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Pseudouridine Modification of U5 RNA in Ribonucleoprotein Particles Assembled In Vitro

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The formation of pseudouridine (Ψ) in U5 RNA during ribonucleoprotein (RNP) assembly was investigated by using HeLa cell extracts. In vitro transcribed, unmodified U5 RNA assembled into an RNP particle with the same buoyant density and sedimentation velocity as did U5 small nuclear RNP from extracts. The greatest amount of Ψ modification was detected when a combination of S100 and nuclear extracts was used for assembly. Ψ formation was inhibited when ATP and creatine phosphate or $MgCl_2$ were not included in the assembly reaction, paralleling the inhibition of RNP particle formation. A time course of assembly and Ψ formation showed that Ψ modification lags behind RNP assembly and that at very early time points, Sm-reactive U5 small nuclear RNPs are not modified. Two of three Ψ modifications normally found in U5 RNA were present in RNA incubated in the extracts. Mutations in the form of deletions and truncations were made in the U5 sequence, and the effect of these mutations on Ψ formation was investigated. A mutation in the area of stem-loop I which contains the Ψ moieties or in the Sm binding sequence affected Ψ formation.

With the recent development of in vitro systems for the splicing of pre-mRNA, there has been a rapid elucidation of the mechanism of pre-mRNA splicing (reviewed in references 10 and 29). Several small nuclear ribonucleoprotein particles (snRNPs) are cofactors in this process (reviewed in references 12 and 35). These particles, U1, U2, U5, and U4/U6 snRNPs, are abundant, stable, and located in the nucleus. The U RNA sequences and secondary structures are highly conserved, and the RNAs contain a number of modified nucleotides (33). These snRNPs form a complex during splicing, called the spliceosome, that has been extensively studied (12). In addition, U5 and U4/U6 snRNPs interact in a presplicing complex that is dependent on the presence of ATP (2).

The spliceosomal snRNAs bind a core of seven polypeptides, several of which are recognized by autoimmune patients anti-Sm antibodies (20, 22). The Sm proteins bind to a conserved sequence $A(U)_nG$, found in U1, U2, U4, and U5 RNAs (21). These proteins range in molecular weight from 9,000 to 29,000. In addition to these core proteins, a number of snRNP-specific proteins have been identified (22). Recently, Bach et al. (1) revealed that the protein composition of the 20S U5 snRNP is complex and includes six U5-specific proteins in addition to the common Sm proteins. U5 particles isolated by a different procedure had sedimentation values of 8S to 10S on glycerol gradients and did not contain these proteins (1).

The RNAs contained in these snRNPs are highly modified (33). These modifications include, but are not limited to, a trimethylguanosine (TMG) cap of the 5′ end and pseudouridine (Ψ), a modified form of uridine. The TMG cap appears to be necessary for the nuclear targeting of newly assembled snRNPs (8, 13). A function for Ψ in snRNAs has not yet been determined, but in tRNAs, Ψ is necessary for the interaction of tRNA with the ribosome (6, 28). *hisT* mutants of *Salmonella typhimurium* lack Ψ at positions 38, 39, and 40 in tRNA (4, 5, 26). This mutation causes a derepression of the histidine operon because the undermodified $tRNA^{His}$ produces inefficient reading of the histidine codons present in the histidine leader peptide mRNA (14).

This study combines the use of extracts and in vitro-transcribed, unmodified U5 RNA to study the formation of Ψ under various conditions and with mutant substrates. The results show that the modifications are specific, that in vitro, RNP formation precedes Ψ formation, and that there is a requirement for Sm protein binding to the RNA for Ψ formation.

MATERIALS AND METHODS

Synthesis of a human U5a gene. Six overlapping oligodeoxynucleotides, representing both strands of the human U5a gene sequence (Fig. 1 [33]), were phosphorylated, hybridized, and ligated as described previously (36) and inserted in the vector pGEM1 that had been treated as outlined previously (31). Random clones were sequenced (34) and one clone, pHU5a2, had the full U5 sequence and only an additional G residue between the start of SP6 polymerase transcription and the U5 sequence. A *Mae*I site at the 3′ end of the U5 gene was deliberately engineered such that RNA transcripts from *Mae*I-cut pHU5a2 have the same 3′ end as does human U5a RNA. Another clone had a deletion of the first 80 bp of the U5a gene (pHU5aΔ1-80). In vitro transcription from *Mae*I-cut DNA resulted in an RNA of 41 nucleotides (nt) due to an extra 5 bp between the SP6 promoter and the U5 gene (the RNA has the extra sequence GAAUA at the 5′ end).

In vitro mutagenesis of the U5 sequence. Regions of the U5 sequence were deleted by the gapped-duplex method of Kramer et al. (17). The *Sph*I-to-*Sal*I fragment from pHU5a2 was inserted into M13mp19RF, and phage DNA from this clone was used as a template in directing mutant strand synthesis as described previously (18). The oligonucleotides used were 5′-CCTCTCCACGGATATGCGATCTGA-3′, 5′-GAAATCTTTAGTGCGAAAGATTTA-3′, and 5′-AAGGC AAGGCTCTTGGGTTAAGAC-3′. Three clones, with the appropriate regions deleted, were identified and named mHU5aΔ39-43, mHU5aΔ29-53, and mHU5aΔ89-94.

