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Effect of 3′ terminal adenylic acid residue on the uridylation of human small RNAs in vitro and in frog oocytes

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ABSTRACT

It is known that several small RNAs including human and *Xenopus* signal recognition particle (SRP) RNA, U2 small nuclear RNA (snRNA) and 7SK RNAs are posttranscriptionally adenylated, whereas U6 snRNA and ribosomal 5S RNA are posttranscriptionally uridylated on their 3′ ends. In this study, we provide evidence that a small fraction of U6 snRNA and 5S ribosomal RNA molecules from human as well as *Xenopus* oocytes contain a single posttranscriptionally added adenylic acid residue on their 3′ ends. These data show that U6 snRNA and 5S rRNAs are posttranscriptionally modified on their 3′ ends by both uridylation and adenylation. Although the SRP RNA, 7SK RNA, 5S RNA, and U6 snRNA with the uridylic acid residue on their 3′ ends were readily uridylated, all these RNAs with posttranscriptionally added adenylic acid residue on their 3′ ends were not uridylated in vitro, or when U6 snRNA with 3′ A_{OH} was injected into *Xenopus* oocytes. These results show that the presence of a single posttranscriptionally added adenylic acid residue on the 3′ end of SRP RNA, U6 snRNA, 5S rRNA, or 7SK RNA prevents 3′ uridylation. These data also show that adenylation and uridylation are two competing processes that add nucleotides on the 3′ end of some small RNAs and suggest that one of the functions of the 3′ adenylation may be to negatively affect the 3′ uridylation of small RNAs.

Keywords: 3′ adenylation; 3′ uridylation; 7SK RNA; SRP RNA; U6 snRNA

INTRODUCTION

Most RNAs in eukaryotic cells are processed on their 3′ ends. These processing events include CCA turnover on the 3′ end of tRNAs (Deutscher, 1990) and cleavage/polyadenylation in the case of mRNAs (Keller, 1995; Manley, 1995). In the case of some small RNAs (Denis & Wegnez, 1973; Rinke & Steitz, 1985; Reddy et al., 1987; Lund & Dahlberg, 1992) and guide RNAs (Arts & Benne, 1996; Sollner-Webb, 1996; Alfonzo et al., 1997; Stuart et al., 1997), the 3′ end is posttranscriptionally extended by uridylation. In the case of eukaryotic U1–U5 small nuclear RNAs (snRNAs), it is known that several nucleotides are removed from the 3′ end of the primary transcripts to generate mature RNA molecules. These precursor RNAs bind snRNP proteins and the ribonucleoprotein (RNP) particles are used as substrates for RNA processing (Hernandez, 1985; Kleinschmidt & Pederson, 1987; Zieve et al., 1988; Wendelburg & Marzluff, 1992; Yang et al., 1992). The removal of these 3′ end nucleotides is carried out by a multiprotein complex termed “Exosome” that contains multiple exonucleases and RNA helicases (Mitchell et al., 1997; Allmang et al., 1999a, 1999b; Van Hoof & Parker, 1999). In addition to removal of 3′ end nucleotides, there are at least two activities that rebuild the 3′ ends, namely adenylation and uridylation, which add nucleotides to the 3′ end of human small RNAs. It appears that the actual 3′ end sequence of at least some cellular RNAs is a summation of the exonuclease and rebuilding activities.

There is an accumulation of evidence showing that several cellular RNAs from evolutionarily distant species contain a single posttranscriptionally added adenylic acid residue on their 3′ ends. Our earlier studies showed that approximately 65% of the human SRP RNA, 7SK RNA, and U2 snRNA molecules contain a posttranscriptionally added adenylic acid residue on their 3′ ends (Sinha et al., 1998). A fraction of U2 and SRP RNAs from *Xenopus* oocytes and *Saccharomyces cerevisiae* also contain this posttranscriptional adenylation (Perumal et al., 2000). In addition to these RNAs,

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Abbreviations: PCR: polymerase chain reaction; RNP: ribonucleoprotein; snRNA: small nuclear RNA; SRP: signal recognition particle; TUTase, terminal uridyl transferase

there are many RNAs (e.g., U6 snRNA and 5S rRNA) in which a small fraction of the RNAs contain 3′ adenylic acid residues (Sinha et al., 1998). Adenylation where one or two adenylic acid residues are posttranscriptionally added is known to occur in many RNAs, including some stable small RNAs of *Escherichia coli* (Attardi, 1985; Deutscher, 1990; Clayton, 1992; Li et al., 1998). An enzymatic activity that posttranscriptionally adds a single adenylic acid residue to signal recognition particle (SRP) RNA has recently been identified and shown to be distinct from mRNA poly(A) polymerase (Sinha et al., 1999). These data show that posttranscriptional adenylation, where a single adenylic acid residue is added to the 3′ end of RNAs, is widespread in nature and conserved through evolution.

In addition to 3′ adenylation, many small RNAs are uridylated on their 3′ ends. For example, the guide RNAs involved in the editing of some trypanosomal mRNAs contain long posttranscriptionally added 3′ uridylic acid tails; this 3′ U tail is also constantly turning over (Peris et al., 1994). The posttranscriptional uridylation, its turnover, and the involvement of terminal uridyl transferase (TUTase) in the biogenesis of trypanosomal guide RNAs have been extensively investigated (Arts & Benne, 1996; Sollner-Webb, 1996; Alfonzo et al., 1997; Stuart et al., 1997). Among RNAs from higher eukaryotes, the 3′ uridylation of U6 snRNA has been most extensively studied. Uridylation on the 3′ end of U6 snRNA is readily detectable in vitro upon incubation of mammalian cell extracts with [α-^{32}P]-UTP (Reddy et al., 1987; Hirai et al., 1988). This 3′ uridylation on the 3′ end of U6 snRNA occurs also in vivo, and approximately 10% of the mammalian U6 snRNA has been shown to contain U stretches up to 20 nt long (Rinke & Steitz, 1985; Lund & Dahlberg, 1992). The putative enzyme that uridylates the human U6 snRNA, designated TUTase, has been partially purified and characterized (Trippe et al., 1998). Another RNA that is uridylated is ribosomal 5S RNA; it appears that the 3′ uridylic acid residues of ribosomal 5S RNAs are constantly turning over posttranscriptionally (Denis & Wegnez, 1973). When HeLa cell extracts are incubated in the presence of [α-^{32}P]-UTP, many small RNAs including U6 and 5S RNAs get labeled on their 3′ ends (Sinha et al., 1998). Therefore, 3′ uridylation is a common occurrence and many cellular RNAs are posttranscriptionally uridylated on their 3′ ends.

This study was undertaken to understand the functional correlation between adenylation and uridylation that occur on the 3′ end of small RNAs. There are several reports on the posttranscriptional uridylation and adenylation in the case of small RNAs; however, it has not been shown that some small RNAs are both uridylated and adenylated on their 3′ ends. We studied four small RNAs, SRP RNA, 7SK RNA, ribosomal 5S RNA, and U6 snRNA, that are readily uridylated on their 3′ ends in vitro and found that a small fraction of ribosomal 5S RNA and U6 snRNA molecules also contain a posttranscriptionally added adenylic acid residue on their 3′ ends. We tested several small RNAs with posttranscriptionally added 3′ adenylic acid residue as substrates for uridylation in vitro and in vivo and found that the presence of the posttranscriptionally added adenylic acid residue prevented the uridylation of several small RNAs.

