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Metabolism of pre-messenger RNA splicing cofactors: modification of U6 RNA is dependent on its interaction with U4 RNA

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ABSTRACT

The requirements for the formation of pseudouridine (Ψ) in U4 and U6 RNAs, cofactors in the splicing of pre-messenger RNA, were investigated *in vitro* using HeLa nuclear (NE) and cytoplasmic (S100) extracts. Maximal Ψ formation for both RNAs was extract order-dependent. Maximal Ψ formation in U4 RNA required incubation in S100 followed by the addition of NE, paralleling the *in vivo* maturation pathway of U4 RNA. In contrast, maximal formation of Ψ in U6 RNA required incubation in NE followed by the addition of S100 extract. Since U6 RNA does not exit the nucleus *in vivo* the contribution of S100 was investigated. In experiments where the extracts were treated with micrococcal nuclease to digest endogenous snRNAs, the efficient formation of Ψ in U6 RNA was dependent on the presence of U4 RNA, but not in U5 RNA or tRNA. When mutant U4 RNAs that inhibit or strengthen the interaction between U4 RNA and U6 RNA were substituted for wild-type U4 RNA, the results confirmed the need for the interaction between these two RNAs for Ψ formation in U6 RNA. U6 RNA isolated from glycerol gradients after incubation in extracts had four times as much Ψ when associated with U4 RNA.

INTRODUCTION

Small nuclear ribonucleoprotein particles (snRNPs) are essential cofactors in the splicing of premessenger RNA (pre-mRNA; 1). The snRNPs are composed of highly conserved small nuclear RNAs (snRNAs), U1, U2, U4, U5 and U6, and associated proteins. A common core of proteins, the Sm proteins, are found in all the snRNPs, while additional proteins are specific to particular snRNPs (2,3). The snRNPs involved in splicing are highly modified, and these modifications include base methylations, 3′-end processing, 5′-end capping and pseudouridine (Ψ) formation (4,5). U4 and U6 RNAs are extensively base paired and form one snRNP which enters the spliceosome complexed with U5 snRNP as part of a tri-snRNP. This interaction between U4 and U6 RNA is essential for spliceosomal assembly and subsequent spliceosomal function (6–8). Several lines of evidence indicate U6 RNA is a catalytic component of the spliceosome, while U4 RNA appears to function in ferrying U6 into the spliceosome, and perhaps in keeping U6 RNA inactive until needed in splicing (9).

The formation of Ψ in these snRNAs has been the subject of several reports. Using *in vitro* transcribed snRNAs and extracts from HeLa cells, there is evidence for multiple Ψ synthase activities that specifically recognize U1, U2 and U5 snRNAs (10,11). In addition, for U2 snRNA which contains 13 Ψ residues, the formation of Ψ is not dependent upon earlier Ψ formation at another site (11). U5 snRNA contains three Ψ residues but only two sites were modified in HeLa S100 (essentially cytoplasmic) extracts. Modification at the third site required HeLa nuclear extract (NE), again suggesting multiple Ψ synthase activities for a single snRNA (12). Efficient Ψ modification at all sites in U5 snRNA requires Sm protein binding, while Ψ modification at some sites in U2 RNA does not require particle assembly (11,13).

The function of Ψ in snRNAs is unknown, but it is important to note that Ψ is found in regions of snRNAs that are necessary for snRNP function in the splicing of pre-mRNA (14,15). When U2 snRNA that contains no modifications was added to U2-depleted extracts, pre-mRNA splicing was not restored, however, U2 RNA isolated from HeLa cells was able to restore splicing. When either unmodified or fully modified U5 snRNA was added to U5-depleted extracts, splicing was restored (16). This difference may be due to the fact that U2 snRNP contains a large percentage of Ψ in contrast to U5 snRNP, and its absence may have a greater impact on the function of U2 snRNP than on the function of U5 snRNP (16).

In tRNA, Ψ appears to be required for the efficient reading of codons during the translation process (17). *HisT* in *Escherichia coli* codes for a Ψ synthase that modifies certain positions in the anticodon of tRNAs. The *hisT* gene product is necessary for normal growth of *E.coli* on minimal media since the *hisT* mutant has excessive need for uracil that interferes with cell division (18,19). Most Ψ residues in rRNAs are found near the functional centers of the ribosome (20,21). Recently, it has been shown that acetylated Ψ, but not acetylated uridine, can transfer an acetyl group to the N-terminus of a peptide, suggesting that Ψ participates in the acyl transfer reaction in the ribosome (22).

