Formation of 2',3'-Cyclic Phosphates at the 3' End of Human U6 Small Nuclear RNA in Vitro

IDENTIFICATION OF 2',3'-CYCLIC PHOSPHATES AT THE 3' ENDS OF HUMAN SIGNAL RECOGNITION PARTICLE AND MITOCHONDRIAL RNA PROCESSING RNAs

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Approximately 90% of human U6 small nuclear RNA (snRNA) contains uridine cyclic phosphate (U<p) at its 3'-end (Lund, E., and Dahlberg. J. E. (1992) Science 255, 327–330). We studied the formation of U<p at the 3' end of human U6 snRNA using an in vitro system where uridylic acid residues are added from UTP precursor and U<p is formed. Analysis of U6 snRNAs with varying number of uridylic acid residues showed that each of these species contains U<p where the phosphate originated from α-phosphate of UTP precursor. The cyclic phosphate formation occurred on U6 snRNA in extracts where essential spliceosomal snRNAs were specifically degraded, thereby indicating that U<p formation is not coupled to pre-mRNA splicing. A subpopulation of human signal recognition particle and mitochondrial RNA processing RNAs isolated from HeLa cells also contained cyclic phosphates at their 3' ends. These data suggest that U<p in U6 snRNA is unlikely to be related to its participation in splicing of pre-mRNAs. It appears that cyclic phosphate is an intermediate product in the metabolism of these small RNAs.

Many small RNAs are known to participate in important cellular functions. U1, U2, U4, U5, and U6 small nuclear ribonucleoproteins (snRNPs) are required co-factors for splicing of nuclear pre-mRNAs (1–4). RNaseP, U7 RNA, and many snRNAs are necessary for the site-specific cleavage of pre-tRNA (5), histone pre-mRNA (6), and site-specific methylation of pre-ribosomal RNAs (7, 8), respectively. SRP particles that contain 7SL RNA recognize the signal peptide of the secretory proteins and participate in the secretion of these proteins (9). MRP RNA is found mostly in the nucleolus and is required for the accurate formation of the ribosomal 5.8 S RNA (10, 11). In addition, there are many other small RNAs that are believed to play important roles in both eukaryotic and prokaryotic cells (1, 12, 13).

Most RNAs undergo post-transcriptional modifications at their 3' ends. These modifications include polyadenylation of eukaryotic mRNAs (14) and -CCA addition/turnover on tRNAs (15). Most eukaryotic small RNAs transcribed by RNA polymerase III, such as ribosomal 5 S, U6, SRP, MRP, RNaseP, 7SK, and plant U3 RNA, terminate with 4–5 uridylic acid residues. Although some of them, like 5 S RNAs, retain their 3' ends and are found in the functional ribosomes with -UUUU-OH or -UUUOU-OH, several others are processed at their 3' ends and contain sequences slightly different from the original transcripts. Rinke and Steitz (16) identified a small fraction (∼10%) of the total U6 snRNAs that has longer 3' ends with multiple uridylic acid residues and associates with La protein. Studies in our lab showed that the 3' end of U6 snRNA in snRNPs specifically get labeled in vitro when [α-<sup>32</sup>P]UTP is added to the cell extracts (17). This 3'-uridylation of U6 snRNA has also been studied by Hirai et al. (18), Lund and Dahlberg (19), and Tazi et al. (20). Lund and Dahlberg (19) showed that about 90% of the U6 snRNA in human cells contains U<p at its 3' end, and Brunel and co-workers concluded that this U<p formation is coupled to its involvement in splicing of pre-mRNAs (20, 21).

