# Formation of Pseudouridine in U5 Small Nuclear RNA†

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ABSTRACT: The formation of pseudouridine (Ψ) on U5 small nuclear RNA (U5 snRNA) was studied using an *in vitro* modification system. Labeled U5 RNA, synthesized *in vitro* and therefore unmodified, was incubated in reactions containing S100 and/or nuclear extracts (NE) from HeLa cells, and the levels of Ψ were determined. There are three Ψ residues found in human U5 RNA, at positions 43, 46, and 53. Incubation of unmodified U5 RNA in reactions containing either S100 or NE supports Ψ formation at positions 43 and 46, which are found in a loop in the predicted secondary structure of U5 RNA. However, Ψ formation at position 53, which is found in a stem, is dependent on the presence of NE during the incubation. The order of extract addition does not have a significant effect on the formation of Ψ at position 53 as long as NE is present. The most efficient Ψ formation was observed with a combination of S100 and NE which allowed for efficient small nuclear ribonucleoprotein particle (snRNP) assembly and Ψ formation. When 9S and 20S U5 snRNPs were isolated by velocity sedimentation gradient centrifugation after incubation in the combined extracts, there was little difference in the Ψ levels at any of the positions for the two distinct particles. Mutations in the U5 RNA sequence do affect Ψ formation. U5 RNAs that have mutated Sm binding sites or are truncated prior to the Sm binding site have very low levels of Ψ formation at positions 43 and 46 and no detectable Ψ formation at position 53. A deletion of five nucleotides from 39 to 43 abolishes Ψ formation at positions 43 and 46, but the modification of position 53 is unaffected.

Small nuclear ribonucleoprotein particles (snRNPs)[1] are essential cofactors in the splicing of premessenger RNA (Baserga & Steitz, 1993). These snRNPs are composed of highly conserved small nuclear RNAs (snRNAs) U1, U2, U4, U5, and U6 and associated proteins (Luhrmann *et al.*, 1990; Reddy & Busch, 1988). Some of these proteins are specific to a particular snRNP, but others, termed the Sm proteins, are found in all the snRNPs involved in splicing (Luhrmann *et al.*, 1990; Behrens *et al.*, 1993). The synthesis of these spliceosomal snRNPs includes assembly of the particle, trimming at the 3′ end of the snRNA, extensive internal modifications, and 5′ end capping. Several of these steps apparently occur in the cytoplasm (Zieve & Sauterer, 1990).

The formation of pseudouridine (Ψ) in these snRNAs has been the subject of several recent 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 RNAs (Patton, 1993a). Efficient Ψ formation in U5 RNA requires Sm protein binding (Patton, 1991), but that is not the case for Ψ formation in U2 RNA (Kleinschmidt *et al.*, 1989; Patton *et al.*, 1994). Additionally, Ψ formation at a particular site in U2 RNA, which has 13 Ψs, is not dependent on prior Ψ formation at another site (Patton *et al.*, 1994). This autonomous formation may require multiple U2 snRNA Ψ synthases, suggesting a substantial commitment by the cell to the modification of these essential components of the splicing machinery. A previous study of Ψ formation in U5 RNA showed that even though there were three known sites of modification, only two of these sites were actually found to be modified in S100 extracts from HeLa cells (Patton, 1991), again suggesting multiple activities that modify a single snRNA.

The function of Ψ in snRNAs is unknown, but it is noteworthy that Ψ is found in regions of snRNAs known to be necessary for the function of snRNPs in the splicing of premessenger RNA (Steitz *et al.*, 1988; Guthrie & Patterson, 1988). Ψ appears to be required for the efficient reading of codons during the translation process (Johnston *et al.*, 1980). Tsui *et al.* (1991) found that the *hisT* function is required for normal growth of *Escherichia coli* on minimal media due to a requirement for uracil that interferes with cell division. *HisT* codes for a Ψ synthase that modifies certain positions in the anticodon of tRNAs (Mullenbach *et al.*, 1976). In addition, Ψ is required in the anticodon (GΨA) of $tRNA^{Tyr}$ for UAG and UAA suppression in the translation of tobacco mosaic virus RNA (Zerfass & Beier, 1992). Recently, it has been hypothesized that Ψ in *E. coli* 23S rRNA participates in the peptidyl transfer reaction in the ribosome (Lane *et al.*, 1992).

