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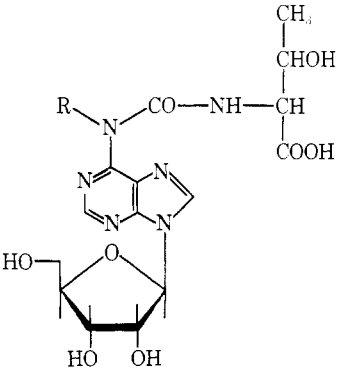
N-[(9-β-D-Ribofuranosylpurin-6-yl)-*N*-methylcarbamoyl]threonine, a Modified Nucleoside Isolated from *Escherichia coli* Threonine Transfer Ribonucleic Acid†

F. Kimura-Harada, D. L. von Minden, James A. McCloskey, and S. Nishimura*

ABSTRACT: A hitherto undiscovered modified nucleoside was isolated from *Escherichia coli* $tRNA^{Thr}$ and its structure determined to be *N*-[(9-β-D-ribofuranosylpurin-6-yl)-*N*-methylcarbamoyl]threonine (mt^6A). Its chemical and physical properties, including ultraviolet spectra, thin-layer chromatographic and electrophoretic mobilities, and the liberation of N^6-methyladenosine and threonine upon treatment with alkali, are similar to those of *N*-[(9-β-D-ribofuranosylpurin-6-yl)carbamoyl]threonine, previously found in unfractionated yeast tRNA. Trimethylsilylation produced a mixture of the pentasilyl derivative of mt^6A (molecular ion, *m/e* 786) and the trisilyl derivative of N^6-methyladenosine (molecular ion, *m/e* 386), which confirmed both the molecular weight and the presence of methyl at the N^6 position of the base in the underivatized nucleoside.

N-[(9-β-D-Ribofuranosylpurin-6-yl)carbamoyl]threonine(I) was first isolated from unfractionated yeast tRNA and characterized by Hall and his coworkers (Chheda *et al.*, 1969; Schweizer *et al.*, 1969). A survey of its distribution in individual *Escherichia coli* tRNAs indicated that it is present in $tRNA_3^{Ser}$ (AGU, AGC), $tRNA_1^{Met}$, $tRNA^{Lys}$, $tRNA^{Ile}$, $tRNA^{Thr}$, and $tRNA^{Asn}$, presumably in the position next to the 3′-hydroxyl end of the anticodon in all cases, but not in other tRNAs (Ishikura *et al.*, 1969; Kimura-Harada *et al.*, 1972). There is a striking similarity among tRNAs that contain t^6A,[1] since they always recognize codons starting with A.



I, R = H
II, R = CH_3

It is also known that several other modified nucleosides with structures similar to that of t^6A are present in these *E. coli* tRNAs (Ishikura *et al.*, 1969; Cory and Marcker, 1970;

† From the Biology Division, National Cancer Center Research Institute, Chuo-ku, Tokyo, Japan (F. K. and S. N.), and from the Institute for Lipid Research and Department of Biochemistry, Baylor College of Medicine, Houston, Texas 77025 (D. L. M. and J. A. M.). *Received March 31, 1972.* This work was supported in part by grants from the Princess Takamatsu Cancer Research Fund and the Japanese Ministry of Education (S. N.), the Robert A. Welch Foundation, and U. S. Public Health Service Grants GM 13901 (J. A. M.) and GM 02055 (D. L. M.).

[1] Abbreviations used are: t^6A, *N*-[(9-β-D-ribofuranosylpurin-6-yl)-carbamoyl]threonine; mt^6A, *N*-[(9-β-D-ribofuranosylpurin-6-yl)-*N*-methylcarbamoyl]threonine; m^7G, 7-methylguanosine; s^4U, 4-thiouridine; A_{260} or A_{280} unit, an amount of material with an absorbance of 1.0 at 260 mμ or 280 mμ when dissolved in 1 ml of water and measured with a 1-cm light path.

Kimura-Harada *et al.*, 1972; Nishimura, 1972). In this communication, we report that one such nucleoside, isolated from *E. coli* tRNA^{Thr}, is the N^6-methyl-substituted derivative of t^{6}A, *i.e.*, *N*-[(9-β-D-ribofuranosylpurin-6-yl)-*N*-methylcarbamoyl]threonine (II) (Nishimura, 1972).

