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Localization of modified nucleotides in *Schizosaccharomyces pombe* spliceosomal small nuclear RNAs: Modified nucleotides are clustered in functionally important regions

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ABSTRACT

The specific and dynamic RNA:RNA interactions between pre-mRNA and small nuclear RNAs (snRNAs), especially U2, U5, and U6 snRNAs, form the catalytic core and are at the heart of the spliceosome formation. The functionally important regions in the snRNAs correspond to the highly modified regions in snRNAs from human, rat, and plant cells. To better understand the importance of the modifications of snRNAs, we identified and localized the modified nucleotides in the five spliceosomal snRNAs of *Schizosaccharomyces pombe* cells. Twenty-two modified nucleotides, including base methylations, 2′-*O*-methylations, and pseudouridines, were found in the five spliceosomal snRNAs. The conservation of modified nucleotides between human and *S. pombe* snRNAs is striking. In addition, most of the modified nucleotides are in or around positions that form hydrogen bonds with the pre-mRNA or with other snRNAs. The results are consistent with the suggestion that modified nucleotides are clustered around functionally important regions of the spliceosomal snRNAs. These data provide the basis for further functional studies on posttranscriptional modifications in spliceosomal snRNAs.

Keywords: Cap; fission yeast; methylation; modified nucleotides; pseudouridine; snRNA; splicing

INTRODUCTION

Most cellular RNAs, including small nuclear RNAs, undergo posttranscriptional modifications that include modifications of bases, sugar, and sometimes the phosphate moieties in RNA. Transfer RNAs are the most modified among the different classes of RNAs and more than 90 different modifications have been identified in various RNAs (reviewed in Limbach et al., 1994). In addition to transfer, ribosomal, and messenger RNAs, small nuclear RNAs also contain posttranscriptional modifications. Work from our and other laboratories has shown that the five abundant spliceosomal snRNAs from higher eukaryotes, including rodent, human, and plant cells, contain posttranscriptional modifications (reviewed in Reddy & Busch, 1988; Solymosy & Pollak, 1993).

There are several studies showing that modified nucleotides are important for optimal function of tRNAs (reviewed in Bjork et al., 1987). Although no clear functional role has been suggested for any of the modified nucleotides in rRNA, their importance is suggested by models of the *Escherichia coli* ribosome in which the modifications are clustered around the mRNA–tRNA–peptide complex in the catalytic center of the ribosome (Lane et al., 1992; Brimacombe et al., 1993). These suggestions are more relevant in light of Noller's observations that peptidyl transferase activity is unusually resistant to protein extraction procedures, consistent with the hypothesis that rRNA itself is the catalyst (Noller et al., 1992). In one instance, the gene that is responsible for a ribose methylation at a universally conserved nucleotide in the peptidyl transfer center of the ribosomal RNA was found to be essential for cell growth (Sirum-Connolly & Mason, 1993). Persson et al. (1992) showed that the gene for a tRNA modifying enzyme, m^5U54-methyltransferase, is essential for viability in *E. coli*. Acetylated pseudouridine (Ψ), but not acetylated U, was found to be capable of acetylating the N-terminus of a peptide in vitro (Wood et al., 1995). The Ψ in tRNA appears to be required for the efficient

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reading of the codons during translation (Johnston et al., 1980). These and many other studies show that posttranscriptional modifications in RNAs play important and some times essential roles.

Evidence obtained from various laboratories showed that, although a large portion of the snRNA sequences were involved in binding to specific proteins, a small portion of snRNA sequences were found to participate in hydrogen bonding with conserved regions in pre-mRNAs and other spliceosomal snRNAs. These specific and dynamic RNA:RNA interactions, especially those involving pre-mRNA with U2, U5, and U6 snRNAs, form the catalytic core and are at the heart of the spliceosome formation (reviewed by Steitz, 1992; Wise, 1993; Sharp, 1994; Umen & Guthrie, 1995). While determining the primary sequences of snRNAs, we localized the modified nucleotides in all the spliceosomal snRNAs of Novikoff hepatoma cells. The modified nucleotides in every snRNA were clustered in a portion of the RNA and were not distributed randomly throughout the snRNA sequences (reviewed in Reddy & Busch, 1988; Steitz et al., 1988). It is also evident that the functionally important regions in snRNAs correspond to the highly modified regions in the snRNAs. For example, the rat U2 snRNA has 23 posttranscriptional modifications, all of which are in the 5′ half of the molecule (Shibata et al., 1975). The sequence of U2 snRNA that forms hydrogen bonds with the pre-mRNA branch point site is highly modified. Although the U5 snRNA does not contain many modified nucleotides, virtually all the modifications in rat U5 snRNA are confined to the invariant loop structure in the middle of the RNA. The invariant U residues in the middle of this loop structure hydrogen bond with both the 5′ and 3′ exons, thus holding both the exons together in place for ligation (Newman & Norman, 1992; Steitz, 1992). Extensive information from mammalian cells, plants, and Dinoflagellates is available on the modified nucleotides in U5 snRNA; in all cases, the conserved nucleotides in this conserved loop structure are extensively modified (Szkukalek et al., 1995). The U6 snRNA sequence that hydrogen bonds with the U2 snRNA or with the pre-mRNA also contains a large number of modified nucleotides.