RESULTS

Human SRP RNA and Alu RNA with 3′ A_{OH} are not uridylated in vitro

Human SRP RNA has 3′ end heterogeneity in which 65% of the SRP RNA contains a posttranscriptionally added A residue and 35% of the molecules have 3′ U_{OH} (Sinha et al., 1998). We tested these two naturally occurring SRP RNA populations for their ability to serve as substrates for 3′ uridylation in vitro. Because three transcriptionally encoded uridylic acid residues are removed before 3′ adenylation (Sinha et al., 1998), we wanted to test whether human SRP RNA can also be uridylated in vitro. The endogenous SRP RNA in the HeLa cell extract was degraded by treatment with micrococcal nuclease. Then, SRP RNA with 3′ U_{OH} was added to this extract and in the presence of [α-^{32}P]-UTP, this added SRP RNA was uridylated (Fig. 1A, lane 1). In the case of SRP RNA with 3′ A_{OH}, there was no detectable uridylation (Fig. 1A, lane 2). The Alu portion of the human SRP RNA was also tested in this in vitro system. Alu RNA with 3′ U_{OH} was uridylated (Fig. 1A, lane 3) and in contrast, there was only ~15% uridylation in the case of Alu RNA with 3′ A_{OH} (Fig. 1A, lane 4). These data show that human SRP RNA and Alu RNA with 3′ A_{OH} are poor substrates for 3′ uridylation.

The low level of 3′ uridylation observed in the case of Alu RNA with 3′ A_{OH} (see Fig. 1A, lane 4) is possibly due to either uridylation on 3′ A or on 3′ U that is a contaminant in the RNA preparation, or removal of 3′ A by exonuclease(s) in the extract. If this uridylation occurred on 3′ A, digestion of labeled Alu RNA with T2 RNase will result in labeled Ap. The 3′-end-labeled RNA from Figure 1A, lane 4, was purified, digested with T2 RNase, and analyzed by two-dimensional chromatography. All the radioactivity was found in Up (Fig. 1B), suggesting that the low level of uridylation found in the case of Alu 3′ A_{OH} RNA is due to uridylation on 3′ U_{OH}. These data suggested that 3′ A_{OH}-containing Alu RNA is not a substrate for uridylation.

Uridylation occurs only on U residues of 7SK RNA in vitro

Human SRP and Alu RNAs are readily adenylated in vitro (Fig. 2A, lane 1) and our earlier studies (Sinha

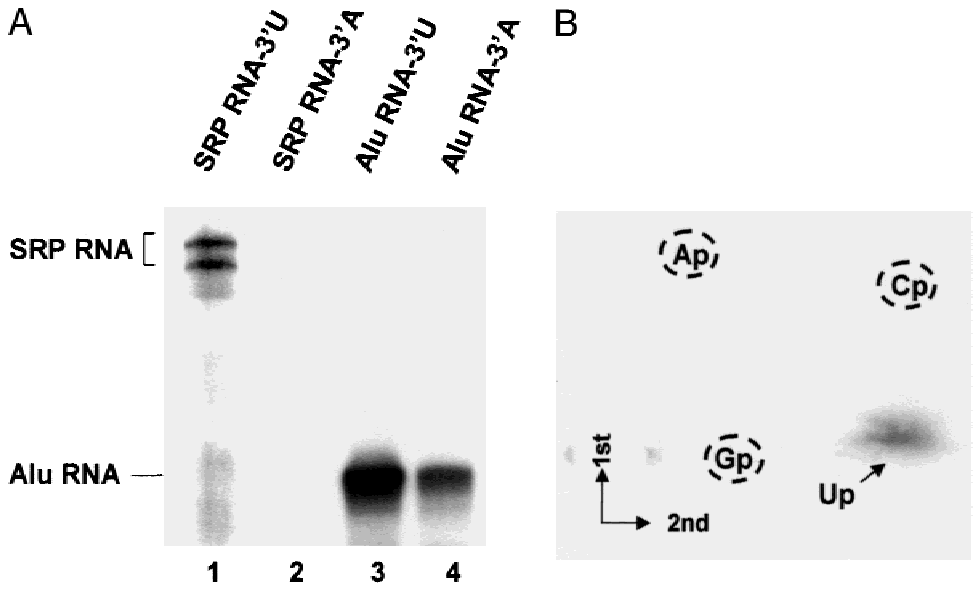


FIGURE 1. **A**: Uridylation of human SRP and Alu RNAs in vitro. Human SRP RNA and Alu RNA with 3′ U_{OH} or 3′ A_{OH} were prepared in vitro by transcription of appropriate DNA templates with T7 RNA polymerase and equal moles of purified RNAs were incubated with HeLa cell extracts in the presence of [α-^{32}P]-UTP. The incubation conditions are described in Materials and methods. The labeled RNAs were purified, fractionated on polyacrylamide gels, dried, and subjected to autoradiography. **B**: Analysis of the 3′ uridylated Alu RNA. The 3′ uridylated Alu RNA from **A**, lane 4, was isolated, purified, and digested to completion by T2 RNase. The digestion products were fractionated by two-dimensional chromatography and subjected to autoradiography. The unlabeled nucleotides visualized under UV light are shown as standard mononucleotide markers.

et al., 1998) showed that the nuclear extract (Dignam et al., 1983) is more efficient for adenylation of SRP RNA compared to the HeLa cell S-100 extract (Weil et al., 1979). However, for uridylation in vitro, the S-100 extract (Weil et al., 1979) was found to be more suitable (Fig. 2A, lane 2). In addition to U6 snRNA and 5S rRNA, 7SK RNA is also posttranscriptionally uridylated on its 3′ end in vitro (Fig. 2A, lane 2). At steady state, ~65% of the human 7SK RNA molecules contain posttranscriptionally added 3′ adenylic acid residue and the remaining 35% of the 7SK RNA molecules contain uridylic acid residue (see Fig. 2B, top panel; also see Sinha et al., 1998). We wanted to test whether 3′ A_{OH}-containing 7SK RNA can be uridylated in vitro. Figure 2B, bottom panel, shows the analysis of the 3′ end mononucleotide of 7SK RNA that was uridylated when HeLa cell extract (Weil et al., 1979) was incubated in the presence of [α-^{32}P]-UTP. Even though ~65% of the 7SK RNA molecules contain 3′ A_{OH} (see Sinha et al., 1998 and Fig. 2B, top panel), when 7SK RNA is uridylated in vitro, uridylation takes place only on the 3′ U_{OH}-containing 7SK RNA (see Fig. 2B, bottom panel). These data show that the uridylating enzyme works only on the 3′ U_{OH}-containing RNAs, and 3′ A_{OH}-containing 7SK RNAs are not substrates for uridylation. Because adenylated RNAs cannot be uridylated, in RNAs where most molecules are adenylated, only a small fraction of RNA molecules with 3′ U_{OH} will be available for uridylation. These data again show the high specificity of the uridylating enzyme to 3′ U_{OH}-containing small RNAs.

A small fraction of human U6 snRNA and 5S rRNA contains posttranscriptionally added adenylic acid residue

U6 snRNA synthesized by RNA polymerase III (Kunkel et al., 1986; Reddy et al., 1987), contains a monomethylphosphate cap structure on its 5′ end (Singh & Reddy, 1989) and the primary transcript contains $-UUUUU_{OH}$ on its 3′ end (Reddy et al., 1987; Das et al., 1988). It is known that U6 snRNA in higher eukaryotes is posttranscriptionally uridylated both in vivo and in vitro (Rinke & Steitz, 1985; Reddy et al., 1987). In our earlier studies in which we analyzed the 3′ end of different small RNAs after pCp labeling, a small fraction (<10%) of U6 snRNA and 5S RNAs contained 3′ A_{OH} (Sinha et al., 1998). We wanted to test whether this 3′ A_{OH} is due to posttranscriptional adenylation or to 3′ processing in which one of the transcribed adenylic acid residues, for example adenylic acid corresponding to position 102 of the 106-nt-long human U6 snRNA, ends up as the 3′ end. This was done as described in Materials and methods by oligo-ligation-mediated polymerase chain reaction (PCR), cloning and sequencing of individual clones corresponding to U6 snRNA and 5S rRNA. Figure 3A,B shows the representative clones for 3′ A_{OH}-containing U6 snRNA from human and *Xenopus* oocytes. Although the major population of human U6 snRNA containing 3′ hydroxyl group consists of $-AUAUUUU_{OH}$ followed by the ligated oligo (Fig. 3A, right panel), a minor fraction contained -$AUAUUUUA_{OH}$ (Fig. 3A, left panel). Only ~10% of the human U6 snRNA