In this report, nuclear extract (NE) was found to catalyze the *in vitro* formation of Ψ at the third site in U5 RNA. In addition, it is shown that mutation in the loop containing the sites of modification has a differential effect on the modification at the three sites but mutation or deletion of the Sm binding region severely inhibits Ψ formation at all the sites. Finally, the levels of Ψ in 9S and 20S U5 snRNPs were determined, and little difference was found in the levels of this modification at any of the sites in the two types of particles, suggesting Ψ is not part of a signal for the formation of the 20S U5 snRNP.

## EXPERIMENTAL PROCEDURES

Human U5 RNA was synthesized *in vitro* (Melton *et al.*, 1984) using SP6 polymerase and *Bfa*I-cut pHU5a2 DNA (Patton, 1991). The mutant U5 RNAs were synthesized from *Hin*fI-cut pHU5a2 (Δ79-116), *Bfa*I-cut mHU5aΔ39-43 (Δ39-43), and *Bfa*I-cut mHU5aΔ89-94 (Δ89-94); these last two

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[1] Abbreviations: snRNA, small nuclear RNA; snRNP, small nuclear ribonucleoprotein particle; Ψ, pseudouridine; NE, nuclear extract; nt, nucleotide; TLC, thin-layer chromatography.

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are U5 RNA mutant clones that have been previously described in detail (Patton, 1991). The *in vitro* transcription reactions contained [$^{32}$P]UTP (50 $\mu$Ci, 600 Ci/mmol), 50 $\mu$M UTP and GTP, 250 $\mu$M ATP and CTP, and 1 mM m$^7$GpppG. All RNAs were gel purified on 10% polyacrylamide/8.3 M urea gels prior to incubation in the modification reactions (Patton *et al.*, 1989).

The *in vitro* modification reactions and isolation of RNA were carried out as previously described (Patton *et al.*, 1987; Patton, 1991, 1993a) with S100 and/or NE isolated from HeLa cells (Dignam *et al.*, 1983). Reactions (100 $\mu$L) typically contained $5 \times 10^6$ cpm of gel-purified $^{32}$P-labeled RNA. Velocity sedimentation gradient centrifugation conditions were as described (Patton, 1991, 1993b). Briefly, the sample (reaction had $12.5 \times 10^6$ cpm of U5 RNA per 100 $\mu$L) was layered on a 10–30% glycerol gradient (Patton, 1991) and centrifuged in a SW41 rotor at 40 000 rpm for 18 h at 4 °C. The assay for Ψ formation has been described (Nishimura, 1972; Patton, 1991, 1993a,b; Patton *et al.*, 1994) and involves gel purification of the RNA from the reaction, digestion with RNase T1, gel purification of the T1 fragments, nuclease P1 digestion, and thin-layer chromatography (TLC) on cellulose plates in 2-propanol:concentrated HCl:water (70:15:15, v/v/v).

## RESULTS

*Dependence of Ψ Formation at Position 53 on Nuclear Extract.* Previous studies have shown that the *in vitro* assembly/modification system that employs extracts from HeLa cells can be used to investigate the formation of Ψ in snRNAs (Patton *et al.*, 1987; Kleinschmidt *et al.*, 1989; Patton, 1991, 1993a,b; Patton *et al.*, 1994). When U5 RNA was incubated in S100 extracts, Ψ was formed at positions 43 and 46 but not at position 53 [see Figure 1 and Patton (1991)]. In order to determine whether the activity that modifies the uridine at position 53 was missing from the S100, NE either alone or in combination with S100 extract was added to the incubation reactions.