In addition to U6 snRNA, many RNAs are known to contain 2',3'-cyclic phosphate structures. The autolytic products of the hammerhead cleavage of the satellite RNA of tobacco ringspot virus (22) and avocado sunblotch viroid RNA (23, 24) contain 2',3'-cyclic phosphates. Several other autolytic motifs, such as hairpin, also cleave to give 2',3'-cyclic-phosphate (25). The cleavage of pre-tRNA by an endonuclease results in a 5'-half-molecule containing 2',3'-cyclic phosphate (26–28). The self-excision of a plant pre-tRNA also results in RNAs containing 2',3'-cyclic phosphate (29). Shumyatsky et al. (30) analyzed Ehrlich ascites carcinoma (mouse) cell RNAs fractionated on sucrose density gradients and analyzed poly(A)+ RNA isolated from light, intermediate, and heavy fractions. Cyclic phosphates (pC>p and pU>p) were found in the poly(A)+ fraction obtained from the intermediate fraction. The synthesis of these cyclic phosphate-containing RNAs was inhibited by low concentrations of α-amanitin, indicating that cyclic phosphate may be present at the 3' end of mRNAs. Nilsen and his colleagues studied the human pre-rRNA processing in vitro and found 2',3'-cyclic phosphate cleavage intermediates (31). These data show that the formation of cyclic phosphate in RNA is widespread in nature.

In this study, we further characterized the formation of cyclic phosphate at the 3' end of U6 snRNA. We also report that cyclic phosphate is present at the 3' end of other small RNAs. In addition, the U>p appears to be an intermediate after the removal of a nucleoside from RNAs containing 3'-OH by an exonuclease.

MATERIALS AND METHODS

Chemicals and Isotopes—Fine chemicals, including nucleoside 2',3'-cyclic phosphates, were obtained from Sigma. [α-<sup>32</sup>P]ATP, [α-<sup>32</sup>P]CTP, and [α-<sup>32</sup>P]UTP were obtained from Amersham Corp., and [32P]orthophosphate was obtained from ICN.

Labeling of HeLa Cells—For preparation of uniformly labeled RNAs,
HeLa cells were incubated at 37 °C with [32P]orthophosphate for 16 h in phosphate-free medium (32). The 4–8 S RNA was prepared by centrifugation of the whole cell RNA on a sucrose density gradient and pooling the fractions corresponding to 4–8 S RNAs (33). The RNAs were fractionated and purified on a 10% denaturing polyacrylamide gel containing 7 M urea.

Hybrid Selection—DNA dot hybridizations were carried out as described by Kafatos et al. (34). 5 μg each of cloned DNA was immobilized on nitrocellulose discs and hybridized at 42 °C with uniformly [32P]-labeled HeLa 4–8 S RNAs. Hybridization solution contained 50% formamide, 80 mM Tris, pH 7.5, 600 mM NaCl, 4 mM EDTA, 1.5 × Denhardt’s reagent, 0.01% SDS, and 5 μg/ml yeast tRNA. The filters were washed at 42 °C with 3 × SSC, 1.5 × SSC, and 0.5 × SSC containing 0.1% SDS. The hybridized RNAs were eluted with sterile water at 100 °C for 5 min, precipitated in carrier yeast tRNA, and electrophoresed on denaturing polyacrylamide gels.

Digestion with Nuclease P1 and Other Enzymes—The RNAs were digested with nuclease P1 at a 1:1,500 (w/w) enzyme to substrate ratio at 37 °C for 30 min. Usually, 30 μg of RNA was digested with 20 ng of nuclease P1 in 25 μl of 10 mM acetate buffer, pH 5.0. The digests were either spotted on DEAE-cellulose paper and subjected to electrophoresis or spotted on cellulose plate and subjected to chromatography. Digestion with T1 RNAse was done in 10 ml Tris-HCl, pH 7.4, at a 1:10 (w/w) enzyme to substrate ratio at 37 °C for 60 min. Labeled standard pUC, prepared by labeling U–p with polynucleotide kinase and γ-[32P]ATP.