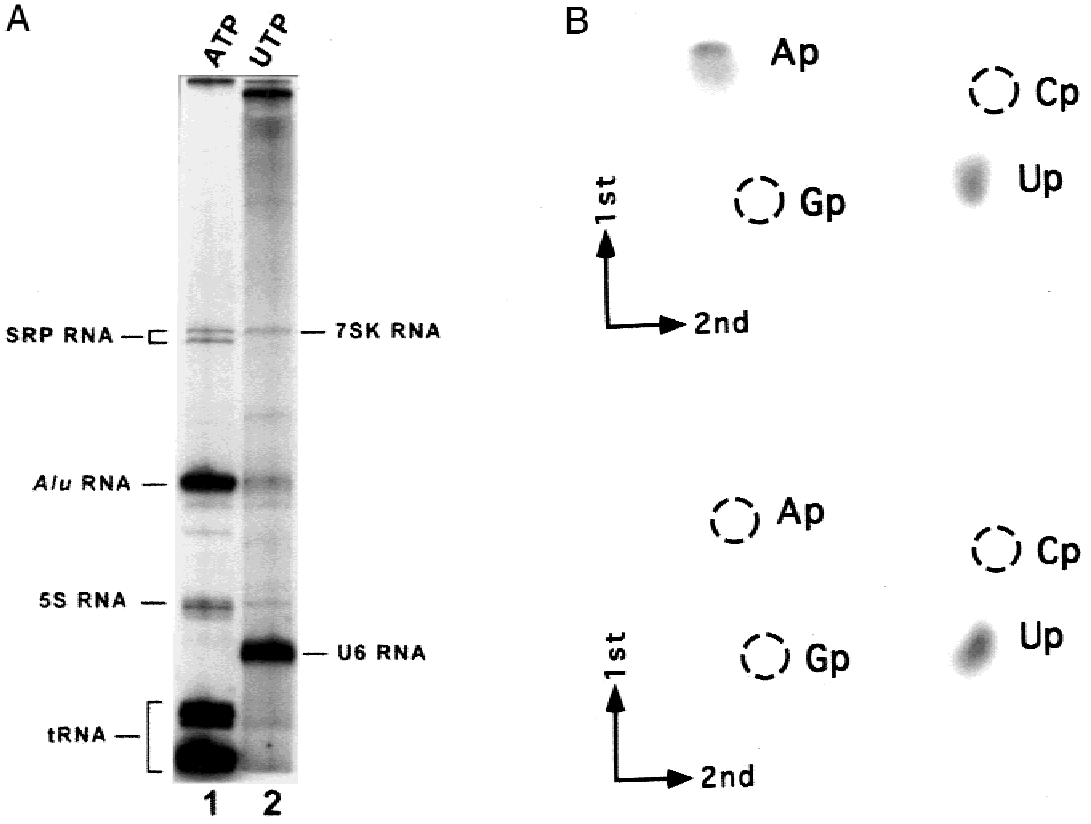


FIGURE 2. **A**: Human 7SK RNA is uridylated in vitro. The HeLa cell extract prepared by the method of Weil et al. (1979) was incubated with [α-^{32}P]-UTP (lane 2) or HeLa cell extract prepared by the method of Dignam et al. (1983) was incubated with [α-^{32}P]-ATP (lane 1) and labeled RNAs were fractionated and displayed on a polyacrylamide gel. The Dignam extract used in lane 1 was also supplemented with 1 μg of human Alu RNA, which is adenylated well in vitro (Chen et al., 1998). The two SRP RNA bands are the result of sequence variants present in human cells. Although 7SK RNA is 330 nt long, it migrates along with 300-nt-long SRP RNA in some acrylamide gels runs. The 7SK RNA was identified by hybrid-selection with cloned human 7SK DNA. **B**: Analysis of human 7SK RNA uridylated in vitro. Top panel: Purified 7SK RNA from HeLa cells was 3′-end labeled with 5′ [^{32}P]-pCp (England et al., 1980) and the labeled RNA was digested to completion with T2 RNase and fractionated by two-dimensional chromatography (Silberklang et al., 1979). Bottom panel: 7SK RNA labeled in vitro when HeLa cell extract was incubated in the presence of [α-^{32}P]-UTP (see Fig. 4A, lane 2) was purified, digested to completion with T2 RNase, and fractionated by two-dimensional chromatography (Silberklang et al., 1979). The radioactivity was visualized and quantitated by phosphorimaging. The broken circles are unlabeled mononucleotides visualized under UV light.

contains 3′ hydroxyl group and the remaining 90% contains 2′, 3′-cyclic phosphate (Lund & Dahlberg, 1992). Because we analyzed only the U6 snRNA containing 3′ hydroxyl groups (Table 1), the percentage of U6 snRNA with 3′ A_{OH} is ~0.4% out of the total U6 snRNA. These data provide evidence that some human U6 snRNA molecules contain posttranscriptionally added adenylic acid residue on their 3′ ends. Similar data were obtained for *Xenopus* U6 snRNA (Fig. 3B), human 5S rRNA (Fig. 3C), and *Xenopus* 5S rRNA (data not shown). These data provide conclusive evidence that a small fraction of human U6 and 5S RNAs undergo posttranscriptional adenylation. Because it is well established that 5S and U6 snRNAs are posttranscriptionally uridylated (Denis & Wegnez, 1973; Rinke & Steitz, 1985; Lund & Dahlberg, 1992; Trippe et al., 1998), it is clear that the 3′ ends of U6 and 5S rRNAs in higher eukaryotes are acted upon by two different processing reactions, namely uridylation and adenylation.

U6 snRNA with 3′ A is not uridylated in vitro

We wanted to investigate the correlation between adenylation and uridylation on the 3′ end of small RNAs. Initially, we used HeLa cell extracts capable of uridylating the endogenous U6 snRNA present in HeLa cell extracts (Gu et al., 1997). Subsequently, we developed an in vitro system utilizing HeLa cell extract (Weil et al., 1979) in which uridylation is dependent on the addition of in vitro-transcribed U6 RNA substrate (Fig. 4A). The HeLa cell extract itself was capable of uridylating endogenous U6 snRNA and 5S rRNA, and can be readily detected upon incubation in the presence of [α-^{32}P]-UTP (Fig. 4A, lane 1). When this extract was first treated with micrococcal nuclease and used after inactivation of the micrococcal nuclease with EGTA, no labeling of the RNAs was detected because the endogenous RNAs were degraded (Fig. 4A, lane 2). Addition of T7-transcribed U6 RNA with 3′ U_{OH} resulted in uridylation