$^{32}$P-U5 RNA was incubated in reactions containing either S100 alone, NE alone, or a combination of the two extracts either initially combined or added sequentially. In the reactions where extracts were added sequentially, the U5 RNA was incubated in the reaction with the first extract for 30 min, and then the second extract was added and incubated for an additional 30 min. With the combined S100 and NE the two extracts were mixed at the start, and another aliquot of mixed extract was added after 30 min. The RNA was isolated from the reactions and electrophoresed on a 10% polyacrylamide/8.3 M urea gel, and the $^{32}$P-U5 RNA was purified by elution from the gel slice. These RNA samples were then digested with RNase T1 and the T1 fragments separated on a 20% polyacrylamide/8.3 M urea gel. The 13- and 7-nucleotide (nt) fragments, the only two T1 fragments that can contain Ψ (see Figure 1), were eluted from the gel, digested with nuclease P1, and chromatographed on TLC plates (see Experimental Procedures). The autoradiograph of the TLC is shown in Figure 2. There is no Ψ in the control lanes, since this RNA was not incubated with either extract. However, with any of the extracts alone or with any combination of extracts there was Ψ formation in the 13-nt fragment, the RNase T1 fragment that contains nucleotides 43 and 46. The level of Ψ formation in this fragment was high (see Figure 2 and Table 1) when any extract was included in the reaction. The highest level was seen with the S100 and then NE combination of extracts (Figure 2, lane 3) and the S100 + NE combination (Figure 2, lane 5). In these experiments, when U5 RNA was incubated in the presence of S100 and NE together, the formation of Ψ in the 13-nt fragment was nearly

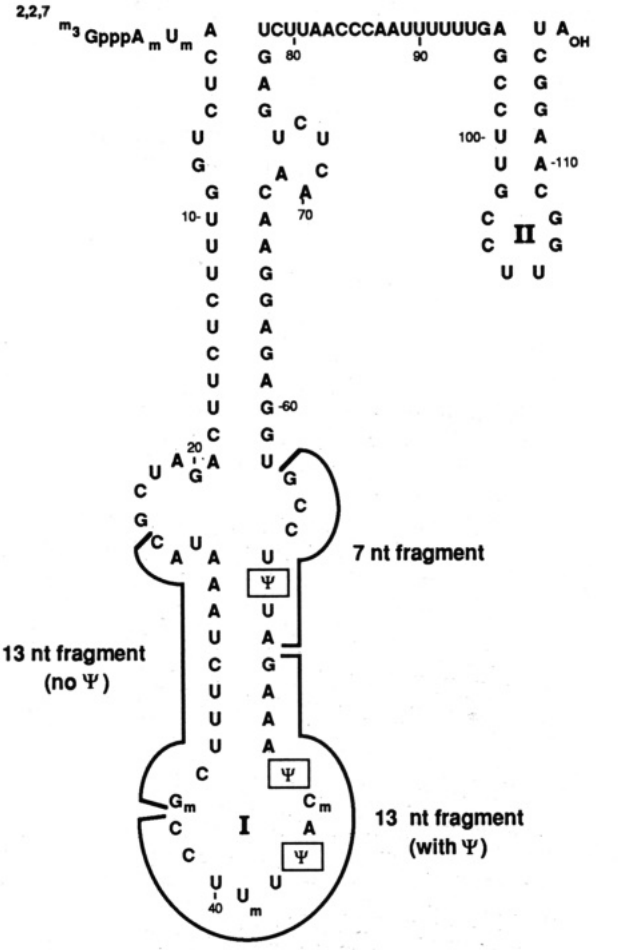


FIGURE 1: Primary and proposed secondary structure of human U5a RNA (Reddy & Busch, 1988). The positions of Ψ modification are boxed, and RNase T1 fragments are indicated with brackets.

