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Accurate and efficient *N*-6-adenosine methylation in spliceosomal U6 small nuclear RNA by HeLa cell extract *in vitro*

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ABSTRACT

Human U6 small nuclear RNA (U6 snRNA), an abundant snRNA required for splicing of pre-mRNAs, contains several post-transcriptional modifications including a single m^{6}A (*N*-6-methyladenosine) at position 43. This A-43 residue is critical for the function of U6 snRNA in splicing of pre-mRNAs. Yeast and plant U6 snRNAs also contain m^{6}A in the corresponding position showing that this modification is evolutionarily conserved. In this study, we show that upon incubation of an unmodified U6 RNA with HeLa cell extract, A-43 residue in human U6 snRNA was rapidly converted to m^{6}A-43. This conversion was detectable as early as 3 min after incubation and was nearly complete in 60 min; no other A residue in U6 snRNA was converted to m^{6}A. Deletion studies showed that the stem–loop structure near the 5′ end of U6 snRNA is dispensable for m^{6}A formation; however, the integrity of the 3′ stem–loop was necessary for efficient m^{6}A formation. These data show that a short stretch of primary sequence flanking the methylation site is not sufficient for U6 m^{6}A methyltransferase recognition and the enzyme probably recognizes secondary and/or tertiary structural features in U6 snRNA. The enzyme that catalyzes m^{6}A formation in U6 snRNA appears to be distinct from the prolactin mRNA methyltransferase which is also present in HeLa nuclear extracts.

INTRODUCTION

U6 snRNA, an essential component for splicing of pre-mRNAs (1–3), contains several post-transcriptional modifications (Fig. 1). These include formation of γ-monomethyl phosphate cap structure (4,5), UMP addition on the 3′ end (6–8), formation of a 2′,3′ cyclic phosphate at the 3′ end (9), base methylations including a *N*-6-methyladenosine (m^{6}A) formation at position 43 and several 2′-*O*-ribose methylations (10,11). The *N*-6-methylation of adenine at position A-43 of human U6 snRNA is of interest in many respects. (i) *N*-6-methyladenine residues are found in not only U6 snRNA but also in many other RNAs including pre- and mature mRNAs, rRNAs, tRNAs, U2 snRNA and U4B snRNA. (ii) The position 43 in mammalian U6 snRNA is in a highly conserved region (3). (iii) The mutation of this nucleotide is lethal in yeast (12). (iv) Furthermore, cross-linking studies showed that this region of U6 snRNA encompassing m^{6}A-43 is involved in base-pairing with pre-mRNA (13–15). Recent data showed that A-43 of U6 snRNA is actually involved in base-pairing with the pre-mRNA sequence near the 5′ splice-site (16,17). These studies show that m^{6}A in U6 snRNA is present in a functionally important nucleotide; however, the function of m^{6}A residues in snRNAs, mRNAs or in other RNAs is not known.

The synthesis of m^{6}A in mRNAs has been studied by several investigators. The m^{6}A in mRNAs has been found in a variety of organisms including higher eukaryotes and in viral RNAs (reviewed in 18). The number of m^{6}A residues varies among different messenger RNAs. In HeLa, Novikoff and L-cells, the average mRNA molecule contains three m^{6}A residues. Rous sarcoma and influenza viruses have 1–12 m^{6}A residues per genomic RNA subunit (reviewed in 18). The methylation of *N*-6-adenine in mRNAs occurs prior to their polyadenylation, and the m^{6}A residues are conserved during processing and are present in mature mRNAs (19). In all known cases, the m^{6}A residues are found in two conserved sequences, Am^{6}AC or Gm^{6}AC (20–22). Cespany *et al.* (23) have confirmed and extended the consensus sequence for methylation to RGm^{6}ACU, where R is usually a guanine residue. In addition to the primary sequence, efficiency of m^{6}A methylation in mRNA is influenced by the overall context in which the consensus sequence is located (24). However, the primary sequence around the methylation site A-43 in human U6 snRNA is UACm^{6}AGA showing that the methylation site in U6 snRNA does not match the consensus sequence found in mRNAs.