Materials and Methods

Preparation of Purified E. coli tRNA^{Thr}. Unfractionated tRNA was prepared from *E. coli* B cells harvested in the late-log phase of growth as described by Zubay (1962), except that treatment with alkali was omitted. *E. coli* tRNA thus obtained was first fractionated by DEAE-Sephadex A-50 column chromatography at pH 7.5, as previously described (Nishimura *et al.*, 1967; Nishimura, 1971). The fractions enriched with tRNA^{Thr} were then fractionated by DEAE-Sephadex A-50 column chromatography at pH 4.0 as described by Yoshida *et al.* (1971). A major tRNA^{Thr} fraction which was obtained was further purified by successive application of reverse-phase partition column chromatography (Kelmers *et al.*, 1965) and benzoylated DEAE-cellulose column chromatography (Gillam *et al.*, 1967). The purity of the preparation was estimated to be more than 80%, as judged from its amino acid acceptor activity.

Large-Scale Isolation of New Modified Nucleoside from E. coli tRNA^{Thr}. *E. coli* tRNA^{Thr} (600 A_{260} units) was hydrolyzed by 200 units of RNase T_2 in 1 ml of 0.05 M sodium acetate buffer (pH 4.7) at 37° for 18 hr. The hydrolysate was applied to Whatman No. 3MM paper as a streak 15 cm wide, and chromatographed by the descending technique with a solvent system of 1-propanol–concentrated NH_4OH–water (55:10:35, v/v). The ultraviolet-absorbing band, which migrates almost to the solvent front, contained the 2′:3′-cyclic phosphate of "N" but was free from 2′:3′-cyclic phosphate of t^{6}A. The band was cut out, and eluted with water. The eluate, which contained "N">p, was again incubated with 48 units of RNase T_2 (ten times as much as that used for general tRNA hydrolysis) for 18 hr at 37° to open the cyclic phosphate moiety. The 3′-phosphate thus formed was further treated with *E. coli* alkaline phosphomonoesterase to obtain the nucleoside, by the procedure reported previously (Harada *et al.*, 1971). The purified nucleoside was finally obtained by paper electrophoresis at 20 V/cm with 0.05 M triethylammonium bicarbonate buffer (pH 7.5) for 50 min, using Toyo-roshi No. 51A paper. By this procedure, only N moved to the anode due to the presence of a negative charge, whereas other nucleosides present as contaminants remained at the origin. Approximately 6 A_{280} units of N was obtained.

Paper and Thin-Layer Chromatography. Two-dimensional paper or thin-layer chromatography as used for detection of minor components in tRNA was carried out as previously described (Saneyoshi *et al.*, 1969; Harada and Nishimura, 1972). The solvent systems used were: first dimension, solvent 1 [isobutyric acid–0.5 M NH_4OH (5:3, v/v)]; second dimension, solvent 2 [2-propanol–concentrated HCl–water (70:15:15, v/v)]. Other solvent systems used for characterization of modified nucleosides were: solvent 3, ethanol–1 M ammonium acetate buffer, pH 7.5 (7:3, v/v); solvent 4, 1-propanol–concentrated NH_4OH–water (55:10:35, v/v); solvent 5, *tert*-butyl alcohol–1 M NH_4OH (8:2, v/v); solvent 6, 2-propanol–concentrated NH_4OH–water (7:1:2, v/v); solvent 7, 1-butanol–acetic acid–water (4:1:2, v/v).

Mass Spectrometry of Trimethylsilyl Derivative. Conversion to the trimethylsilyl derivative (McCloskey *et al.*, 1968) was carried out by placing 0.1 A_{280} unit of N in a mass spectrometer direct probe glass sample tube, followed by addition of 80 μl of *N,O*-bis(trimethylsilyl)trifluoroacetamide and 1% trimethylsilylchlorosilane. The tube was placed in a glass vial, which was sealed and heated for 20 min at 80°. The reagents were removed under vacuum immediately before introduction of the sample by direct probe into the mass spectrometer.

