2'-O-Methyl ribothymidine: a component of rabbit liver lysine transfer RNA

H.J.Gross*, M.Simsek/, M.Raba*, K.Limburg*, J.Heckman/ and U.L.RajBhandary

* Max-Planck Institut für Biochemie, D-8033 Martinsried bei München, BRD, and /Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA

Received 16 October 1973

ABSTRACT

One of the lysine transfer RNAs of rabbit liver is shown to contain 2'-O-methyl ribothymidine in place of ribothymidine. This represents the first demonstration of the presence of 2'-O-methyl ribothymidine in a nucleic acid.

INTRODUCTION

The nucleotide sequence of some sixty different tRNAs from various sources are now known. Almost all of these tRNAs contain the modified nucleoside ribothymidine (T) in a specific location and in the sequence G-T-ψ-C-G(A)-[1]. In the few instances where it is absent, T is replaced either by U, its precursor[2-6], or in the special case of eukaryotic cytoplasmic initiator tRNAs, by A[7-11]. We now provide the first example in which T is present in one of the rabbit liver lysine tRNAs in the form of 2'-O-methyl T.

Among the modified nucleosides known to-date, 2'-O-methyl T represents the second in a class which contains modifications in both the base and the sugar residues. The only previously identified one is N^4-methyl, 2'-O-methyl cytidine found by Nichols and Lane[12] in the 16S ribosomal RNA of E. coli.

Studies to be reported elsewhere show that the lysine tRNA which contains 2'-O-methyl T is fully active in protein synthesis in bacterial and in eukaryotic cell-free protein synthesizing systems.

MATERIALS AND METHODS

Rabbit liver $tRNA_3^{lys}$ was purified using two steps of column chromatography: (i) chromatography on DEAE-Sephadex A-50[13], which yielded three peaks of lysine acceptor activity and (ii) chromatography of the third peak of lysine tRNA on benzoylated DEAE-cellulose[14] which separated the lysine tRNA from 5S RNA and other contaminating tRNAs. The $tRNA_3^{lys}$ used in this work was at least 90% pure.

Conditions for complete digestion of tRNA with pancreatic RNase or T_1-RNase and subsequent separation and characterization of the oligonucleotides were all as described previously[15,16]. The incubation mixture for digestion with T_2-RNase (Sankyo Chemical Co., Japan) contained 2 A_{260} units of $tRNA_3^{lys}$, 4 units of T_2-RNase and 10 mM ammonium acetate, pH 4.5 in a total volume of 200 µl. Incubation was at 37° for 5 hours. The mixture was evaporated to dryness, and the residue was taken up in 2-3 µl of water and used for thin layer chromatography.

Thin layer chromatography was carried out on glass plates (Brinkman Instruments Inc, Celplate-22) coated with cellulose using the following solvent systems: A, isobutyric acid - conc. NH_4OH - water (577:38:385 v/v, pH 4.3); B, t-butyl alcohol - conc. HCl - water (70:15:15, v/v); C, isobutyric acid - conc. NH_4OH - water (66:1:33, v/v); D, ethyl acetate - n-propyl alcohol - water (4:1:2, v/v, upper phase); E. n-butyl alcohol - conc. NH_4OH - water (86:5:14, v/v); and F, ammonium sulphate (600 g) in 1 l. of 0.1 M sodium phosphate, pH 6.8 and 20 ml of n-propyl alcohol.

For two-dimensional thin layer chromatography of nucleotides solvent A was used in the first dimension and after thorough drying of the thin layer plate, solvent B was used in the second dimension.

Paper chromatography was carried out on Whatman No. 1 paper using the following solvents: 1, isopropyl alcohol - conc. NH_4OH - water (7:1:2, v/v); and 2, isobutyric acid - conc. NH_4OH - water (66:1:33, v/v, pH 3.7).

