necessary for U4 RNA modification, U6 RNA in the extracts was targeted for oligodeoxynucleotide-mediated RNase H digestion. The combined extracts were pretreated with oligodeoxynucleotides antisense to U6 RNA (see Fig. 1) or to U1 (αU1) and then ^{3}H-labeled U4 RNA was added, the reactions incubated for 30 min and the amount of ^{3}H released determined. The results are shown in Figure 3. There was a significant reduction in formation of Ψ when the oligodeoxynucleotide antisense to the region of U6 that participates in stem II formation (nt 60–72 on U6 RNA) was preincubated with the extracts [lanes marked αU6(60–72) and αU6all]. There was no effect on Ψ formation in U4 RNA when the αU1 oligodeoxynucleotide was used, which targets the 5′-end of U1 RNA, or when the αU6(45–59) oligodeoxynucleotide was used. There was a slight but reproducible reduction in ^{3}H released when an αU6(25–38) oligodeoxynucleotide was used, but the change versus no oligodeoxynucleotide was not significant. The levels of U6 RNA present in the lanes in the gel in Figure 3 are much lower for the anti-U6-treated samples, but variable. αU6(25–38) shows the least amount of U6 RNA but inhibition of ^{3}H release is not as low as when all the oligodeoxynucleotides or when αU6(60–72) was used. It is possible that when just one oligodeoxynucleotide was used, incompletely digested U6 RNAs can still interact with U4 and allow for Ψ formation. αU6(45–59) did not inhibit Ψ formation in U4 and it is possible this oligodeoxynucelotide did not provide a good substrate for RNase H, since there is a significant amount of U6 RNA remaining. A separate experiment using a different preparation of extract and an oligodeoxynucleotide antisense to U2 RNA gave the same results (data not shown).

Table 3. U4 RNA associating with U6 RNA is enriched in Ψ

Treatment		Percent Ψ (± SD)[a]	Percent of theoretical (± SD)
15 min incubation	16S	0.43 (0.02)	6 (0.3)
	8S	0.07 (0.01)	1 (0.1)
30 min incubation	16S	0.96 (0.02)	13 (0.3)
	8S	0.69 (0.01)	9 (0.1)
60 min incubation	16S	3.13 (0.05)	43 (0.7)
	8S	1.78 (0.03)	24 (0.4)
60 min incubation with antisense U6 oligos	8S	0.70 (0.02)	10 (0.3)

[a]The method outlined in Table 1 was used to obtain percent Ψ and percent of theoretical. In this experiment the 'no extract' control was 0.35% Ψ. The glycerol gradients used in this experiment were 10–30% centrifuged on a SW41 rotor at 35 000 r.p.m. at 4°C for 15.5 h. Markers for 18 and 8S were centrifuged on identical gradients in the same run.

Fluorography of the gel shown in Figure 3 showed that the ^{3}H-labeled U4 RNA was equal in all the lanes and not degraded, so the reason for lowered counts was not due to an altered half-life of the substrate U4 RNA in the oligodeoxynucleotide-treated extracts. These data suggest that U6 RNA is necessary for Ψ modification of U4 RNA.

If interaction with U6 RNA is necessary for formation of Ψ in U4 RNA then U4 RNA that is associated with U6 RNA should be enriched in Ψ at early time points of the *in vitro* modification reaction. Glycerol gradient centrifugation of the *in vitro* reactions was used to separate U4 associated with U6 from U4 RNA that is not. The amount of Ψ found in the U4 RNA that sedimented at 16S (associated with U6 RNA) and at 8S (U4 RNA alone) was determined for different lengths of incubation and for reactions treated with anti-U6 RNA oligodeoxynucleotides. The results (Table 3) show that U4 RNA found at 16S was enriched in Ψ relative to the U4 RNA found at 8S for all time points. In addition, the amount of Ψ found at 8S after treatment with anti-U6 oligodeoxynucleotides was reduced. There was no U4 RNA at 16S when U6 was destroyed, so no Ψ assay could be made. The amount of Ψ found in U4 RNA not complexed with U6 is significant at the 1 h time point, but this is probably due to recycling of U4 RNA in the extracts and not to a relaxing of the requirement for interaction with U6 RNA.