To understand the mechanism of this faithful and sequence-dependent methyl transfer reaction which accurately methylates the *N*-6-position of adenine residues in mRNA, a cell-free system was developed (25). Using this *in vitro* system, the factors involved in m^{6}A formation in mRNAs have been partially purified from HeLa cells (26,27). While significant progress has been made in characterizing the m^{6}A formation in mRNAs, not much is known about the m^{6}A formation in small nuclear RNAs. In this study, we studied accurate m^{6}A residue formation in human U6 snRNA using an *in vitro* system. The data presented

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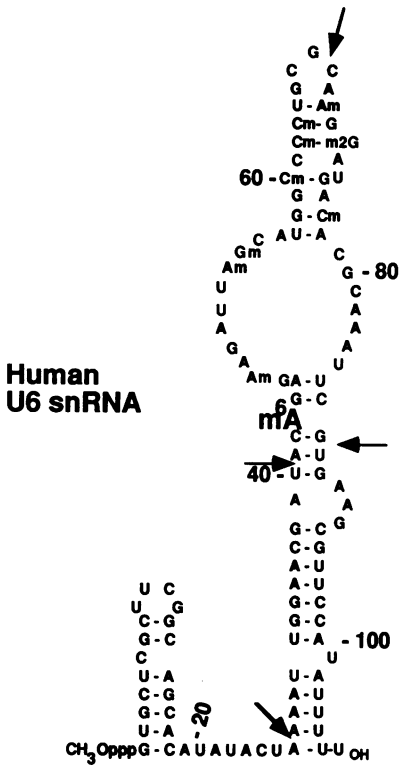

Figure 1. Proposed secondary structure of human U6 snRNA with post-transcriptional modifications. The secondary structure is from Epstein *et al.* (1980). The arrows indicate the sites of deletion mutants that were used to study substrate requirements in Figure 5. The m^6A at position 43 is shown in bold letters.

here show that the integrity of the 3′ end stem–loop structure is essential for efficient m^6A formation in U6 snRNA. In addition, the formation of m^6A in U6 snRNA and mRNA involve different methyltransferases.

MATERIALS AND METHODS

Preparation of RNAs by *in vitro* transcription

Unmodified RNAs were synthesized from linearized DNA templates by T7 RNA polymerase in the presence of [α-^{32}P]ATP or [α-^{32}P]GTP. The restriction enzymes used were *Dra*I for human U6 and 7SK, *Sma*I for human U2 and *Bam*HI for human U4 snRNA. The 3′ deletion mutants of U6 snRNA 1–67 and 1–88 were generated from the DNAs linearized by *Hin*pI and *Fok*I, respectively. The 5′ end deletion mutant of U6 snRNA, (Δ1–25 described in 28), was transcribed *in vitro* using HeLa whole cell S-100 extract which was prepared according to Weil *et al.* (29). The HeLa cell nuclear extract was prepared according to Dignam *et al.* (30).

In vitro modification of U6 snRNA

Approximately 20 000 c.p.m. (~4 fmol) of *in vitro*-transcribed unmodified U6 snRNA or other RNAs were incubated with HeLa cell S-100 extract at 30°C for 1 h in 50 µl of reaction mixture containing 50 mM Tris–HCl (pH 8.0), 150 mM KCl, 10 mM $MgCl_2$ and 4 mM *S*-adenosyl-L-methionine. For RNAs that are

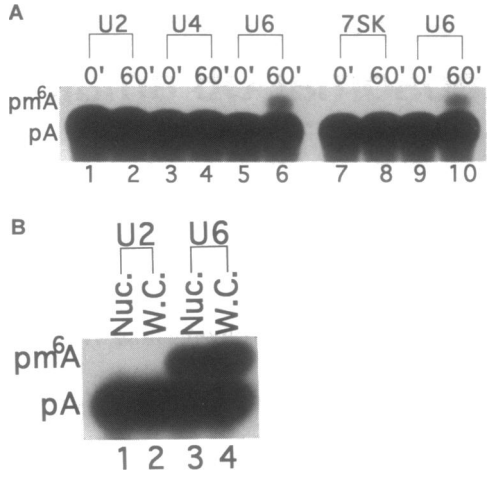

Figure 2. *In vitro* m^6A formation in U6 snRNA. Human U6, U2, U4 and 7SK RNAs were synthesized *in vitro* using T7 RNA polymerase and these labeled RNAs were incubated with whole cell extract prepared from HeLa cells. RNAs were digested with nuclease P1, fractionated by chromatography on a cellulose plate and subjected to autoradiography. (**A**) The RNAs were incubated for 60 min (lanes 2, 4, 6, 8 and 10) or 0 min (lanes 1, 3, 5, 7 and 9) with HeLa whole cell S-100 extract. (**B**) U2 and U6 snRNAs were incubated with either nuclear extract (lanes 1 and 3) or whole cell S-100 extract (lanes 2 and 4).