In this report, the formation of Ψ in U4 and U6 RNA was studied and was found to be HeLa extract order-dependent, with at least two Ψ synthase activities required for Ψ formation in U4

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RNA. In addition, the efficient formation of Ψ in U6 RNA is dependent on its interaction with U4 RNA.

MATERIALS AND METHODS

SP6 transcriptions of the *Dra*I-cut human U4 RNA and U4 RNA mutants pSP6-U4, pSP6-U4 ΔStemI (deleting nucleotides 56–63), pSP6-U4 ΔStemII (deleting nucleotides 1–16), pSP6-U4 Δ5′Stem–loop (deleting nucleotides 19–55) and of the *Xba*I-cut human U4 mutant pSP6-U4 ΔSm (deleting nucleotides 91–145) were performed as described (10–12). The U4 clones were a generous gift from Albrecht Bindereif, Humbolt University, Germany (6). Human U6 RNA was transcribed *in vitro* using T7 polymerase and *Dra*I-cut pHU6-1 as described (23) and human U5 RNA was transcribed using SP6 RNA polymerase and *Bfa*I-cut pHU5a2 (12). Pre-tRNA^{Ser} was transcribed with T7 RNA polymerase and *Ava*I-cut pUC19pSer and was a gift from C. Guerrier-Takada and S. Altman, Yale University (unpublished). The *in vitro* transcription reactions contained, depending upon the application, [α-^{32}P]UTP (50 μCi, 800 Ci/mmol) or [5-^{3}H]UTP (1–50 μCi, 17 Ci/mmol), 50 μM GTP, 250 μM ATP and CTP. When U4 RNA was synthesized, m7GpppG was included in the reaction mixture at 1 mM. Low specific activity [5-^{3}H]UTP labeled RNAs were made with 50 μM UTP and 1 μCi [5-^{3}H]UTP to facilitate the determination of the amount of RNA synthesized. RNAs to be used as substrates in the ^{3}H release/Norit A charcoal binding assay (19) were synthesized in the presence of 50 μCi [5-^{3}H]UTP with no additional UTP added.

The *in vitro* modification reactions were carried out as previously described in 300 μl total volume using HeLa S100 and NE extracts (10–12,24). Briefly, the reaction mixture contained 60% HeLa S100 and/or NE extract (25) by volume, 0.5 mM ATP, 20 mM creatine phosphate, 3.2 mM $MgCl_2$ and 2 mM dithiothreitol. RNAs were incubated 30 min at 37°C in either HeLa S100 or NE or a combination of the two extracts. That incubation was followed by the addition of another aliquot of reaction/extract mix (see particular experiments for details), and incubated for an additional 2.5 h at 37°C. 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 poly A/poly C to a final concentration of 1 mg/ml, prior to the addition of ^{32}P-labeled RNA. ^{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) (10–12,24). For total Ψ, gel purified RNA was subjected directly to nuclease P1 digestion and TLC. The formation of Ψ on [5-^{3}H]UTP labeled RNA was assayed in a ^{3}H release/Norit A charcoal binding assay, where the release of ^{3}H to solvent water is an indication of Ψ formation (12,19).

Glycerol gradients (10–30%) were prepared and centrifuged at 4°C in an SW41 rotor for 18 h at 40 000 r.p.m. (12). 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 and 0.5 mM dithiothreitol. After centrifugation the gradients were fractionated from the bottom.

RESULTS

HeLa extract order-dependence for Ψ formation

U4 and U6 RNAs contain three Ψ residues each, located at positions 4, 72 and 79 in human U4 RNA, and at positions 31, 40 and 86 in U6 RNA (Fig. 1) (3). In order to determine the conditions necessary for maximum *in vitro* Ψ formation, the extract-order dependence for Ψ formation in U4 RNA was investigated. [5-^{3}H]UTP labeled U4 RNA was incubated in HeLa NE or S100 extract or a combination of the extracts, followed by another aliquot of NE or S100 or a combination of the two extracts, and further incubation. The amount of ^{3}H that does not bind to Norit A charcoal, ^{3}H released from uridine to the bulk solvent when the C·C glycosidic bond is formed, is a function of Ψ formation (10,12,19). The highest levels of Ψ formation in U4 RNA were observed for incubation in S100 followed by incubation in NE, and S100 incubation followed by the addition of another aliquot of S100, at 19.0 and 15.3%, respectively (Table 1). The fact that the highest amount of Ψ was observed with HeLa cytoplasmic extract followed by nuclear extract was expected given that after U4 RNA is transcribed it exits into the cytoplasm, is assembled into an RNP and modified, and then enters the nucleus, where it may undergo further modification before being incorporated into the spliceosome.