DEAE-cellulose paper electrophoresis was carried out essentially as described before[17]. Electrophoresis was in pyridine acetate, pH 3.5 and for 3.5 hours at 35 volts/cm.

2'(3')-O-methyl T was synthesized using a procedure identical to that used for the corresponding uridine derivatives[18]. The synthetic material was purified from unreacted T by paper chromatography in solvent 2, and from traces of N^3-methyl T by subsequent paper chromatography in solvent 1. The synthetic 2'(3')-O-methyl T was characterized by (a) its ultraviolet absorption spectra, (b) resistance to oxidation with sodium metaperiodate as analyzed using the periodate-Schiff spray reagent[19]

and (c) by its mass spectrum which indicated the presence of only one O-methyl group per T residue.

RESULTS

Initial evidence which indicated the absence of T in rabbit liver $tRNA_3^{lys}$ and further suggested that the absence of T was not due to its replacement by U were as follows: (i) The spot corresponding to Tp was absent in T_2-RNase digests of the tRNA as analyzed by two-dimensional thin layer chromatography. (ii) In the few tRNAs which contain U in place of T, the U residue can be enzymatically methylated to T[2,4-6]. Rabbit liver $tRNA_3^{lys}$ could not be methylated by extracts of E. coli, yeast or wheat germ. Control experiments using E. coli tRNA (a gift of M. Gefter) isolated from a mutant deficient in T[5,6] showed that the extracts used contained the U→T methylase activity. Thus, the absence of T in rabbit liver $tRNA_3^{lys}$ is probably not due to its replacement by U. (iii) Two-dimensional thin layer chromatography of T_2-RNase digests of $tRNA_3^{lys}$ did indicate the presence of an unidentified ultraviolet absorbing spot. This spot (R_{Up} = 0.95 in solvent A and R_{Up} = 0.88 in solvent B) has been shown to contain 2'-O-methyl T in the dinucleotide U*pψp (U*, 2'-O-methyl T). This dinucleotide is found in the sequence A-G-G-G-U*-ψ- in pancreatic RNase digests and in the sequence U*-ψ-C-A-m^1A-G- in T_1-RNase digests of the tRNA.

Isolation of U*-ψ: A-G-G-G-U*-ψ- (3.6 A_{260} units) was incubated with a mixture of T_1-RNase and E. coli alkaline phosphatase. The products were 1 mole of A-G, 2 moles of G and 1 mole of U*-ψ. U*-ψ was isolated by paper chromatography of the digest in solvent 1.

The unusually high chromatographic mobility of U*-ψ (higher than that of guanosine) suggests the presence of modifications in the ring and/or the ribose hydroxyl group. The ultraviolet absorption spectrum of U*-ψ is shown in Fig. 1A. The bathochromic shift observed in alkali (Fig. 1A) indicates that one of the components present is ψ.

Isolation of U*: U*-ψ (1 A_{260} unit) was incubated with snake venom phosphodiesterase (25 μg) at 37° for 24 hours. Even under these prolonged incubations, only about 50% of the U*-ψ was

converted to U* and pψ. U* was isolated by paper chromatography of the digest in solvent 1 (R_f = 0.76). Fig. 1B shows that the ultraviolet absorption spectrum of U* is identical to that of T. The observed hypochromic shift in alkali rules out the presence of any additional modification in the N^3- position of U*.

Evidence for 2'-O-alkyl substitution in U*: U*-ψ-C-A-m^1A-G- was dephosphorylated at the 3'-end with E. coli alkaline phosphatase and subsequently labelled at the 5'-end with ^{32}P- using T_4-polynucleotide kinase and γ-^{32}P-ATP[11]. The ^{32}pU*-ψ-C-A-m^1A-G was treated with T_2-RNase, pancreatic RNase or with alkali (0.3 N KOH at 37° for 18 hours). The digests were subjected to electrophoresis on DEAE-cellulose paper, and ^{32}P- containing oligonucleotides were detected by autoradiography. Fig. 2 shows that the electrophoretic mobility of the ^{32}P-labelled product obtained upon treatment with alkali is identical to that obtained by treatment with either T_2-RNase or pancreatic RNase and quite different from that of pUp. Since the phosphodiester bond linking U* to ψ is resistant to these nucleases, the above result indicates that this bond is also resistant to digestion with alkali and suggests the presence of an alkyl-substitution in the 2'-hydroxyl group of U*.