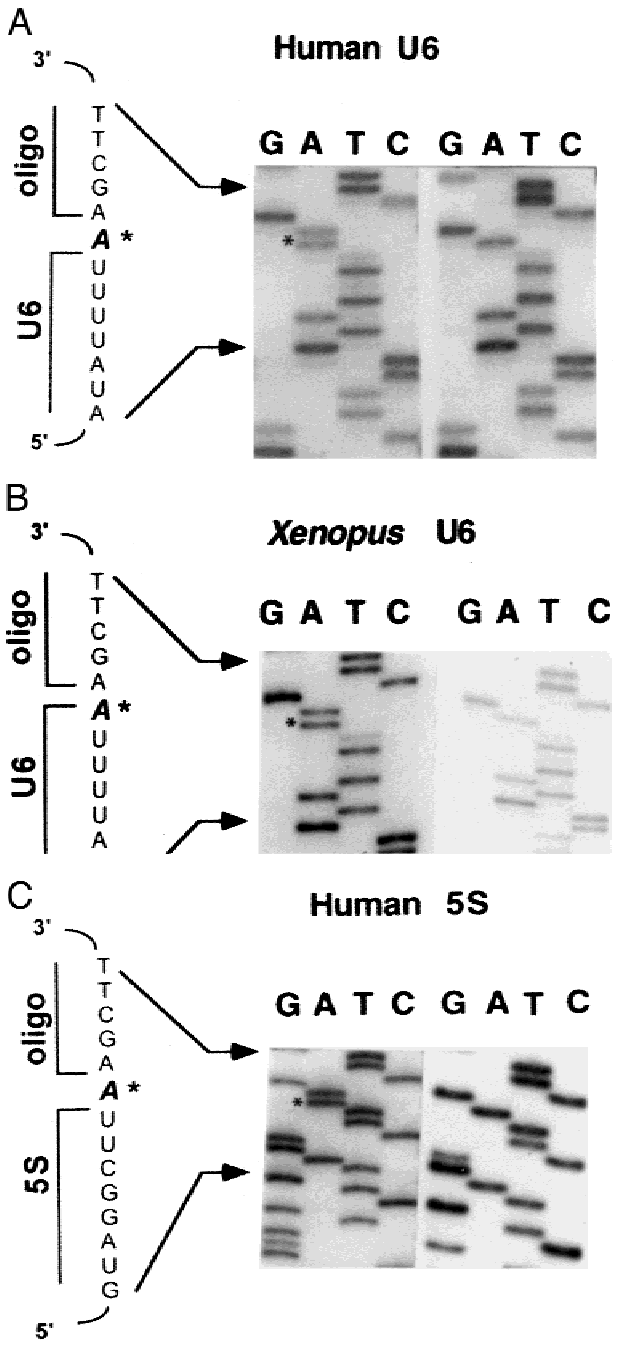


FIGURE 3. The 3′ end sequence of cloned DNAs corresponding to human U6 snRNA and 5S RNAs. The cDNAs corresponding to human U6 (**A**), *Xenopus* U6 (**B**), and human 5S (**C**) were obtained as described in Materials and methods. The oligonucleotide corresponding to the internal PCR primer was used to determine the sequence near the 3′ ends. The left panels in each case correspond to the cDNA clones containing the posttranscriptionally added adenylic acid residue, which is shown in bold and italics. The sequences were determined by the method of Sanger et al. (1977).

of the exogenously added U6 RNA (Fig. 4A, lane 4). However, there was less than 20% uridylation when U6 RNA with 3′ A_{OH} was added to the reaction mixture compared to the uridylation obtained with U6 snRNA with 3′ U_{OH} (Fig. 4A, lane 3). Similar results were obtained in three independent experiments (data not shown). The difference in electrophoretic mobility between endogenous U6 snRNA and T7-transcribed U6 snRNA is probably due to two factors: the endogenous U6 snRNA contains many modified nucleotides, including 5′ monomethylphosphate cap structure, that may contribute to altered mobility, or more uridylic acid residues are added to endogenous U6 snRNA (Gu et al., 1997).

Although U and A residues are added posttranscriptionally to the 3′ end of some small RNAs in vivo, we wanted to employ U6 snRNA with 3′ G_{OH} and 3′ C_{OH} as substrate RNAs and see the uridylation of these artificial substrate RNAs in vitro. It is worth mentioning that there are other small RNAs (for example U1 and U2 snRNAs) which naturally contain 3′ G_{OH} and 3′ C_{OH} and it would be important to know whether these RNAs are potential substrates for uridylation by the uridylating enzyme. U6 snRNA with 3′ G_{OH} and 3′ C_{OH} (Fig. 4A, lanes 5 and 6, respectively) were poor substrates for uridylation with less than 20% uridylation compared to control RNA with 3′ U_{OH} (Fig. 4A, compare lanes 5 and 6 with lane 4). These data show that human uridylating enzyme has preference for U_{OH}-containing RNAs as substrates.

We also carried out similar experiments using 5S rRNA (Fig. 4B). Ribosomal 5S RNA with U_{OH} or A_{OH} as its 3′ end was tested for uridylation in vitro. Here again, only 20% or less uridylation was obtained with A_{OH}-containing 5S RNA (Fig. 4B, lane 4) compared to that with the U_{OH}-containing 5S RNA (Fig. 4B, lane 3). Although equal quantities of 5S rRNA of discrete size were added to the incubation mixture, in addition to the correct size 5S RNA band, a minor labeled RNA with slightly faster migration was consistently observed (Fig. 4B, lanes 3 and 4). We presume that this faster migrating band is a 5S RNA truncated at its 5′ end.

The 3′ uridylated U6 snRNA-3′A obtained in Figure 4A, lane 3, was purified, digested with T2 RNase, and analyzed by two-dimensional chromatography. If the low levels of uridylation observed with 3′ A_{OH}-containing U6 snRNA occurred on the 3′ A_{OH}, radioactivity upon T2 RNase digestion will be expected in Ap; however, all the radioactivity was found in Up and there was not detectable radioactivity in Ap (Fig. 4C, left panel). Therefore, it is concluded that the uridylation observed with U6-3′A snRNA (see Fig. 4A, lane 3) is probably due to uridylation of a minor population of U6 snRNA with 3′ U_{OH} present in the RNA preparations and/or to removal of 3′ A and subsequent uridylation on 3′ U_{OH}. These data show that uridylation occurs only on U6 snRNA containing U_{OH} on the 3′ end, whereas A_{OH} on the 3′ end of U6 snRNA prevents uridylation.

Similar results were obtained in the case of 5S RNA. The 3′-end-labeled 5S RNA from Figure 4B, lane 4, was purified, digested with T2 RNase and analyzed by two-dimensional chromatography. All the radioactivity

TABLE 1. Sequence of cDNA clones corresponding to the 3′ end of human U6 snRNA.

Human U6 snRNA gene (Kunkel et al., 1986)	–GTTCCAUATTTTACAC– 102	Number of clones analyzed
Human U6 snRNA		
Primary transcript	–GUUCCAUAUUUU$_{OH}$	
Actual 3′ end sequences	–GUUCCAUAU$_{OH}$	1
	–GUUCCAUAUU$_{OH}$	1
	–GUUCCAUAUUU$_{OH}$	10
	–GUUCCAUAUUUU$_{OH}$	38
	–GUUCCAUAUUUUA$_{OH}$	4
	–GUUCCAUAUUUUU$_{OH}$	15
	–GUUCCAUAUUUUUU$_{OH}$	12
	–GUUCCAUAUUUUUUU$_{OH}$	9
	–GUUCCAUUUUUUUUU$_{OH}$	1
	–GUUCCAUAUUUUUUUU$_{OH}$	4
	–GUUCCAUAUUUUUUUUU$_{OH}$	1

The cDNA clones were prepared as described in Materials and methods and randomly selected clones were sequenced by the method of Sanger et al. (1977).

in the case of 5S RNA-3′ A_{OH} RNA was found in Up (Fig. 4C, right panel). These data show that the low level of uridylation found in the case of 5S RNA with 3′ A_{OH} is due to uridylation on 3′ U_{OH}. From these data, it is concluded that 3′ A_{OH}-containing U6 snRNA and 5S rRNA are not substrates for uridylation.