The requirement for modified bases for snRNP function in splicing has not been established. Segault et al. (1995) used an in vitro snRNP reconstitution/splicing complementation system to show that U2 snRNP reconstituted from U2 snRNA that was isolated from HeLa cells could restore splicing to a U2 snRNP-depleted splicing extract. The U2 snRNA that was synthesized in vitro and unmodified after assembly did not restore splicing, suggesting that the modifications of U2 snRNA are necessary for U2 snRNP function. However, in the case of yeast U2, U6 snRNAs, and nematode U6 snRNA, unmodified in vitro synthesized RNAs were functional in the in vitro reconstitution system (Fabrizio et al., 1989; McPheeters et al., 1989; Yu et al., 1993).

Because of the relative ease of genetic manipulation of yeast cells, it would be easier to study the function of snRNA modifications in the yeast system. *Schizosaccharomyces pombe* is an ideal organism to start with due to the abundance and small size of its snRNAs. Wise and her co-workers showed previously that *S. pombe* U2 snRNA contains modified nucleotides (Brennwald et al., 1988). It is important to first identify and localize these modified nucleotides. In this report, we identified and determined the location of modified nucleotides in the five spliceosomal snRNAs of the *S. pombe* cells. The results are consistent with the suggestion that modified nucleotides are clustered around functionally important regions of spliceosomal snRNAs. These data provide the basis for further functional studies on posttranscriptional modifications in spliceosomal snRNAs.

RESULTS

S. pombe spliceosomal snRNAs contain several modified nucleotides

To detect the modified nucleotides, uniformly labeled individual snRNAs were prepared as described in the Materials and methods. Each snRNA was digested with nuclease P1, and the digest was subjected to two-dimensional chromatography. *S. pombe* U2 snRNA contained several modified nucleotides, including 2′-*O*-methylated nucleotides, Ψ, and m^6A (Fig. 1, left). The *S. pombe* U6 snRNA contained methylphosphate cap structure, m^6A, and 2′-*O*-methylated nucleotides (Fig. 1, right). Uniformly labeled U1, U4, and U5 snRNAs from *S. pombe* were also analyzed by two-dimensional chromatography. Although U1 and U5 snRNAs contained modified nucleotides, there were no detectable modified nucleotides in U4 snRNA (data not shown).

The m^6A residues are conserved in yeast U2 and U6 snRNAs

Human U6 snRNA contains one m^6A at position 43. Analysis of the uniformly labeled yeast U2 and U6 snRNAs indicated the presence of m^6A residues (Fig. 1). To localize the m^6A residue in *S. pombe* U6 snRNA, uniformly labeled U6 snRNA was digested with RNase A and the resulting oligonucleotides were separated by electrophoresis on a 20% polyacrylamide/7 M urea gel. The oligonucleotides were isolated, digested with T2 RNase, and analyzed for the presence of m^6A residue (Fig. 2). Only the nonanucleotide corresponding to nt 37–45 of *S. pombe* U6 snRNA contained m^6A (Fig. 2, lane 5); however, m^6A was not detectable when this oligonucleotide was digested with nuclease P1 (Fig. 2,

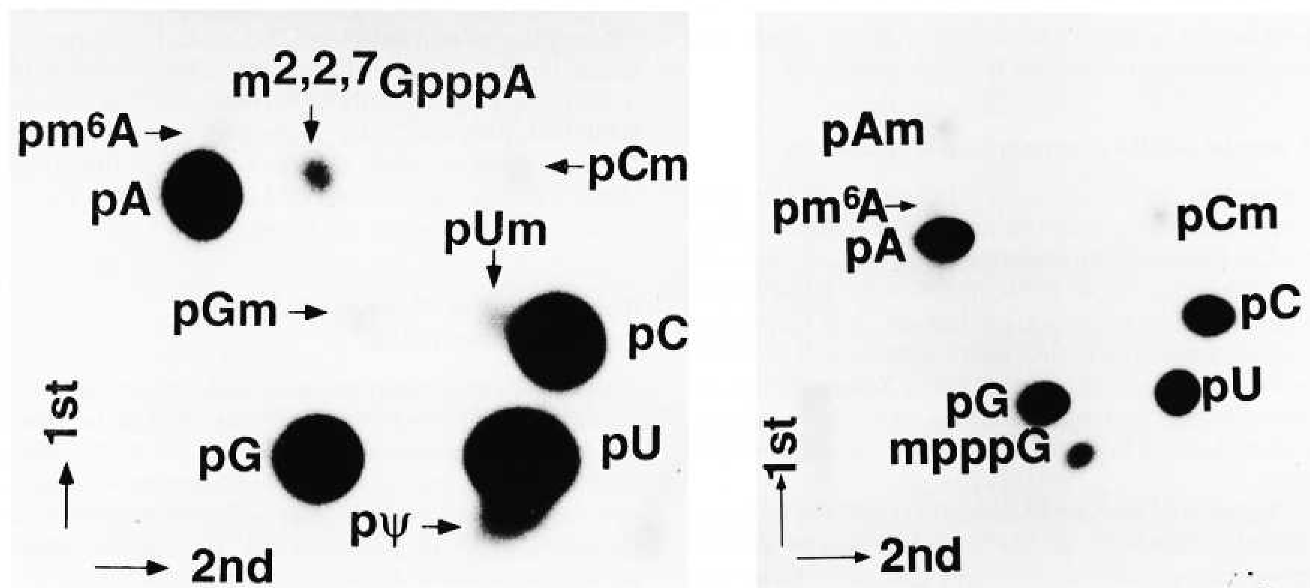

FIGURE 1. Identification of modified nucleotides in yeast (*S. pombe*) U2 and U6 snRNA by two-dimensional chromatography. [^{32}P]-orthophosphate labeled *S. pombe* U2 and U6 snRNAs obtained by immunoprecipitation with anti-trimethylguanosine or anti-methylphosphate cap antibodies were purified and digested with nuclease P1 to completion. The digest was subjected to two-dimensional chromatography on cellulose plates according to Silberklang et al. (1979). Dried plates were subjected to autoradiography. Identification of the modified nucleotides was based on published data and comigration with unlabeled standards in the same chromatographic system. Left: U2 snRNA. Right: U6 snRNA. On the left, the unlabeled spot adjacent to pU is inorganic phosphate.

lanes 7 and 9). These data show that the m^{6}A is the 5′-most A in this oligonucleotide, which corresponds to position 37 of *S. pombe* U6 snRNA and position 43 of human U6 snRNA.