Identification of U* as 2'-O-methyl T: Final evidence that U* is 2'-O-methyl T was provided by a comparison of the chromatographic mobility of U* with synthetic 2'(3')-O-methyl T in several systems. The results (Table 1) show that U* behaves identically to 2'(3')-O-methyl T in all the systems examined.

TABLE I

R_T in Solvent System	U	T	U*	2'(3')-O-methyl T Synthetic
1	.84	1.00	1.20	1.20
C	.825	1.00	1.28	1.28
D	.576	1.00	1.88	1.87
E	.407	1.00	2.0	2.0
F	1.23(1.18+)	1.00	0.79	0.82

+Mobility of U when cochromatographed along with U*. An internal marker of U was added to U* in all the chromatographic systems examined.

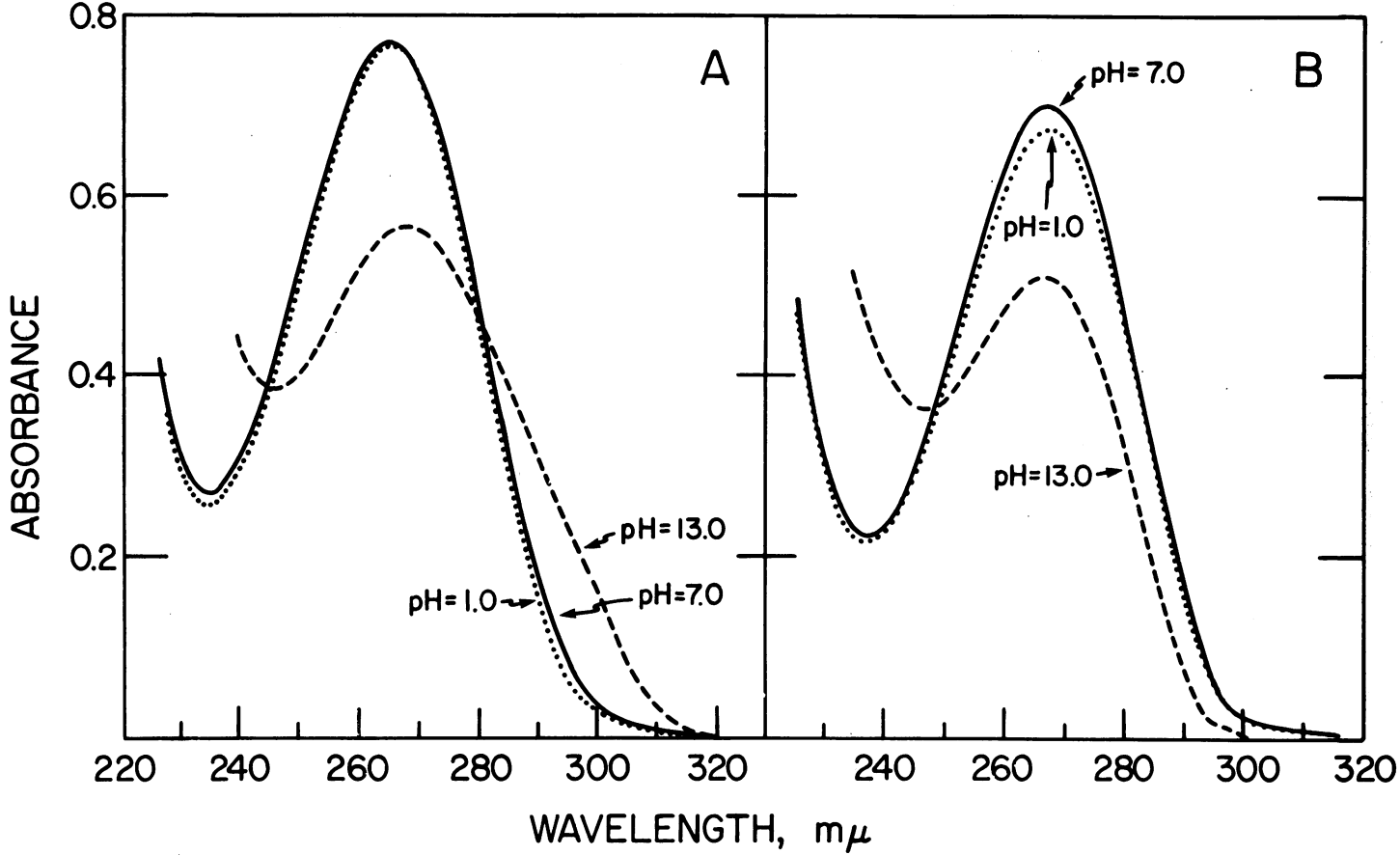

Fig. 1. Ultraviolet absorption spectra of U*pψ (A) and U* (B).