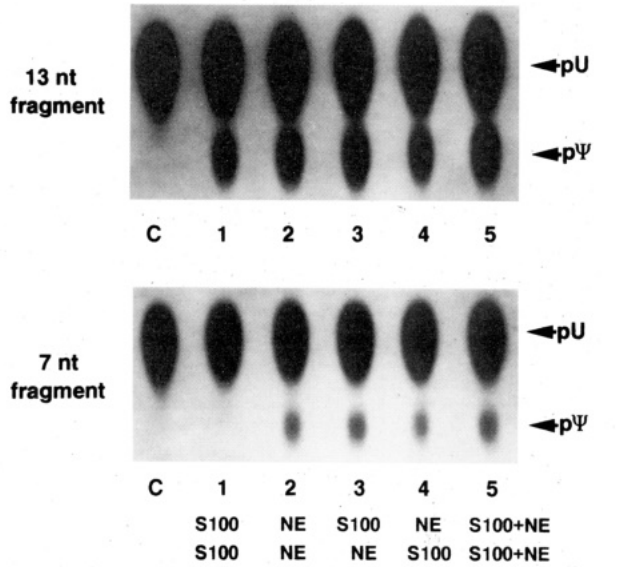


FIGURE 2: Formation of Ψ on U5 RNA in extracts from HeLa cells. $^{32}$P-U5 RNA was incubated in extracts, the 13- and 7-nt RNase T1 fragments from the U5 RNA were isolated, and the nuclease P1 digested samples were chromatographed on TLC plates as described in the Experimental Procedures. A portion of an autoradiograph of each TLC is shown in the two panels. The positions of pU and pΨ are indicated on the right of each panel, and the types of extracts included in the reactions are indicated at the bottom of the figure. C is $^{32}$P-U5 RNA that was not incubated with extract.

Table 1: Ψ Formation in U5 RNA RNase T1 Fragments in S100 and Nuclear Extracts

| | 13-nt RNase T1 fragment | | 7-nt RNase T1 fragment | |
|---|---|---|---|---|
| extracts used | % Ψ[a] (± sd) | % of theoretical[b] (± sd) | % Ψ (± sd) | % of theoretical (± sd) |
| S100/S100 | 7.9 (0.1) | 39.6 (0.4) | 0.3 (0.1) | 1.0 (0.1) |
| NE/NE | 10.1 (0.1) | 50.4 (0.4) | 5.5 (0.2) | 16.6 (0.5) |
| S100/NE | 13.8 (0.1) | 68.8 (0.5) | 5.6 (0.2) | 17.1 (0.5) |
| NE/S100 | 9.1 (0.1) | 45.4 (0.4) | 4.8 (0.2) | 14.4 (0.5) |
| S100 + NE/ S100 + NE | 13.7 (0.1) | 68.6 (0.4) | 5.6 (0.2) | 17.1 (0.5) |

[a] To obtain the % Ψ, the uridine and Ψ spots were scraped from the TLC plates, counted in scintillant for 30 min, and corrected for background, and the counts were used to obtain a ratio of Ψ counts to the total counts in uridine plus Ψ spots (Ψ/U + Ψ) and converted to percent. In addition, a "no extract" control value for % Ψ (0.4% for the 13-nt fragment and 0.5% for the 7-nt fragment) was subtracted from the values for RNAs incubated in extracts. [b] The percent of theoretical is obtained by comparing the observed % Ψ and the theoretical percent of Ψ (Ψ/U + Ψ) expected from the known sequence of this RNase T1 fragment from human U5 RNA. The theoretical percent for the 13-nt fragment is 20% (since there are actually two 13-nt fragments, 2 Ψ out of 10U + Ψ), and the value for the 7-nt fragment is 33% (1 Ψ out of 3U + Ψ).