longer in size, proportionately more radioactivity was used for the *in vitro* methylation reactions. The reaction was terminated by the addition of 0.25 ml of 0.3% SDS in 50 mM sodium acetate (pH 5.1) containing 0.14 M NaCl. The RNAs were extracted with equal volume of phenol, precipitated, digested with nuclease P1 and analyzed as described in the figure legends.

Analysis of modified nucleotides

m^6A nucleotides were separated from other nucleotides on a cellulose plate by thin layer chromatography according to Silberklang *et al.* (31). The first dimension was developed with isobutyric acid/water/NH_4OH (66:33:1, v/v/v). The second dimension, if necessary, was developed with 0.1 M sodium phosphate buffer (pH 6.8)/ammonium sulfate/*n*-propanol (100:60:2, v/w/v). The cap structure in U6 snRNA was separated by electrophoresis on DEAE–cellulose paper at pH 3.5. Autoradiography was done at –70°C using Hyperfilm-MP (Amersham) with Lightning Plus screens (Dupont). The radioactivity was quantitated using Betascope 603 (Betagen).

RESULTS

U6 snRNA serves as a substrate for m^6A formation *in vitro*

In the 106 nt long U6 snRNA isolated from rat, mouse or human cells, there is a single m^6A residue corresponding to nucleotide 43 (Fig. 1; ref. 10). In addition to U6 snRNA, human U2 and U4B snRNAs also contain one m^6A residue each. 7SK RNA isolated from Novikoff hepatoma cells does not contain any m^6A residues (32). Therefore, human U2, U4, U6 and 7SK RNAs were synthesized *in vitro* using T7 RNA polymerase and these labeled RNAs were incubated with HeLa whole cell S-100 extract. The RNAs were purified, digested with nuclease P1, fractionated by chromatography on a cellulose plate and subjected to autoradiography. Only in the case of U6 snRNA was detectable m^6A

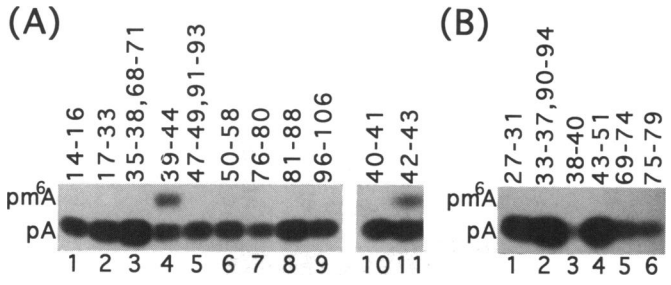

Figure 3. Localization of *in vitro* synthesized m^{6}A in U6 snRNA. *In vitro*-synthesized U6 RNA labeled with [α-^{32}P]ATP was incubated with HeLa whole cell S-100 extract, digested with either T1 RNase or RNase A and subjected to fingerprinting. (**A**) The oligonucleotides obtained with T1 RNase digestion were digested with nuclease P1 and fractionated on a cellulose plate (lanes 1–9). The oligonucleotide corresponding to nucleotides 39–44 was further digested with U2 RNase, followed by the digestion with nuclease P1. (**B**) The oligonucleotides obtained with RNase A digestion were digested with nuclease P1 and fractionated on a cellulose plate. There was no detectable m^{6}A in any of the oligonucleotides even after longer exposure (data not shown). Note that methylated form of A-43 cannot be detected since RNase A removes the ^{32}P-labeled phosphate of A-43.