U6 snRNA with 3′ adenylic acid residue is not a substrate for uridylation in vivo

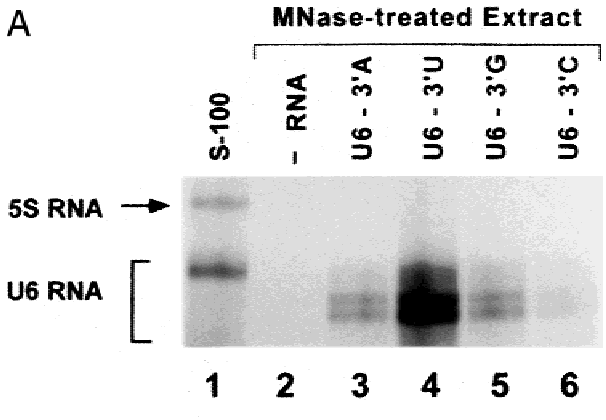
In view of the above interesting results obtained in vitro, we tried to test the effect of posttranscriptionally added 3′ adenylic acid residue on the uridylation of U6 snRNA in the *Xenopus* oocyte system. We chose to inject internally labeled ribosomal 5S and U6 snRNAs and follow the addition of uridylic acid residues on their 3′ ends. It is known that U6 snRNA, when injected into oocyte nucleus, remains in the nucleus and multiple uridylic acid residues are added on its 3′ end (Terns et al., 1992). Although 5S rRNA is also uridylated, the uridylation is by turnover and limited to one or two uridylic acid residues; therefore, the mobility of 5S rRNA on the gel when subjected to electrophoresis does not show any change (Shumyatsky et al., 1993). The addition of multiple uridylic acid residues during incubation in frog oocytes makes the U6 snRNA longer on the 3′ end and hence the RNA migrates more slowly when subjected to electrophoresis on polyacrylamide gels (Fig. 5, lanes 1–5). One can clearly see the progressively slower migration of U6 snRNA as duration of incubation in oocytes increases and the distance between the 106-nt-long U6 snRNA and the 121-nt-long 5S RNA that were injected decreases upon incubation (Fig. 5, lanes 1–5). In contrast, the U6 snRNA molecules with 3′ adenylic acid residue first undergo a slow shortening (compare the mobility at 2, 5, and 9 h) followed by later lengthening (as seen by two bands at 20 h) (see Fig. 5, lanes 6–10). The lengthening of U6 snRNA observed at 20 h is possibly due to turnover of the 3′ A_{OH} resulting in 3′ U_{OH}-containing U6 snRNA. These results show that U6 snRNA, once posttranscriptionally adenylated, is not a suitable substrate for uridylation. The 3′ adenylic acid has to be removed first, before further uridylation can take place. Thus, the extent of 3′ uridylation can be modulated by 3′ adenylation. These results provide evidence consistent with a mechanism to regulate the extent of 3′ uridylation of RNAs by 3′ adenylation of some small RNAs.

Uridylation occurs only on 3′ U residues of U6 snRNA in vivo

As shown previously, more than 90% of the human U6 snRNA molecules contain 3′ U on their 3′ ends. However, a small but significant number of U6 snRNA molecules contain posttranscriptionally added A residues (Sinha et al., 1998; also see Fig. 1). If 3′ A-containing U6 snRNA molecules are utilized as substrates for uridylation, then one would expect some U6 snRNA molecules where A residue is in the middle of the posttranscriptionally added 3′ U tail. However, out of 96 independent U6 snRNA clones we analyzed, no adenylic acid residue was found interspersed among U residues (see Table 1). These data again are consistent and support the suggestion that 3′ adenylic acid residue prevents further uridylation. The 3′ A has to be removed to generate 3′ U_{OH}-containing U6 snRNA before uridylation can take place. These data also show that the in vitro results obtained with U6 snRNA showing uridylation only on U_{OH}-containing RNAs reflect the in vivo situation.

Uridylation as a possible mechanism to rebuild 3′ ends

It is well established that there is 3′ processing in which nucleotides are removed on the 3′ end and there is



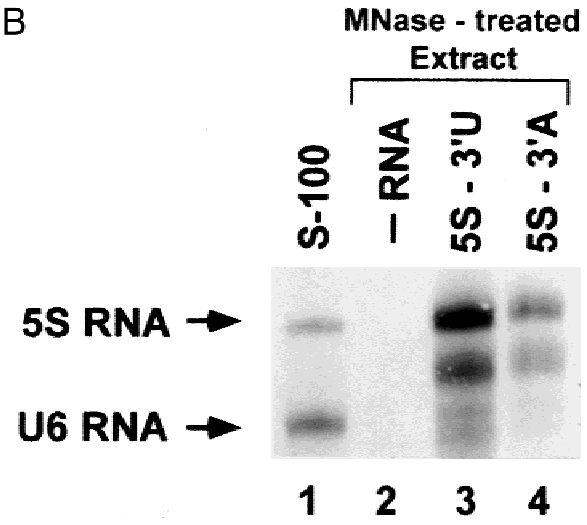
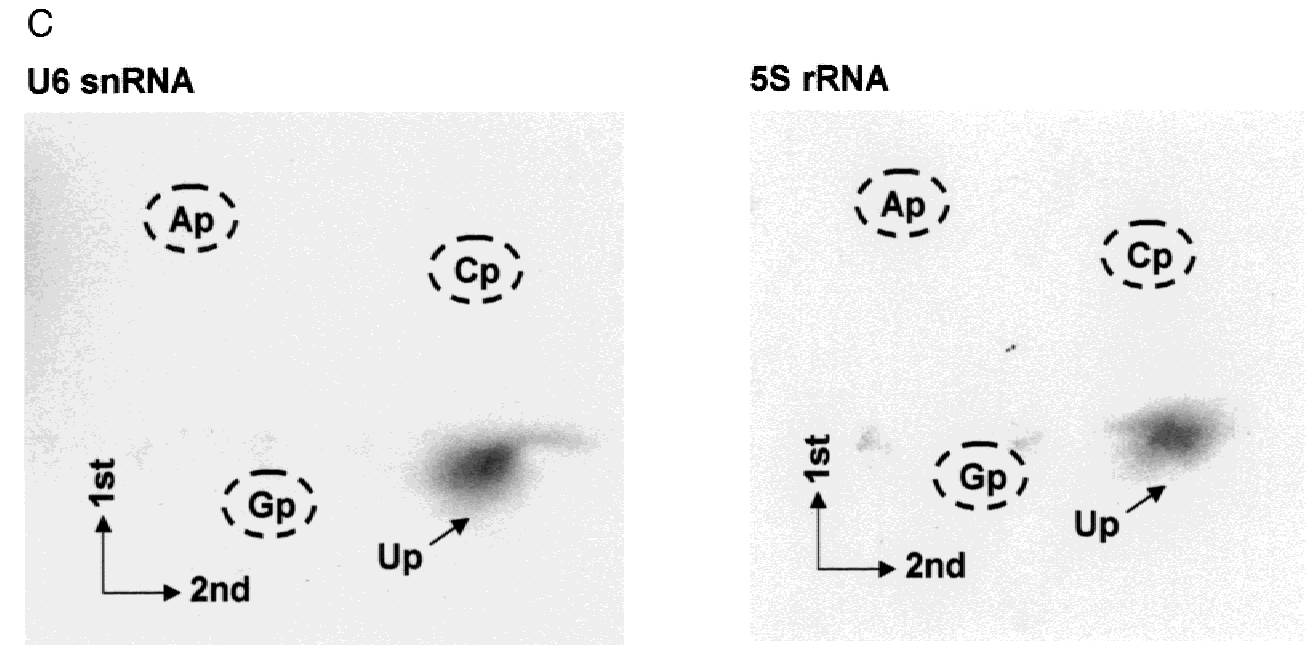