In vitro synthesized U6 RNA or native U6 RNA isolated from HeLa cells was added to either extracts treated with the anti-U6 oligodeoxynucleotides or MN-treated extracts to determine if formation of Ψ in U4 RNA can be restored. No restoration of activity was observed with either method of extract treatment. In addition, no restoration of activity was seen when total RNA from nuclear or S100 extracts was used. It is possible that although U6 RNA is necessary for Ψ formation in U4 RNA it is not sufficient; additional cofactors could be required other than the enzymes. Alternatively, it is possible that the proper set of reconstitution conditions for assembly of U6 RNP have not been arrived at.

Number of Ψ synthase activities necessary for complete U4 RNA pseudouridylation

U6 RNA is necessary for Ψ formation but how many enzymes recognize U4 RNA associated with U6 RNA? To define the number of activities involved in Ψ formation in U4 RNA we used 5-FU-containing wild-type and mutant U4 RNAs as inhibitors of Ψ formation in unmodified ^{32}P-labeled U4 RNA substrates. 5-FU-containing RNAs have been shown to be specific and potent inhibitors of both tRNA and snRNA Ψ synthases (9,30) and are quite stable in the extracts (9,10). When 5-FU-containing wild-type RNAs are preincubated in extracts prior to addition of unmodified ^{32}P-labeled substrate RNA, Ψ formation will be inhibited at all sites in the substrate RNA (see Fig. 4). However, when deletion or point mutations are made in the 5-FU-containing RNAs at sites where Ψ is normally formed there are two possible results. If there are separate synthases that are specific for sites of Ψ formation, then Ψ will be formed in the substrate RNA at the position where there is a mutation in the 5-FU-containing U4 RNA. If the mutant 5-FU-containing U4 RNA inhibits formation of Ψ in the substrate U4 RNA to the same extent as 5-FU-containing wild-type U4 RNA then this would argue that there is just one Ψ synthase that acts at all sites in U4 RNA (see Fig. 4).

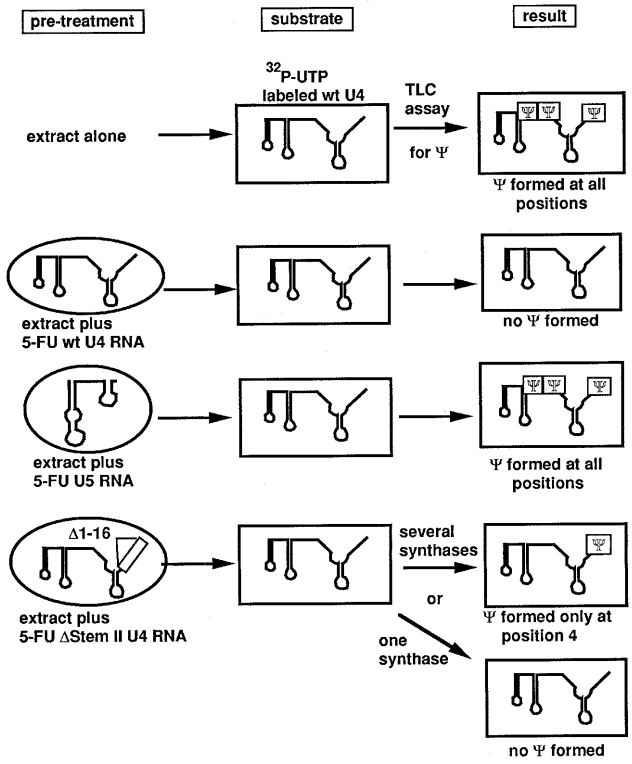

Figure 4. Diagram of the competition experiment and expected results. Each pretreatment is different for each of the conditions, whereas the substrate added to the reaction after pretreatment is the same. The results predicted for the condition where the extract is treated with the 5-FU ΔStem II U4 RNA will be different depending on whether there are several Ψ synthases that are specific for particular positions in U4 or whether there is only one synthase that modifies all three positions.

Table 4. Competitive inhibition of U4 Ψ synthase activities: Ψ formation when 5-FU-containing U4 RNAs are present in the reaction

5-FU RNA[a]	21 nt (72,79) Percent Ψ[b] (± SD)	Percent of theoretical[c] (± SD)	5 nt (4) Percent Ψ[b] (± SD)	Percent of theoretical[c] (± SD)
None	34.4 (0.69)	85.9 (1.72)	9.8 (0.26)	89.1 (2.34)
Wild-type U4	6.8 (0.14)	17.0 (0.34)	3.0 (0.10)	27.3 (0.94)
ΔSm	11.2 (0.28)	28.1 (0.70)	2.0 (0.08)	18.2 (0.71)
ΔStem I	5.9 (0.12)	14.8 (0.30)	3.3 (0.12)	30.0 (1.04)
ΔStem II	9.0 (0.18)	22.4 (0.45)	11.3 (0.29)	102.7 (2.58)
Δ5′Stem–loop	3.0 (0.07)	7.6 (0.19)	0.8 (0.03)	7.2 (0.30)
U5	34.7 (0.94)	86.8 (2.35)	n.d.[d]	n.d.

