Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 8280–8283, November 1989
Biochemistry

γ-Monomethyl phosphate: A cap structure in spliceosomal U6 small nuclear RNA

(mRNA splicing/RNA processing/RNA modification)

RAVINDER SINGH AND RAM REDDY

Baylor College of Medicine, Department of Pharmacology, 1 Baylor Plaza, Houston, TX 77030

Communicated by Aaron J. Shatkin, July 28, 1989

ABSTRACT **U6 small nuclear RNA (snRNA), a component of eukaryotic spliceosomes, is required for splicing of nuclear pre-mRNAs. Whereas trimethylguanosine cap-containing U snRNAs are transcribed by RNA polymerase II, the U6 RNA is transcribed by RNA polymerase III and contains a nonnucleotide cap structure on its 5′ end. We characterized the cap structure of human U6 snRNA and show that the γ phosphate of the 5′ guanosine triphosphate is methylated. The mobilities of *in vivo*-modified γ phosphate from the 5′ end of HeLa U6 RNA were identical to the synthetic monomethyl phosphate (CH_3-O-P) in two-dimensional chromatography and two-dimensional electrophoresis. The cap structure of U6 RNA is distinct from all other cap structures characterized thus far.**

In eukaryotes, RNAs transcribed by RNA polymerase II, such as mRNAs, small nuclear RNAs (snRNAs), and most viral RNAs, are blocked on their 5′ terminus by a guanosine cap: m^7GpppN in the case of mRNAs and $m_3^{2,2,7}$GpppN in the case of U1–U5 snRNAs (reviewed in refs. 1–3). U2 RNA cap hypermethylation requires the Sm-binding site (4). In contrast, certain viral RNAs have protein covalently attached to their 5′ terminus (reviewed in ref. 1).

U6 snRNA, a member of the U snRNA family, is a component of eukaryotic spliceosomes (5–7) and is required for splicing of nuclear pre-mRNAs (5, 6, 8, 9). In yeast *Saccharomyces cerevisiae*, U6 RNA is encoded by a single-copy gene and is essential for cell viability (9). Based on the presence of an intervening sequence in the U6 gene of fission yeast (10) at position 52, corresponding to stem I in the U4–U6 RNA complex, it has been argued (11) that U6 RNA may be directly involved in catalysis during pre-mRNA splicing. While trimethylguanosine cap-containing U snRNAs are transcribed by RNA polymerase II (reviewed in ref. 12), U6 snRNA is unique in that it does not contain the Sm antigen binding site and it is the only known capped RNA transcribed by RNA polymerase III (13–15). Since the observation that the 5′ end of rat U6 snRNA is blocked by a cap structure, designated XpppG (13), the structure of X has not been characterized. In this study, we characterized the cap structure of human U6 snRNA and provide evidence that the γ phosphate of the 5′ guanosine triphosphate is linked to a methyl group through an ester bond.

MATERIALS AND METHODS

Isolation of U6 Cap Core. HeLa cells were labeled with [^{32}P]phosphate (0.5 mCi/ml; 1 Ci = 37 GBq) in monolayer cultures for 16 hr, and the U6 snRNA was obtained by fractionating whole HeLa cell 4–8S RNA on 10% polyacrylamide gels. The labeled U6 RNA was sequentially digested with nuclease P1 and alkaline phosphatase. This digest was used for electrophoresis on DEAE-cellulose paper at pH 3.5 to obtain the U6 cap core. The structure of this cap core was shown to be XpppG, where X was identified as a nonnucleotide compound (13). The XpppG was digested with tobacco acid pyrophosphatase (Promega), and the digest was fractionated by chromatography and electrophoresis. The labeled pG and P_i, used as standards, were obtained by treating [α-^{32}P]GTP with venom phosphodiesterase and alkaline phosphatase, respectively. The monomethyl [^{32}P]phosphoric ester (CH_3-O-P) was prepared by incubating 1 μCi of [^{32}P]orthophosphate in 10 μl of 10 mM Tris·HCl (pH 8) with 1 ml of methanol at 65°C for 16 hr. The conversion of orthophosphate to monomethyl phosphate was >90% as confirmed by its comigration with unlabeled monomethyl phosphate obtained from Sigma and release of P_i by alkaline phosphatase (data not shown). The formation of methyl phosphate from orthophosphate and methanol has been reported (16).