Similar analysis was conducted for *S. pombe* U2 snRNA and an m^{6}A residue was found in a position corresponding to nt 30; human U2 snRNA also contains m^{6}A at position 30 (Reddy et al., 1981a). These

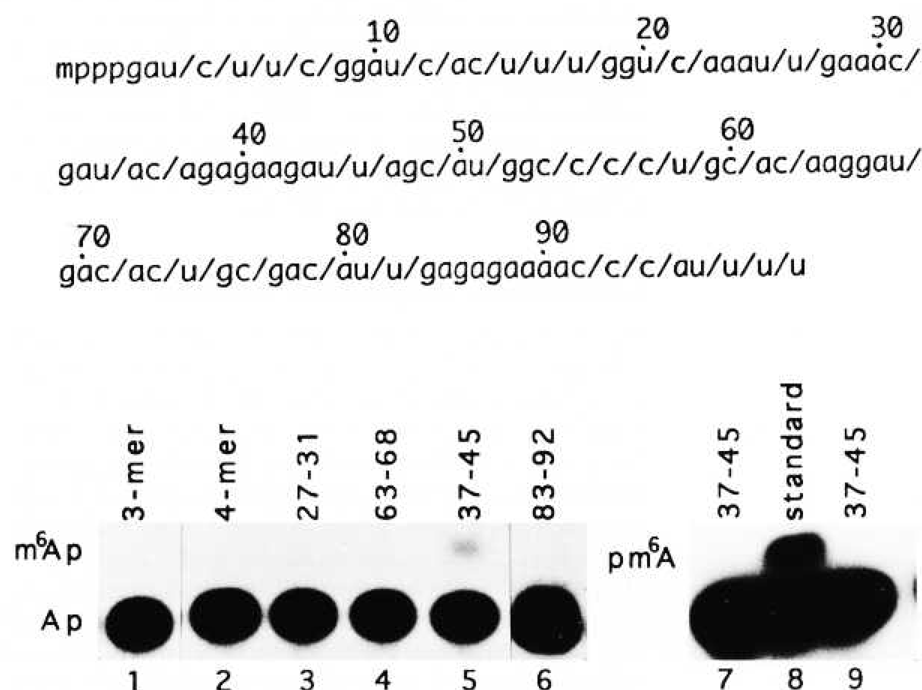

FIGURE 2. Localization of m^{6}A residue in yeast (*S. pombe*) U6 snRNA. Top: Nucleotide sequence of *S. pombe* U6 snRNA. The RNase A cleavage sites are marked. Bottom: Uniformly [^{32}P]-labeled *S. pombe* U6 snRNA was digested with RNase A and fractionated by electrophoresis on a 20% polyacrylamide/7 M urea gel. Adenosine-containing oligonucleotides were isolated, digested with T2 RNase (lanes 1–6) or with nuclease P1 (lanes 7–9), and subjected to chromatography on a cellulose plate. Positions of oligonucleotides are indicated above the lanes. The 3-mer oligonucleotides correspond to nt 1–3, 18–20, 32–34, 47–49, 52–54, 69–71, and 77–79. The 4-mer oligonucleotides correspond to nt 8–11 and 22–25. The portion of the autoradiogram corresponding to only the AMP and m^{6}AMP is shown.

data show that m^6A residues present in human U2 and U6 snRNAs are also present in the corresponding positions of *S. pombe* U2 and U6 snRNAs. Thus, this modification is conserved through evolution.

S. pombe snRNAs contain Cap 0 structures

Depending on the ribose methylations on the transcription initiation nucleotide and adjacent nucleotides, the Cap structures are designated Cap 0, Cap 1, or Cap 2. Cap 0 has no sugar methylation, Cap 1 has ribose methylation in initiation nucleotide, and Cap 2 has methylations in initiation nucleotide as well as in the second nucleotide (Banerjee, 1980). Yeast mRNAs are known to contain Cap 0 and mRNAs in higher eukaryotes are known to contain Cap 2 structures (Banerjee, 1980).

Digestion of uniformly labeled U1 snRNA with nuclease P1, T2 RNase, or T2 RNase followed by alkaline phosphatase yielded $m^{2,2,7}$GpppA, $m^{2,2,7}$GpppAp, and $m^{2,2,7}$GpppA, respectively (Fig. 3, lanes 1–3).