70% of the theoretical Ψ content (which is 20% of the uridines in this fragment). However, when S100 was the only extract used, only 40% of the theoretical amount of Ψ was formed. The addition of NE increases the efficiency of Ψ formation when compared to S100 alone, but both extracts support Ψ formation in the 13-nt fragment. This increase in the efficiency of Ψ formation in the 13-nt fragment with the combination of the two extracts could be due to an increase in the total amount of the Ψ synthase that modifies the uridines in that fragment, coupled with the slightly increased efficiency of RNP formation seen with the combination of extracts (Patton, 1991). *In vitro* U5 snRNP formation with NE alone was low (~10–20%; data not shown) when compared with assembly in S100 alone or in a combination of the two extracts. The low efficiency not withstanding, enough U5 snRNP was assembled *in vitro* to allow for the purification of full-length U5 RNA from the reactions and for the analysis of the Ψ content of the RNase T1 fragments.

There was no Ψ formation in the 7-nt fragment when just S100 was included in the reaction. The presence of Ψ at position 53 was completely dependent on the presence of NE in the reaction. There is no significant difference in the levels of Ψ between the treatments that contained NE. These results suggest that there is an additional Ψ synthase activity found in NE. They also suggest that the lack of activity in the S100 is not due to inhibition of an activity present in the extract, since Ψ was found in those samples that included S100 in addition to NE.

In an attempt to increase the level of the activity that is responsible for Ψ formation at position 53, an additional extract of the nucleus was prepared by a subsequent higher salt extraction [normal extraction buffer (Dignam *et al.*, 1983) with additional 0.12 M KCl]. Although there was some formation of Ψ at positions 43 and 46 (11% of theoretical; data not shown) when this extract was used alone, there was no Ψ detected at position 53. The inefficiency of Ψ formation at position 53 when NE was used was not due to incomplete extraction of the activity that is responsible for this modification from the nuclei. Changes in other parameters of the reaction such as salt or thiol concentration might improve the efficiency of Ψ formation at this position since these are factors known to affect the activity of tRNA Ψ synthases (Kammen *et al.*, 1988).

Table 2: Ψ Formation in Mutant U5 RNAs

| | 13-nt RNase T1 fragment | | 7-nt RNase T1 fragment | |
|---|---|---|---|---|
| type of U5 RNA used | % Ψ[a] (± sd) | % of theoretical[b] (± sd) | % Ψ (± sd) | % of theoretical (± sd) |
| wild type | 6.6 (0.1) | 33.0 (0.4) | 3.2 (0.1) | 9.7 (0.4) |
| Δ39-43[c] | 0.1 (0.1) | 0.4 (0.1) | 2.6 (0.1) | 7.9 (0.4) |
| Δ89-94 | 1.8 (0.1) | 8.8 (0.2) | 0.2 (0.1) | 0.5 (0.2) |
| Δ79-116 | 1.2 (0.1) | 6.2 (0.2) | 0.1 (0.1) | 0.4 (0.2) |

[a] The % Ψ was calculated as described in the legend to Table 1. In this experiment the % Ψ values for the "no extract" control were 0.8% for the 13-nt RNase T1 fragment and 1.0% for the 7-nt RNase T1 fragment. [b] The percent of theoretical was determined as described in the legend to Table 1. [c] An 8-nt RNase T1 fragment was isolated for this RNA since the deletion of nts 39-43 creates an 8-nt fragment. The % Ψ was found to be 1.4% *without* a correction for "no extract". There was no visible Ψ spot on the TLC.