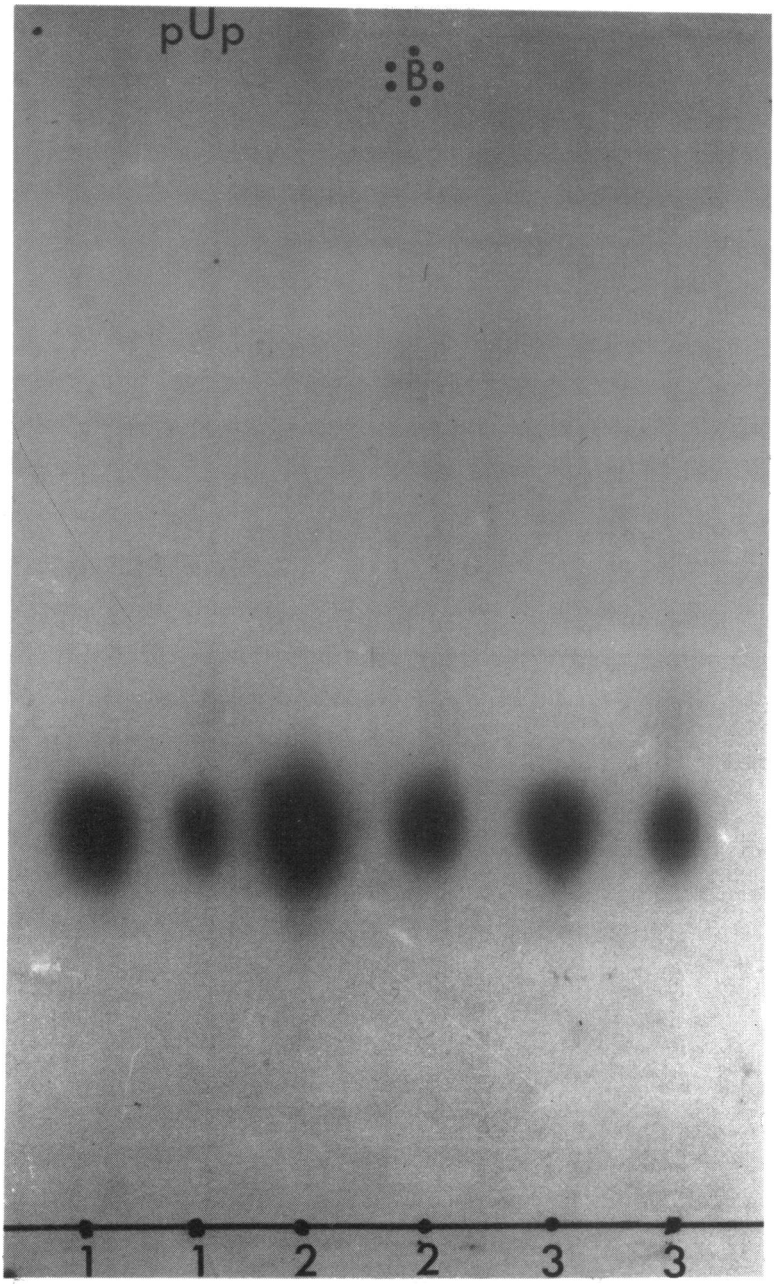

Fig. 2. 1, T_2-RNase digest; 2, pancreatic RNase digest; and 3, alkali digest on duplicate samples of ^{32}pU*-ψ-C-A-m^{1}A-G. (B), blue dye marker. Also shown is the mobility of pUp added as a marker during electrophoresis to all the samples.

Location of U* in rabbit liver $tRNA_3^{lys}$: With the single exception of rabbit liver $tRNA^{Phe}$[20], the modified nucleoside m^1A, when present in a tRNA is located exclusively in the sequence T-ψ-C-G(A)-m^1A-, four nucleotides away from T. In the rabbit liver $tRNA_3^{lys}$, the presence of U* in the sequence U*-ψ-C-A-m^1A-G-, which is homologous to T-ψ-C-G-m^1A-, strongly suggests that U* replaces T in this tRNA. This has been confirmed (manuscript in preparation) by the isolation and sequence analysis of a 30 nucleotide long 3'-terminal fragment containing this sequence and obtained by cleavage of rabbit liver $tRNA_3^{lys}$ at the site occupied by the modified nucleoside m^7G[11].

DISCUSSION

Although the modified nucleoside T has been found in virtually every tRNA of known sequence[1], analysis of T content in bulk tRNA preparations from several sources[21-23] has yielded values significantly lower than 1 mole per mole of tRNA. Methods used for the analysis of T content in these tRNAs have involved degradation of tRNA with either T_2-RNase[24] or with alkali or with a mixture of snake venom phosphodiesterase and alkaline phosphatase[22]. Our work, which has demonstrated the presence of 2'-O-methyl T in a purified rabbit liver tRNA, and the observation that the dinucleotide phosphate U*-ψ was markedly resistant to degradation with snake venom phosphodiesterase suggest that the low values for T content in bulk tRNAs from some sources could, in part, be due to the presence of T in a further modified form, i.e., as 2'-O-methyl T.