Ribonuclease H Digestion of HeLa Cell Extract—The RNase H digestion of U1 or U2 snRNAs was done according to Black et al. (35). The oligonucleotides used in this study were 5′-TGCGAGTAACTATG-3′ (complementary to U1 snRNA nucleotide 1–14) and 5′-GAAACATGATACACTTGAA-3′ (complementary to U2 snRNA nucleotide 27–45).

Fractionation of Nucleotides—Electrophoresis on 57–80-cm-long DEAE-cellulose paper was carried out at 500 V for 4–5 h in Savant high voltage electrophoresis unit using 5% acetic acid/ammonium hydroxide/formamide, 80 mM Tris, pH 7.5, 600 mM NaCl, 4 mM EDTA, 1.5 M urea. Oligonucleotides used in this study were 5′-TGCGAGTAACTATG-3′, 5′-GAACAGATACGAAGACATG-3′, 5′-TACACTTGA-3′, 5′-TGGCCAGTAACTATG-3′, and 5′-GGAGATACACCTTGAA-3′.

RESULTS

Cyclic Phosphate Formation in Vitro—As shown earlier by Lund and Dahlberg (19), U6 snRNA from HeLa cells labeled in vivo with [32P]orthophosphate contained pU–p (Fig. 1D). Incubation of HeLa cell extract with [α–32P]UTP resulted in rapid labeling of U6 snRNA, which was the major labeled RNA (Fig. 1A, lane 3). The labeled U6 snRNA was then digested with different enzymes and subjected to chromatography. Digestion with nuclease P1 yielded pU and pU–p (Fig. 1B, lane 3), and digestion with nuclease P1 followed by alkaline phosphatase resulted in pP and U–p (Fig. 1B, lane 4). When the nuclease P1 digest was fractionated by two-dimensional chromatography, radioactivity was observed in pU–p, pU, and pUP (Fig. 1C). In several independent experiments, the amount of pU–p and pUP were 10–30% and 1–2% of the total radioactivity, respectively. The isolation and subsequent digestion of pU–p with alkaline phosphatase yielded two labeled spots corresponding to pP, and U–p (data not shown). These data show that some of the U6 snRNA 3′ ends formed in vitro contain U–p; in addition, these data show that both the 5′- and 3′-phosphate residues in pU–p originated from the labeled α-phosphate of the precursor UTP.

Addition of [α–32P]CTP or [α–32P]ATP to HeLa cell extract resulted in labeling of RNA 3′ ends (Fig. 1A, lanes 1 and 2). Digestion of labeled tRNA with nuclease P1 yielded only pC or PA (Fig. 1B, lanes 1 and 2), and no detectable cyclic phosphate was found. These data show that formation of cyclic phosphate is specific to U6 snRNA and does not occur in all cases where nucleotides are added to the 3′ end of RNAs.

Cyclic Phosphate Is Present in U6 snRNA with Variable Number of Uridylic Acid Residues—Previous data showed that there are multiple U6 snRNAs differing in the number of uridylic acid residues at the 3′ end (16, 37). When labeled U6 snRNA was fractionated on a short polyacrylamide gel (Fig. 1A, lane 3), multiple U6 snRNA species did not separate. To facilitate separation of U6 snRNA differing in number of uridylic acid residues at the 3′ end, the U6 snRNA labeled in vitro (Fig. 1A, lane 3) was digested with T1 RNAse to completion and then fractionated by electrophoresis on a long 20% polyacrylamide gel (Fig. 1A, lane 2). A U6 snRNA T1 RNAse digestion fragment corresponding to the 3′ end with four U residues was electrophoresed to serve as a standard (Fig. 1A, lane 1). The position of this fragment was designated as 0, because this fragment with four uridylic acid residues represents the U6 snRNA transcript as transcribed by RNA polymerase III (37–39). Thirteen labeled bands were visualized with varying intensities; these fragments correspond to seven additional U residues to five deleted nucleotides when compared with U6 snRNA transcribed by RNA polymerase III. These labeled RNA fragments were purified and analyzed for the presence of cyclic phosphate. Both longer and shorter U6 snRNA forms contained U–p (Fig. 2B). These data show that the presence of U–p is not restricted to mature U6 RNA with 4–5 uridylic acid residues.