Chromatographic and Electrophoretic Analysis. Chromatography on PEI-cellulose plates (Fig. 1 *A*, *D*, and *G*) was carried out as described (17). The first dimension was developed with water up to the origin, with 0.25 M acetic acid until the solvent migrated 10 cm, and with 0.88 M formic acid until the solvent front had migrated an additional 12 cm; the second-dimension solvent was 0.22 M Tris·HCl (pH 8). Chromatography on cellulose plates (Fig. 1 *B*, *E*, and *H*) was done as described (18). The first-dimension solvent was isobutyric acid/water/NH_4OH, 66:33:1 (vol/vol), and the second dimension solvent was 0.1 M sodium phosphate buffer, pH 6.8/ammonium sulfate/1-propanol, 100:60:2 (vol/wt/vol). Electrophoresis was carried out as described (19) (Fig. 1 *C*, *F*, and *I*). The first and second dimensions were at pH 3.5 on cellulose acetate and DEAE-cellulose paper, respectively. The total amount of radioactivity analyzed in the case of Fig. 1 *A–F* was approximately 1000 cpm each; in Fig. 1 *G–I*, equal counts (1000 cpm each) from *in vivo* labeled cap components and synthetic standards were applied. Autoradiography was done for 72 hr at −70°C by using XAR-5 film and Lightning Plus screens.

RESULTS

To characterize the cap structure of U6 snRNA, the relative mobilities of various nucleotides and their modified counterparts during chromatographic and electrophoretic separations (13, 17, 18) were analyzed. The 2′-*O*-methylated nucleotides, in relation to their unmethylated counterparts, migrate faster during electrophoresis on DEAE-cellulose paper at pH 3.5 and in two-dimensional chromatography on PEI-cellulose sheets (13, 17). In contrast, the 2′-*O*-methylated nucleotides migrate faster in the first dimension but slower in the second dimension on cellulose plates (18). It has been argued that these effects of hydrophobic modifications on the

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: snRNA(s), small nuclear RNA(s).

mobilities of nucleotides probably result from an increase in both mass and hydrophobicity, as well as a slight increase in pK_b of the residues after methylation (18).

The cap core obtained from the U6 RNA was digested with tobacco acid pyrophosphatase and electrophoresed on DEAE-cellulose paper at pH 3.5. In addition to pG and P_i, one spot with electrophoretic mobility greater than P_i was observed (13). This modified γ phosphate from the ^{32}P-labeled U6 snRNA, designated Xp, was previously analyzed by chromatography and electrophoresis (13). The mobilities of Xp and XpppG during chromatography on cellulose plates, PEI-cellulose plates, and electrophoresis on DEAE-cellulose paper (13, 15) suggested that X could be a methyl group. Therefore, we analyzed several phosphate derivatives, including CH_3-O-P, by electrophoresis and chromatography. We used ^{32}P-labeled pG, P_i, and CH_3-O-P as synthetic standards to compare with labeled Xp, P_i, and pG derived from the HeLa cell U6 RNA cap core. Fig. 1 *A* and *B* show the analyses of U6 cap components in two different two-dimensional chromatography systems. The mobility of Xp