Table 1. HeLa extract order-dependence for Ψ formation in U4 and U6 RNAs

Incubation conditions[a]	c.p.m. of ^{3}H released (±sd)[b]	% of theoretical (±sd)[c]
U4 RNA		
S100/NE	622 (20.8)	14.2 (0.47)
NE–S100	523 (22.7)	11.9 (0.52)
S100–NE	829 (101.7)	19.0 (2.33)
NE–NE	319 (27.3)	7.3 (0.62)
S100–S100	674 (32.4)	15.3 (0.74)
U6 RNA		
S100/NE	444 (14.1)	6.4 (0.20)
NE–S100	664 (21.1)	9.6 (0.31)
S100–NE	156 (39.7)	2.3 (0.59)
NE–NE	426 (27.0)	6.2 (0.39)
S100–S100	223 (11.0)	3.2 (0.16)

[a]Incubation conditions are as follows: S100/NE, extracts added in combination followed by another aliquot of combined extract; NE–S100, incubated first in NE followed by S100; S100–NE, S100 first, then NE; NE–NE, NE followed by another aliquot of NE; S100–S100, S100 then another aliquot of S100.

[b]Counts were corrected for background by a 'no extract' control. In this experiment 15 c.p.m. ^{3}H was subtracted from the counts released by the RNAs incubated in extracts.

[c]The percent of theoretical is obtained by determining the actual number of counts due to Ψ formation compared with the theoretical number of counts that could be released based on the percent of Ψ (Ψ/U + Ψ) expected from the known sequence of human U4 and U6 RNAs and the number of counts of ^{3}H-labeled U4 or U6 RNA added to the reaction. The theoretical percent Ψ for U4 RNA is 7.32% (3 Ψ/41 U + Ψ), while the theoretical percent Ψ for U6 is 11.53% (3 Ψ/26 U + Ψ).

We also determined the extract order-dependence for Ψ formation in U6 RNA using this same ^{3}H release assay. In contrast to U4 RNA, the maximal amount of Ψ was observed for the incubation conditions of NE followed by S100 extract addition, at 9.6% of theoretical (Table 1). This result was