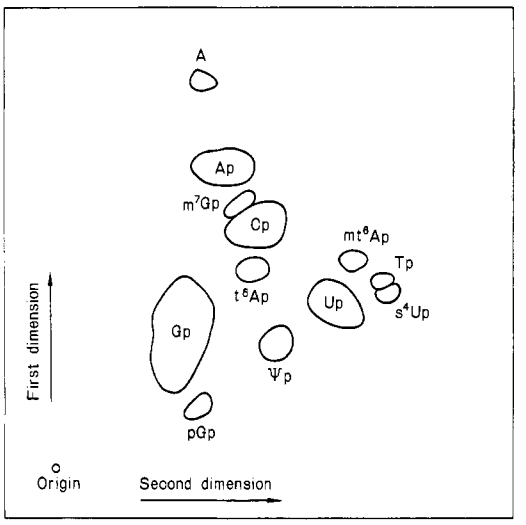


FIGURE 1: Two-dimensional tlc of RNase T_2 digest of *E. coli* tRNA^{Thr}.

Mass spectra were acquired using an LKB 9000 instrument, with ionizing electron energy of 70 eV, and ion source temperature 250°.

Amino Acid Analysis for Detection of Threonine. Approximately 1 A_{280} unit of N was heated for 3 hr at 100° in 0.2 M NaOH to liberate the amino acid as described by Chheda (1969). The neutralized alkaline hydrolysate was then examined for the presence of an amino acid using an automatic amino acid analyzer, JEOL JLC-5 AH.

Materials. RNase T_2 and *E. coli* alkaline phosphomonoesterase were obtained from Sankyo Co. Ltd., and Worthington Biochemical Corp., respectively. Thin-layer glass plates coated with Avicel SF cellulose were purchased from Funakoshi Pharmaceutical Co., Tokyo. Toyo-roshi No. 51A paper equivalent to Whatman No. 1 was obtained from Toyo-roshi Co. Ltd., Tokyo. Whatman No. 3MM paper was a product from W & R Balston Ltd. Authentic N^6-methyladenosine was chemically synthesized by the method of Jones and Robins (1963), and has been used in previous work (Saneyoshi *et al.*, 1969). *N,O*-Bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylsilylchlorosilane which was used for trimethylsilylation of N was purchased from Regis Chemical Co., Chicago, Ill.

Results

Presence of a New Component in the RNase T_2 Digest of E. coli tRNA. Purified *E. coli* tRNA^{Thr} was extensively hydrolyzed by RNase T_2, and the digest was analyzed either by two-dimensional thin-layer chromatography, or two-dimensional paper chromatography. As seen in Figure 1, the chromatogram of the digest showed that the 3′-phosphate[2] of a new

[2] It is not certain whether N>p is converted to Np during or after chromatography in the second dimension, or whether Np is initially formed by incubation with RNase T_2.

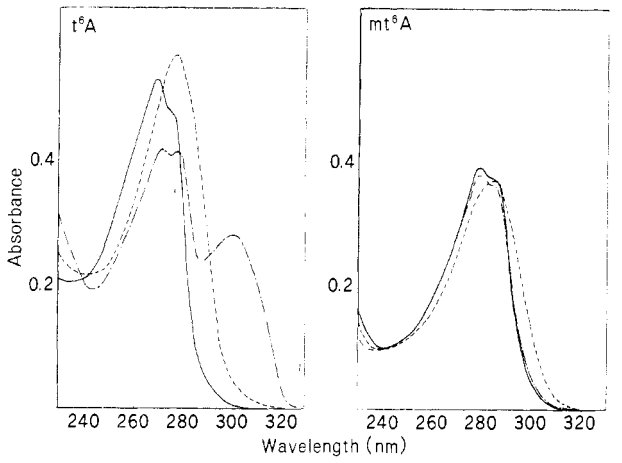


FIGURE 2: Ultraviolet absorption spectra of mt⁶A and t⁶A. 1 N HCl (- - - - - - - - -), pH 7.0 (——), and 0.1 N NaOH (·—·—·—·—).