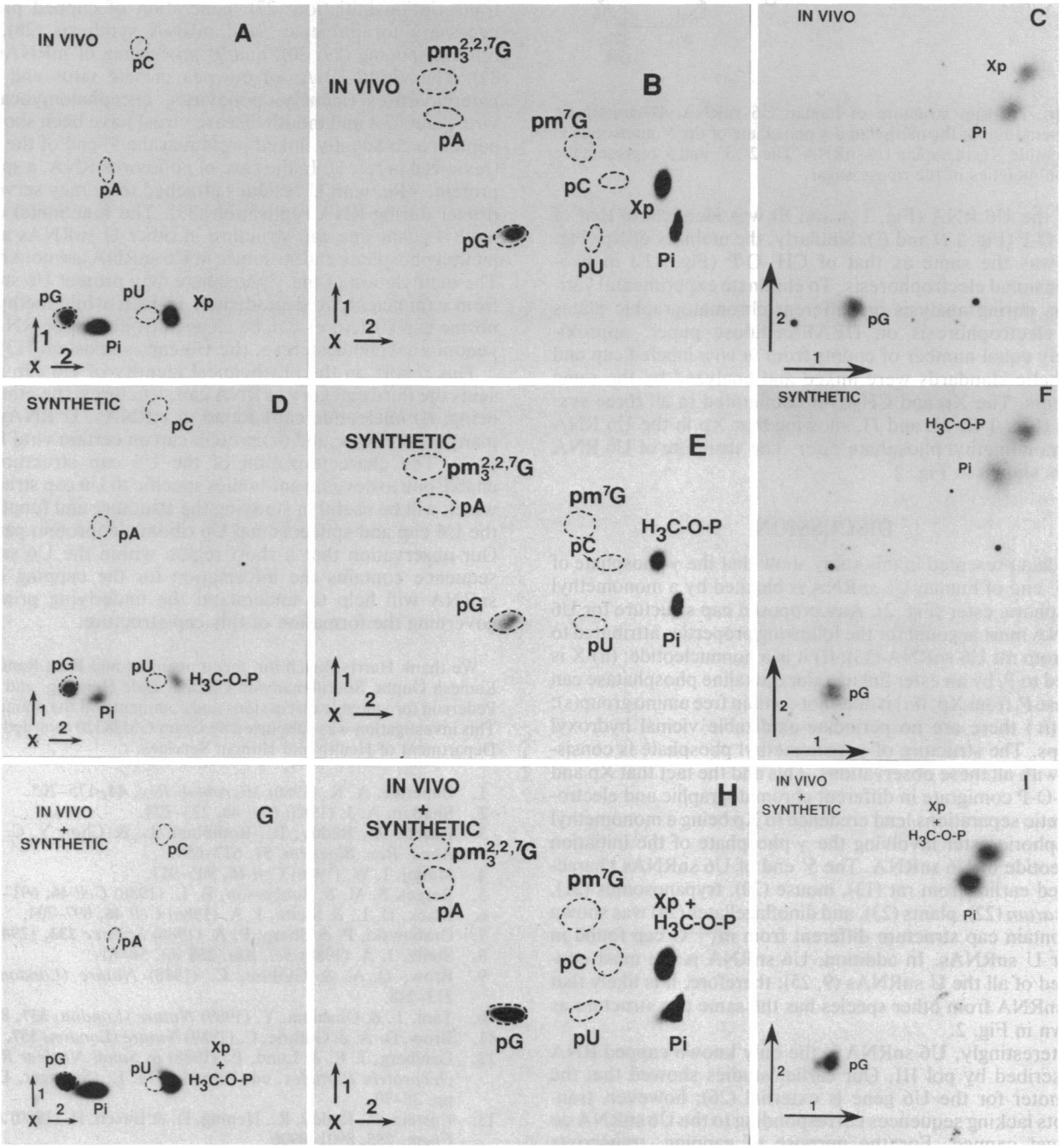

FIG. 1. Characterization of U6 cap structure by chromatography and electrophoresis. (*A*, *D*, and *G*) Two-dimensional chromatography on PEI-cellulose plates. (*B*, *E*, and *H*) Two-dimensional chromatography on cellulose plates. (*C*, *F*, and *I*) Two-dimensional electrophoresis on cellulose acetate and DEAE-cellulose paper. (*Top*) Analyses of tobacco acid pyrophosphatase digestion products of HeLa cell U6 cap core. (*Middle*) Analyses of standard pG, P_i, and CH_3-O-P. (*Bottom*) Analyses of a mixture of U6 snRNA cap components and standard pG, P_i, and CH_3-O-P spotted together. The unlabeled pG, pA, pC, and pU were also included in the samples as internal standards and are indicated by broken circles. Unlabeled pm^{7}G and labeled pm$_3^{2,2,7}$G were analyzed in the two-dimensional chromatography system of Silberklang *et al.* (18), and their mobilities are indicated in *B*, *E*, and *H*. pG, P_i, and CH_3-O-P were also analyzed separately, and their chromatographic and electrophoretic mobilities were the same as shown here. The X indicates the origin, and arrows show the first and second dimensions.

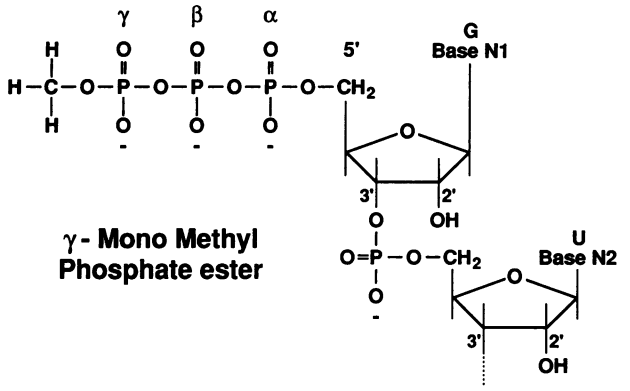

FIG. 2. Cap structure of human U6 snRNA. Diagrammatic representation of the methylated γ phosphate of the 5′ nucleotide G (nucleotide N1) of human U6 snRNA. The 2′, 3′, and 5′ represent the carbon moieties of the ribose sugar.