*Ψ Formation in U5 RNA Mutants.* In order to begin to understand the substrate requirements for Ψ formation at position 53, several mutants of U5 RNA were tested in the *in vitro* system. Wild-type and mutant U5 RNAs were transcribed *in vitro* either from pHU5a2 cut with *Bfa*I (wild-type U5 RNA) or *Hin*fI (Δ79-116 U5 RNA) or from clones generated by *in vitro* mutagenesis, mHU5aΔ39-43 (Δ39-43 U5 RNA) or mHU5aΔ89-94 (Δ89-94 U5 RNA) which have been described previously (Patton, 1991). The Δ39-43 mutant U5 RNA is missing a portion of the loop of U5 RNA that contains two of the Ψs, at nt 43 and 46, and deletes one of the sites of Ψ formation. It has been shown previously that this mutation abolished Ψ formation on U5 RNA when S100 extracts alone were included in the reaction (Patton, 1991). The other two mutant U5 RNAs are missing the Sm binding site but still have the entire first stem loop intact. The first stem loop is the region of U5 RNA that contains the three Ψs (see Figure 1). The Δ89-94 and Δ79-116 U5 RNAs do not bind Sm proteins as judged by the absence of a reaction of the mutant U5 RNAs with anti-Sm monoclonal antibody after incubation in extracts (Patton, 1991). It has been shown previously that both contain very low levels of Ψ after incubation in modification reactions containing S100 extracts (Patton, 1991).

These $^{32}$P-labeled wild-type and mutant U5 RNAs were incubated in reactions with S100 and NE combined, the RNA from the reactions was isolated, and the 13- and 7-nt RNase T1 fragments were isolated for both the wild-type and mutant U5 RNAs. For the Δ39-43 U5 RNA an 8-nt fragment was also isolated since this represents the new RNase T1 fragment that was generated due to the 5-nucleotide deletion from nts 39–43. The Ψ content of each of these fragments was determined as outlined above, and the results are shown in Table 2. For the 13-nt fragment there was significant Ψ formation with wild-type U5 RNA, but no Ψ was detected in the Δ39-43 U5 RNA 13- or 8-nt fragments. The 13-nt fragment (nts 25–37) from the Δ39-43 U5 RNA is one of the two 13-nt fragments normally found in U5 RNA, and it should not contain Ψ. However, the 8-nt fragment from the Δ39-43 U5 RNA should still have the potential for containing Ψ since the uridine at position 46 is still present. With the Δ89-94 and Δ79-116 mutant U5 RNAs, a low level of Ψ formation is detected, but it is significantly reduced when compared with the level found in the 13-nt fragment from wild-type U5 RNA.

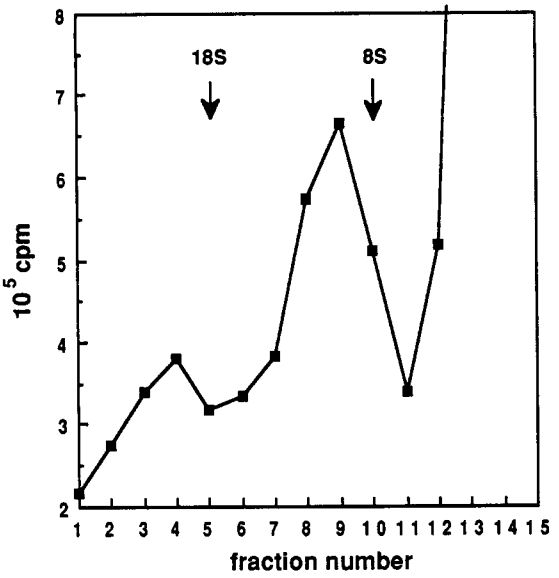


FIGURE 3: Velocity sedimentation gradient analysis of U5 snRNP assembled *in vitro*. $^{32}$P-U5 RNA was incubated in modification reactions that contained a combination of S100 and NE, layered on a 10–30% glycerol gradient and centrifuged as described in Experimental Procedures. The bottom of the gradient is on the left, and the positions of protein sedimentation markers are at the top of the panel. Fractions 3–5 were pooled for the 20S U5 snRNP sample, and fractions 8–10 were pooled for the 9S U5 snRNP sample. The RNA from each pool was isolated, and the level of Ψ in each RNase T1 fragment was determined as described in Experimental Procedures. The results are shown in Table 3.