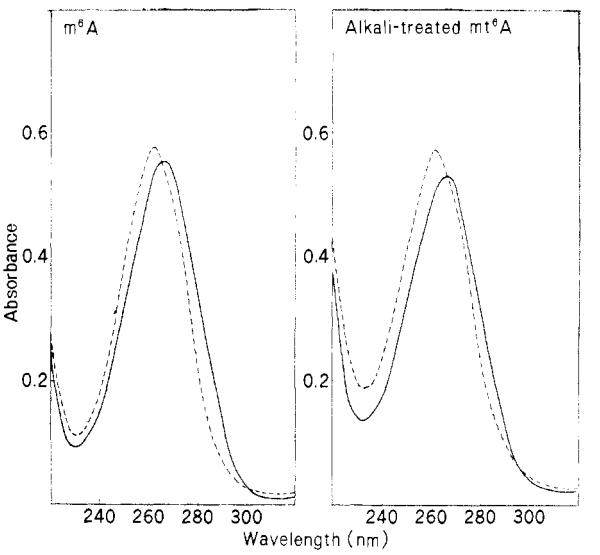


FIGURE 3: Ultraviolet absorption spectra of alkali-treated mt⁶A and authentic m⁶A. pH 7.5 (——) and 0.1 N HCl (- - - - - - -).

minor nucleoside, N, now characterized as *N*-[(9-β-D-ribofuranosylpurin-6-yl)-*N*-methylcarbamoyl]threonine, was detected in a position adjacent to uridylate and ribothymidylate. The chromatogram also showed that $tRNA^{Thr}$ contains 1 mole each of ribothymidine, pseudouridine, and 4-thiouridine, and approximately 0.4 mole of *N*-[(9-β-D-ribofuranosylpurin-6-yl)carbamoyl]threonine (t^6A), as minor components per tRNA molecule. It should be noted that the amount of Np isolated was approximately 0.6 mole/tRNA molecule, assuming the same molar extinction coefficient as that of t⁶Ap. The new component was not detected in purified *E. coli* $tRNA^{Ile}$, $tRNA^{Met}$, $tRNA^{Asn}$, $tRNA^{Lys}$, and $tRNA_3^{Ser}$, which are known to contain t⁶A, when analyzed by the same method of two-dimensional chromatography.

Comparison of Properties of N with t⁶A. The new nucleoside was isolated on a large scale as described in Materials and Methods. Figure 2 shows its ultraviolet (uv) absorption spectra in comparison to those of t⁶A. It is evident that the spectra of both compounds are rather similar but not identical. It is noteworthy that the λ_{max} of N both in alkaline and acidic media is shifted to a higher wavelength than that of t⁶A, and that the second peak observed at 300 nm in t⁶A at alkaline pH disappears. Table I shows the R_F value upon thin-layer chromatography using several solvent systems. N behaved differently from t⁶A, moving faster in the lipophilic solvent systems, indicating that it is more hydrophobic than t⁶A. Table I also shows that upon electrophoresis, N migrates to the anode at the same rate as t⁶A, indicating that it possesses a negative charge at pH 7.5 as does t⁶A.

Formation of N⁶-Methyladenosine and Threonine from N by Treatment with Alkali. According to Chheda *et al.* (1969), t⁶A is decomposed to adenosine and threonine by heating at 100° for 40 min in 0.1 M NaOH. Since N was considered likely to be a derivative of t⁶A, it was treated with alkali under the similar conditions. Ultraviolet absorption spectra of alkali-treated material are shown in Figure 3, and are identical with those of authentic *N*⁶-methyladenosine. In addition, the thin-layer chromatographic mobilities of the uv-absorbing product derived from it were the same as those of *N*⁶-methyladenosine (Table II). It was concluded that the product derived from N was *N*⁶-methyladenosine, in parallel to the production of adenosine from t⁶A.

Automatic amino acid analysis of the alkaline hydrolysate showed that threonine was the only major amino acid present. The molar ratio of threonine to the original was found to be 0.48, assuming that molar extinction coefficient is equal to that of t⁶A. In addition serine and glycine were detected, each approximately one-tenth in amount as compared to threonine. At present, it is not certain whether or not amino acids detected in such small quantities are actual components of tRNA.