FIGURE 4. **A**: Effect of different 3′ end nucleotides on uridylation of U6 snRNA in vitro. HeLa cell (S-100) extract, first treated with micrococcal nuclease (MNase), was used for uridylation assays in lanes 2–6. The incubation was carried out in the presence of 50 μCi of [α-^{32}P]-UTP. Lane 1: S-100 extract; lanes 2–6: S-100 extract treated with micrococcal nuclease. The U6 snRNA with different nucleotides on the 3′ end (corresponding to position 106) was added to different tubes and are shown on top of each lane. The labeled RNAs, after a 1.5-h incubation, were purified, fractionated on a polyacrylamide/7 M urea gel, and subjected to autoradiography. All other details are as described in Materials and methods. **B**: Effect of different 3′-end nucleotides on uridylation of 5S RNA in vitro. HeLa cell (S-100) extract, first treated with micrococcal nuclease (MNase), was used for uridylation assays in lanes 2–4. The incubation was carried out in the presence of 50 μCi of [α-^{32}P]-UTP. Lane 1: S-100 extract; lanes 2–4: S-100 extract treated with micrococcal nuclease. The 5S rRNA with different nucleotides in position 121 added to different tubes are shown on top of each lane. All other details are as described in Figure 2A. **C**: Analysis of the 3′ uridylated U6 and 5S RNAs. The 3′ uridylated U6 snRNA RNA from Figure 4A, lane 3, and 3′ uridylated 5S RNA from Figure 4B, lane 4, were isolated, purified, and digested to completion by T2 RNase. The digestion products of U6 snRNA (left panel) and 5S RNA (right panel) were fractionated by two-dimensional chromatography and subjected to autoradiography. The unlabeled nucleotides visualized under UV light are shown as standard mononucleotide markers.



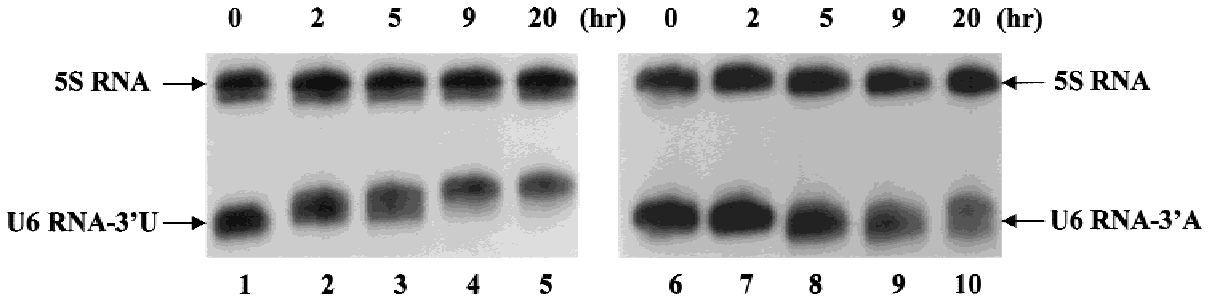


FIGURE 5. Effect of 3′ adenylic acid residue in U6 snRNA on uridylation in frog oocytes. Uniformly labeled U6 snRNA with 3′ U_{OH} (lanes 1–5) and U6 snRNA with 3′ A_{OH} (lanes 6–10) were injected into frog oocyte nuclei along with uniformly labeled 5S rRNA as an internal control. After varying periods of incubation, the RNAs were isolated, fractionated on a polyacrylamide/7 M urea gel, and subjected to autoradiography.

extensive posttranscriptional uridylation on the 3′ end of U6 snRNA (Rinke & Steitz, 1985; Reddy et al., 1987; Lund & Dahlberg, 1992; Terns et al., 1992; Gu et al., 1997). The 3′ processing is a dynamic process where uridylation and deuridylation occur simultaneously (Gu et al., 1997). Booth and Pugh (1997) reported the presence of a nuclease specific for the 3′ end of U6 snRNA. One possible function of the 3′ uridylation is to rebuild 3′ ends that are shortened by 3′ exonucleases. If true, the sequence that is rebuilt by uridylation will contain only U residues no matter what the sequence was in the primary transcript. The data obtained in the case of human U6 snRNA is consistent with this hypothesis. The nucleotide corresponding to position 102 in the human U6 snRNA gene and in the RNA primary transcript is A (see Table 1 and Fig. 6, right panel). However, in one interesting clone out of nearly 100 human U6 snRNA clones sequenced, the nucleotide corresponding to position 102 is uridylic acid instead of A (see Fig. 6, left panel). These data show that trimming is not confined to the 3′ uridylic acid residues and rebuilding of the 3′ end is only by uridylation, no matter what the nucleotide was in the primary RNA transcript. Therefore, detection of U6 snRNA transcripts in which adenylic acid residue corresponding to position 102 has been replaced with uridylic acid strongly supports the possibility that one of the functions of 3′ uridylation is to rebuild the 3′ ends trimmed by exonucleases.

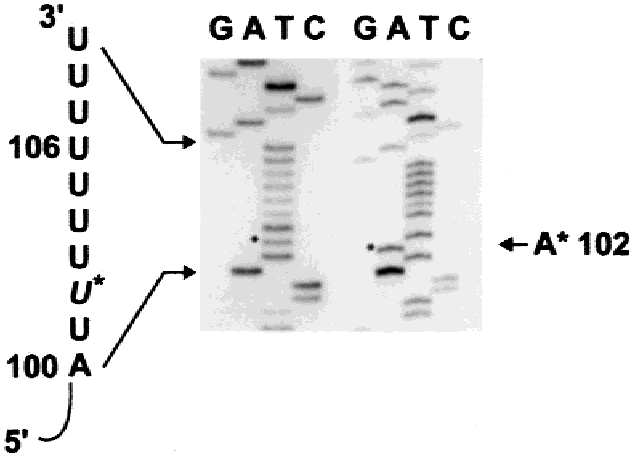


FIGURE 6. Nucleotide sequence of U6 snRNA with uridylic acid residue at position 102. The cDNAs corresponding to human U6 snRNA were obtained as described in Materials and methods. The oligonucleotide corresponding to the internal PCR primer was used to determine the sequence near the 3′ ends. The left panel corresponds to the cDNA clone containing the posttranscriptionally added uridylic acid residue at position 102, which is shown in bold and italics. The right panel is wild-type human U6 snRNA with A at position 102 indicated with an asterisk. The sequences were determined by the method of Sanger et al. (1977).

All previously published data and the results presented in this study are supportive of a mechanism where two competing processes work on the 3′ end of small RNAs. Uridylation is one process that adds uridylic acid residues to 3′ U_{OH}-containing small RNAs; this has been shown to be true for U6 snRNA and 5S rRNA in vivo and in vitro. Adenylation is the other process where a single adenylic acid residue is added to the 3′ end; this has been shown to occur in vivo on the 3′ ends of SRP, 7SK, U2, 5S, and U6 snRNAs. However, the 3′ A_{OH}-containing RNAs cannot be uridylated. Thus, the extent of 3′ adenylation modulates the 3′ uridylation. These data are supportive of a hypothetical model that is summarized and presented in Figure 7. As shown in this figure, uridylation and deuridylation are reversible; similarly, adenylation and deadenylation are also reversible. Although 3′ U_{OH}-containing RNAs can be adenylated, 3′ A_{OH}-containing RNAs cannot be uridylated.