With the 7-nt fragment, which contains nt 53, a different situation exists. There is significant Ψ formation in the 7-nt fragment from both the wild-type U5 and Δ39-43 mutant U5 RNAs, whereas the Δ89-94 and Δ79-116 U5 RNAs have little or no Ψ in this fragment. These results suggest that mutations that affect the binding of Sm proteins have a significant effect on the Ψ formation at nt 53. In addition, it suggests that the activities that are responsible for the modification of position 53 require Sm protein binding but will tolerate a change in local secondary structure.

*Ψ Content of 9S and 20S U5 snRNP.* In NE, U5 snRNP is found as a 20S RNP and with subsequent manipulation can be converted into a 9S snRNP (Bach *et al.*, 1989). The loss of U5 RNA specific proteins accompanies the conversion of the 20S snRNP into the smaller particle (Bach *et al.*, 1989). In the *in vitro* modification reaction the $^{32}$P-U5 RNA is assembled into both 9- and 20S particles (Patton, 1991). It is possible that the Ψ content of these two particles might be different, in other words, Ψ formation at position 53 might be necessary for 20S particle formation, and that the low level of Ψ formation at position 53 was indicative of a low level of 20S U5 snRNP formation *in vitro*. To test this possibility, $^{32}$P-U5 RNA was incubated in combined S100 and NE and the snRNP separated on a 10–30% glycerol gradient by velocity sedimentation gradient centrifugation. The RNA from the peaks for 20S and 9S U5 snRNPs (see Figure 3) was isolated. The Ψ content of the isolated 13- and 7-nt fragments after RNase T1 digestion was determined as outlined in the above experiments, and the results are shown in Table 3. There does not appear to be a large difference between the Ψ content of the U5 RNA from the 9S versus the 20S snRNP in either the 13- or 7-nucleotide fragment, but the RNA from the 20S particles had consistently higher Ψ content in both T1 fragments. This higher Ψ content in the RNA from 20S U5 snRNP particles had also been observed previously when the total Ψ content was compared (Patton, 1991).

Table 3: Ψ Levels in RNA from 9S and 20S U5 snRNP

| type of U5 snRNP RNA assayed | 13-nt RNase T1 fragment | | 7-nt RNase T1 fragment | |
|---|---|---|---|---|
| | % Ψ[a] (± sd) | % of theoretical[b] (± sd) | % Ψ (± sd) | % of theoretical (± sd) |
| 9S | 8.5 (0.1) | 42.5 (0.4) | 3.8 (0.1) | 11.4 (0.2) |
| 20S | 13.3 (0.1) | 66.7 (0.7) | 4.7 (0.1) | 14.2 (0.2) |

[a] The % Ψ was calculated as described in the legend to Table 1. In this experiment the % Ψ values for the "no extract" control were 0.5% for the 13-nt RNase T1 fragment and 0.8% for the 7-nt RNase T1 fragment. [b] The percent of theoretical was determined as described in the legend to Table 1.

## DISCUSSION

The *in vitro* formation of Ψ in U5 RNA was examined, and the results suggest that at least two separate activities located in the nucleus and the cytoplasm of HeLa cells are responsible for the modification of uridines at positions 43, 46, and 53. Multiple activities for the formation of Ψ on snRNAs in general (Patton, 1993) and on U2 RNA in particular (Patton *et al.*, 1994) have also been described recently, but these activities were all found in the S100 extract, which is predominantly cytoplasmic. In a previous study of the formation of Ψ in U5 RNA (Patton, 1991) evidence for the modification of uridines at positions 43 and 46 was presented. In this report it was shown that the modification of the uridine at position 53 is dependent on the presence of nuclear extract from HeLa cells. This result suggests a nuclear location for the activity that is responsible for Ψ formation at position 53 of U5 RNA. This activity cannot be detected in the S100 extract alone, and therefore it is unlikely that its presence in the NE is due to contamination from the cytoplasm during extract preparation. Nor is it likely that the activity is present in the S100 and inhibited in some manner since inclusion of S100 extract in reactions containing NE does not inhibit the formation of Ψ at position 53. In fact, the most efficient Ψ formation was observed with a combination of the two extracts.