Mass Spectrometry of the Trimethylsilyl Derivative. Attempts to directly record the mass spectrum of free mt⁶A were unsuc-

TABLE I: Relative Thin-Layer Chromatographic (tlc) and Electrophoretic Mobilities of mt⁶A, t⁶A, m⁶A, and A.[a]

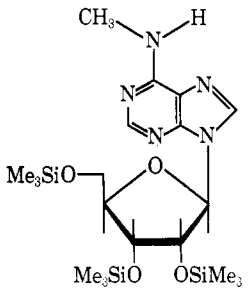
Compound	Tlc, R_F in Solvent System 2	3	4	Electrophoresis Migration from Origin (cm)
mt⁶A	0.61	0.51	0.74	+1.5
t⁶A	0.30	0.36	0.67	+1.5
m⁶A	0.33	0.68	0.77	
A	0.21	0.51	0.64	0
Ap				+3.1

[a] Thin-layer electrophoresis was carried out using a glass plate coated with Avicel SF-cellulose at 20 V/cm for 15 min in 0.05 M triethylammonium bicarbonate buffer (pH 7.5).

TABLE II: Relative Thin-Layer Chromatographic Mobilities of Alkali-Treated mt⁶A, m⁶A, and A.

	Tlc, R_F in Solvent System 3	5	6	7
Alkali-treated mt⁶A	0.70	0.60	0.73	0.62
m⁶A	0.70	0.60	0.73	0.61
A	0.54	0.40	0.51	0.42

cessful because of its high polarity. Therefore conversion to the more volatile trimethylsilyl derivative was carried out. The mass spectrum of the trimethylsilylation product is shown in Figure 4, and indicates extensive conversion to N^6-methyladenosine during silylation. Ions associated principally with the trimethylsilyl derivative are *m/e* 786, 771, 742, 669, 147, and 117. Otherwise, the remainder of major peaks which fall below the molecular ion of *O*-2′,3′,5′-tris(trimethylsilyl)-N^6-methyladenosine (III) (*m/e* 497) correspond both



III

in mass and relative intensity to the mass spectrum of authentic III (J. A. McCloskey and R. N. Stillwell, unpublished experiments, 1968).

Trimethylsilylation was carried out using various silylating reagents under different conditions of temperature and time, in order to minimize the facile conversion to N^6-methyladenosine. However, degradation could not be prevented, and the optimal conditions were those used to obtain the data represented in Figure 4. A limited number of experiments were carried out to prepare the trimethylsilyl derivative of t^6A as a model compound for mass spectrometry, but no volatile product was obtained.

Discussion

The preceding results indicate that the new nucleoside is the N^6-methyl-substituted derivative of t^6A, *i.e.*, *N*-[(9-β-D-ribofuranosylpurin-6-yl)-*N*-methylcarbamoyl]threonine (mt^6A), structure II. This conclusion is based primarily on the following comparisons. (1) At pH 7.5, mt^6A possesses a negative charge and the same electrophoretic mobility as t^6A (Table I). (2) mt^6A and t^6A produce similar uv spectra, with the former exhibiting a higher λ_{max}, the usual result of alkylation at the N^6 position of adenosine derivatives (Hall, 1971). (3) Treatment with alkali liberates threonine and N^6-methyladenosine, while under the same conditions t^6A produces threonine and adenosine.

Strong evidence in support of structure II is provided by the mass spectrum of the trimethylsilyl derivative (Figure 4). The identity of *m/e* 786 as the molecular ion is confirmed by the required characteristic peak 15 mass units lower (*m/e* 771) due to loss of a methyl radical from a trimethylsilyl group (McCloskey *et al.*, 1968). The molecular weight of 786 corresponds to structure IV, as expected for the pentasilyl derivative of mt^6A (II). The loss of 44 mass units (*m/e* 742) is interpreted as resulting from expulsion of CO_2 from the threonyl ester moiety, with rearrangement and retention of $SiMe_3$. The latter process occurs commonly in the mass spectrometry of nucleic acid components (McCloskey *et al.*, 1968; Lawson *et al.*, 1971; White *et al.*, 1972). Loss of 117 mass units (*m/e* 669) can be reasonably attributed to either cleavage as indicated, with the resulting charge on the α carbon of the threonyl moiety stabilized by the adjacent nitrogen. Likewise,