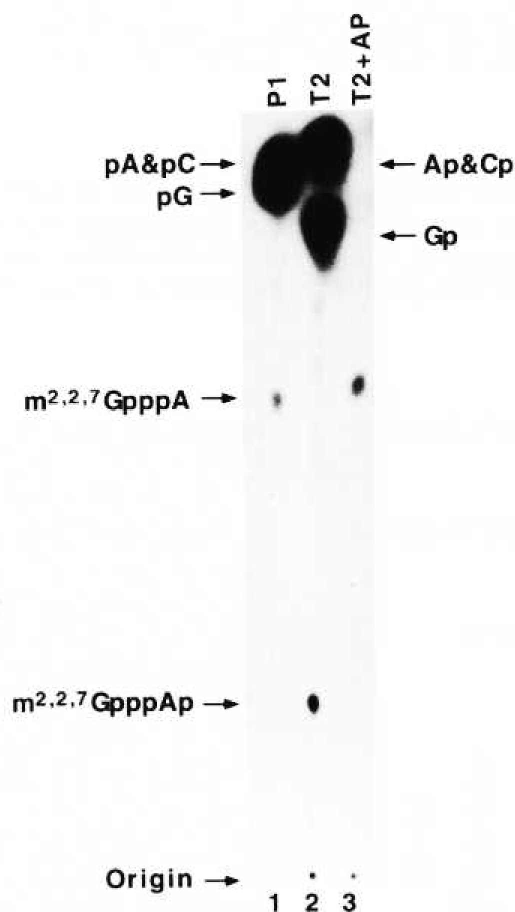

FIGURE 3. Analysis of Cap structure in *S. pombe* U1 snRNA. Uniformly labeled U1 snRNA was digested with various enzymes as indicated on top of each lane (AP, alkaline phosphatase), fractionated by electrophoresis on a DEAE-cellulose paper and subjected to autoradiography.

These data are consistent with the presence of Cap 0 structure in *S. pombe* snRNAs. The experiments with other spliceosomal snRNAs U2, U4, and U5 gave the same results (data not shown). The presence of $m^{2,2,7}$Gppp Cap structure in *S. pombe* snRNAs was established previously by Wise and her colleagues (Brennwald et al., 1988). Results obtained in this study show that the first and second nucleotides in the *S. pombe* snRNAs are not 2′-*O*-methylated.

Identification of ribose methylations in spliceosomal RNAs

The 2′-*O*-ribose methylations are the most frequent modifications found in snRNAs as well as in other RNAs. The characteristic property of 2′-*O*-ribose methylation is that the 3′-phosphodiester linkage is not susceptible to cleavage by ribonucleases such as RNase A, T1, or T2, which form 2′,3′-cyclic phosphate as an intermediate during hydrolysis of RNA.

Figure 4 shows the analysis of the five spliceosomal snRNAs after digestion with T2 RNase. The five spliceosomal snRNAs digested with T2 RNase were separated by electrophoresis on DEAE-cellulose paper. All the ribose-methylated nucleotides appeared as dinucleotides or larger and hence migrated more slowly than the mononucleotides. Other than the Cap structure, there were no detectable T2 RNase-resistant structures in U1 and U4 snRNAs (lanes 2 and 3), indicating that these two RNAs do not contain any ribose methylations. The identity of ribose methylations in U2, U5, and U6 snRNAs was determined by further digestion of the T2 RNase resistant di- and trinucleotides by other nucleases as described by Choi and Busch (1978). These di- and trinucleotides were localized to known T1 RNase and/or RNase A digestion fragments. For example, U5 snRNA contained three T2 RNase-resistant dinucleotides (Fig. 4, lanes 4 and 6). All three dinucleotides were found in one T1 RNase fragment (Fig. 4, lane 7). The results obtained by this procedure were sufficient to localize all the ribose-methylated nucleotides in U2, U5, and U6 snRNAs.

Localization of pseudouridine residues

We used a method developed recently by Bakin and Ofengand (1993) to localize the Ψ residues. The approach takes advantage of the unique stability of N_3-CMC-Ψ to alkaline hydrolysis. The CMC group at the N_3 position of Ψ efficiently blocks reverse transcription and results in a strong stop band one base downstream from Ψ sites, whereas U and other nucleotide adducts of CMC are cleaved readily under alkaline conditions and will not affect reverse transcription. As shown in Figure 5, lane 2, strong stops were observed at positions that are one base 3′ to 34, 37, 41, 43, 44, and 58 Ψ residues in *S. pombe* U2 snRNA treated with CMC