The formation of Ψ at position 53 is not dependent on Ψ formation at positions 43 and 46, since the Δ39-43 mutant U5 RNA has nearly wild-type levels of Ψ at position 53 even though there is no Ψ at positions 43 and 46. This independent formation of Ψ in snRNAs was also seen with U2 RNA when the Ψ modification process was studied *in vitro* (Patton *et al.*, 1994). Multiple activities with nonoverlapping substrate specificities could easily allow for an autonomous mode of Ψ formation. The Ψs at positions 43 and 46 are located in a terminal loop of the U5 RNA secondary structure (Figure 1), and the Ψ at position 53 is located in a stem of that loop. The activities that modify these nucleotides are probably responding to both the sequence and secondary structure contexts of the modification site. Both the sequence and the secondary structure adjoining the sites of Ψ formation were also major determinants in the modification of U2 RNA *in vitro* (Patton *et al.*, 1994).

The presence of Ψ at position 53 is not a requirement for 20S particle formation *in vitro*. It was possible that the low level of Ψ formation at the nucleotide 53 site was reflecting the fraction of the total U5 snRNP assembled *in vitro* into the larger 20S particle. The RNA from the 20S and 9S particles both had Ψ at position 53, and although the level found in the RNA from 20S particles was greater than that in RNA from 9S particles, the levels were only a fraction of the predicted amount found in completely modified U5 RNA isolated from HeLa cells (Reddy & Bush, 1988). Therefore,

20S particle formation is not dependent on Ψ formation at nucleotide 53 since only 14% of the U5 RNA from the 20S U5 snRNP has Ψ at that site.

In an earlier report, it was shown that the amount of U5 RNA specific Ψ formation activity found in the NE was significantly lower than that found in the S100 extract (Patton, 1991). On the surface this earlier report seemingly contradicts the results presented here, but the differences can be attributed to the type of assay for Ψ formation used in the study. Previously, the $^3$H-release assay for Ψ formation (Mullenbach *et al.*, 1976) was used to detect the total amount of Ψ formation on $^3$H-labeled *in vitro* synthesized U5 RNA (Patton, 1991). This is a simple assay based on the release of $^3$H from the 5 position of the uridine ring when Ψ was formed and was used to gauge total activity in a reaction without regard for the integrity of the U5 RNA or competing reactions that might destroy the $^3$H-labeled substrate. In the assay used in the present study, full-length U5 RNA was isolated from a denaturing gel after incubation in the modification reactions. The total counts for any of the samples was equalized to allow comparisons of activity. Some of the treatments, such as incubation with NE alone or with NE as the first extract, resulted in significant degradation of the U5 RNA so that the yield of full-length U5 RNA was low. In the TLC assay used in this report, the effect of degradation was minimized since the RNA was isolated before it was assayed. However, degradation would result in an artificially low level of $^3$H release for U5 RNA in the $^3$H-release assay when only NE was included in the reaction (Patton, 1991).

How does the Ψ formation in U5 RNA fit into the complex maturation process of U5 snRNP? Since the formation of Ψ at all of the sites in U5 appears to require the binding of Sm proteins, it is reasonable to assume that the U5 snRNP is formed prior to Ψ formation on the U5 RNA. Therefore, the modification of positions 43 and 46 probably occurs during the cytoplasmic phase of the maturation process with subsequent formation of Ψ at position 53 when the particle gets back into the nucleus. Actual testing of this hypothesis awaits microinjection experiments or the purification of the activities responsible, which will allow for more detailed tests of substrate requirements.

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