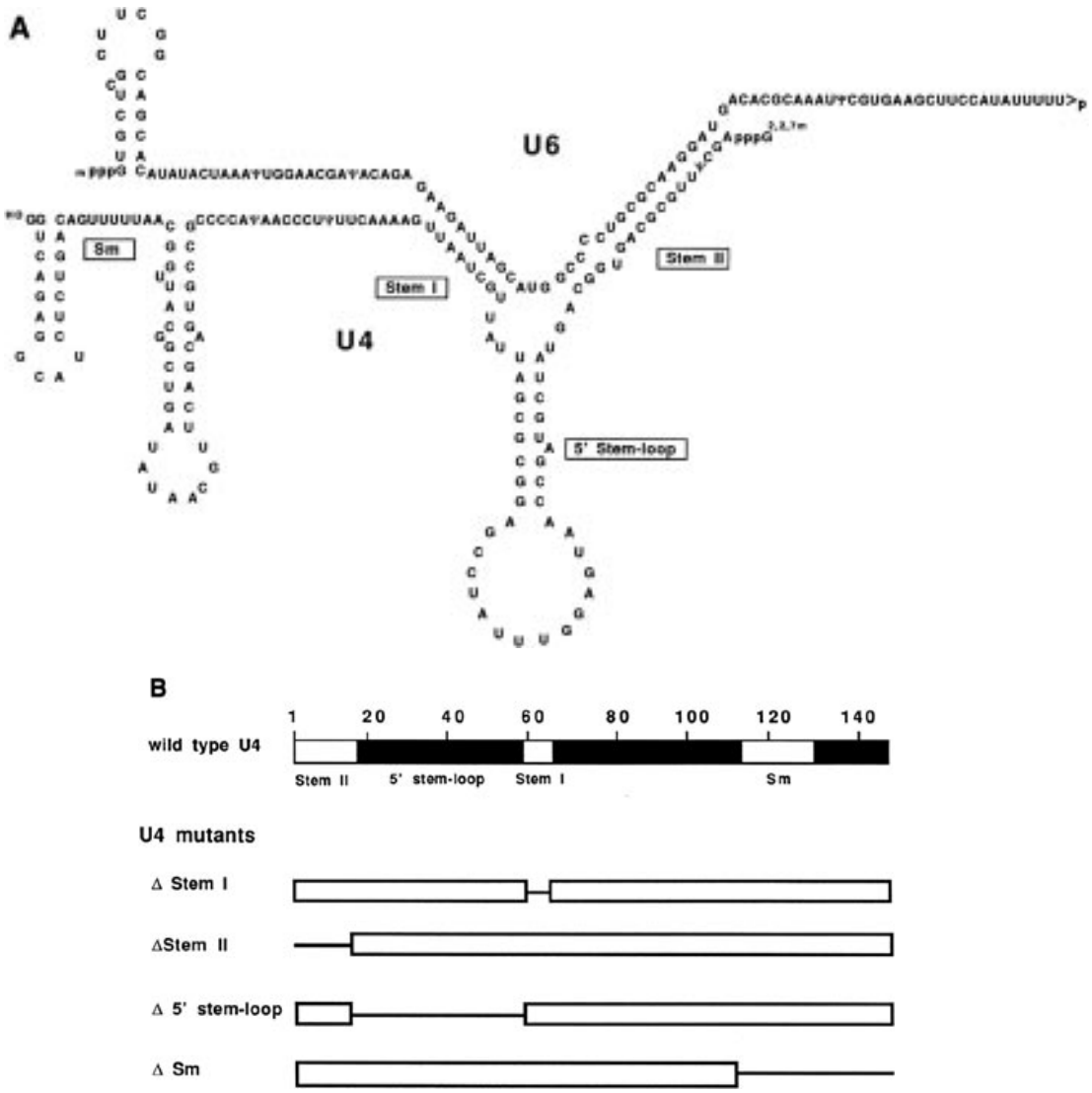

Figure 1. Secondary structures of U4 and U6 RNAs and diagram of U4 mutants. (**A**) Primary sequence and secondary structures of human U4 and U6 RNA are shown as well as the positions of Stems I and II, the regions of intermolecular base-pairing (3,6–8). (**B**) Wild-type U4 is diagrammed with the regions identified in A marked for easy reference. The portions of U4 that have been deleted in the mutants is denoted by a line in the figure, whereas the shaded blocks indicate the portions of the U4 RNA left intact (6).

unexpected since in the cell, U6 RNA never exits the nucleus. The S100 fraction could contain either Ψ synthase enzyme that leaked from the nucleus into the cytoplasm or additional uncomplexed U4 snRNP or both. There is extensive base pairing between U4 and U6 RNAs and this interaction may be necessary for Ψ formation in U6 RNA.

Although the ^{3}H release assay was useful for identifying the combination of extracts or the order of extract addition that resulted in the maximal Ψ formation in U4 and U6 RNA, the results reveal nothing about the extent of the modification process or the specificity of the reaction. The percent of theoretical is low using the ^{3}H release assay since this value was calculated from the total number of counts added to the reaction and a significant portion of RNA is degraded during the incubation making it unavailable as substrate for the modification reaction. In order to determine the specificity and extent of the modification reaction, we used ^{32}P-labeled RNAs and a TLC assay. Since the RNA is gel purified after incubation in extracts and only full-length snRNA was isolated, an accurate assessment of the extent of Ψ formation can be determined. Using this assay we were able to determine that the amount of Ψ formed in U4 RNA was ~70% of theoretical (data not shown; see Table 2 for how percent of theoretical was calculated) after incubation in a combination of S100 and NE or S100 alone. However with U6 RNA the reaction was less complete, ranging from 5 to 17% of theoretical suggesting that a component essential for efficient Ψ formation in U6 RNA was a limiting factor in these extracts.

The site-specific Ψ formation in U4 RNA using the two conditions that gave the greatest amount of ^{3}H released (S100 followed by NE, and S100 followed by S100) was determined using the TLC assay. [^{32}P]U4 RNA was incubated in the extracts, purified on a 10% polyacrylamide/8.3 M urea gel, eluted and RNase T1 digested. The RNase T1 fragments of 21 nucleotides (nt), containing Ψ at positions 72 and 79, and a 5 nt RNase T1

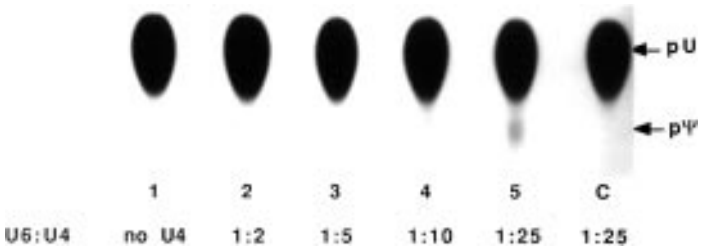

Figure 2. Effect of the presence of U4 RNA on Ψ formation in U6 RNA. ^{32}P-labeled U6 RNA was incubated with either none or increasing amounts of ^{3}H-labeled U4 RNA and the U6 RNA isolated. The RNA was subjected to digestion with nuclease P1 and chromatographed on TLC plates as described in Materials and Methods. A portion of the autoradiograph is shown in the panel above with the positions of pU and pΨ indicated to the right of the panel. The molar ratio of U6 to U4 RNA is indicated at the bottom of the panel. Lane C is U6 RNA that was not incubated in the extracts.