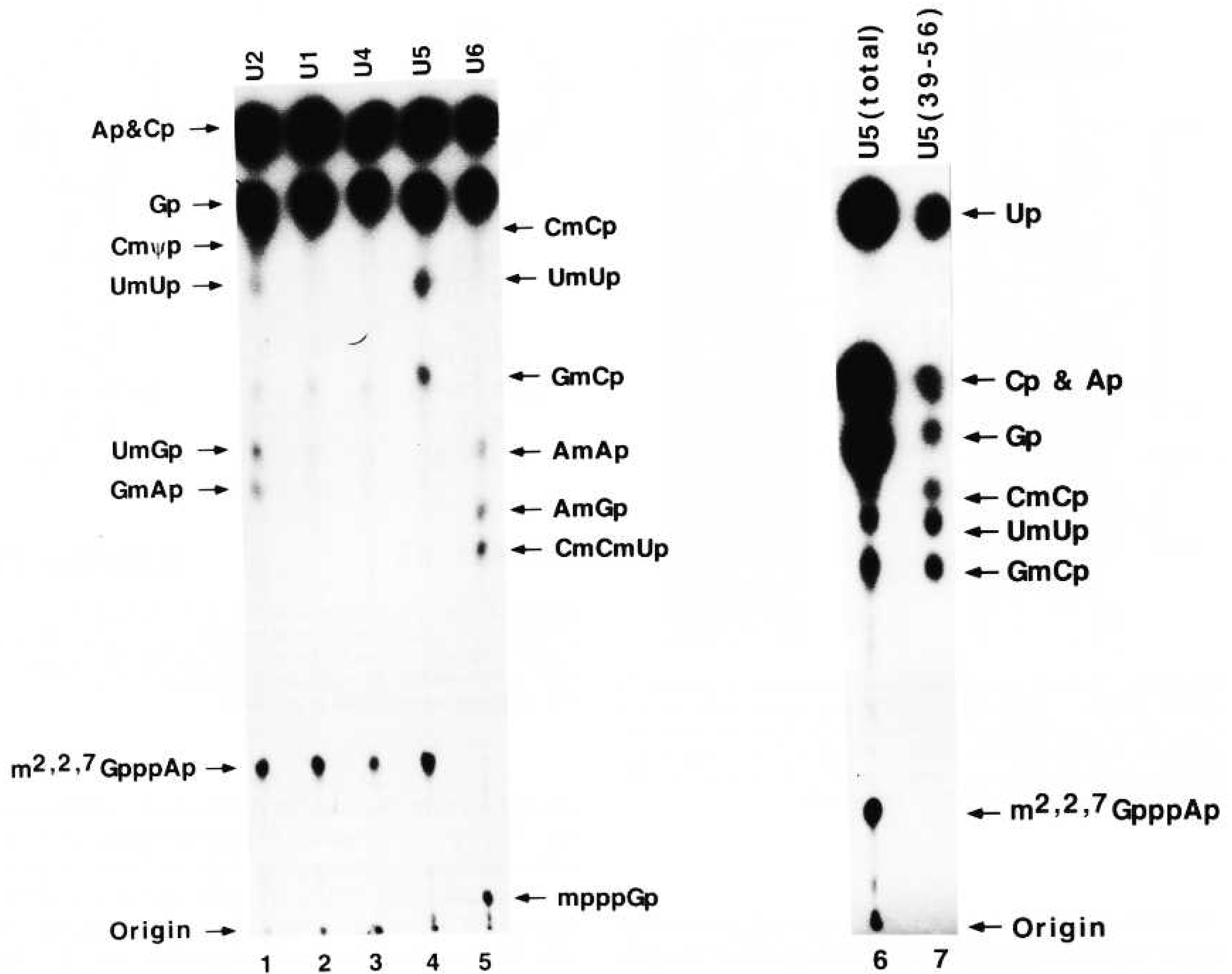

FIGURE 4. Analysis of T2 RNase-resistant oligonucleotides. Spliceosomal snRNAs from *S. pombe* were digested with T2 RNase, fractionated by electrophoresis on a DEAE-cellulose paper, and subjected to autoradiography. The identification of sugar-methylated nucleotides is based on further analysis of these T2 RNase-resistant oligonucleotides and is consistent with their expected Rf values in this system. The spot co-migrating with GmCp was observed in submolar yields with many RNAs, and is probably due to unstable aromatic ring opening in the $m^{2,2,7}$GpppA cap structure (Shibata et al., 1975)

as compared to control (lane 1), indicating that these six residues are Ψ residues. A similar strategy showed that U1 snRNA contains one Ψ corresponding to nt 3 (Fig. 5, lane 4). We also detected two Ψ residues corresponding to nt 37 and 51 in *S. pombe* U5 snRNA; we did not find any Ψ residues in *S. pombe* U4 and U6 snRNAs (data not shown).

Human Ψ-forming enzymes are not capable of forming Ψ on the opposite strand of U5 snRNA

All of the Ψ residues found in *S. pombe* snRNAs exist in corresponding positions of human snRNAs. The only exception is the Ψ corresponding to nt 37 in *S. pombe* U5 snRNA, which is found at position 53 in human U5 snRNA (Fig. 6). Interestingly, when one considers the universally conserved secondary structure of U5 snRNA, this Ψ residue is present in the same position but on opposite strands of the human and *S. pombe* U5 snRNAs. We wanted to know whether or not the enzyme(s) that converts U → Ψ has strand specificity and distinguishes uridine residues on different strands.

We took wild-type human U5 snRNA and a mutated human U5 snRNA in which the A-U base pair is reversed to mimic the *S. pombe* situation. Both of these RNAs synthesized in vitro were incubated in an in vitro system capable of Ψ formation, and the Ψ formed in different positions was analyzed. There was no Ψ present in the 7-mer from the mutant U5 snRNA (Fig. 7, lane 5) and there was no Ψ in the 13-mer from the 41-nt fragment of the mutant U5 RNA treated with the deoxyoligonucleotide (see the Materials and methods, and Fig. 7, lane 6). From these data, we know that there is no formation of Ψ at position 29 in the mutant

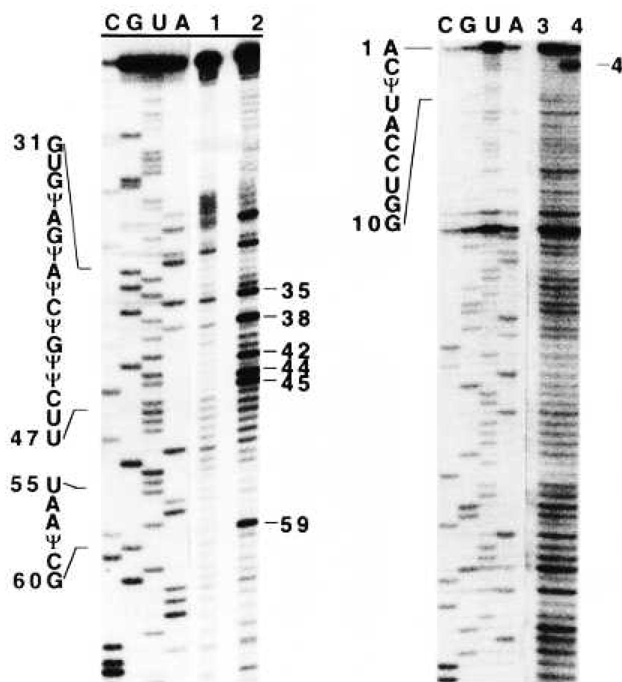

FIGURE 5. Determination of pseudouridine residues in *S. pombe* U1 and U2 snRNAs. The U1 and U2 snRNA samples where Ψ residues are modified were prepared as described in the Materials and methods by the method of Bakin and Ofengand (1993). Lanes 1 and 3, control U2 and U1 snRNAs not treated with CMC; lanes 2 and 4, CMC-treated U2 and U1 snRNAs, respectively.