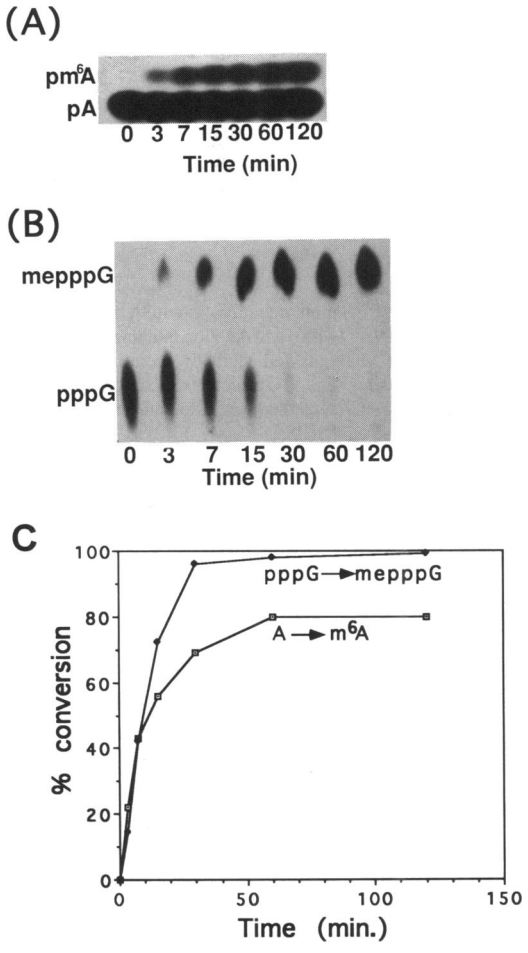

Figure 4. Kinetics of *in vitro* m^{6}A formation in U6 snRNA. *In vitro*-synthesized U6 snRNA, uniformly labeled either with [α-^{32}P]ATP (A) or [α-^{32}P]GTP (B), was incubated with HeLa whole cell S-100 extract for various periods of time. The RNAs after appropriate period of incubation were purified, digested with nuclease P1 and fractionated either on a cellulose plate (to analyze m^{6}A) or a DEAE-cellulose paper [to analyze the γ-methylphosphate (mepppG) cap structure]. (**A**) Time course of m^{6}A formation in U6 snRNA *in vitro*. (**B**) Time course of the cap structure formation in human U6 snRNA *in vitro*. (**C**) The radioactivities in (A) and (B) were quantitated using Betascope 603. The counts obtained were plotted in this panel.

formed (Fig. 2A, lanes 6 and 10); there was no detectable m^{6}A in U2 snRNA (Fig. 2A, lane 2), U4B snRNA (Fig. 2A, lane 4) or human 7SK RNA (Fig. 2A, lane 8). Since 7SK RNA does not contain any m^{6}A residues, it was not surprising that 7SK RNA did not serve as a substrate for m^{6}A formation. Human U2 snRNA contains one m^{6}A at position 30 and human U4B snRNA contains one m^{6}A at position 100 (33); however, there was no detectable m^{6}A formed in these RNAs in this *in vitro* system. We also tested nuclear and whole cell extracts from HeLa cells, and here again m^{6}A was not formed with U2 snRNA as a substrate (Fig. 2B, lanes 1 and 2), whereas U6 snRNA was methylated by both nuclear and whole cell S-100 extracts (Fig. 2B, lanes 3 and 4). These data suggest that formation of m^{6}A residues in U2 and U6 snRNAs is probably mediated by different m^{6}A methyltransferases.

Only A-43 of human U6 snRNA is converted to m^{6}A

In order to ascertain the fidelity of m^{6}A formation *in vitro*, substrate U6 snRNA labeled with [α-^{32}P]ATP was incubated with the HeLa cell S-100 extract, and purified RNA was digested with T1 RNase and subjected to fingerprinting (34). All the T1 RNase oligonucleotides containing A residues were eluted, digested with nuclease P1, chromatographed and subjected to autoradiography. Of the nine oligonucleotides that were analyzed, only the oligonucleotide corresponding to 39–44 contained m^{6}A (Fig. 3A, lane 4).

Oligonucleotide ApUpApCpApGp corresponding to 39–44 of human U6 snRNA contains three A residues, and two of them, corresponding to positions 41 and 43, can theoretically yield pm^{6}A upon digestion with nuclease P1. To determine whether these two positions are methylated or only A-43 is methylated, the T1 RNase oligonucleotide corresponding to 39–44 was digested with U2 RNase, and the digestion products UpAp corresponding to nucleotides 40–41 and CpAp corresponding to nucleotides 42–43 were isolated and analyzed for the presence of m^{6}A. Only the CpAp fragment contained m^{6}A (Fig. 3A, Lane 11) and there was no detectable m^{6}A in fragment UpAp (Fig. 3A, lane 10). These data show that only A-43 in the T1 RNase oligonucleotide ApUpApCpApGp is converted to m^{6}A and the A-41 residue is not modified in this *in vitro* system.