RNA transcription and in vitro assembly. RNA was transcribed by SP6 polymerase as described by Melton et al. (25) from *Mae*I-cut DNA except that for the Δ79-116 mutant RNA, the pHU5a2 DNA was digested with *Hin*fI. The

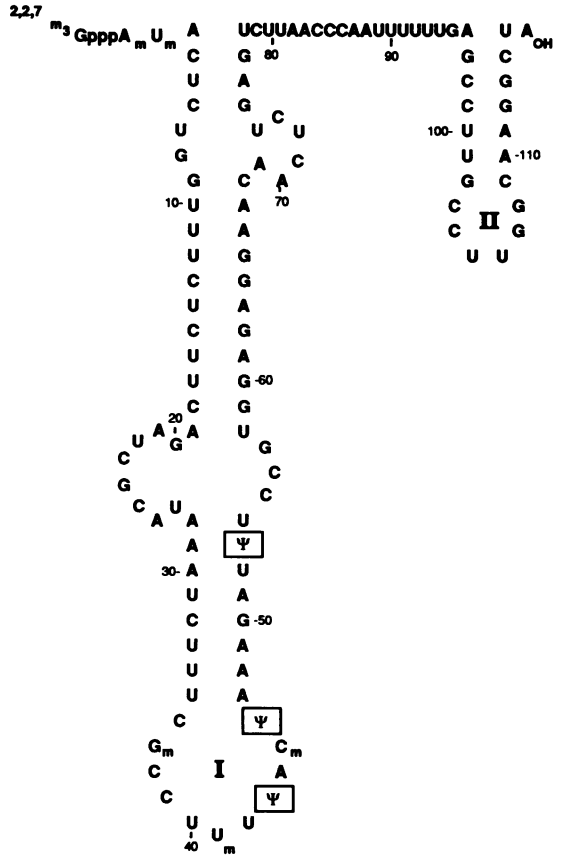

FIG. 1. Primary and proposed secondary structure of human U5a RNA (33).

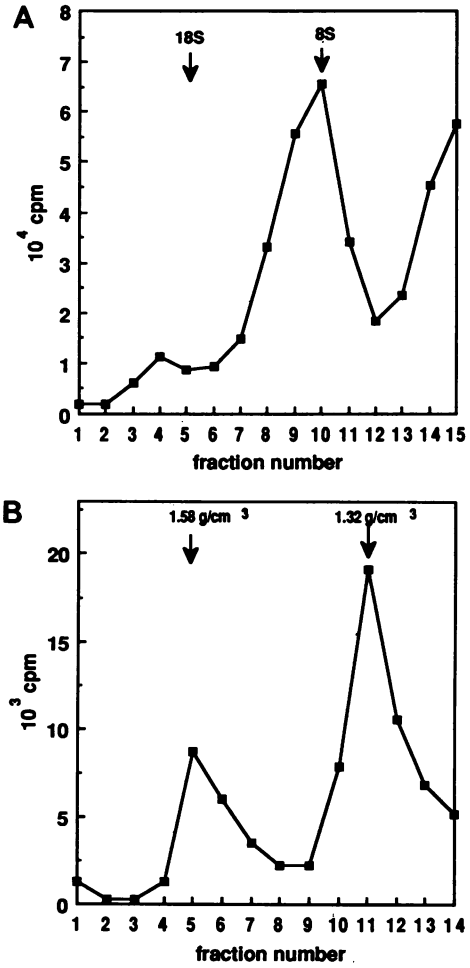

FIG. 2. Sedimentation and buoyant density gradient analysis of U5 snRNP. (A) The in vitro assembly reaction mixture, containing ^{32}P-labeled U5 RNA (0.3 pmol), was analyzed on 10 to 30% glycerol gradients as described in Materials and Methods. The bottom of the gradient is on the left. (B) A 30-min in vitro assembly reaction mixture of ^{32}P-labeled U5 RNA (35 fmol) was analyzed on a cesium sulfate gradient as described in Materials and Methods. The densities of the two peaks are indicated and were obtained by weighing aliquots of four of the fractions and plotting a curve for the linear gradient.

reactions conditions were as follows: 40 μg of DNA per ml, 1 mM m^{7}GpppG, 250 μM CTP and ATP, 25 μM UTP and GTP, RNasin (1,600 U/ml), and 2,000 Ci of [α-^{32}P]UTP and/or -GTP per ml, depending on whether it was necessary to analyze the RNA for the presence of Ψ (GTP was not used if Ψ was analyzed). When U5 RNA labeled with ATP was needed, [α-^{32}P]ATP was used and the concentration of cold ATP was reduced to 25 μM, while that of UTP was raised to 250 μM. When ^{3}H-labeled RNA was required, [5, 6-^{3}H]UTP (23 Ci/mmol) was used as the only source of UTP in the reaction (at least 50 μM). All RNAs were gel purified (on 10% polyacrylamide–urea gels) before use in any reaction.

U5 snRNP was assembled in vitro as described previously (31) but with some modifications. The modifications involved lowering the $MgCl_2$ concentration (1) and adding HeLa nuclear extract (NE) (7) in addition to the S100 extract during the assembly reaction. The typical 250-μl reaction mixture included 75 μl of S100 extract, 12.5 μl of 10 mM ATP, 10 μl of 0.5 M creatine phosphate, 5 μl of 80 mM $MgCl_2$, 0.5 μl of RNasin (40 U/μl), gel-purified U5 RNA (or equivalent moles of gel-purified, mutant U5 RNA), and water was added to a total volume of 175 μl. This reaction mixture was incubated for 30 min at 37°C, then 75 μl of NE (7) was added, and the mixture was incubated for an additional 30 min at the same temperature. In some experiments (noted in text), the NE was added at the same time as the S100 extract.

Assays for Ψ. Two assays were used to detect Ψ formation. The first is a modification of a method used to study the formation and function of Ψ in tRNA (4, 9, 15, 26). [5, 6-^{3}H]UTP is used during transcription, and the tritium ion at C-5 is exchanged with the bulk solvent as U is converted to Ψ in the unmodified U5 RNA. The ^{3}H released is measured by adding 5 volumes of Norit A (15% [wt/vol] in 0.2 N HCl) to the reaction mixture, mixing, and incubating at 37°C for 30 min. The charcoal was pelleted in a microfuge (14,000 rpm for 30 s), and the supernatant was filtered through a 0.22-μm-pore-size cellulose acetate SPIN-X centrifuge filter unit (Costar, Cambridge, Mass.). A portion (300 μl out of 380 μl) of the total eluate was mixed with scintillation fluid and counted.