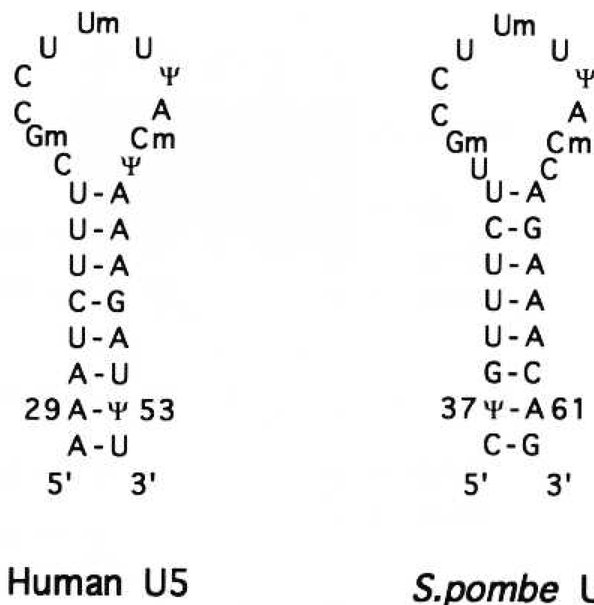

FIGURE 6. Partial secondary structure of human and *S. pombe* U5 snRNA with posttranscriptional modifications. The human U5 snRNA structure is from Krol et al. (1981) and the modified nucleotides determined in this study are included in *S. pombe* U5 snRNA secondary structure (Small et al., 1989).

U5 RNA and there is no Ψ formed around position 53 in the mutant, even though uridines bracket this position in human U5 RNA. However, Ψ was formed at positions 43 and 46 in both the wild-type and mutant U5 RNA (Fig. 7, lanes 2 and 4), and Ψ is formed at position 53 in the wild-type U5 RNA (lane 3). The levels of Ψ formation at positions 43 and 46 are the same for the wild-type and the mutant U5 RNAs, 12.7% and 14.2%, respectively. The maximum amount of Ψ that can be formed in this fragment is 20%. The level of Ψ formation in the 7-mer of the wild-type U5 RNA is low (3.1% when the maximum possible is 33%); however, this low level is consistent with what was seen in earlier work (Patton, 1994). As expected, no Ψ formation was observed in the 11-mer from wild-type U5 RNA (lane 1). These data show that the human enzyme(s) responsible for Ψ formation in this region of U5 RNA modifies the U residue at the right position and is strand-specific.

DISCUSSION

Previously, we identified the methylphosphate Cap structure in small RNAs from human (Singh & Reddy, 1989) and other higher eukaryotic cells (Reddy et al., 1992). The data presented in this study provide the first definitive evidence for the presence of monomethylphosphate Cap structure in yeast cells. In addition, the $m^{2,2,7}$Gppp Cap structure in *S. pombe* snRNAs, which was initially characterized by Wise and her colleagues (Brennwald et al., 1988), is Cap 0, which is similar to that found in *S. pombe* mRNAs and other lower eukaryotic RNAs transcribed by RNA polymerase II. These data show that the type of Cap structure in snRNAs and mRNAs transcribed by RNA polymerase II is the same, namely, Cap 0 in lower eukaryotes and Cap 2 in higher eukaryotes.

Wise and her colleagues characterized *S. pombe* spliceosomal snRNAs extensively and determined their primary sequences (Brennwald et al., 1988; Small et al., 1989; Porter et al., 1990). They also analyzed in vivo-labeled *S. pombe* U2 snRNA and showed that it contains several modified nucleotides (Brennwald et al., 1988). Our data confirm this observation and extend it by localizing these modified nucleotides in the U2 snRNA as well as in other *S. pombe* spliceosomal RNAs. Table 1 summarizes the modified nucleotides present in the five spliceosomal snRNAs of *S. pombe* and HeLa cells. Five human snRNAs have a total of 56 modified nucleotides compared to 22 in the case of *S. pombe* snRNAs. This observation is similar to that in the case of tRNAs and rRNAs, where there are more modified nucleotides in human RNAs compared to the corresponding yeast RNAs (Bjork et al., 1987; Maden et al., 1995).

The most interesting observation is where these modified nucleotides are in the *S. pombe* snRNAs. Figure 8 (top) shows the portions of human U2 and U6 snRNAs hydrogen bonded to a human pre-mRNA;