Since T1 RNase digestion results in the transfer of the labeled phosphate from GpA sequences to Gp, the above data from T1 RNase digestion products do not provide information regarding A residues corresponding to positions 35, 39, 45, 47, 50, 73 or 91 of human U6 snRNA which are 3′ to the G residues. To confirm that m^{6}A residue is not formed in these positions, U6 snRNA labeled with [α-^{32}P]ATP was digested with RNase A, and the resulting oligonucleotides were analyzed for m^{6}A after digestion with nuclease P1. There was no detectable m^{6}A in any of the RNase A oligonucleotides that were analyzed (Fig. 3B, lanes 1–6). (Note that the methylated form of A-43 cannot be detected in these fragments since RNase A digestion transfers the phosphate of A-43 to G-42.) These data conclusively show that A-43 in U6 snRNA is converted to m^{6}A, the same A residue in

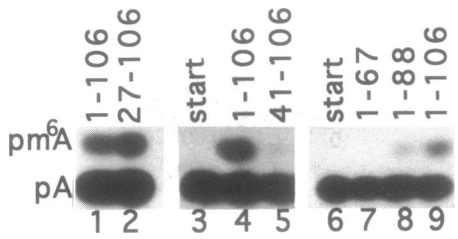

Figure 5. Effect of deletion mutations on m^{6}A formation in U6 snRNA. Full-length U6 snRNA (1–106) and deletion mutants of U6 snRNAs, generated as described in the Materials and Methods, were incubated for 60 min with HeLa whole cell extract. The RNAs were extracted and digested with nuclease P1. The digests were separated on a cellulose plate and subjected to autoradiography and quantitated.

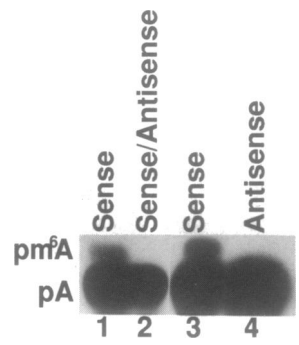

Figure 6. Effect of antisense U6 snRNA on the m^{6}A formation *in vitro*. Labeled U6 snRNA was annealed with excess of unlabeled antisense U6 snRNA, and the hybrid was purified on a non-denaturing polyacrylamide gel and used as a substrate *in vitro*. Lanes 1 and 3: labeled U6 snRNA; lane 2: labeled U6 snRNA/unlabeled antisense U6 snRNA complex; lane 4: labeled antisense U6 snRNA. These RNAs were incubated with HeLa cell extract, digested with nuclease P1, fractionated by chromatography and subjected to autoradiography.

U6 snRNA that is converted to m^{6}A *in vivo*, and no other A residue in U6 snRNA is converted to m^{6}A.

Kinetics of m^{6}A formation *in vitro*

In vitro-synthesized U6 snRNA, uniformly labeled either with [α-^{32}P]ATP or [α-^{32}P]GTP, was incubated with HeLa whole cell S-100 extract for various periods of time. The radioactivity in m^{6}A-43 (Fig. 4A) and in the 5′ mepppG cap (Fig. 4B) in U6 snRNA was quantitated, and the percentage of U6 snRNA modified after various time periods of incubation is shown in Figure 4C. We observed that the m^{6}A formation began without a lag and that ~80% of the A-43 residues in U6 snRNA were converted to m^{6}A residue in 60 min (Fig. 4C). The actual efficiency is probably >80% because any degradation of RNA during incubation with the HeLa cell S-100 extract will make the RNA unsuitable or less than an ideal substrate for modification. The methylphosphate cap formation also was monitored under the same conditions, and here again the cap formation was detectable as early as 3 min and was nearly 95% complete in 30 min (Fig. 4C). Although these two methylations are carried out by different methyltransferases (35), the kinetics of methylation under the *in vitro* conditions were very similar (Fig. 4C).