The second assay involved using [^{32}P]UTP-labeled U5 RNA. The RNA was incubated in the reaction, centrifuged on either glycerol or cesium sulfate gradients, and isolated from the peak fractions. The [^{32}P]RNA was electrophoresed on denaturing polyacrylamide gels as described previously (31). Bands were eluted from the gel as described elsewhere

TABLE 1. Ψ formation under various conditions

Expt	Components in reaction	cpm of ^3H released (SD)[a]
1	Complete reaction[b]	782 (9.2)
	No nuclear extract	652 (29.3)
	No S100 extract	101 (4.4)
2	Complete reaction at:	
	37°C	577 (34.8)
	1°C	13 (8.4)
	15°C	19 (5.2)
	25°C	160 (11.2)
	30°C	346 (3.2)
	45°C	202 (1.2)
3	Complete reaction	793 (11.0)
	No ATP or CP added[c]	102 (0.6)
	No $MgCl_2$ added	130 (5.2)
	No ATP, CP, or $MgCl_2$ added	34 (3.4)

[a] Average of three separate assays of the same reaction. The counts were corrected for ^3H released in the absence of extract. The counting efficiency of the scintillant was 42%, and that of the counter was 51%.

[b] The typical reaction is composed of 30% (vol/vol) S100 extract and 30% (vol/vol) NE. When no S100 was added, the reaction had 60% NE, and when no NE was added, the reaction had 60% S100 extract.

[c] The complete reaction normally contains 0.5 mM ATP, 20 mM creatine phosphate (CP), and 1.6 mM $MgCl_2$.

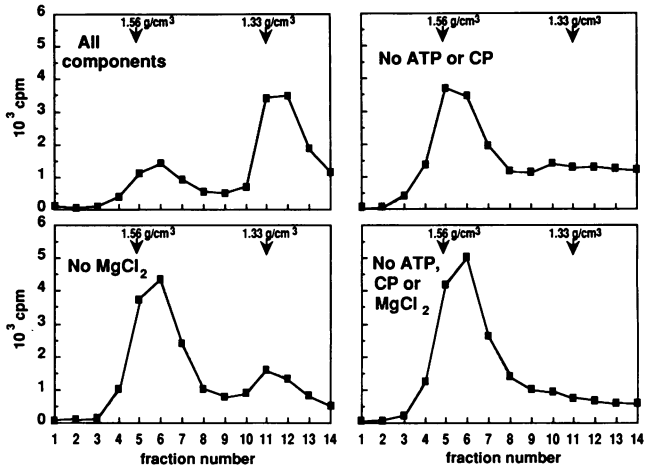

FIG. 3. ATP dependence of in vitro U5 snRNP formation. Assembly reaction mixtures containing ^{32}P-labeled U5 RNA (12 fmol) incubated for 30 min were analyzed by cesium sulfate gradient centrifugation. The reaction mixture components are listed in Materials and Methods.

(30, 32), and the isolated RNA was digested with nuclease P1 (100 μg/ml) in 20 mM sodium acetate (pH 5.2) for 60 min at 37°C. The samples were chromatographed on thin-layer cellulose (TLC) plates (Eastman) in one dimension (isopropanol-concentrated HCl-water, 70:15:15 [vol/vol/vol] [27]). After chromatography, the plate was dried overnight and autoradiographed.

For the experiment depicted in Fig. 5B, the RNA isolated from the gels was digested with RNase T_2 and chromatographed on TLC plates with isobutyric acid–0.5 M NH_3 (5:3 [vol/vol] [27]) in the first dimension and isopropanol-concentrated HCl-water (70:15:15 [vol/vol/vol]) in the second dimension.

Gradient centrifugation. Cesium sulfate (1.25- to 1.75-g/cm^3 step) and glycerol gradients (10 to 30% linear) were prepared and centrifuged as described previously (24, 31). The glycerol gradients used for analysis of the assembly reaction mixtures containing NE were modified and contained 150 mM KCl, 20 mM Tris-HCl (pH 7.5), 1.5 mM $MgCl_2$, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol as the buffer (1). In addition, the reactions were diluted with 1 volume of 150 mM KCl–20 mM Tris-HCl (pH 7.5)–1.5 mM $MgCl_2$ before layering on the glycerol gradient.

Isolation of RNA and antibody reactions. RNA from the assembly reactions was isolated and analyzed on denaturing polyacrylamide gels as described previously (31). The hypermethylation of U5 RNA was analyzed with a 2,2,7-TMG-specific polyclonal antibody provided by R. Luhrmann (Marburg, Germany) as described previously for U1 RNA (31). The antibody reactivity of the in vitro-assembled U5 snRNP was determined as described previously (30) with an anti-Sm monoclonal antibody (19).

RESULTS

In vitro assembly of U5 snRNP. The same system that was used for the in vitro assembly of U1 snRNP (30–32) and U2 snRNP (16) was used to assemble in vitro-transcribed U5 RNA (see Materials and Methods) into an RNP. The particle had a sedimentation coefficient of 9S, but since no 20S particle containing labeled U5 RNA was observed, the incubation conditions were adjusted (see Materials and Methods). When S100 extract was added prior to or in combination with NE, a 20S peak was observed (Fig. 2A). No 20S peak was detected when NE was used exclusively or when the RNA was incubated with NE prior to the addition of S100 extract (data not shown). In this particular reaction (Fig. 2A), 5.7×10^5 cpm of ^{32}P-labeled U5 RNA was used, which was equivalent to 12.5 ng (total reaction volume was 500 μl); of that amount, 0.6 ng was assembled into 20S particles (fractions 3 to 5) and 4.2 ng was assembled into 9S particles (fractions 8 to 11). Native U5 snRNP from HeLa cells sedimented at ~20S in glycerol gradients, with a minor peak at 9S (data not shown).