Splicing Is Not Required for U–p Formation in U6 snRNA—Tazi et al. (20) studied the formation of U–p at the 3′ end of U6 snRNA and concluded that U–p formation is coupled to an event arising during splicing. Complementary oligonucleotide-directed RNAse H cleavage was employed to degrade U1 or U2 snRNAs, which are essential for splicing of pre-mRNAs (35). Fig. 3A shows the analysis of snRNAs after targeted degradation. There was no detectable intact U1 snRNA when oligonu-
cleotide complementary to U1 snRNA was added (Fig. 3A, lane 2), and a similar result was obtained when U2 snRNA was targeted (Fig. 3A, lane 3). These data show that essential snRNAs were effectively degraded by this procedure.

Next, the U1 and U2 snRNA-depleted extracts were used for 3' end labeling of U6 snRNA in vitro (Fig. 3B). The labeled U6 snRNAs from the control (Fig. 3B, lane 1) and snRNA-depleted extracts (Fig. 3B, lanes 2 and 3) were purified and analyzed for the presence of U->p. There was U->p formation in each case, and the amounts of pU->p in the control, U1, and U2 oligonucleotide-treated extracts were 29, 23, and 22% of total radioactivity, respectively (Fig. 3C, lanes 1–3). These data show that the formation of U->p at the 3' end of U6 snRNA does not require its participation in the splicing of pre-mRNAs. Because the cyclic phosphate formation is occurring in RNP particles, the marginal reduction in pU->p formation upon U1 and U2 oligonucleotide treatment may be due to nonobligate linkage of cyclic phosphate formation to pre-mRNA splicing (see "Discussion").

**SRP and MRP RNAs Contain Cyclic Phosphate at Their 3' Ends**—HeLa cell 4–8 S RNA uniformly labeled with [32P]orthophosphate was fractionated on a 50-cm-long polyacrylamide gel and subjected to autoradiography (Fig. 4A). Two distinct bands of roughly equal intensity were observed for SRP RNA (also known as 7SL RNA), the RNA component of the SRP. The faster migrating band was designated SRP-1 RNA, and the slower band was designated SRP-2 RNA. Similar results were obtained with MRP RNA (also known as 7SM or 7–2 RNA). There were two bands of similar intensity where one was designated MRP-1 RNA and the other was designated MRP-2 RNA.

Hybrid selection was carried out to confirm that these four distinct bands correspond to SRP or MRP RNAs. Human SRP RNA resides on nitrocellulose paper hybrid-selected both SRP-1 and SRP-2 RNAs (Fig. 4B, lane 2), and human MRP RNA hybrid-selected both MRP-1 and MRP-2 RNAs (Fig. 4B, lane 3). These data provide evidence for the identity of these RNAs. Under similar experimental conditions, U6 snRNA also fractionated into two major bands and several slower migrating bands of less intensity (Fig. 4B, lane 4), whereas 7SK RNA electrophoresed as one band (Fig. 4B, lane 1).

The SRP and MRP RNAs were purified, digested with nuclease P1, and fractionated on a DEAE-cellulose paper. The SRP RNA is not known to contain any modified nucleotides (33); therefore, only pppG and mononucleotides were expected. However, in addition to pppG and mononucleotides, another labeled compound comigrating with pU->p was observed. This spot was more prominent in the SRP-1 RNA species than SRP-2 RNA (Fig. 4C, lanes 3 and 4). Similar results were obtained when MRP-1 and MRP-2 RNAs were analyzed after nuclease P1 treatment. The faster migrating MRP-1 RNA species contained significantly greater quantities of pU->p than