Substrate specificity of U6 snRNA m^{6}A methyltransferase

U6 snRNA primary sequence is available from many evolutionary distant organisms and is a highly conserved RNA (3,33,36) and all available data for mammalian U6 snRNA are consistent with the secondary structure shown in Figure 1. We incubated different portions of U6 snRNA to determine the ability of these truncated U6 snRNAs to serve as substrates for A-43 → m^{6}A-43 conversion (Fig. 5; Table 1). U6 snRNA lacking the stem–loop near the 5′ end was as efficient a substrate (Fig. 5, lane 2) as the full-length U6 RNA (Fig. 5, lane 1). However, U6$_{41-106}$ was not methylated efficiently (lane 5). Deletion of 19 nt of the 3′ end stem–loop structure (U6$_{1-88}$) reduced the efficiency to 20–45% of the wild-type (Fig. 5, lane 8) and U6 snRNA$_{1-67}$ did not serve as substrates at all (Fig. 5, lane 7). These data show that the m^{6}A methyltransferase recognizes some structural feature in the second stem–loop of U6 snRNA (see Fig. 1) and the integrity of this stem–loop is necessary for optimal and efficient m^{6}A formation at position 43 of human U6 snRNA.

Table 1. Efficiency of m^{6}A formation at nucleotide 43 of human U6 snRNA

Substrate RNA	Efficiency of m^{6}A formation
U6 1–106	84
U6 27–106	80
U6 41–106	5
U6 1–67	2
U6 1–88	47

U6 snRNA labeled *in vitro* with [α-^{32}P]ATP was used as the substrate and since U6 snRNA contains 32 adenosine residues, the radioactivity in A-43 is 1/32 of the total radioactivity in U6 snRNA used for the assay. The radioactivity in pm^{6}A and pA was quantified and a ^{32}P c.p.m. ratio of 31:1 in pA:pm6A is taken as 100% efficiency.

We also prepared unlabeled antisense U6 snRNA and allowed the labeled U6 snRNA to form a duplex *in vitro*. This double-stranded RNA was not recognized as a substrate by the methyltransferase (Fig. 6, lane 2). As expected, the U6 snRNA was used as a substrate for m^{6}A formation (Fig. 6, lanes 1 and 3), but the antisense U6 snRNA was not (Fig. 6, lane 4). These data again suggest that m^{6}A-methyltransferase recognizes and requires secondary/tertiary structural features in U6 snRNA and the primary sequence alone is not sufficient to act as a substrate.

Comparison of factors involved in m^{6}A formation in prolactin mRNA and human U6 snRNA

We prepared unlabeled PRL60, U2 and U6 snRNAs by transcription with T7 RNA polymerase *in vitro* and used these RNAs as competitors by preincubating the HeLa cell extract and then testing these extracts for their ability to form m^{6}A in a labeled U6 snRNA. Compared with the control where no competitor RNA was added (Fig. 7, lane 1), only preincubation with unlabeled U6 snRNA inhibited m^{6}A formation (Fig. 7, lane 3). Prolactin mRNA (Fig. 7, lane 2) or U2 snRNA (Fig. 7, lane 4) did not inhibit the m^{6}A formation in U6 snRNA. These data also suggest

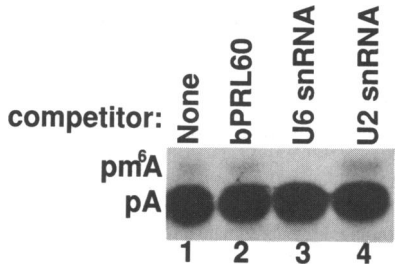

Figure 7. Effect of different competitor RNAs on the m^{6}A formation *in vitro*. The HeLa cell extract containing 10 μg of protein was first incubated for 20 min with no RNA (lane 1) or with 30 pmol each of the competitor RNA indicated above the lane (lanes 2–4). Labeled U6 snRNA was incubated with these extracts, the purified RNAs were digested with nuclease P1, fractionated by chromatography and subjected to autoradiography.

that m^{6}A formation on U6 snRNA is catalyzed by a different enzyme than that which methylates mRNA.