[a]The mole ratios for substrate U4 RNAs to the 5-FU-containing RNAs were 1:6.
[b]Percent Ψ was calculated as described in the legend to Table 1. For this experiment the 'no extract' control value was 0.30%.
[c]Percent of theoretical was calculated as described in the legend to Table 1 except that the theoretical percentage for the 21 nt RNase T1 fragment is 40% (2Ψ/5U + Ψ) and the value for the 5 nt fragment is 11% (1Ψ/9U + Ψ, since there are four 5 nt RNase T1 fragments).
[d]n.d., no counts detected.

The deletion mutants ΔSm, ΔStem I, ΔStem II and Δ5′Stem–loop U4 RNAs, wild-type U4 RNA and U5 RNA were synthesized *in vitro* with 5-FUTP, gel purified and incubated in S100 for 10 min at 37°C at a molar ratio of 1:6 (unmodified ^{32}P-labeled U4 RNA:5-FU-containing RNAs). The properties of these deletion mutants were previously described (see Fig. 1; 5). Wild-type U4 RNA and all the mutant U4 RNAs participate in U4–U6 interaction in a reconstitution assay, except for the ΔStem II mutant, and all of the U4 RNAs bind Sm proteins, except for the ΔSm mutant (5). Unmodified ^{32}P-labeled U4 RNA was then added to S100 containing the individual 5-FU-containing RNAs and incubated for 30 min at 37°C, followed by addition of NE that had also been preincubated with individual 5-FU-containing U4 RNAs. The reaction mixtures were further incubated for 2.5 h at 37°C, the ^{32}P-labeled U4 RNA purified on a 10% polyacrylamide, 8.3 M urea gel, eluted and RNase T1 digested. The RNase T1 fragment of 21 nt, containing Ψ at positions 72 and 79, and the 5 nt fragment, containing Ψ at position 4, were gel purified on a 20% polyacrylamide, 8.3 M urea gel, eluted and subjected to nuclease P1 digestion and TLC. The results are shown in Table 4. When extracts were treated with 5-FU-containing wild-type U4 RNA Ψ formation in the ^{32}P-labeled U4 RNA was reduced to 17% of theoretical for positions 72 and 79 and 27% for position 4. The control (no 5-FU-containing RNA added) showed 86 and 89% of theoretical for the two T1 fragments respectively, showing that with no inhibitor present this *in vitro* modification system approaches complete modification of all three positions in the U4 RNA substrate. When 5-FU-containing U4 mutant RNAs ΔStem I and ΔStem II were used, Ψ formation was also severely inhibited at positions 72 and 79, giving only 15 and 22% of theoretical respectively. These results were expected, since the 5-FU-containing RNA deletion mutants all contain nt 72 and 79 and can compete effectively for Ψ synthases that recognize these positions. The formation of Ψ at position 4 when using these two mutants gave very different results. The ΔStem I mutant inhibited Ψ formation at position 4 just as well as wild-type U4 RNA (30% of theoretical), but the 5-FU ΔStem II mutant RNA did not inhibit Ψ formation at position 4 at all, exhibiting 103% of theoretical for this position. The ΔStem II mutant is missing nt 1–16 and cannot effectively inhibit the Ψ synthase that would modify uridine to Ψ at position 4. These results reveal that at least two Ψ synthase activities are required for full modification of U4 RNA, with one activity for position 4 and at least one activity for positions 72 and 79. ΔStem II U4 RNA does not interact with U6 RNA in a reconstitution assay (5) and yet it functions as an inhibitor of Ψ formation in this assay. It is possible that there is some interaction between this U4 mutant and U6, but this interaction was not detected by native RNP gels (5).

As expected, 5-FU-containing U5 RNA did not inhibit Ψ formation at positions 72 and 79. The 5-FU-containing Δ5′Stem–loop mutant U4 RNA inhibited even better than wild-type U4 5-FU-containing RNA (Table 4), probably due to the fact that this RNA has been characterized as having a 2-fold higher binding capacity for U6 RNA when compared with wild-type U4 RNA (5). This result is consistent with a requirement for U6 RNA interaction for Ψ formation in U4 RNA.