from the U6 RNA (Fig. 1 *A* and *B*) was identical to that of CH_3-O-P (Fig. 1 *D* and *E*). Similarly, the mobility of Xp (Fig. 1*C*) was the same as that of CH_3-O-P (Fig. 1*F*) in two-dimensional electrophoresis. To eliminate experimental variations during analysis on different chromatographic plates and electrophoresis on DEAE-cellulose paper, approximately equal number of counts from *in vivo* labeled cap and synthetic standards were mixed and analyzed by the same systems. The Xp and CH_3-O-P comigrated in all three systems (Fig. 1 *G*, *H*, and *I*), showing that Xp in the U6 RNA is a monomethyl phosphate ester. The structure of U6 RNA cap is shown in Fig. 2.

DISCUSSION

The data presented in this study show that the γ phosphate of the 5′ end of human U6 snRNA is blocked by a monomethyl phosphoric ester (Fig. 2). Any proposed cap structure for U6 snRNA must account for the following properties attributed to Xp from rat U6 snRNA (13): (*i*) it is a nonnucleotide; (*ii*) X is linked to P_i by an ester linkage since alkaline phosphatase can release P_i from Xp; (*iii*) it does not contain free amino group(s); and (*iv*) there are no periodate-oxidizable vicinal hydroxyl groups. The structure of γ-monomethyl phosphate is consistent with all these observations. This and the fact that Xp and CH_3-O-P comigrate in different chromatographic and electrophoretic separations lend credence to Xp being a monomethyl phosphoric ester involving the γ phosphate of the initiation nucleotide of U6 snRNA. The 5′ end of U6 snRNAs characterized earlier from rat (13), mouse (20), trypanosomes (21), *Physarum* (22), plants (23), and dinoflagellates (24) was shown to contain cap structure different from $m_3^{2,2,7}G$ cap found in other U snRNAs. In addition, U6 snRNA is the most conserved of all the U snRNAs (9, 25); therefore, it is likely that U6 snRNA from other species has the same cap structure as shown in Fig. 2.

Interestingly, U6 snRNA is the only known capped RNA transcribed by pol III. Our earlier studies showed that the promoter for the U6 gene is external (26); however, transcripts lacking sequences corresponding to the U6 snRNA do not get capped. For the purpose of capping, transcripts containing as few as 25 nucleotides corresponding to the 5′ end of U6 snRNA were as good substrates as the full-length U6 snRNA (our unpublished results), indicating that 1–25 nucleotides of U6 snRNA contain information necessary and sufficient for capping. These data indicate that information for the formation of U6 cap resides within the transcribed portion of the U6 gene and may explain why U6 is the only known capped RNA transcribed by pol III. The 5′ region of U6 snRNA can potentially form a stem–loop structure (6, 9, 13, 25), as validated by chemical modification data (20) as well as phylogenetic comparison of known U6 sequences (25), indicating that the integrity of this stem–loop structure is important for the U6 snRNA function.

The cap structure of mRNAs has been shown to enhance the stability of mRNAs by protecting against 5′ exonucleolytic degradation and to increase translational efficiency by facilitating the formation of the initiation complex (reviewed in refs. 1 and 2). Recent studies indicate that cap structure plays additional roles in mRNA biogenesis. These include transcription initiation (27), generation of capped primers necessary for influenza viral mRNA synthesis (28), pre-mRNA splicing (29, 30), and 3′ processing of mRNAs (31, 32). The virion RNAs of cowpea mosaic virus and three picornaviruses (namely, poliovirus, encephalomyocarditis virus, and foot and mouth disease virus) have been shown to contain a covalently linked protein at the 5′ end of the RNA (reviewed in ref. 1). In the case of poliovirus RNA, a specific protein, VPg, with U residues attached to it, may serve as a primer during RNA replication (33). The function(s) of trimethyl guanosine cap structure in other U snRNAs and of methyl phosphate cap structure in U6 snRNA are not known. The methylation of the γ phosphate may protect U6 snRNA from exonucleolytic degradation. In fact, while methylguanosine cap structures can be cleaved from capped RNAs by venom phosphodiesterase, the U6 cap is resistant (13).

This report on the biochemical identity of U6 cap represents the third category of RNA cap structures, the other two being: (*i*) nucleotide caps found in mRNAs, U RNAs, and many viral RNAs; and (*ii*) protein cap on certain viral RNAs (1–3). The characterization of the U6 cap structure will enable one to develop antibodies specific to U6 cap structure, which will be useful in studying the structure and function of the U6 cap and spliceosomal U6 ribonucleoprotein particle. Our observation that a short region within the U6 snRNA sequence contains the information for the capping of U6 snRNA will help to understand the underlying principles governing the formation of this cap structure.