DISCUSSION

We present here an *in vitro* cell-free system that is capable of forming m^{6}A accurately and efficiently in human U6 snRNA. The efficiency of m^{6}A formation in U6 snRNA *in vitro* is ~70% after a 30 min incubation. This *in vitro* system will be very useful in characterizing the factors that are involved in m^{6}A formation. Although U6 snRNA contains many post-transcriptional modifications including m^{2}G, Am, Cm, Gm, Ψ, m^{6}A and mepppG, only m^{6}A formation at position 43 and methylphosphate cap formation on the 5′ end were readily detectable *in vitro*. If other modifications found *in vivo* take place in this *in vitro* system, they must be very inefficient compared with m^{6}A formation and mepppG cap formation. Patton and the associates (37,38) characterized the formation of pseudouridine in human U2 and U5 snRNAs *in vitro*, and the conversion of U to Ψ was <10%, although these RNAs *in vivo* contain fully modified nucleotides in these positions (39). It is possible that the enzymes and/or other factors necessary for these other modifications in U6 snRNA are not optimal under our *in vitro* conditions.

Since m^{6}A residues are found in many RNAs including mRNAs, rRNAs, tRNAs and snRNAs, it is important to know how many different m^{6}A methyltransfrases are present in the cells. The data presented in this study show that the factors required for m^{6}A formation in prolactin mRNA and U6 snRNA are not the same. There are two lines of evidence to support this conclusion: (i) preincubation of the HeLa cell extract with prolactin mRNA did not inhibit the m^{6}A formation in U6 snRNA, while preincubation with U6 snRNA resulted in complete inhibition (Fig. 7); (ii) the consensus sequence flanking the prolactin mRNA required for m^{6}A formation (18,24) is not present in U6 snRNA. However, our study does not rule out the possibility that one or more factors may be common to m^{6}A formation in U6 snRNA and prolactin mRNA. Further purification of the factors involved in m^{6}A formation in these two different RNAs is necessary to resolve this issue.

The data presented here also show that m^{6}A formation in U6 snRNA is dependent on secondary and/or tertiary structure of U6 snRNA. All the known mammalian U6 snRNA sequences fit into a model consisting of two stem–loops in which nucleotides 27–106 are involved in the 3′ terminal stem–loop (see Fig. 1). To define the m^{6}A formation signal in U6 snRNA, we compared m^{6}A formation in several deletion mutants of U6 snRNA (Fig. 5). U6$_{1-67}$ RNA lacking the 39 nt of the 3′ end was unable to serve as a substrate for m^{6}A formation. U6 snRNA lacking 5′ 40 nt or 3′ 18 nt were <50% efficient in directing the formation of m^{6}A residue, while U6$_{27-106}$ RNA lacking the 5′ stem–loop was as efficient a substrate as the full-length U6 RNA (Fig. 5, lane 2; Table 1), indicating that nucleotides 27–106 comprising the second stem–loop in U6 snRNA are important and sufficient to direct m^{6}A formation. The double-stranded U6 snRNA, composed of U6 snRNA hybridized to antisense U6 snRNA, was not capable of forming the m^{6}A residue (Fig. 6), suggesting that the U6 snRNA m^{6}A methyltransferase may recognize the overall secondary and/or tertiary structure of U6 snRNA but it is not capable of methylating this adenosine residue when it is sequestered in a duplex.

The function of the m^{6}A residue in U6 snRNA is still unknown. Studies using inhibitors of methylation suggest that m^{6}A residues in mRNA may play a role in transport of mRNA (40,41). However, the m^{6}A in U6 snRNA is unlikely to play a role in transport, since U6 snRNA is not transported to the cytoplasm from the nucleus (42,43). As described in the Introduction, nucleotide A-43 in U6 snRNA is involved in base-pairing with the 5′ splice site of pre-mRNA. Engel and von Hippel (44) reported that monomeric m^{6}A can exist in one of two isomeric forms in solution and the *cis*-isomer, which is 20-fold more dominant than the *trans*-isomer, is unable to form a Watson–Crick base pair with thymine. One possible role of m^{6}A in U6 snRNA could relate to its influence on the strength and/or specificity of the interaction between this critical region in U6 snRNA and the complementary sequence in mRNA. Such a role for m^{6}A remains entirely speculative. We found that *Saccharomyces pombe* U6 snRNA contains several modified nucleotides such as m^{6}A and mepppG cap (data not shown). The retention of these modifications in U6 snRNA through evolution suggests an important role for these nucleotides. In any event, the data presented in this study support the existence of a unique methyltransferase responsible for this conserved post-transcriptional modification in the highly conserved sequence of U6 snRNA.

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