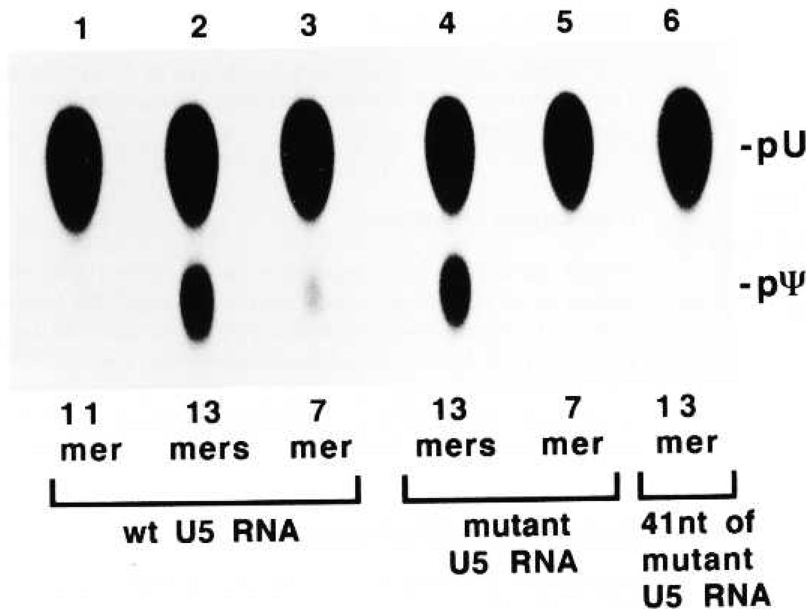

FIGURE 7. Formation of Ψ in wild-type and mutant U5 snRNAs in vitro. ^{32}P-phosphate-labeled RNAs were incubated in modification reactions. The indicated RNase T1 fragments were isolated and the nuclease P1 digested samples were chromatographed on TLC plates as described in the Materials and methods. A portion of an autoradiograph of the TLC is shown. Positions of pU and pΨ are indicated on the right and the RNase T1 fragments are indicated at the bottom of the figure.

Figure 8 (bottom) shows the portions of *S. pombe* U2 and U6 snRNAs hydrogen bonded to a *S. pombe* pre-mRNA. The conservation of modified nucleotides between human and *S. pombe* U2 and U6 snRNAs is striking. In addition, most of the modified nucleotides are in or around positions that hydrogen bond with the pre-mRNA or with other snRNAs. This is true of the 5′ end of U1 snRNA and the conserved loop in U5 snRNA. Both of these regions containing modified nucleotides are known to hydrogen bond with pre-mRNAs. Therefore, one can conclude that the modified nucleotides in snRNAs are present in portions that are functionally important and participate in hydrogen bonding with pre-mRNA or with other snRNAs. Similar conclusions regarding the correlation between the posttranscriptional modifications in snRNAs and their functional importance have been drawn by several investigators (Reddy & Busch, 1988; Steitz et al., 1988; Ares & Weiser, 1995; Szkukalek et al., 1995). This situation is similar to ribosomal RNAs, where the modifications are clustered around the mRNA–tRNA–peptide complex in the catalytic center of the ribosome (Brimacombe et al., 1993).

TABLE 1. Posttranscriptional modifications in human and *S. pombe* spliceosomal snRNAs.

RNA	Source	Cap	2′-O-m	m^{6}A	m^{2}G	Ψ
U1	Human	m^{2,2,7}GpppN1mN2m	3	0	0	2
	S. pombe	m^{2,2,7}GpppN1N2	0	0	0	1
U2	Human	m^{2,2,7}GpppN1mN2m	10	1	0	12
	S. pombe	m^{2,2,7}GpppN1N2	4	1	0	6
U4	Human	m^{2,2,7}GpppN1mN2m	4	0	0	3
	S. pombe	m^{2,2,7}GpppN1N2	0	0	0	0
U5	Human	m^{2,2,7}GpppN1mN2m	5	0	0	3
	S. pombe	m^{2,2,7}GpppN1N2	3	0	0	2
U6	Human	mpppGN2	8	1	1	3
	S. pombe	mpppGN2	4	1	0	0
Total	Human		30	2	1	23
	S.pombe		11	2	0	9

[a] Modified nucleotides in human snRNAs can be found in Reddy and Busch (1988) and references therein. *S. pombe* results are from this study.

As detailed in the Introduction, it is clear that modified nucleotides play important and sometimes essential roles. The functions of the modified nucleotides in the spliceosomal snRNAs is not known. Posttranscriptional modifications are known to strengthen or weaken the hydrogen bonding depending on the types of modifications. Modification of U to Ψ results in an extra imino group that creates an additional locus for hydrogen bonding. Poly-Ψ has a much higher melting temperature (*Tm*) than poly-U (Pochon et al., 1964). Griffey et al. (1985) showed that Ψ could form hydrogen bonds to bridging water molecules or to vicinal ribose 2′-hydroxyl groups. Therefore, the Ψ residues found in U1, U2, and U5 snRNAs may either strengthen the interactions between the snRNAs and pre-mRNA, or stabilize the secondary structure of the snRNAs. The 2′-*O*-methylation, on the other hand, introduces a positive charge and increases the hydrophobicity of RNA chains. The 2′-*O*-methylation may prevent the 2′-hydroxyl group from forming hydrogen bonds with phosphodiester bridges in RNA and with peptide bonds in proteins (Nichols & Lane, 1966). Monomeric m^{6}A exists in two isomeric forms in solution. The *cis*-isomer, which is 20-fold more dominant than the *trans*-isomer, is unable to form a Watson–Crick base pair with thymine (Engel & von Hippel, 1974). It is very likely that each modification in these RNAs confers a slight ad-