fragment containing Ψ at position 4, were further gel purified on a 20% polyacrylamide/8.3 M urea gel, eluted, and subjected to nuclease P1 digest and TLC. The results are shown in Table 2, with the highest amount of Ψ at positions 72 and 79 observed with S100 followed by NE addition, with 87% versus 56% of theoretical when only S100 extract was used. More interestingly, there was a 4-fold increase in Ψ formation at position 4 (18% versus 62% of theoretical) when NE was included in the reaction. This suggests that at least two Ψ synthase activities are needed for Ψ formation in U4 RNA, with one of these activities located within the nucleus. Another possibility is that NE might supply a non-enzymatic factor necessary for Ψ formation in U4 RNA. Alternatively, the S100 extract might contain an inhibitor to Ψ formation and that the presence of NE mitigates the effect of that inhibitor. Whichever is the case, the extent of modification in these extracts is quite high for U4 RNA. The same could not be said for the modification of U6 RNA at specific sites and the possible involvement of U4 RNA in the Ψ formation in U6 RNA was investigated.

Table 2. Site specific Ψ formation in U4 RNA

RNase T1 fragment (position of Ψ)[a]	Incubation conditions	% Ψ (±sd)[b]	% of theoretical (±sd)[c]
21 nt (72,79)	S100–NE	17.4 (0.19)	87.0 (0.93)
21 nt (72,79)	S100–S100	11.2 (0.26)	56.0 (0.60)
5 nt (4)	S100–NE	6.2 (0.19)	62.0 (1.88)
5 nt (4)	S100–S100	1.8 (0.08)	18.0 (0.84)

[a]The 21 nt RNase T1 fragment was reconstructed from a 12 and 9 nt RNase T1 fragment. RNase T1 not only has specificity for G, but also for poly C tracts.
[b]To obtain the % Ψ, the plate was exposed to X-ray film and the autoradiograph used to identify the uridine and Ψ spots. These spots were scraped from the TLC plates, counted in scintillant for 30 min, corrected for background, and the counts were used to obtain a ratio of Ψ counts to the total counts in uridine plus Ψ spots. In addition, a 'no extract' control value for % Ψ (0.20%) was subtracted from the values for RNAs incubated in extracts.
[c]The percent of theoretical is obtained by comparing the observed % Ψ and the theoretical percent of Ψ ([Ψ/U + Ψ] × 100) expected from the known sequences of the RNase T1 fragments from human U4 RNA. The theoretical percent for the reconstructed 21 nt fragment is 20% (2 Ψ/10 U + Ψ), and the value for the 5 nt fragment is 10% (1 Ψ/10 U + Ψ, since there are five 5 nt RNase T1 fragments).

Requirement of U4 RNA for Ψ formation on U6 RNA

In order to determine if U6 RNA requires the interaction of U4 RNA for Ψ formation, it was necessary to eliminate endogenous snRNAs from the extracts using micrococcal nuclease (MN). MN is a non-specific nuclease that digests all RNAs in the extracts. By eliminating the endogenous snRNAs from the extracts we can determine if U4 RNA that we add back to the extract contributes to Ψ formation on U6 RNA. MN at a final concentration of 1 U/µl was added to the extracts, incubated for 30 min at 37°C, and then the MN was inhibited with EGTA. Poly A and poly C RNA were also added to counter the effects of substrate masking (27). Then [^{32}P]U6 RNA was added with or without increasing amounts of [5-^{3}H]U4 RNA to the MN treated NE, incubated for 30 min, followed by the addition of MN treated S100, and incubated for an additional 2.5 h. The isolated [^{32}P]U6 RNA was gel purified, subjected to a nuclease P1 digestion, and chromatographed on TLC plates to determine total Ψ formation (Fig. 2). With no U4 present in the extracts the amount of Ψ found in the U6 RNA is quite low (0.3% of theoretical; Table 3), but as the amount of ^{3}H-labeled U4 RNA added to the reaction was increased, the formation of Ψ in U6 RNA also increased to a high of 8.2% when a 25× molar excess of U4 RNA was added to the treated extracts (Table 3).