DISCUSSION

This article presents data to show that some small RNAs like human U6 snRNA and 5S rRNAs are adenylated as well as uridylated on their 3′ ends. Data are also presented to show that 3′ A_{OH}-containing RNAs are not substrates for 3′ uridylation; these data were obtained in the in vitro system with HeLa cell extracts and also in the frog oocyte system. In addition, data are presented

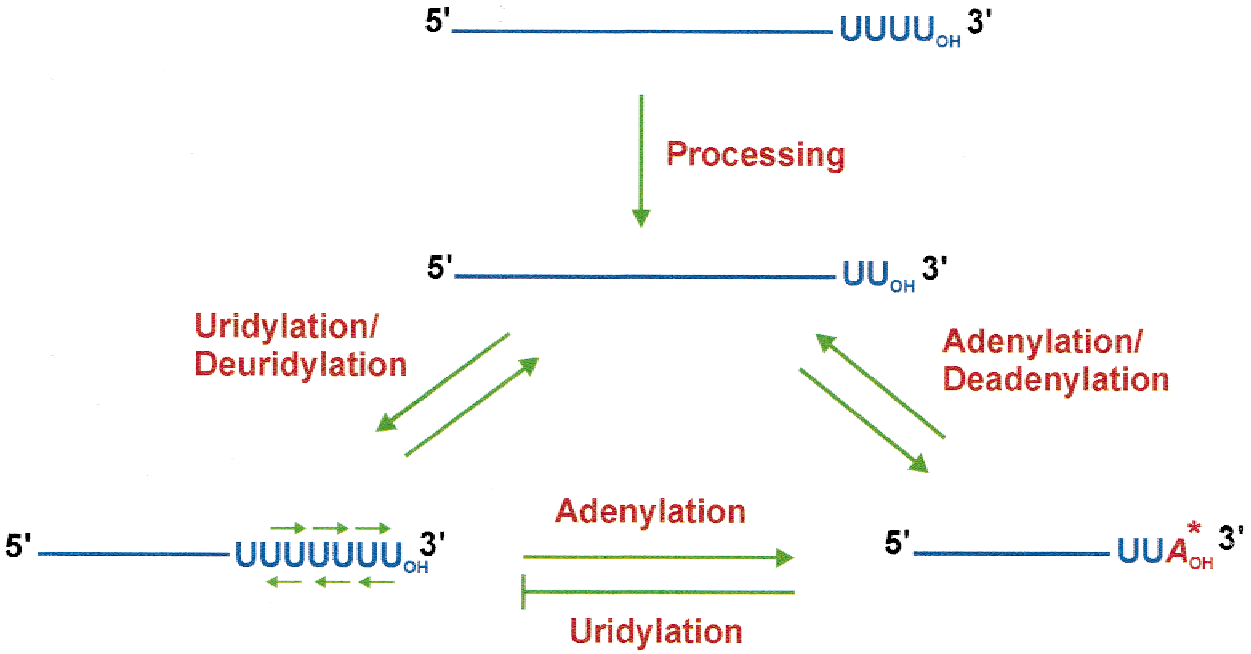


FIGURE 7. A model depicting the 3′ end deletions/additions occurring on the 3′ end of small RNAs. This model is consistent with all the available evidence. Step 1: The RNAs bind with appropriate proteins to form the ribonucleoprotein particles. Step 2: The 3′ ends of RNAs are trimmed where one or more nucleotides are removed. Step 3: These RNAs can be rebuilt by uridylation; thus, this reaction is reversible. Step 4: The RNAs are also adenylated and deadenylated; this reaction also is reversible. Step 5: The RNAs containing adenylic acid residues cannot be uridylated. This reaction is not reversible. RNAs containing adenylic acid on the 3′ end have to be deadenylated first before further uridylation can take place. The number and shape of the proteins is arbitrary and used for the purposes of illustration.

that are supportive of the notion that 3′ uridylation is a mechanism to rebuild the 3′ ends trimmed by exonucleases.

It is clear from the data presented in this study that RNAs containing posttranscriptionally added 3′ A are not substrates for uridylation. A simple and straightforward explanation for this observation is that the human TUTase enzyme has preference, if not an absolute requirement, for 3′ U_{OH}-containing RNAs as a substrate for uridylation. The data obtained in this study suggest, but do not prove, that this might be the case. Purification of the human uridylating enzyme or a complex containing the uridylating enzyme and characterization of its substrate specificity is necessary to test this possibility. The fact that adenylation and uridylation occur on the same RNA raises the question of whether these two processes are related and also perhaps regulated. One possibility is that these two processes are totally unrelated; the second possibility is that these two processes are related and regulated.

The following data are consistent and supportive of the second possibility. If these two events are related, one would expect the RNAs with low posttranscriptional adenylation to be good substrates for uridylation; this is indeed the case. Human U6 snRNA and 5S rRNAs have a low percentage (<10%) of 3′ posttranscriptional adenylation (Sinha et al., 1998). Rapid and readily detectable uridylation is observed in the case of U6 and 5S rRNAs (see Fig. 2A, lane 1; see also Denis & Wegnez, 1973; Rinke & Steitz, 1985; Reddy et al., 1987; Lund & Dahlberg, 1992). Conversely, RNAs with a high percentage of adenylation, for example U2 snRNA, are not uridylated in vitro to any significant extent (Sinha et al., 1998). We, as well as other investigators (Sinha et al., 1998; Lund & Dahlberg, 1992), showed that posttranscriptionally added 3′ adenylic acid residue and 3′ uridylic acid residues are constantly turning over. In other words, the 3′ end of these RNAs are constantly trimmed on the 3′ end and rebuilt. However, the 3′ adenylic acid residue turns over slowly, whereas uridylation has a faster turnover (Chen et al., 1998; Sinha et al., 1998). Thus, even a low percentage of 3′ adenylation may result in significant reduction of the uridylation of RNAs. It is possible that 3′ adenylation with a low turnover rate and uridylation with a high turnover rate are two intimately related processes designed to rebuild and keep the 3′ ends of RNAs intact. Whether adenylation negatively regulates 3′ uridylation of RNAs needs to be verified by direct experimentation. We recently showed that 3′ adenylation of SRP RNA is carried out by an enzyme distinct from mRNA poly(A) polymerase (Sinha et al., 1999). Isolation and characterization of the adenylating enzyme and the human uridylating enzyme will help in answering this im-

portant question. In addition to adenylation on the 3′ end, there might be other mechanisms that are operative to negatively regulate 3′ uridylation of small RNAs. Lund and Dahlberg (1992) showed that nearly 90% of human U6 snRNA contains 2′, 3′-cyclic phosphate on its 3′ end. Data from our laboratory showed that a small fraction of other RNAs including SRP RNA and 7SK RNA contain 2′, 3′-cyclic phosphate on their 3′ ends (Gu et al., 1997). Because a 3′ hydroxyl group is required for addition of nucleotides, these RNA molecules with 2′, 3′-cyclic phosphate on their 3′ ends are not substrates for 3′ uridylation. Therefore, it is possible that there are multiple mechanisms that can result in negative regulation of 3′ uridylation in small RNA.

Posttranscriptional uridylation is known to occur on the 3′ end of many RNAs. These RNAs include U6 snRNA and 5S ribosomal RNA from higher eukaryotes, and guide RNAs from *Trypanosomes* that are involved in editing of pre-mRNAs. In the case of guide RNAs, the posttranscriptionally added U tail has been shown to hybridize to one side of the editing site in pre-mRNA (Arts et al., 1995; Leung & Koslowsky, 1999). In the case of human U6 snRNA, proteins that bind the U tail have been identified and it is suggested that U tail helps in the U4/U6 complex formation (Achsel et al., 1999; Mayes et al., 1999). Another proposed function for the 3′ U tail is that it inhibits the action of 3′ exonucleases (Preiser et al., 1993; Ford & Wilusz, 1999). However, uridylation in the case of many other RNAs including ribosomal 5S RNA is limited to the addition of one to a few U residues and the function of this limited uridylation is not known.