The cesium sulfate gradients of particles assembled in vitro in combined S100 extract and NE are shown in Fig. 2B. The presence of a peak at 1.3 g/cm^3 is indicative of a salt-resistant particle. The peak at 1.6 g/cm^3 is U5 RNA with no protein bound, either because it had not yet bound protein or because it had formed nonspecific complexes that are easily dissociated by the cesium salt (31). For assembly reactions incubated for a full hour at 37°C, 50% or more of the input counts per minute were found in the 1.3-g/cm^3 peak (fractions 9 to 12). U5 snRNPs from HeLa cell extracts are also found at 1.3 g/cm^3 (data not shown). The particles react with the Sm monoclonal antibody (see Fig. 4 and 9), and the incubated U5 RNA reacts with the TMG antibody (gift of R. Luhrmann). As was found with the in vitro assembly of U1 snRNP (31), only a portion (approximately 40%) of the U5 RNA contained in the particles assembled in vitro had TMG caps (data not shown).

Formation of Ψ during in vitro assembly. It has been shown that the in vitro assembly system supports Ψ formation when unmodified U1 and U2 RNA transcripts are incubated in the extract (16, 31). The modified assembly system used in these experiments also shows that the U5 RNA is a substrate for modification. In the ^3H release assay (26), more activity was detected in the S100 fraction than in the NE (Table 1) when [^3H]UTP-labeled U5 RNA was added

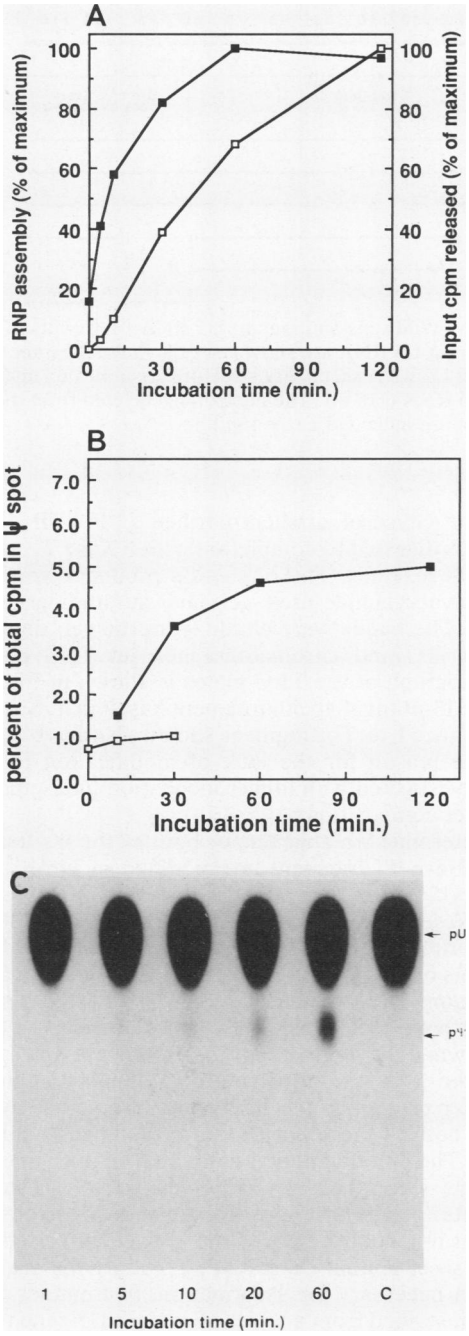

FIG. 4. Time course of Ψ formation and RNP assembly. (A) Aliquots (0.22 pmol) withdrawn from a reaction mixture containing [^{3}H]UTP-labeled U5 RNA (total of 1.3 pmol) at the times indicated was split into four parts; three parts were used to determine the amount of ^{3}H released (□), and one part was analyzed on a cesium sulfate gradient. The percentage of the total counts on the gradient found in the 1.3-g/cm^3 peak was used to calculate the percentage of the maximum RNP assembly, which was at 60 min in this experiment (■). The averaged maximum amount of input counts released (120 min) was used to calculate the percentage of maximum for the ^{3}H release samples. The standard deviation for the samples was below 1% of maximum for 0, 5, 10, and 60 min. For 30 and 120 min, the standard deviations were 1.8 and 4.5%, respectively. (B) Aliquots (0.12 pmol) of [^{32}P]UTP-labeled U5 RNA (total of 0.72 pmol) were incubated for the indicated times and then analyzed on cesium sulfate gradients. The peaks at 1.6 (□) and 1.3 (■) g/cm^3, if present, were analyzed as described in the text. The ratio of the counts found in the Ψ spot to the total counts (U and Ψ) was calculated for each sample. The values used in the calculations were averages of three separate 10-min counts of the same vial with an averaged background subtracted. The standard percent error on any sample was no more than 11. The maximum possible percentage of total counts that can be observed is 7.5 (3 Ψ out of 40 total U+Ψ). (C) Aliquots (0.1 pmol) removed from the assembly of [^{32}P]UTP-labeled U5 RNA (total of 0.5 pmol) were reacted with the Sm monoclonal antibody (see Materials and Methods), and the isolated RNA was assayed for Ψ by thin-layer chromatography. Lane C, control (RNA that was not incubated in the extracts).

to the reactions. The optimal temperature is 37°C, and it appears the formation of Ψ is dependent on the presence of ATP, creatine phosphate, and $MgCl_2$ (Table 1). When these same reactions, except with ^{32}P-labeled U5 RNA, are subjected to cesium sulfate gradient centrifugation, the inhibition of stable 1.3-g/cm^3 RNP formation is evident when ATP, creatine phosphate, and $MgCl_2$ are not present during incubation (Fig. 3). These data suggest that Ψ formation could be dependent on RNP formation or vice versa.