An important question which might be raised is the role of 2'-O-methylation of T in the function of rabbit liver $tRNA_3^{lys}$. As noted above, this tRNA is fully active in protein biosynthesis. The presence of 2'-O-methyl T is clearly not essential for the lysine acceptor activity, since another species of rabbit liver lysine tRNA which lacks 2'-O-methyl T (unpublished observations) also accepts lysine. The possibility that O-methylation of T in $tRNA_3^{lys}$ is essential for a specialized function of this tRNA *in vivo* must, therefore, be left open.

Finally, the presence of 2'-O-methyl T in a tRNA raises several other questions as to its biosynthesis, in particular the specificity of the enzyme responsible for the methylation of T to

2'-O-methyl T. An answer to this will require, at the least, a knowledge of the relative distribution of 2'-O-methyl T in tRNAs and of the nucleotide sequences of some of these tRNAs. Studies along these lines are in progress.

ACKNOWLEDGEMENTS

H.J. Gross thanks the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 51) and Professor H. Dannenberg for generous support for this project and Dr. Mineo Saneyoshi, Tokyo, for his kind permission to use his unpublished procedure for two-dimensional thin layer chromatography of nucleotides. We thank Dr. K. Agarwal for help in the synthesis of 2'(3')-O-methyl T, Dr. J.P.H. Verheyden for sending us a preprint of his procedure for methylation of U, Dr. G.F. Temple for providing us with rabbit globin mRNA and mouse ascites cell-free extracts and Drs. K. Biemann and C. Hignite for mass spectral analysis.