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**Fig. 2. Isolation and analysis of U6 snRNAs differing in length for cyclic phosphate.** A, the 3' end labeled U6 snRNAs were digested with T1 RNase to completion and resolved on a 50-cm-long 20% polyacrylamide gel (lane 2). Lane 1, 11-nucleotide-long 3' end T1 RNase digestion fragment of U6 snRNA transcribed by T7 RNA polymerase. B, T1 RNase digestion fragments from A (lane 2) were eluted and purified from the gel, digested with nuclease P1, and subjected to chromatography on cellulose plate (0.1 M sodium phosphate buffer, pH 6.8/20% acrylamide gel (Fig. 5B, lane 2)). T1 RNase digestion fragments from A (lane 2) were eluted and purified from the gel, digested with nuclease P1, and subjected to chromatography on cellulose plate (0.1 M sodium phosphate buffer, pH 6.8/ammonium sulfate/1-propanol (100:60:2, v/w/v)). The labeled U6 snRNAs were effectively degraded by this procedure.

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**Fig. 3. Formation of cyclic phosphate on U6 snRNA in HeLa cell extracts lacking intact essential spliceosomal RNAs.** A, the profile of small RNAs isolated from HeLa cell extracts after incubation with no oligonucleotides (lane 1), U1 oligo (lane 2), and U2 oligo (lane 3). B, HeLa cell extracts treated as above were incubated with [α-32P]UTP, and the labeled RNAs were resolved on a 10% polyacrylamide gel and visualized by autoradiography. C, U6 snRNA from B were purified, digested with nuclease P1, and subjected to one-dimensional chromatography on a cellulose plate (0.1 M sodium phosphate buffer, pH 6.8/ammonium sulfate/1-propanol (100:60:2, v/w/v)); the labeled nucleotides were visualized by autoradiography.
MRP-2 RNA (Fig. 4C, lanes 1 and 2). As controls, human U6 snRNA and ribosomal 5 S RNA were also analyzed after nuclease P1 digestion. As expected, a spot consistent with pUp was observed in U6 snRNA (Fig. 4C, lane 6), whereas there was no detectable pUp in 5 S RNA (Fig. 4C, lane 5). The nuclease P1 digests of SRP-1 RNA and MRP-1 RNA were also subjected to two-dimensional chromatography, and in both cases pUp was observed (Fig. 5, A and B). These data show that in addition to U6 snRNA, a subset of SRP and MRP RNAs also contain UP.

**DISCUSSION**

The main findings of this study are: 1) UP is not restricted to mature U6 snRNA with 4–5 uridylic acid residues because longer and shorter U6 snRNA species contain UP; 2) Splicing is not required for the formation of UP at the 3′ end of U6 snRNA. These data and data from other investigators are consistent with a mechanism shown in Fig. 6 where trimming and addition of uridylic acid residues to the 3′ end of U6 snRNA is constantly occurring in a dynamic equilibrium. In addition, this mechanism also implies that UP is an intermediate in the trimming process. The evidence for this mechanism is as follows.

This mechanism (Fig. 6) predicts three populations of U6 snRNA: 3′-OH, 3′-p, and 2′,3′-p. These products are formed in vitro and have been identified by us in this study and also by other investigators (19, 20). Because [α-32P]UTP is the source of the radioactivity and is added to the 3′-OH of U6 snRNA, the necessary RNA substrates with 3′-OH should be present in vitro. These substrates varied from 1 to 10 U residues (see Fig. 2A, lane 2). We found that the 3′-p in pUp was also labeled (Fig. 1B, lane 4); and this is possible only by the addition of UMP and the subsequent removal of a nucleoside. Therefore, these data provide evidence for a trimming reaction in vitro involving deletion of a nucleoside. Though the yield was rather low (1–5%), we also found pUp (Fig. 1C), which is a result of decyclization of UP. Removal of the 2′,3′-cyclization is required to make the 3′-OH group available for the elongation reaction. The fact that U6 snRNAs-1 to -5 species were found to be labeled in vitro (Fig. 2A, lane 2) shows that the U6 snRNAs where nucleotides are deleted are also used for elongation in vitro. These data are also consistent with the observation of Lund and Dahlberg (19), who showed that the terminal nucleotides of U6 snRNA are turning over in a nontemplate-dependent manner.