The 5-FU-containing ΔSm mutant U4 RNA, which does not bind Sm proteins (5), did not inhibit as well as the wild-type at positions 72 and 79 (28%), but did inhibit slightly better than the wild-type at position 4 (18 versus 27% of theoretical respectively). This is consistent with a requirement for Sm protein binding for Ψ formation at positions 72 and 79 of U4 RNA, since these two positions are closer to the Sm binding site of U4 RNA.

To confirm that more than one Ψ synthase activity is required for Ψ formation in U4 RNA point mutants were made at positions 4 and 72 of U4 RNA and 5-FU-containing RNAs transcribed *in vitro* (see Materials and Methods). A point mutant corresponding to position 79 was not created since positions 72 and 79 are located on the same 21 nt RNase T1 fragment and it would be difficult to discern between modifications at these two sites. Wild-type U4 5-FU-containing RNA inhibited formation of Ψ at position 4 of U4 RNA, giving a value of 9% of theoretical in this experiment (Table 5). As expected, the U4 A72 5-FU-containing RNA inhibited U4 Ψ formation at position 4 similarly to wild-type U4 5-FU-containing RNA (12% of control), since the mutation was at position 72 not position 4. However, when U4 A4 5-FU-containing mutant RNA was used, 54% of theoretical for Ψ formation at position 4 was observed. This was the expected result if there are separate specific Ψ synthases, given that 5-FUTP is not incorporated at position 4 in this point mutant. Again, the U5 5-FU-containing competing RNA did not inhibit Ψ formation in U4 RNA, although we only see a 47% of theoretical value at this position when this RNA is used as inhibitor.

Table 5. Competitive inhibition of U4 Ψ synthase activities: Ψ formation when 5-FU-containing U4 RNA point mutants are present in the reaction

5-FU RNA[a]	21 nt (72,79)		5 nt (4)	
	Percent Ψ[b] (± SD)	Percent of theoretical[c] (± SD)	Percent Ψ[b] (± SD)	Percent of theoretical[c] (± SD)
None	34.2 (0.48)	85.5 (1.19)	9.3 (0.23)	84.5 (2.09)
Wild-type U4	4.9 (0.14)	12.3 (0.36)	1.0 (0.04)	9.1 (0.32)
U4 A4	4.0 (0.15)	10.0 (0.36)	5.9 (0.18)	54.5 (1.64)
U4 A72	12.1 (0.33)	30.3 (0.83)	1.3 (0.05)	11.8 (0.45)
U5	38.2 (1.18)	95.5 (2.96)	5.2 (0.12)	47.3 (1.09)

[a]The mole ratios for substrate U4 RNAs to the 5-FU-containing RNAs were 1:6.
[b]Percent Ψ was calculated as described in the legend to Table 1. For this experiment the 'no extract' control value was 1.20%.
[c]Percent of theoretical was calculated as described in the legend to Table 4.

When 5-FU-containing A4 mutant U4 RNA was used to treat extracts, Ψ formation at positions 72 and 79 was 10% of theoretical, almost identical to that seen with wild-type U4 5-FU-containing RNA (Table 5). The 5-FU-containing A72 mutant RNA, on the other hand, was 30% of theoretical, which shows moderate inhibition and suggests that Ψ formation was still occurring at position 79 (compare U4 wild-type 5-FU-containing RNA and A4 5-FU-containing RNA with A72 5-FU-containing RNA results in the 21 nt column). This suggests, but does not prove, that sites 72 and 79 do not share a Ψ synthase. As expected, U5 5-FU-containing RNA did not compete, revealing the specificity of inhibition.

There is variation in the control values between each of these inhibition experiments (deletion versus point mutants), but since each experiment has a set of controls (no 5-FU-containing RNA, wild-type 5-FU-containing U4 and 5-FU-containing U5) for comparison within the experiment, meaningful conclusions can be drawn for each set of data.