Table 3. Effect of interaction with U4 RNA on U6 RNA Ψ formation in micrococcal nuclease treated extracts

U6:U4 molar ratio	% Ψ (±sd)[a]	% of theoretical (±sd)[b]
no added U4	0.03 (0.001)	0.3 (0.01)
1:2	0.16 (0.002)	1.4 (0.02)
1:5	0.28 (0.004)	2.4 (0.04)
1:10	0.45 (0.005)	3.9 (0.05)
1:25	0.95 (0.009)	8.2 (0.09)

[a]The %Ψ was calculated as described in the legend to Table 2. In this experiment the 'no extract' control was 0.22%.
[b]The percent of theoretical was obtained as described in the legend to Table 2. The theoretical percent for U6 RNA is 11.53% (3 Ψ/26 U + Ψ).

Other RNAs do not have the same effect on Ψ formation in U6 RNA. The same type of experiment using MN treated extracts was carried out using U4 RNA, U5 RNA and pre-tRNA^{Ser} as the added RNA. The results are in Table 4 and it is clear that U4 RNA is the only RNA tested that stimulated the formation of Ψ in U6 RNA. Adding another snRNA, such as U5 RNA, did not increase the amount of Ψ in U6 RNA over the amount observed when no additional RNA was added (compare 8.9% versus 9.3% of theoretical for these two samples). Likewise for the addition of

pre-tRNA^{Ser} to the reaction, there was no stimulation of Ψ formation in U6 RNA. In fact there appears to be a slight decrease in the level of Ψ in U6 RNA, for unknown reasons. The data show that the increase in efficiency of Ψ formation in U6 RNA is limited to U4 RNA and it is not due to an RNA mass effect.

In order to confirm the above results, U4 RNA mutants (Fig. 1B) that either inhibit or increase the binding between U4 RNA and U6 RNA were utilized in a MN treated extract experiment. The U4 mutant ΔStem II RNA has previously been shown not to interact with U6 RNA, nor does it take part in spliceosome assembly (6). U4 ΔStem I RNA can interact with U6 RNA, although it does not become part of the spliceosome (6). The U4 Δ5′Stem–loop mutant RNA has a 2-fold higher binding capacity for U6 RNA, and is not incorporated into the spliceosome, while U4 ΔSm mutant RNA does interact with U6 RNA and is incorporated into the spliceosome (6). ^{32}P-Labeled U6 RNA and ^{3}H-labeled U4 wild-type or mutant RNAs were added at a molar ratio of 1:25 (U6:U4 RNA) to MN treated NE, followed by the addition of MN treated S100. RNA was isolated from the reaction and [^{32}P]U6 RNA was gel purified, nuclease P1 digested and chromatographed on TLC plates to determine total Ψ formation (Table 5). The U4 ΔStem II mutant RNA which does not interact with U6 RNA showed an ~80% decrease in the amount of Ψ formed in U6 RNA when compared with the amount of Ψ formed when wild-type U4 RNA was added to the reaction. There was actually less Ψ formed when this mutant was present than when no U4 RNA was added to the reaction. When the ΔStem I and the ΔSm mutant RNAs were added to the reaction they exhibited an ~50% decrease in the amount of Ψ formed in U6 RNA compared with when wild-type U4 was added. Although these latter two U4 mutant RNAs still interact with U6 RNA, the data indicates a requirement for the intact U4–U6 complex, and/or the presence of Sm proteins in the U4–U6 particle for efficient Ψ formation in U6 RNA. When Δ 5′ Stem–loop mutant RNA, which has a 2-fold increase in binding to U6 RNA, was added to the modification reaction the result was similar to that seen with wild-type U4 RNA, which supports the need for U4 RNA interaction. When no U4 RNA was added to the micrococcal nuclease treated extracts, very little Ψ formation was observed in U6 RNA, while in the mock treated extracts Ψ formation was significantly higher than those reactions with wild-type U4 RNA added. Mock treated extracts are identical to micrococcal nuclease treated extracts, except no micrococcal nuclease is added to the reactions and therefore all the endogenous U4 RNA is still intact, in an RNP form, and available for interaction with U6 RNA. The amount of U4 snRNP that can be assembled in the MN treated extracts is probably less than the level of U4 snRNP found in the mock treated extracts.

Since the extent of Ψ formation in U6 RNA is not 100% of theoretical we determined whether the Ψ formed is evenly distributed between the three possible nucleotides at positions 31, 40 and 86 or whether one site is modified preferentially. U6 RNA modified in the presence of U4 RNA was isolated from the modification reactions by gel elution, digested with RNase T1, the 6mer (containing residue 40), 8mer (containing residue 86) and 17mers (containing residue 31) were isolated, and the Ψ content assayed by TLC. The results show that no Ψ can be detected at position 31, that the formation of Ψ is 4.6% of theoretical at position 40, and it is 19.1% of theoretical at residue 86. So it would appear one site is modified preferentially under the conditions employed in these studies. When this same site specific analysis of Ψ formation was carried out on U6 RNA incubated with ΔSm U4 RNA the amount of Ψ found at residue 40 decreased to 0.9% of theoretical.