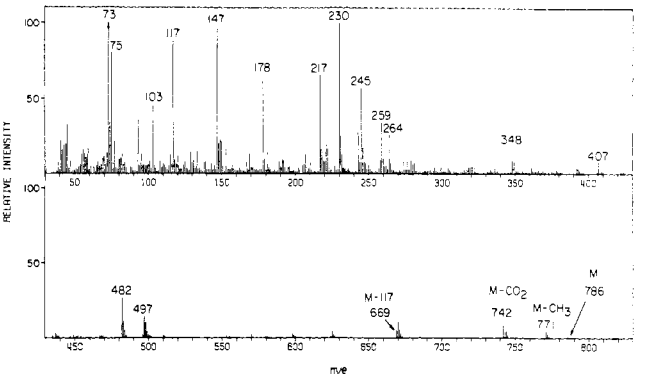
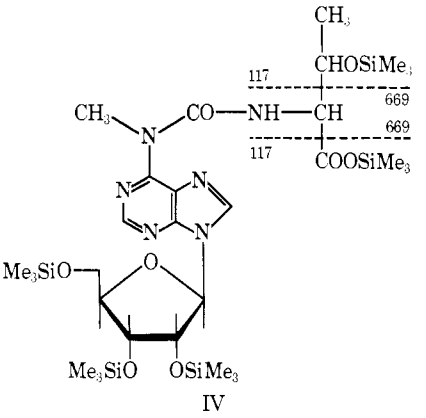


FIGURE 4: Mass spectrum of the trimethylsilylation product of mt^6A.

the intense peak at *m/e* 117 can result from the same processes, with the charge retained and stabilized on either oxygen containing fragment. The only other major peaks not associated mainly with contamination by compound III are *m/e* 75 ($HOSi^+Me_2$) and 147 ($Me_2Si^+OSiMe_3$), neither of which contain structural information. The remainder of major ions which are labeled in Figure 4 are associated with III. In accordance with previous interpretations of mass spectra of silylated nucleosides, *m/e* 178 and 264 are fragments containing the base N^6-methyladenine, while *m/e* 103, 217, 230, 245, 259, and 348 are derived exclusively from the ribose moiety (McCloskey *et al.*, 1968).



IV

It should be noted that the spectrum of III differs substantially from that of the derivative of isomeric 1-methyladenosine, which by contrast incorporates a trimethylsilyl function at N^6 (McCloskey, 1972).

The structure assignment of mt^6A based on the previously discussed data assumes the methyl group in the native nucleoside to be located at N^6, since under the isolation conditions used isomerization is unlikely. However, rearrangement would in principle be possible during the experiments involving treatment with alkali, in parallel to the base-catalyzed conversion of 1-methyladenosine to its N^6-methyl isomer (Brookes and Lawley, 1960; Jones and Robins, 1963; Macon and Wolfenden, 1968). This possibility also seems unlikely however, since the 1-methyl analog of mt^6A would be expected to have a positive charge at pH 7.5 as in the case of 1-methyladenosine (Brookes and Lawley, 1960), while the opposite behavior is observed on electrophoresis (Table I). Further, the relatively large amount of silylated N^6-methyladenosine produced during trimethylsilylation of mt^6A would

not be expected to result from rearrangement of a 1-methyl structure, since the analogous conversion does not occur during trimethylsilylation of 1-methyladenosine or 1-methyl-2′-deoxyadenosine (J. A. McCloskey and R. N. Stillwell, unpublished experiments, 1968).

mt^{6}A is easily converted to N^6-methyladenosine by alkaline treatment. The detection of N^6-methyladenosine in yeast tRNA in earlier experiments was later considered to be the result of its formation from 1-methyladenosine on alkaline treatment during the isolation procedure (Hall, 1965). In the same sense, one should recognize the possibility of formation of N^6-methyladenosine from mt^{6}A in tRNA hydrolysates prepared by alkaline hydrolysis. The purified *E. coli* tRNA^{Thr} used for the isolation of mt^{6}A contained mt^{6}A and t^{6}A in a ratio of 6 to 4. The sum of amounts of mt^{6}A and t^{6}A is approximately equal to that of ribothymidylate obtained from tRNA^{Thr}. It is very likely that *E. coli* tRNA^{Thr} contains either t^{6}A or mt^{6}A in the position adjacent to the 3′-hydroxyl end of the anticodon, although we presently have no sequential data to prove this supposition. It may well be that the tRNA^{Thr} used in these experiments is a mixture of isoaccepting species, and that mt^{6}A is specifically present in one of them.