We thank Harris Busch for encouragement and Kurt Randerath, Ramesh Gupta, Subrahmanyam Chirala, Dale Henning, and Thoru Pederson for valuable discussions and comments on the manuscript. This investigation was supported by Grant GM38320 awarded by the Department of Health and Human Services.

1. Banerjee, A. K. (1980) *Microbiol. Rev.* **44**, 175–205.
2. Shatkin, A. J. (1985) *Cell* **40**, 223–224.
3. Busch, H., Reddy, R., Rothblum, L. & Choi, Y. C. (1982) *Annu. Rev. Biochem.* **51**, 617–654.
4. Mattaj, I. W. (1986) *Cell* **46**, 905–911.
5. Berget, S. M. & Robberson, B. L. (1986) *Cell* **46**, 691–696.
6. Black, D. L. & Steitz, J. A. (1986) *Cell* **46**, 697–704.
7. Grabowski, P. & Sharp, P. A. (1986) *Science* **233**, 1294–1299.
8. Steitz, J. A. (1988) *Sci. Am.* **258** (6), 56–61.
9. Brow, D. A. & Guthrie, C. (1988) *Nature (London)* **334**, 213–218.
10. Tani, T. & Ohshima, Y. (1989) *Nature (London)* **337**, 87–90.
11. Brow, D. A. & Guthrie, C. (1989) *Nature (London)* **337**, 14–15.
12. Dahlberg, J. E. & Lund, E. (1988) in *Small Nuclear Ribonucleoprotein Particles*, ed. Birnstiel, M. L. (Springer, Berlin), pp. 38–70.
13. Epstein, P., Reddy, R., Hening, D. & Busch, H. (1980) *J. Biol. Chem.* **255**, 8901–8906.
14. Kunkel, G. R., Maser, R. L., Calvet, J. P. & Pederson, T. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 8575–8579.
15. Reddy, R., Henning, D., Das, G., Harless, M. & Wright, D. (1987) *J. Biol. Chem.* **262**, 75–81.
16. Bieleski, R. L. & Young, R. E. (1963) *Anal. Biochem.* **6**, 54–68.
17. Gupta, R. C., Randerath, E. & Randerath, K. (1976) *Nucleic Acids Res.* **3**, 2915–2921.
18. Silberklang, M., Gillum, A. M. & RajBhandary, U. L. (1979) *Methods Enzymol.* **59**, 58–109.

19. Brownlee, G. G., Sanger, F. & Barrell, G. B. (1968) *J. Mol. Biol.* **34,** 379–412.
20. Miura, K., Tsuda, S., Harada, F. & Ueda, T. (1983) *Nucleic Acids Res.* **11,** 5893–5901.
21. Mottram, J., Perry, K. L., Lizardi, P. M., Luhrmann, R., Agabian, N. & Nelson, R. G. (1989) *Mol. Cell. Biol.* **9,** 1212–1223.
22. Skinner, H. B. & Adams, D. S. (1987) *Nucleic Acids Res.* **15,** 371.
23. Kiss, T., Antal, M. & Solymosy, F. (1987) *Nucleic Acids Res.* **15,** 543–560.
24. Reddy, R., Spector, D., Henning, D., Liu, M.-H. & Busch, H. (1983) *J. Biol. Chem.* **258,** 13965–13969.
25. Roiha, H., Shuster, E. O., Brow, D. A. & Guthrie, C. (1989) *Gene* **82,** 137–144.
26. Das, G., Henning, D., Wright, D. & Reddy, R. (1988) *EMBO J.* **7,** 503–512.
27. Shatkin, A. J. (1976) *Cell* **9,** 645–653.
28. Ulmanen, I., Broni, B. & Krug, R. M. (1983) *J. Virol.* **45,** 27–35.
29. Konarska, M. M., Padgett, R. A. & Sharp, P. A. (1984) *Cell* **38,** 731–736.
30. Edery, I. & Sonenberg, N. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 7590–7594.
31. Georgiev, O., Mous, J. & Birnstiel, M. L. (1984) *Nucleic Acids Res.* **12,** 8539–8551.
32. Hart, R. P., McDevitt, M. A. & Nevins, J. R. (1985) *Cell* **43,** 677–683.
33. Flanegan, J. B., Pettersson, R. F., Ambros, V., Hewlett, M. J. & Baltimore, D. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 961–965.