Table 4. Ψ formation in U6 RNA with unrelated RNAs in micrococcal nuclease treated extracts

Type of RNA added[a]	%Ψ (±sd)[b]	% of theoretical (±sd)[c]
U4	1.95 (0.019)	16.91 (0.16)
U5	1.03 (0.011)	8.93 (0.10)
Pre-tRNA^{Ser}	0.61 (0.008)	5.29 (0.07)
No added RNA	1.07 (0.013)	9.28 (0.11)
Mock	1.42 (0.022)	12.32 (0.19)

[a]The mole ratios for U6 to the RNAs added were 1:25 for U4 RNA, 1:30 for U5 RNA and 1:9 for pre-tRNA^{Ser}, however, all of the samples had at least 2 μg of unlabeled yeast tRNA which was used for efficient precipitation of the probe.
[b]The %Ψ was calculated as described in the legend to Table 2. In this experiment the %Ψ for the 'no extract' control was 0.28%.
[c]The % of theoretical was calculated as described in the legend to Table 2.

All of the mutant U4 RNAs, except for the ΔSm mutant, have the same stability as wild-type U4 RNA in these extracts (data not shown). So the failure of the ΔStem II mutant U4 to stimulate the formation of Ψ in U6 RNA was not due to the instability of the U4 mutant in the extracts. It is interesting to note that even though the ΔSm U4 RNA is not stable in the extracts, since it does not bind the Sm core proteins (6), it nevertheless stimulated the formation of Ψ in U6 to over 50% of that seen with wild-type U4 RNA (see Table 5).

Table 5. Ψ Formation in U6 snRNA with mutant U4 RNAs in micrococcal nuclease treated extracts

Type of U4 RNA used	% Ψ (±sd)[a]	% of theoretical (±sd)[b]
No added U4	0.19 (0.003)	1.6 (0.02)
Wild-type	0.62 (0.006)	5.4 (0.06)
ΔStemI	0.31 (0.004)	2.7 (0.03)
ΔStemII	0.10 (0.001)	0.9 (0.01)
ΔSm	0.33 (0.004)	2.9 (0.03)
Δ5′Stem–loop	0.53 (0.006)	5.0 (0.06)
Wild-type (mock)	1.31 (0.013)	11.4 (0.11)

[a]The % Ψ was calculated as described in the legend to Table 2. In this experiment the % Ψ for the 'no extract' control was 0.52%.
[b]The % of theoretical was calculated as described in the legend to Table 2.

If the association of U4 RNA is required for the efficient formation of Ψ in U6 RNA *in vitro* then if we isolate the U4–U6 snRNP complex the U6 RNA should be enriched in Ψ relative to U6 RNA not associated with U4 RNA. In order to test this hypothesis we subjected the modification reactions, containing ^{32}P-labeled U6 RNA and either endogenous (mock) or exogenously added U4 RNA (MN and the no extract control), to sedimentation velocity centrifugation on glycerol gradients to separate U4–U6 complexes from U6 RNA alone. After incubation under modification conditions, the samples were layered on 10–30% glycerol gradients and centrifuged as described in Materials and Methods. The RNAs from fractions corresponding to ~16S (fraction 6) and

~7S (fraction 9) were isolated from the gradients (total of 14 fractions). The total amount of Ψ in the U6 RNA was determined and the results are presented in Table 6. The amount of Ψ found in U6 RNA associated with U4 RNA in the ~16S fraction was 4-fold higher than that found for free U6 RNA. This is true for both micrococcal nuclease treated extracts, where U4 RNA was added back after nuclease treatment, as well as for the mock treated extracts where native U4 snRNP can interact with the added ^{32}P-labeled U6 RNA.

The percent of theoretical for Ψ formation in U6 RNA is quite high from ~16S (58.7 and 24.4% respectively for mock and MN treated samples) versus the levels for U6 RNA from ~7S (15.0 and 3.1% respectively). This is consistent with the hypothesis that the formation of Ψ in U6 RNA is dependent on its association with U4 RNA. The percents of theoretical for the U6 RNA found at ~16S are high compared with previous experiments and this is due to the additional purification step employed in this experiment. Most of the counts on the gradients were found in the ~7S sample and therefore the higher levels seen with pure U4–U6 particles is normally obscured by the abundance of U6 RNA from ~7S which has lower levels of Ψ.