It should be emphasized that the presence of mt^{6}A is specific to *E. coli* tRNA^{Thr}. It was not detected in other *E. coli* tRNAs such as tRNA^{Met}, tRNA^{Ile}, tRNA^{Asn}, tRNA^{Lys} and tRNA$_3^{Ser}$, which contain t^{6}A. This indicates that the enzyme system involved in biosynthesis of mt^{6}A specifically recognizes tRNA^{Thr}.

We have previously isolated N^6-methyladenosine from *E. coli* tRNA$_1^{Val}$ as a naturally occurring minor nucleoside (Saneyoshi *et al.*, 1969) located at the position adjacent to 3′-hydroxyl end of the anticodon (Yaniv and Barrell, 1969; Harada *et al.*, 1969, 1971; Kimura *et al.*, 1971). Questions which must now be posed are: (1) does a single enzyme participate in the formation of both mt^{6}A and N^6-methyladenosine in the reaction to replace an N^6 proton with a methyl group in these two compounds? (2) Is the N^6-methyladenylate residue a precursor molecule of mt^{6}A in tRNA^{Thr}, or on the contrary, is the t^{6}A residue in tRNA^{Thr} later converted to mt^{6}A? Answers to those questions remain to be clarified.

The biological significance of the specific presence of mt^{6}A specifically in *E. coli* tRNA^{Thr} is unknown at the present time. It should be noted that mt^{6}A cannot base pair with U due to the replacement of all hydrogen atoms in the N^6 position, whereas t^{6}A theoretically can form base pairs. Since the purified tRNA^{Thr} used for the isolation of mt^{6}A accepts threonine to the extent of greater than 80% (under the assumption that 1 A_{260} unit of the tRNA is equal to 1.66 nmoles) it is unlikely that the presence of mt^{6}A is related to the degree of aminoacylation of the tRNA. It can be speculated that the presence of mt^{6}A adjacent to the anticodon of tRNA^{Thr} might affect the function of tRNA during the decoding process in protein synthesis rather than the aminoacylation step, as in the case of modification of the adenosine residue adjacent to the anticodon of *E. coli* suppressor tRNA^{Tyr} (Gefter and Russell, 1969). If so, and if the presence of mt^{6}A is restricted to one of the isoaccepting species of tRNA^{Thr} that recognizes a particular codon or codons, then a different degree of modification adjacent to the anticodon of tRNA^{Thr} might have an effect on the regulatory mechanism of protein synthesis, as previously discussed by Anderson (1969). Recently Ames and his coworkers reported that *Salmonella* tRNA^{His}, which lacks two pseudouridine residues in the anticodon region, is effective in protein synthesis, but not in repression of regulation of the histidine operon (Singer *et al.*, 1972). The function of a modified component in regulatory mechanisms is another interesting possibility in the case of mt^{6}A in *E. coli* tRNA^{Thr}. Studies of the role of mt^{6}A from this point of view still remain to be carried out.

Acknowledgment

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Turnover of Deoxyribonucleic Acid, Histones, and Lysine-Rich Histone Phosphate in Hepatoma Tissue Culture Cells†

Rod Balhorn, Denis Oliver, Philip Hohmann, Roger Chalkley,* and Daryl Granner

ABSTRACT: We have examined the turnover of all the various histone classes during an extended period of cell culture, both in exponential phase and into stationary phase. All of the histone classes appear to be conserved during this period and the decrease in histone specific activity can be accounted for solely in terms of isotope dilution due to the synthesis of new histone molecules. The phosphate groups bound to the F_1 and F_{2a2} histones show similar rate constants of hydrolysis with a half-life of 5 hr. The process of dephosphorylation continues throughout the cell cycle and maintains the same kinetics in G_1 as in S phase.