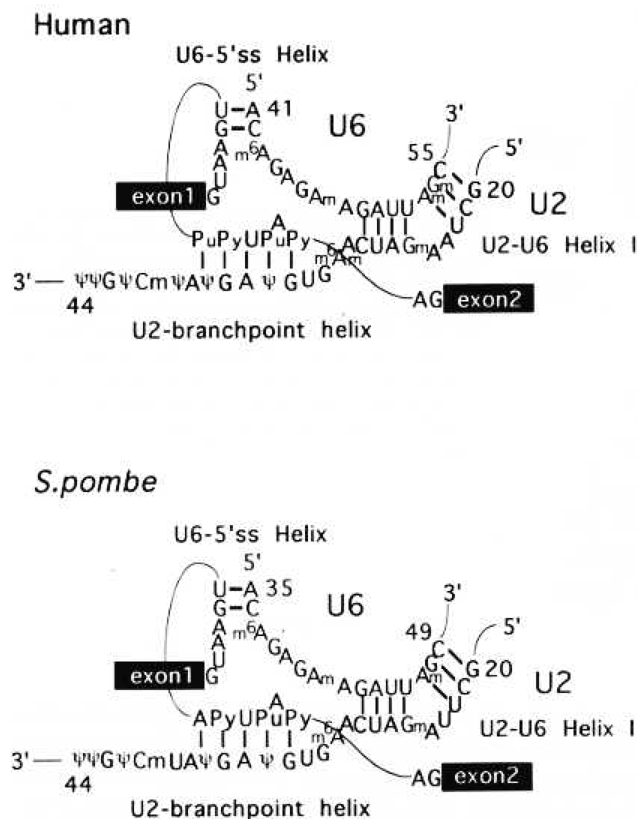

FIGURE 8. Conservation of modified nucleotides between human and *S. pombe* U2 and U6 snRNAs and their clustering in functionally important domains. The model for base pairing between snRNAs and pre-mRNAs is derived from reviews by Steitz (1992), Wise (1993), Sharp (1994), and Madhani and Guthrie (1994). In addition to the specified modifications, the *S. pombe* U2 snRNA contained 2'-*O*-methylated nucleotide Um at positions 9 and 11; *S. pombe* U6 snRNA contained Cm at positions 56 and 57.

vantage to the organism. Otherwise, it is unlikely that these modifications are conserved through evolution. Identification of the modifications in the snRNAs is a first step in understanding the function of these modifications. This study will allow us to purify the enzymes and other co-factors that are involved in these modifications.

MATERIALS AND METHODS

Labeling of *S. pombe* RNA

For preparation of uniformly labeled RNA, *S. pombe* cells were incubated at 30 °C with [^{32}P]-orthophosphate for 16 h in phosphate-free EM medium (Moreno et al., 1991). The uptake of [^{32}P]-orthophosphate was more than 60% at the end of incubation period. The *S. pombe* cells were first broken by vortexing with fine glass beads and total RNA was prepared by the hot phenol-SDS procedure. Whole-cell RNA was fractionated on a sucrose density gradient and fractions corresponding to 4-8S RNA were pooled (Reddy et al., 1981b).

Immunoprecipitations

The immunoprecipitations were conducted as described by Lerner and Steitz (1979) using Pansorbin (Calbiochem) as the source of Protein A.

Purification of snRNAs

Whole-cell 4-8S RNA obtained by sucrose gradient centrifugation or by immunoprecipitation of whole-cell RNA was subjected to electrophoresis on 10% polyacrylamide-7 M urea gels at pH 8.3. Individual snRNAs were extracted from the gels and utilized for further studies. Whenever necessary, snRNAs were repurified on another polyacrylamide gel to remove contaminating RNAs.

Analysis of modified nucleotides

SnRNAs were digested with different enzymes, including RNase T1, T2, or nuclease P1. The digests were subjected to either high-voltage paper electrophoresis on DEAE-cellulose paper at pH 3.5 (Brownlee et al., 1968), or two-dimensional chromatography on cellulose sheets by the method of Silberklang et al. (1979). For chromatography, the first-dimension solvent was isobutyric acid/water/ammonium hydroxide [66:33:1, (v/v/v)], and the second-dimension solvent was 0.1 M sodium phosphate buffer, pH 6.8/ammonium sulfate/1-propanol [100:60:2, (v/w/v)]. The dried paper or cellulose sheet was subjected to autoradiography or quantitated using Betagen.

Localization of pseudouridines

The detection of Ψ was done by CMC-adduct formation method of Bakin and Ofengand (1993). Briefly, 4-8S RNA sample was treated with 0.17 M *N*-cyclohexyl-*N'*-β-(4-methylmorpholinium)-ethylcarbodiimide *p*-tosylate (CMC) for 20 min, followed by treatment of Na_2CO_3 buffer, pH 10.4, for 4 h. The treated sample was recovered and subjected to reverse transcription. Reverse transcription was done using [α-^{32}P]-dATP and primers corresponding to 3' end of individual snRNAs. RNA sequencing was done by the Sanger's dideoxynucleotide method (Sanger et al., 1977) and the reaction samples were analyzed on an 8% polyacrylamide-7 M urea gel.

RNA transcription and in vitro modification

Plasmid DNAs containing wild-type human U5 (pHU5a2, Patton, 1991) or mutant U5 (pHU5a2-29U/53A), which was prepared by using appropriate oligonucleotides and employing PCR, were linearized with *Bfa* I. The RNAs were synthesized using SP6 RNA polymerase in the presence of [α-^{32}P]-UTP and in vitro modification reactions were performed as described earlier (Patton, 1994). In order to generate the 41-nt fragment of mutant U5 RNA, the RNA was isolated from the modification reaction and incubated in HeLa extract (Dignam et al., 1983) in the presence of 1 μg of an oligodeoxynucleotide complementary to nt 41–82 of human U5 RNA to provide a substrate for RNase

H. Wild-type U5 RNA was also incubated in the extract, but the oligonucleotide was not added. Isolation of appropriate fragments and analysis of Ψ formation in different portions of U5 snRNA are as described previously (Patton, 1994).

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