It was shown previously that Ψ formation on U1 RNA lags behind U1 snRNP assembly in this system, with at least 80 to 90% of the total RNP assembly complete after only 30 min, but only 18% of the expected level of Ψ was found in the isolated RNA (31). These data suggest that U1 snRNP assembly is not dependent on Ψ formation. The results of this same experiment with U5 RNA as the substrate are shown in Fig. 4. In Fig. 4A, the ^{3}H release assay was used to monitor Ψ formation and cesium sulfate gradients were used to assay RNP assembly. By 10 min, RNP formation is at more than half the maximum level whereas Ψ formation is barely detectable. In addition, RNP formation levels off at 1 h, but the Ψ level continues to rise. The measure of RNP assembly in Fig. 4A is based on the percentage of the total counts found in the 1.3-g/cm^3 peak, the region of the gradient where stable U5 snRNPs are located. At early time points, the peak at 1.6 g/cm^3, containing U5 RNA not stably bound to protein, is significant. Is the Ψ content of the RNAs from these two different densities the same for each time point? To test this question, [^{32}P]UTP-labeled U5 RNA was isolated from both the 1.6- and 1.3-g/cm^3 peaks of each time point. The zero time point does not have a 1.3-g/cm^3 peak since there was no incubation at 37°C, and the 60- and 120-min time points have no 1.6-g/cm^3 peak because nearly all of the full-length, U5 RNA counts are in stable particles by this time. The RNAs were isolated, run on a polyacrylamide-urea gel, and eluted. The eluted RNAs were digested with nuclease P1 and chromatographed on TLC plates. The resulting autoradiograph was used as a template for scraping the spots from the TLC plates for counting. The results (Fig. 4B) show that the Ψ content is higher in the RNA from the 1.3-g/cm^3 peak and that whereas the Ψ content rises quickly and levels off in this RNA, the amount of Ψ in the 1.6-g/cm^3 peak stays at a very low and constant level. These data suggest stable U5 RNP, not the naked RNA, is the substrate for Ψ formation. This would mean that early during incubation there should be completely unmodified U5 RNA bound to Sm proteins. This is indeed the case, since the RNA isolated from U5 snRNP selected with the Sm monoclonal antibody contains no Ψ at early time points (1-min time point in Fig. 4C).

Where are the sites of modification? Are there Ψs throughout the U5 RNA, or is the modification found only in certain regions? The experiments depicted in Fig. 5 answer this

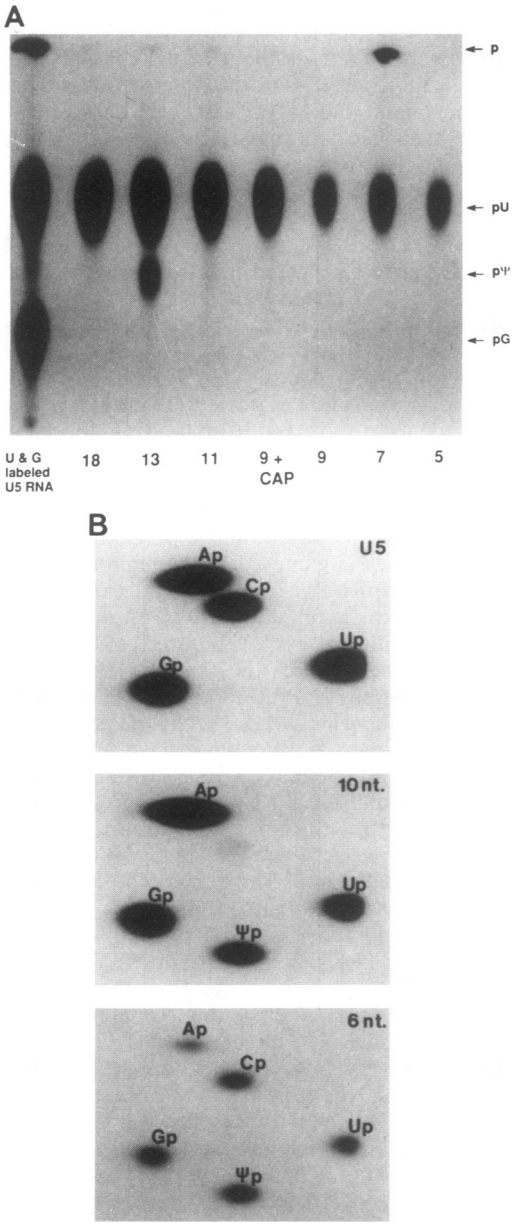

FIG. 5. Detection of Ψ in only one RNase T_1 fragment of in vitro-assembled U5 RNP. (A) Glycerol gradient-purified [^{32}P]UTP-labeled U5 RNA (0.3 pmol used in the reaction) was digested to completion with RNase T_1 and electrophoresed on a 20% polyacrylamide–urea gel. Shown is an autoradiograph of one-dimensional thin-layer chromatography of the nuclease P1 digest of the eluted RNase T_1 fragments. Sizes (in nucleotides) of the T_1 fragments are listed beneath the samples. Positions of pU, pΨ, and pG are shown at the right. The U+G-labeled U5 RNA sample was included on the right to show the position of Gp in this system. (B) [^{32}P]ATP-labeled U5 RNA (0.9 pmol) was incubated in extracts for 2 h, glycerol gradient purified, digested with RNase CL-3, and electrophoresed on a 20% denaturing polyacrylamide gel. The 10- and 6-nt fragments were eluted, digested with RNase T_2, and subjected to two-dimensional thin-layer chromatography (see Materials and Methods). An RNase T_2 digest of U5 RNA that was not incubated in extracts is provided for comparison (U5).