DISCUSSION

The requirement for an RNA cofactor for modification of another RNA, as shown in this report, appears to be an emerging theme in the metabolism of stable RNAs. Recently it was shown that snoRNAs are required for 2′-*O*-methylation of ribose and formation of Ψ in rRNA (31–34). The snoRNAs base pair with rRNA in the region adjacent to the nucleotide that is ultimately modified. Since the different snoRNAs provide the means by which the modification site is recognized it would appear that a single Ψ synthase or methylase might modify all positions on a single rRNA, since the same secondary structure is recognized at all positions of modification (31–34). The results presented in this report suggest that although an RNA cofactor is required, there are several Ψ synthases that participate in formation of Ψ in U4 RNA. It has been shown that efficient *in vitro* formation of Ψ in U6 RNA requires interaction of U6 RNA with U4 RNA (8). Although it is possible that Ψ formation in U1 or U5 RNA might require RNA cofactors, since that possibility was not formally tested (9,16,17) not all snRNAs require RNA cofactors for Ψ formation. MN-treated HeLa S100 extracts support Ψ formation in unmodified U2 RNA (Patton, unpublished data).

U4 RNA is complexed with U6 RNA in HeLa cells and it was expected that this complex would be the substrate for Ψ synthase. The inhibitor experiments using 5-FU-containing mutant U4 RNAs bear this out. Interestingly, the 5-FU-containing ΔStem II mutant U4 RNA was a good inhibitor of Ψ formation at positions 72 and 79, even though this mutant has been characterized as not interacting with U6 RNA (5). It is possible that the methods used to characterize this interaction were too stringent (5) and that there is sufficient interaction in our modification reactions to allow the 5-FU-containing ΔStem II mutant U4 RNA to interact with U6 RNA and serve as a competitive inhibitor of Ψ formation at sites 72 and 79 in the U4 RNA substrate. Of course, since the first 16 nt are missing in this mutant it did not effectively compete when it came to formation of Ψ at position 4 in U4 RNA. On the other hand, the 5-FU-containing Δ5′Stem–loop mutant U4 RNA, which should interact with U6 RNA twice as strongly as wild-type U4 RNA (5), does in fact show greater inhibition of Ψ formation than 5-FU-containing wild-type U4 RNA at all positions in the U4 RNA substrate.

Simply adding back whole RNA or isolated U6 RNA from HeLa extracts as well as U6 RNA synthesized *in vitro* did not restore Ψ formation in U4 RNA (data not shown). This is in contrast to the restoration of Ψ formation in U6 RNA that was seen when *in vitro* synthesized U4 RNA was added back to MN-digested extracts (8). It is possible that the conditions used were not the most efficient for reconstitution of a U6 RNP cofactor that restores activity or that although U6 RNA is necessary, it is not sufficient for Ψ formation in U4 RNA and another RNA-containing cofactor is required.

In this report it was determined that U4 RNA requires at least two different Ψ synthase activities for complete Ψ formation in U4 RNA. These separate Ψ synthases use the same U6 RNA but are specific for certain residues either contained in a U4 RNA/U6 RNA intermolecular stem (position 4 in stem II) or in regions of the U4 RNA that are exposed due to its interaction with U6 RNA (positions 72 and 79). Instead of separate enzymes it could be argued that every position is modified by a shared catalytic subunit interacting with different determinant subunits. The fact that each 5-FU-containing RNA did not inhibit Ψ formation at every site in an unmodified substrate RNA argues against a shared catalytic subunit (9,10). It has already been shown that U1, U2 and U5 RNAs are modified by distinct Ψ synthase activities (9,10). These same snRNAs have also been shown to require multiple Ψ synthase activities for complete Ψ formation, suggesting the possibility that each uridine that is modified to Ψ requires a separate Ψ synthase activity. Expending this much energy and resources to fully modify the snRNAs is metabolically expensive and suggests that Ψ formation is important for the

structure, stability and possibly function of snRNAs when conservation of these modifications is considered.

It is possible that the Ψ synthases could be RNA-containing enzymes. This determination will await isolation or cloning of the activities that modify U4 RNA. Since the data in this report point to at least two separate activities that modify U4 RNA, it would seem more likely that there is one RNA cofactor (U6 RNA plus another possible and as yet unidentified RNA) that recognizes U4 RNA and that this interaction is recognized by the separate enzymes rather than separate RNA-containing enzymes that recognize U4 RNA.

The experiments reported here were done *in vitro*, but how does modification of U4 and U6 RNAs proceed in the cell? A likely scenario is that U4 RNA which appears to require U6 RNA is modified in the nucleus (35) after assembly into an Sm-containing snRNP. U4 RNA that is bound to Sm also allows more efficient Ψ formation in U6 RNA (8). This interaction suggests that modification of U4 and U6 RNAs might occur simultaneously.

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