Table 6. Ψ Formation in U6 RNA isolated by glycerol gradient centrifugation in the presence of U4 RNA

Incubation conditions[a]	Size[b]	% Ψ (±sd)[c]	% of theoretical (±sd)[d]
MN treated	~16S	2.70 (0.019)	24.4 (0.17)
MN treated	~7S	0.36 (0.008)	3.1 (0.07)
Mock treated	~16S	6.77 (0.082)	58.7 (0.71)
Mock treated	~7S	1.73 (0.014)	15.0 (0.12)

[a]U6 and U4 RNAs at a molar ratio of 1:25 were incubated in NE followed by the addition of S100 extract, that were either micrococcal nuclease (MN) or mock treated.
[b]After incubation, the reactions were layered upon 10%–30% glycerol gradient as described in Materials and Methods and fractionated into 14 fractions. The 16S region of the gradient corresponds to fraction number 6 (U4/U6 RNAs), while the 7S region of the gradient corresponds to fraction number 9 (U6 RNA).
[c]The %Ψ was calculated as described in the legend to Table 2. In this experiment the %Ψ for samples from the 'no extract' control gradient was 0.30% for fraction 9 and 0.23% for fraction 6.
[d]The % of theoretical was calculated as described in the legend to Table 2.

DISCUSSION

The requirements and conditions for Ψ formation in the snRNAs involved in splicing are beginning to be elucidated. For U4 RNA, optimal Ψ formation is observed when U4 RNA is first incubated in S100 followed by the addition of NE. This is reminiscent of the metabolism of U4 *in vivo*, since after transcription U4 RNA exits the nucleus, is assembled into a ribonucleoprotein particle, and the 5′ cap hypermethylated, before returning to the nucleus. The fact that optimal Ψ formation for U4 RNA is not observed with the opposite extract incubation condition (NE followed by S100), suggests that U4 RNA needs to form an snRNP before Ψ formation occurs, that the Sm proteins may be necessary for efficient Ψ formation. The formation of Ψ in U5 RNA *in vitro* was shown to be dependent on RNP formation, since U5 mutant RNA that does not contain an Sm binding site does not form Ψ (12,28). Interestingly, the formation of Ψ in a U4 ΔSm mutant was 25% of wild-type U4 RNA so it would appear some Ψ formation can occur in U4 RNA in the absence of Sm protein binding (Zerby and Patton, unpublished data).

The fact that a combination of NE and S100 extracts is needed for optimal Ψ formation in U4 RNA, together with the fact that Ψ formation at position 4 of U4 RNA is increased 4-fold by the addition of NE (after incubating in S100 first), suggests there are at least two Ψ synthase activities required for Ψ formation in U4 snRNA, with one of the activities probably located within the nucleus. The procedure used to isolate HeLa S100 and NE (25) makes it more likely to have leakage of nuclear components into the cytoplasm than to have leakage of cytoplasmic components into the nucleus. Another possibility is that a cofactor necessary for Ψ synthase activity is enriched in the nuclear extract. Alternatively, it is possible that an inhibitor of Ψ formation in U4 RNA is preferentially found in the S100 and incubation with NE releases that inhibition. The determination of the actual number of Ψ synthase activities required for Ψ formation in both U4 and U6 RNAs, as well as the compartmentalization of the activities, awaits future experiments that will employ both point mutants of the two RNAs and microinjection techniques.

Ψ formation in U6 RNA is optimal with incubation in nuclear extract followed by the addition of S100. This was surprising since U6 RNA does not exit the nucleus *in vivo*. Since the Ψ formation in U6 RNA is low, we will need to consider additional manipulations of the *in vitro* system components to boost the level of Ψ formation in this essential splicing cofactor.

Recently it was shown that certain small nucleolar RNAs (snoRNAs) function as guide RNAs in the ribose methylation of pre-rRNA (29). However, even though several tRNA Ψ synthases have been cloned (30–32), the requirement for the interaction of one RNA with another in order for Ψ formation to occur has not been suggested. The need for U6 RNA to interact with U4 RNA would imply the substrate for Ψ formation in U6 RNA is the U4–U6 snRNP. Which is intriguing in light of the fact that U6 snRNA appears to be a catalytic component of the spliceosome, that U4 snRNP may function as an inhibitor of U6 snRNA catalytic function (9), and the possible involvement of Ψ in chemical reactions (20,21). The interaction between these two RNAs might be a possible way of regulating both Ψ formation and U6 RNA catalytic function by not allowing Ψ formation in U6 RNA until associated with U4 RNA.

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