Proc. Nat. Acad. Sci. USA
Vol. 69, No. 7, pp. 1716–1719, July 1972

2′-*O*-Methylation of Adenosine, Guanosine, Uridine, and Cytidine in RNA of Isolated Rat Liver Nuclei

(*S*-adenosyl methionine/wheat-germ RNA/paper chromatography)

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Communicated by C. B. Anfinsen, April 26, 1972

ABSTRACT **Nuclei isolated from rat liver, when incubated with methyl-labeled *S*-adenosylmethionine, incorporated label into 2′-*O*-methyladenosine, 2′-*O*-methylguanosine, 2′-*O*-methyluridine, and 2′-*O*-methylcytidine of endogenous nuclear RNA. Addition of ribosomal RNA from wheat germ to the reaction markedly stimulated 2′-*O*-methylation of total RNA. The relative incorporation of label into the four different 2′-*O*-methyl ribonucleosides was greatly changed by the addition of wheat germ RNA. There was much more 2′-*O*-methylation of the purine ribonucleosides, relative to the pyrimidine ribonucleosides, in the reaction stimulated by wheat germ RNA.**

Newly synthesized RNA is enzymatically modified in mammalian cell nuclei before it is able to function in its cytoplasmic role in protein synthesis (1–3). One way in which newly synthesized RNA is "processed" is the insertion of methyl groups on the 2′-oxygen atom of ribose (sugar-methylation) of all four of the RNA nucleosides, to yield nucleoside 2′-*O*-methyl ethers (4). Although isolated mammalian nucleolar preparations have been reported to contain such sugar-methylating activity (5, 6), the products of sugar-methylation of RNA, namely Am, Gm, Um, and Cm, have not been conclusively identified. We have recently developed a simple paper chromatographic method for the separation of the four commonly occurring sugar-methylated ribonucleosides from each other, as well as from all of the commonly occurring base-methylated ribonucleosides (7). Using this method, we now report, for the first time, that an enzyme system found in isolated rat liver nuclei will transfer a methyl group from *S*-adenosyl methionine to the 2′-oxygen of A, G, U, and C in endogenous nuclear RNA. Moreover, we have found that the addition of wheat germ RNA to the reaction mixture greatly stimulates the transfer of methyl groups from *S*-adenosyl methionine to the 2′-oxygen of RNA nucleosides.

MATERIALS AND METHODS

Sources of reagents were as follows: [*methyl*-^{3}H]AdoMet and Aquasol scintillator, New England Nuclear, Boston, Mass.; snake venom phosphodiesterase (*Crotalus adamanteus*) and *Escherichia coli* alkaline phosphatase, Worthington Biochemical, Freehold, N.J.; Cm and Um, Ash Stevens, Detroit, Mich.; poly(A) and poly(I)·(C), Miles Laboratories, Kankakee, Ill.; tRNA (*E. coli*, Borek strain) General Biochemicals, Chagrin Falls, Ohio; DEAE-cellulose (Whatman, microgranular), Reeve Angel, Clifton, N.J. Am and Gm were generous gifts from Drs. Fritz Rottman and Roland Robins. Wheat-germ ribosomal RNA and alkali-stable dinucleotides were prepared by the method of Singh and Lane (8). Rat liver nuclei were prepared as described (9). DNA was determined with diphenylamine (10).

Standard Assay of Sugar-Methylation of RNA In Vitro. Rat liver nuclei (containing 0.34–1.36 mg of DNA) were incubated at 37° in a 2.5-ml reaction mixture containing the following: 0.16 M Tris·HCl, pH 8.0, 4 mM $MgCl_2$, and 0.1 mM [*methyl*-^{3}H]AdoMet (1 Ci/mmol). The reaction was stopped by the addition of 2.5 ml of sodium dodecyl sulfate, to a final concentration of 0.5%. The mixture was stirred at room temperature for 5 min, and then 10 mg of wheat-germ RNA was added. An equal volume of water-saturated phenol was added, and the mixture was stirred for 30 min at room temperature. Potassium acetate was added to a final concentration of 0.2 M, and the aqueous phase was separated and saved. The phenol phase was re-extracted with 5 ml of 0.1 M Tris·HCl, pH 8.0, and this aqueous extract was pooled with the first aqueous phase. The pooled aqueous phases were treated once more with phenol. The RNA in the final aqueous phase was precipitated at −20° with 4 volumes of ethanol, and the precipitate was washed three times with absolute ethanol, dissolved in 0.02 M potassium acetate, and dialyzed for 48 hr against several changes of 0.02 M potassium acetate. The dialyzed RNA was lyophilized and then hydrolyzed for 24 hr at 37° with 1 ml of 1 M NaOH. The hydrolysate was neutralized with formic acid and then diluted with water to give a final formate ion concentration of 0.02 M. The neutralized hydrolysate was applied to a 1 cm (diam) × 12 cm (length) column of DEAE-cellulose, which had been equilibrated with 0.025 M Tris-formate†, pH 7.8. Before application to the column 5–10 A_{260} units of alkali-stable dinucleotides, prepared from wheat-germ RNA, were added to the neutralized hydrolysate as carrier. The column was washed with 100 ml of 0.025 M Tris-formate, pH 7.8; the mononucleotides were then eluted as a single fraction (Fig. 1) with 100 ml of 0.085 M Tris-formate, pH 7.8–7 M urea (8). The dinucleotides were then eluted as a second fraction (Fig. 1)

Abbreviations: Am, 2′-*O*-methyladenosine; Gm, 2′-*O*-methylguanosine; Um, 2′-*O*-methyluridine; Cm, 2′-*O*-methylcytidine; AdoMet, *S*-adenosyl methionine; m_2^6A, N^6,N^6-dimethyladenosine.