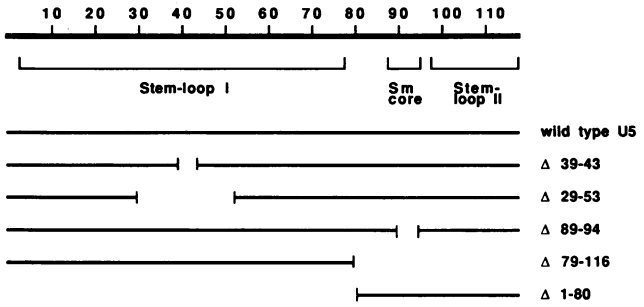

FIG. 6. Wild-type and mutant U5 RNA transcripts. The regions deleted from U5 RNA are shown as gaps in the diagram. The major regions of U5 RNA secondary structure are indicated under the map of the U5 RNA (116 nt). The nucleotides deleted from the U5 RNA transcript are indicated at the right.

question. Glycerol gradient-purified [^{32}P]UTP-labeled U5 RNA was digested to completion with RNase T_1, which cuts only at G residues. These reaction products were run on a 20% polyacrylamide-urea gel and autoradiographed (not shown). The bands were eluted from the gel, digested with nuclease P1, and chromatographed on TLC plates. The autoradiograph of the TLC plates is shown in Fig. 5A, and only the 13-nt (nt 38 to 50) fragment has Ψ in it. Surprisingly, the 7-nt (nt 51 to 57) fragment should also have Ψ but does not. The reason for the lack of modification is currently being investigated with longer incubation times and changes in extract composition.

To determine whether one or both of the Ψs found in the 13-nt RNase T_1 fragment are modified in vitro, [^{32}P]ATP-labeled U5 RNA from gradient-purified RNP was digested with RNase CL-3, which cuts at C residues (3). The two Ψ modification sites (nt 43 and 46) can be separated into two fragments of 10 and 6 nt. For both of the Ψ modification sites in question, an A residue is 3′ to the Ψ, and since the A residues have a 5′ phosphate that is labeled, the Ψ will be labeled when the fragment is cut with RNase T_2 (T_2 leaves 3′ phosphates). If the conversion to Ψ is not complete, there will be Up present on the TLC. Both fragments have Ψ (Fig. 5B), so both of these positions are modified in this in vitro system. The 6-nt fragment is actually a mixture of 6- and 5-nt fragments since the gel did not completely resolve these fragments. That is why spots for Ap and Cp are observed. In the 10-nt fragment, ~50% of the U was converted to Ψ.

Analysis of mutant U5 snRNP. To study the sequences of U5 RNA necessary for Ψ formation, five mutant U5 RNAs were transcribed from a series of mutant U5 gene templates. The transcribed RNAs (see Materials and Methods), compared with the secondary structure of U5 RNA, are shown in Fig. 6.

The same number of moles of wild-type (wt) and mutant U5 RNAs were assembled in S100 extract and NE and analyzed on glycerol gradients (Fig. 7). Wild-type U5 RNA and the Δ39-43 mutant formed a 20S particle, showing that an intact stem-loop I is not necessary for 20S particle formation. None of the other RNAs formed 20S particles. All of the RNAs formed 9S particles, migrating faster than the corresponding RNA alone (4S to 5S; data not shown). The peaks at 20S and 9S could be shifted to 5S when excess cold wt or mutant U5 RNA was added to the reaction mixture (data not shown).

Buoyant density gradient centrifugation showed that in addition to wt and the Δ39-43 mutant, the Δ29-53 mutant is

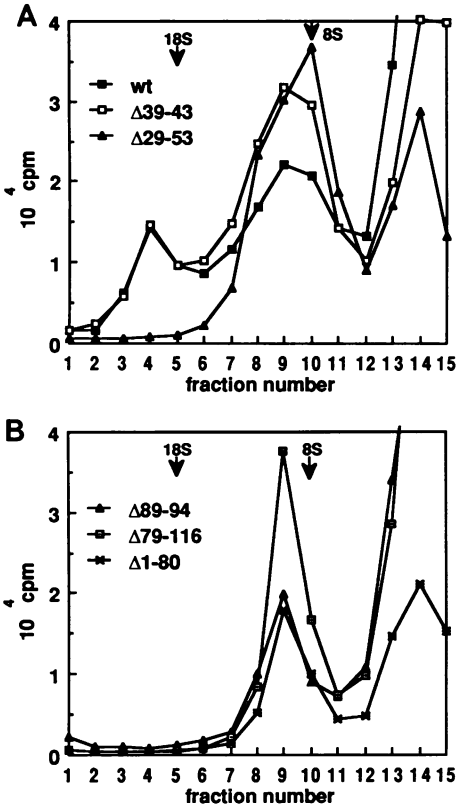

FIG. 7. Sedimentation analysis of mutant U5 RNAs assembled in vitro. Equal amounts (0.2 pmol) of [^{32}P]UTP- and [^{32}P]GTP-labeled U5 wt or mutant transcripts were assembled in vitro in S100 extract and then NE (see Materials and Methods) and analyzed on 10 to 30% gradients as for Fig. 2. The RNAs used in each assembly reaction are indicated within each gradient profile. Positions of protein sedimentation markers are shown at the top.