Histones are a ubiquitous group of basic proteins found in large amounts in eukaryotic chromosomes (Elgin *et al.*, 1971). Considering the large quantity of histone found in the cell, it is ironic that no function has been reliably defined for these proteins. Many groups have studied the phenomenological aspects of histone metabolism and modification in the hope of detecting a correlation between these parameters and the biological activity of the cell under study. Histones can be modified *in vivo* by acetylation (Phillips, 1963; Allfrey *et al.*, 1964; Candido and Dixon, 1971; Shepherd *et al.*, 1971), methylation (Murray, 1964; Paik and Kim, 1967; Gershey *et al.*, 1968; Tidwell *et al.*, 1968), and phosphorylation (Kleinsmith *et al.*, 1966; Ord and Stocken, 1966; Gutierrez and Hnilica, 1967; Ingles and Dixon, 1967; Langan, 1969; Sherod *et al.*, 1970; Balhorn *et al.*, 1971; Sung *et al.*, 1971; Balhorn *et al.*, 1972a,c,d) in a variety of cell systems, though the function of these modifications are unclear. Early studies by Huang and Bonner (1962) and Allfrey *et al.* (1963) suggested that histones might act as repressors of genetic activity and the phosphorylation of specific histone classes was proposed as a possible modulator of this repression (Kleinsmith *et al.*, 1966). Other studies, however, render it unlikely that histones act as specific genetic repressors (Sonnenberg and Zubay, 1965; Johns and Hoare, 1970). As a result we have considered the possibility that phosphorylation of histones might be involved in some other aspect of cellular function. Recent evidence indicates that lysine-rich histone (F_1) phosphorylation is correlated with the process of cell replication in Ehrlich ascites tumor cells (Sherod *et al.*, 1970), regenerating liver (Balhorn *et al.*, 1971), hepatoma (HTC) tissue culture cells (Balhorn *et al.*, 1972d), developing rat liver (Balhorn *et al.*, 1972a), and a series of rat and mouse tumors (Balhorn *et al.*, 1972b). The importance of this association is emphasized by studies with synchronized HTC cells which have shown that F_1 phosphorylation occurs in the S phase of the cell cycle concomitantly with DNA biosynthesis (Balhorn *et al.*, 1972c).

In order to complement the studies of histone phosphorylation we have examined the turnover of F_1-bound phosphate in HTC cells. However, before the lability of histone phosphate could be measured with any degree of certainty, the metabolic stability of the histones themselves had to be determined.

Previous studies of histone turnover have been inconclusive. Histones from rat liver (Piha and Waelsch, 1964; Lawrence and Butler, 1965; Byvoet, 1966; Piha *et al.*, 1966; Murthy *et al.*, 1970), rat brain (Piha and Waelsch, 1964; Piha *et al.*, 1966), mouse mastocytes (Hancock, 1969), and *Euplotes* (Prescott, 1966) appear to be stable and never turn over, while histones from *Tetrahymena* (Lee and Scherbaum, 1966), *Amoebae proteus* (Prescott and Bender, 1963), tobacco cells (Flamm and Birnstiel, 1964), Chinese hamster ovary cells (Gurley and Hardin, 1968, 1969; Gurley *et al.*, 1972), mammary tissue explants (Hohmann *et al.*, 1971), developing chickens (Bondy *et al.*, 1970), and HeLa cells (Sadgopal and Bonner, 1969) have been reported to turn over at variable rates with respect to the various histone classes and subclasses.

We have examined the turnover of histone in both exponential and stationary-phase HTC cells and have found no significant turnover of any of the histone classes over an extended time period. Lysine-rich histone-bound phosphate, on the other hand, was found to turn over with a half-life of ~5 hr. The implications of these results are discussed.

† From the Department of Biochemistry, College of Medicine, University of Iowa, Iowa City, Iowa 52240 (R. B., D. O., P. H., and R. C.), and from the Endocrinology Division, Department of Internal Medicine, College of Medicine, University of Iowa, Iowa City, Iowa 52240 (D. G.). *Received April 12, 1972.* This work was supported by USPHS Grants CA-10871 and CA-12191 and by HEW Grant AI-07690-06. R. C. is a recipient of a Research Career Development award from the USPHS GM 46410 and D. G. is a Veterans Administration Clinical Investigator. D. O. is a postdoctoral fellow of the Biological Sciences Development Plan (Endocrinology) GU-2591.