The predicted intermediates with 3′-OH, 3′-p, and 2′,3′-p are also found in vivo. The identification of U6 snRNA with up to about 20 U residues (16) shows that necessary U6 snRNA substrates for the addition of uridylic acid residues are available in vivo. Because only RNAs with 3′-OH are substrates for the addition of uridylic acid residues, different sizes of these U6 snRNAs must be present in vivo. We also prepared cDNAs of U6 snRNA obtained from immunoprecipitates of La antibodies and sequenced them. Some of the resulting cloned cDNAs contained more as well as less than 4–5 U residues as predicted from mature and predominant form of human U6 snRNA.2 U6 snRNA

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2 Y. Chen and R. Reddy, unpublished data.
snRNA with 3'-p was also found in several species in vivo (19). In yeast U6 snRNA, the 3'-p containing population was predominant. In fruit fly, the 3'-OH, 3'-p, and 2',3'->p populations were present at a ratio of 1:1:1. Therefore, the U6 snRNA in vivo has three populations with 3'-OH, 3'-p, and 2',3'>p. These data are consistent with the proposed mechanism shown in Fig. 6.

The enzymes involved in the trimming and elongation of the 3' end of U6 snRNA are not known. An enzymatic activity capable of forming cyclic phosphate at the 3' end of RNAs has been characterized from HeLa cells (40). However, the RNA substrate for this enzyme requires a 3'-phosphate, which is then converted into a cyclic phosphate. In the case of U6 snRNA, the 3'-cyclic phosphate originates from the 5'-α-phosphate of the pppU used as the precursor. Therefore, enzymes involved in the cyclic phosphate formation in U6 snRNA should be different from this enzyme characterized by Filipowicz and Vinette (40). There are many instances where an endonuclease or self-cleavage of RNA results in RNA fragments with cyclic phosphate at the 3' ends (see Introduction). In fact, any time the ester bond between 3'-phosphate and 5'-hydroxyl group in an RNA molecule is cleaved, the 2',3'-cyclic phosphate is the initial product. However, the cyclic phosphate formation in U6 snRNA 3' end could not be due to the action of an endonuclease. Because our data identified a series of cyclic phosphate-containing U6 snRNAs that differ by one nucleotide in length, it appears that an exonuclease deletes a terminal nucleoside with a cleavage of the ester bond between 3'-phosphate and 5'-hydroxyl resulting in the formation of cyclic phosphate. Data from our lab (17) and other groups (20) have shown that this 3' end modification is occurring in RNP particles, and it is likely that the enzymes responsible for 3' end modification in U6 snRNA recognize one or more proteins associated with U6 snRNA. It would be necessary to reconstitute U6 snRNPs in vitro for characterization and eventual purification of the various enzymes involved in this modification.

Tazi et al. (20) studied the U->p formation at the 3' end of U6 snRNA and concluded that this formation is coupled to its involvement in splicing of pre-mRNA. Our data show that the formation of spliceosome and U6 snRNA participation in the splicing are not required for the U->p formation at its 3' end. The 3' end turnover and U->p formation of U6 snRNA may be occurring within the spliceosome; however, our data indicate that it may be incidental to U6 snRNA being present, and U->p formation is not coupled to its participation in splicing. The observation of Lund and Dahlberg (19) that U6 snRNA in some species has very little U->p at its 3' end is also indicative that U->p is not likely to be functionally relevant or coupled to a step in splicing of pre-mRNAs. In addition, the presence of U->p at the 3' ends of SRP and MRP RNAs, whose function is unrelated to splicing of pre-mRNAs, also suggests that U->p formation is more likely to be related to the metabolism of these small RNAs.

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