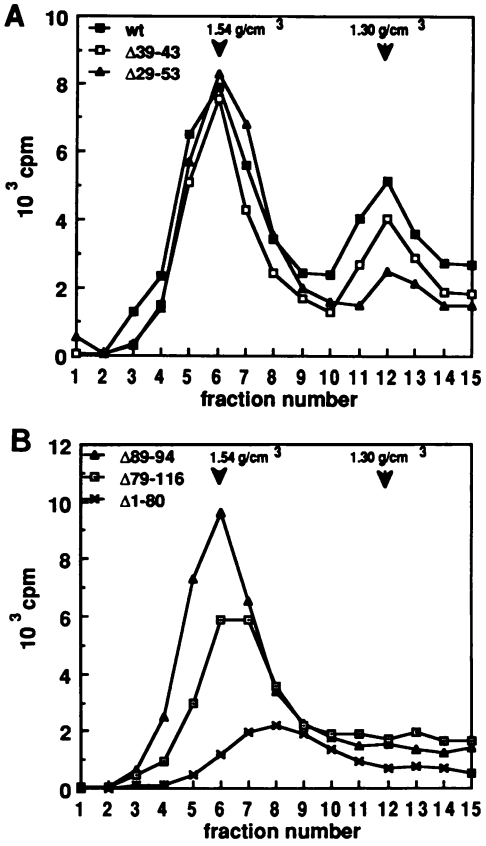

FIG. 8. Buoyant density gradient centrifugation of the mutant U5 RNAs assembled in vitro. Equal amounts (80 fmol) of [^{32}P]UTP-labeled U5 wt or mutant RNAs were assembled in vitro and then analyzed on cesium sulfate gradients (see Materials and Methods). Densities of the peak fractions are shown at the top of each panel.

resistant to salt dissociation (Fig. 8A). The Δ1-80 mutant (Fig. 8B) forms an RNP of intermediate buoyant density and therefore also appears to be resistant to salt, but it is difficult to interpret this result. The Δ89-94 and Δ79-116 mutants (Fig. 8B) show a complete lack of salt-resistant protein-RNA interaction, consistent with the loss of the Sm protein binding site (21, 23, 31). These mutants bind some protein however, evidenced by the increase in the sedimentation rate of the RNA after incubation in extracts, but those interactions are not cesium salt resistant.

Sm antigen binding to mutant U5 RNAs. To ascertain which RNAs bound to Sm antigens, the glycerol gradient-purified 9S U5 snRNPs were reacted with the anti-Sm monoclonal antibody. The wt, Δ39-43, and Δ29-53 RNAs all reacted with the antibody, whereas the Δ89-94 and Δ79-116 mutant RNAs did not (Fig. 9A). A small amount of Δ1-80 RNA can be seen on an overexposure of the autoradiograph (Fig. 9B), indicating this RNA does bind to Sm antigens, but it may not withstand the stringent washing process used to detect protein A-Sepharose binding. The presence of full-length U5 RNAs in the nonbound fractions shows that the RNAs were not degraded during the antibody reaction.

Ψ formation with mutant RNAs. [^{32}P]UTP-labeled wt and mutant U5 RNAs (equal moles per reaction) were also used in the TLC assay. The RNAs from glycerol gradient-purified wt and mutant U5 RNPs were purified, digested with nuclease P1, and chromatographed on TLC plates. The same number of counts was applied for each RNA sample. From the results (Table 2), it is obvious that only wt U5 RNA has a significant amount of Ψ. The amount of Ψ found in the RNA from 9S and 20S U5 snRNPs is not appreciably different. When this same experiment was done after gel purification of the RNA, the wt RNAs were still the only RNAs that contained significant amounts of Ψ (data not shown).

DISCUSSION

It has been shown previously that this assembly system supports the formation of Ψ when unmodified, in vitro-synthesized transcripts are used (16, 31). The present study extends those previous observations and demonstrates the ease with which this complex process can be studied in vitro.

The experiments presented here suggest that RNP formation is necessary for Ψ formation, at least in these extract preparations. RNP formation is inhibited by the lack of ATP, and Ψ formation is also inhibited. The tRNA Ψ synthases isolated from *Escherichia coli* and bovine thymus do not require ATP or Mg^{2+} for activity (9, 15), suggesting that the ATP requirement in this system is related to RNP formation.

The time course of RNP formation versus Ψ formation also supports the notion that the U5 snRNP is the substrate for modification. At very early time points, Sm-reactive U5