This work was also supported by grants GM-17151 NIH, USPHS and Grant NP114, American Cancer Society to U.L.R. and Grant CA-05178, NIH, USPHS to Dr. H.G. Khorana. M.S. was supported by a predoctoral scholarship from Middle East Technical University, Turkey and J.H. by a predoctoral traineeship, Grant GM-00515 from NIH, USPHS.

REFERENCES

1. Dirheimer, G., Ebel, J.P., Bonnet, J., Gangloff, J., Keith, G., Krebs, B., Kuntzel, B., Roy, A., Weissenbach, J. and Werner, C. (1972) Biochimie 54, 127-144.
2. Johnson, L., Hayashi, H. and Söll, D. (1970) Biochemistry 9, 2823-2831.
3. Roberts, R.J. (1972) Nature-New Biol. 237, 44-45.
4. Roe, B., Mignery, R., Marcu, K., Sirover, M. and Dudock, B. (1973) Fed. Proc. Abstracts 32, No. 3, 585.
5. Björk, G.R. and Isaksson, L.A. (1970) J. Mol. Biol. 51, 83-100.
6. Yang, S., Reinitz, E.R. and Gefter, M.L. (1973) Arch. Biochem. Biophys. 157, 55-62.
7. Simsek, M. and RajBhandary, U.L. (1972) Biochem. Biophys. Res. Commun. 49, 508-515.
8. Simsek, M. Ziegenmeyer, J., Heckman, J. and RajBhandary, U.L. (1973) Proc. Nat. Acad. Sci. USA 70, 1041-1045.
9. Petrissant, G. (1973) Proc. Nat. Acad. Sci. USA 70, 1046-1049.
10. Piper, P.W. and Clark, B.F.C. (1973) FEBS Lett. 30, 625-627.
11. Simsek, M. Petrissant, G. and RajBhandary, U.L. (1973) Proc. Nat. Acad. Sci. USA 70, 2600-2604.
12. Nichols, J.L. and Lane, B.G. (1968) Biochim. Biophys. Acta 166, 605-615.
13. Nishimura, S., Harada, F., Narushima, U. and Seno, T. (1967)

Biochim. Biophys. Acta 142, 133-148.
14. Gillam, I., Millward, S., Blew, D., von Tiggerstrom, M., Wimmer, E. and Tener, G.M. (1967) Biochemistry 6, 3043-3056.
15. RajBhandary, U.L. Faulkner, R.D. and Stuart, A. (1968) J. Biol. Chem. 243, 575-583.
16. RajBhandary, U.L., Stuart, A. and Chang, S.H. (1968) J. Biol. Chem. 243, 584-591.
17. Sanger, F., Brownlee, G. and Barrell, B.G. (1965) J. Mol. Biol. 13, 373-398.
18. Wagner, D., Verheyden, J.P.H. and Moffatt, J.G. (1974) J. Org. Chem. in press.
19. Baddiley, J. Buchanan, J.G., Handschumaker, R.E. and Prescott, J.F. (1956) J. Chem. Soc., 2818-2823.
20. Keith, G., Picaud, F., Weissenbach, J., Ebel, J.P., Petrissant, G. and Dirheimer, G. (1973) FEBS Lett. 31, 345-347.
21. Gray, M.W.,and Lane, B.G. (1967) Biochim. Biophys. Acta 134, 243-257.
22. Hall, R.H. (1971) in The Modified Nucleosides in Nucleic Acids, Columbia University Press, New York.
23. Rosenthal, L.J. and Zamecnik, P.C. (1973) Proc. Nat. Acad. Sci. USA 70, 865-869.
24. Kimura-Harada, F., Saneyoshi, M. and Nishimura, S. (1971) FEBS Lett. 13, 335-338.