In this study, we isolated a U6 snRNA clone where several 3′ uridylic acid residues and one adenylic acid residue encoded by the gene have been removed and the 3′ end of U6 snRNA rebuilt by uridylation. It is unlikely that this U6 snRNA with U at position 102 has arisen from a U6 snRNA gene which contains T corresponding to position 102, as all known human U6 snRNA genes contain A at position 102 of U6 snRNA (Kunkel et al., 1986; Tichelaar et al., 1998). These data show that, irrespective of the sequence encoded in the gene, the rebuilding in the case of U6 snRNA is accomplished by uridylation. The most interesting observation made in this study is the high specificity that the uridylating enzyme exhibits in adding uridylic acid residues to 3′ U_{OH}-containing RNAs while not adding any U residues to 3′ A_{OH}-containing RNAs. This specificity has been found in vivo as well in the in vitro system. Several uridylating enzyme activities have been characterized; however, this kind of substrate specificity to 3′ U_{OH}-containing RNAs has not been demonstrated in vitro (Zabel et al., 1981; Andrews et al., 1985). The uridylating enzyme that adds uridylic acid residues is designated terminal uridyl transferase (TUTase), and whether there is a common TUTase acting on the 3′ end of many small RNAs or there are RNA-specific TUTases as suggested by Benecke and his associates (Trippe et al., 1998), is not known. Isolation and characterization of the uridylating enzyme(s) will help in answering this question.

A multiprotein complex containing several exonucleases, designated exosome, has been characterized in human and yeast cells (Mitchell et al., 1997). This exosome is involved in the formation of accurate 3′ ends of many cellular RNAs including small RNAs and it is also responsible for the eventual degradation of RNAs. The trimming of longer 3′ ends that occurs on the 3′ ends of many RNAs appears to be carried out by the exosome complex (Allmang et al., 1999a, 1999b). In addition, 3′ exonuclease exhibiting specificity to U6 snRNA has been reported (Booth & Pugh, 1997). Although the 3′ end of cellular RNAs are constantly degraded by complexes like exosome or other nucleases, the adenylation and uridylation activities repair this damage by rebuilding the RNA 3′ ends. Thus, adenylation and uridylation that we characterized in this study and in our earlier reports will be functionally the opposite of exosome function. The balance between these two events, namely the exosome/nucleases on one side and adenylation/uridylation on the other side may determine the metabolic fate of the cellular RNAs.

Approximately 65% of the human U2 and SRP RNA molecules contain posttranscriptional 3′ adenylation (Sinha et al., 1998). We tested the small RNAs corresponding to SRP and U2 snRNAs from yeast *S. cerevisiae* for the presence of posttranscriptional adenylation. Only a low percentage (2–4%) of the yeast SRP or U2 snRNA molecules contained posttranscriptional adenylation; there was no detectable adenylation of yeast 5S rRNA (our unpubl. data). These data are reminiscent of other RNA processing reactions between yeast and higher eukaryotes. Although only a few yeast genes contain intervening sequences, most genes in higher eukaryotes contain them. Therefore, the presence of posttranscriptional adenylation and uridylation on the 3′ ends of many more RNAs and to a greater extent in higher eukaryotes strongly suggest that these posttranscriptional events must have an important function and probably confer an advantage. Further studies are needed to understand the function(s) of these 3′ processing events.

MATERIALS AND METHODS

Chemicals and isotopes

[α-^{32}P]-dATP, [α-^{32}P]-ATP, and [α-^{32}P]-UTP were purchased from ICN. Micrococcal nuclease was obtained from Sigma Chemical Company. T4 RNA ligase, T7 RNA polymerase, and other restriction enzymes were obtained from New England BioLabs. T2 RNase, Taq DNA polymerase, and PCR products purification kit were from Life Technologies, Inc. The TA-cloning kit was from Invitrogen.

Preparation of HeLa cell S-100 extract and in vitro labeling

HeLa cell extracts were prepared from HeLa cells grown in suspension culture by the procedure of Weil et al. (1979). The final protein concentration of the extract was 5 mg/mL. The micrococcal nuclease treatment was done essentially as described by Parker and Steitz (1989). HeLa cell S-100 extract was incubated for 30 min with micrococcal nuclease at a concentration of 0.5 U/μL in the presence of 0.75 mM $CaCl_2$. After incubation, micrococcal nuclease was inactivated by the addition of 2.5 mM EGTA, and the extract was then used for in vitro uridylation reactions.

For in vitro uridylation, 5 μL of 10$\times$ in vitro uridylation buffer (6 mM each GTP, CTP, and ATP, 250 μM UTP, 10 mM DTT, 200 mM KCl, 60 mM creatine phosphate, and 100 mM Tris-HCl, pH 8.0), 3 mM $MgCl_2$, 40 μL HeLa cell extract, and 50 μCi of [α-^{32}P]-UTP were mixed in a total volume of 50 μL and incubated at 30 °C for 90 min. The amount of in vitro-synthesized RNAs used as substrates for uridylation assay was ~1 μg (20 pmol) for each reaction. Labeled RNAs were extracted using the phenol/chloroform procedure, purified, and fractionated on 10% polyacrylamide gels containing 7 M urea. Whenever necessary, labeled RNAs were excised from the gel, eluted, and purified. These RNAs were then digested with T2 RNase and analyzed by two-dimensional chromatography on cellulose plates as described by Silberklang et al. (1979). 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/n-propanol (100:60:2, v/w/v). The dried plates were subjected to autoradiography or phosphorimaging.

In vitro transcription and purification of substrate RNAs

SRP RNA, Alu RNA, U6 snRNAs, and 5S RNAs with different 3′ ends were transcribed by T7 RNA polymerase using the Ambion MEGA shortscript T7 kit from appropriate DNA templates that were prepared by PCR amplification. RNA products were gel purified and precipitated by ethanol with ~10 μg of glycogen as carrier. These RNAs reproducibly contained over 95% of the desired nucleotide on their 3′ ends. The 3′-end nucleotide of the transcribed RNAs was determined by pCp labeling, purification of the labeled RNA on polyacrylamide gels, digestion of the labeled RNAs with T2 RNase, and fractionation of the digest on a two-dimensional chromatograph to identify the labeled nucleotide(s). The concentration of the RNAs was determined by determining optical density at 260 nm and also by separating on a polyacrylamide gel and staining with methylene blue.

Oocyte injections

Internally labeled RNAs containing about 10,000 cpm (100 fmol in 20–60 nL) were injected into the nucleus of the oocytes (Terns & Goldfarb, 1998). Approximately the same amount of radioactivity from each of the in vitro-synthesized RNAs were mixed so as to obtain similar intensities on film during autoradiography. RNAs were isolated from the oocytes according to the procedure described by Fischer and Lührmann (1990). After various periods of incubation, the oocytes were suspended in homomedium (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1.5% SDS, 300 mM NaCl), and proteinase K was added to a concentration of 1.5 mg/mL, mixed, and incubated for 15 min at 37 °C. Labeled RNAs were extracted using the phenol-chloroform procedure and purified by precipitation with ethanol. The purified RNAs were fractionated on a 10% polyacrylamide gel containing 7 M urea, and the dried gels were subjected to autoradiography.

Cloning of 3′ end A-containing U6 snRNA and 5S rRNA

Total RNAs were extracted from HeLa cells and *Xenopus* oocytes and purified. An oligonucleotide with a partial *Hin*dIII site (AGCTT) on its 5′ end and cordycepin on the 3′ end was ligated to total HeLa cell RNAs or *Xenopus* RNAs (Chen et al., 1998). RT-PCR reaction was carried out using one primer specific to U6 snRNA or 5S rRNA and the other one complementary to the ligated oligonucleotide sequence. If the 3′-end nucleotide of an RNA molecule is A_{OH}, a *Hin*dIII site would be generated. The DNA products obtained by PCR were purified from 2% agarose gel, digested with *Eco*RI and *Hin*dIII restriction enzymes and cloned into pUC19 vector digested with the same restriction enzymes. The RT-PCR product of U6 snRNA was also cloned into TA vector (Invitrogen) directly to generate a pool of U6 cDNA clones. Standard small-scale preparation of DNA was performed using CONCERT Mini-Prep Kits (Life Technologies, Inc.). Randomly selected clones containing the inserts were subjected to sequencing by the method of Sanger et al. (1977).

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