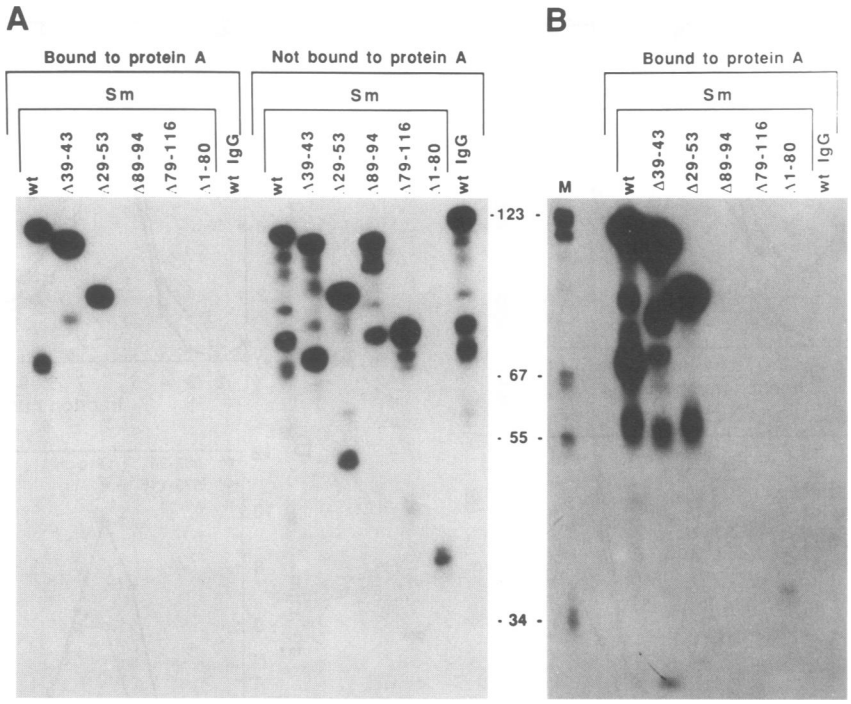

FIG. 9. Presence of Sm antigen in U5 mutant snRNPs. (A) Glycerol gradient-purified (9S) wt and mutant U5 snRNPs were reacted with protein A-Sepharose-bound anti-Sm antibody. The protein A-bound and nonbound RNA of each sample was isolated and electrophoresed on a 10% polyacrylamide denaturing gel, and the gel was autoradiographed. The type of RNA used in each assembly reaction is indicated above the lanes. Lanes wt IgG, wt 9S U5 snRNP incubated with human immunoglobulin G prebound to protein A-Sepharose; lane M, ^{32}P-end-labeled fragments from *Msp*I-cut pHU1. (B) Longer exposure of the bound portion of panel A.

snRNPs are not modified. At 10 min, when the RNP formation is at 60% of maximum, the amount of ^{3}H released was ~10% of the maximum level. But more significantly, the U5 RNA isolated from salt-resistant RNP complexes (1.3 g/cm^3) was more highly modified than the U5 RNA isolated from the naked RNA portion of the same gradient (1.6 g/cm^3). This same type of experiment yielded a significant amount of Ψ in the 1.6-g/cm^3 fraction when U2 RNA was assembled in vitro (16). Since U2 RNA has a significantly higher Ψ content, the modification characteristics of this RNA may be very different from those of U5 RNA. The alleged Ψ moieties (alleged because the positions of all of the Ψ modifications in human U2 RNA have not been determined, but they have been inferred from sequenced rat U2 RNA [33]) are in a portion of U2 RNA that is not bound to snRNP proteins (23). Therefore, the requirement for bound snRNP proteins may not be as stringent when U2 is the substrate for modification. These types of experiments have not been carried out for U1 snRNP, but it is known that U1 snRNP assembly is not dependent on Ψ formation (31).

TABLE 2. Ψ formation with wt and mutant U5 RNAs in the TLC assay

RNA type	% of total cpm in Ψ spot[a]
wt	
9S RNP	0.029
20S RNP	0.032
Δ39-43	
9S RNP	0.004
20S RNP	0.004
Δ29-53	0.003
Δ89-94	0.006
Δ79-116	0.006
Δ1-80	0.003
wt U5 (not incubated)	0.004

[a] To obtain this ratio, the uridine and Ψ spots were scraped from the TLC plates and counted in a scintillant twice for 10 min each time and corrected for background. Total counts were between 40,000 and 44,000 cpm in each of the 9S samples and between 18,000 and 20,000 cpm in the 20S samples. The percent error was never more than 5% (Δ39-43 9S RNP Ψ spot).

The comparison of Ψ formation when wt and mutant U5 RNAs were used as substrates also supports the need for snRNP protein binding, in particular binding of the Sm core proteins. The mutants can be split into two groups, those that bind Sm proteins but do not have all of the Ψ modification sites intact (Δ39-43, Δ29-53, and Δ1-80) and those that have the Ψ modification sites intact but do not bind Sm proteins (Δ89-94 and Δ79-116). The sequence in the vicinity of the modification sites must be intact, or at least the RNA cannot have a deletion as large as the Δ39-43 mutant in order for modification to still occur. This makes sense considering that this region of U5 RNA is extremely well conserved (33). The Δ39-43 mutant leaves one potential Ψ site that is modified in vitro (nt 46), but the deletion inhibits modification at that site. The mutants that do not bind Sm proteins have intact modification sites, but those sites are not modified. Only wt U5 RNA has the modification sites unchanged and binds Sm proteins, and it is the only RNA modified. More subtle mutations will be necessary before the sequence requirements for Ψ formation can be fully understood.

Why should the U5 snRNP but not U5 RNA be the substrate for Ψ modification? Perhaps the enzyme that is

responsible for this modification recognizes one of the Sm proteins and would therefore be at least specific for snRNPs if not specific for U5 snRNP. Hamm et al. (reference 13 and references therein) have shown that TMG capping of microinjected U1 RNA is dependent on Sm protein binding. The requirement for bound Sm proteins may serve the same function in substrate recognition by the enzyme that modifies U to Ψ in U5 RNA.

Does Ψ have a function in snRNAs? Griffey et al. (11) point out that Ψ is potentially more versatile in its hydrogen bonding interactions than is uridine. They found, using double-resonance and two-dimensional ^{15}N nuclear magnetic resonance of N-1-labeled Ψ in several *E. coli* tRNAs, that in the anti conformation for Ψ at position 39, the N-1 proton hydrogen bonds either to a bridging water molecule or to a sugar hydroxyl. They suggest that this small additional stabilization may be important in reducing conformational mobility in the region of a Ψ·A base pair. Therefore, the Ψs found in the 5′ end of U1 RNA may provide increased stability in the interaction between U1 RNA and the pre-mRNA splice site. Ψ may figure prominently in the recognition of snRNAs by snRNP proteins, either as a positive or as a negative affector.

Several directions will be pursued in an effort to understand the formation and role of Ψ in snRNPs. Work on the isolation and purification of an enzymatic activity that modifies snRNAs is under way. In addition, the production of Ψ-deficient snRNP particles that can be tested for interaction with pre-mRNA or other snRNPs is also a top priority.

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