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† The molarity of all Tris-formate buffers is expressed in terms of the Tris, rather than formate, concentration.

with 80 ml of 0.17 M Tris-formate, pH 7.8–7 M urea (8). 3-ml Fractions were collected, and absorbance at 260 nm was measured. 1-ml Aliquots were counted with 18 ml of Aquasol scintillator.

Identification of 2'-O-Methyl Ribonucleosides in Alkali-Stable Dinucleotide Fraction. The fraction containing the alkali-stable dinucleotides from the DEAE-cellulose column was desalted by readsorption to DEAE-cellulose, followed by elution with the volatile buffer, 1 M pyridinium formate, pH 4.5 (11). The pyridinium formate was removed in a flash evaporator, and the alkali-stable dinucleotides were completely degraded to sugar-methylated ribonucleosides, base-methylated ribonucleosides, and nonmethylated ribonucleosides by the combined action of *Crotalus adamanteus* phosphodiesterase and *E. coli* alkaline phosphatase (7, 8, 12). The reaction mixture (1 ml) contained the following: 0.1 M $(NH_4)_2CO_3$, pH 9.2; 5 mM $MgCl_2$; 700 μg phosphodiesterase; 50 μg alkaline phosphatase; and 5–15 A_{260} units of alkali-stable dinucleotides. The mixture was incubated 18 hr at 37°, and the reaction was terminated by treatment at 100° for 5–10 min. The denatured protein was removed by centrifugation, and the digests were lyophilized. Separation of the four commonly occurring sugar-methylated ribonucleosides (Am, Gm, Um, and Cm) from the commonly occurring base-methylated ribonucleosides, as well as individual identification of Am, Gm, Um, and Cm, was performed by ascending paper chromatography; *n*-butyl alcohol–0.8 M boric acid–concentrated ammonia 2000:270:8 was used as developing solvent (7). In this solvent, Am, Gm, Um, and Cm all migrate faster than any of the commonly occurring base-methylated ribonucleosides, of which 14 have been tested (7). In early experiments, Whatman No. 1 chromatography paper, which was not impregnated with ammonium borate, was used (Method *A*); on this paper, the following R_F values are observed: Am, 0.52; Gm, 0.24; Um, 0.35; and Cm, 0.33; the fastest running of the base-methylated ribonucleosides, m_2^6A, has an R_F of 0.17, and most base-methylated ribonucleosides are completely retarded and remain at the origin. However, Method *A* does not resolve Um from Cm. In later experiments, Whatman 3MM paper, dipped in 0.1 M ammonium borate and allowed to dry before application of samples (Method *B*), was used; on this paper the following R_F values are observed: Am, 0.44; Gm, 0.18; Um, 0.34; and Cm, 0.25; the fastest running base-methylated ribonucleoside, m_2^6A, has an R_F of 0.12. Method *B* thus resolves all four sugar-methylated ribonucleosides from each other, as well as from base-methylated ribonucleosides. Radioactivity in various zones of the chromatograms was measured with 20% efficiency, after elution with 2 ml of H_2O and addition of 18 ml Aquasol, in a liquid scintillation counter.

RESULTS

Incorporation of methyl groups into RNA *in vitro*

As reported for isolated Novikoff tumor nucleoli (5), we have found (Fig. 1) that isolated liver nuclei will incorporate radioactivity from methyl-labeled AdoMet into two classes of nucleotides found in macromolecular RNA: (*i*) those that yield mononucleotides upon alkaline hydrolysis of the RNA, and (*ii*) those that yield alkali-stable dinucleotides upon alkaline hydrolysis of the RNA. The labeled mononucleotides obtained by alkaline hydrolysis presumably result from methylation of the purine and pyrimidine bases, while the labeled dinucleotides presumably result from methylation of the 2'-oxygen of ribose. However, since it has been shown that certain dinucleotides that contain base-methylated, rather than sugar-methylated, ribonucleosides are quite resistant to alkaline hydrolysis (13), this method of measuring sugar-methylation of RNA does not give a definitive assay of this process. The attempt to measure sugar-methylation of RNA *in vitro* by measurement of transfer of methyl label from AdoMet to alkali-stable dinucleotides would be particularly invalid if label were transferred to a purine or pyrimidine base on a nucleotide immediately to the right of an unlabeled sugar-methylated nucleotide; in this case, the 5'-phosphodiester bond of a labeled base-methylated nucleotide would be in an alkali-stable form. Further identifica-

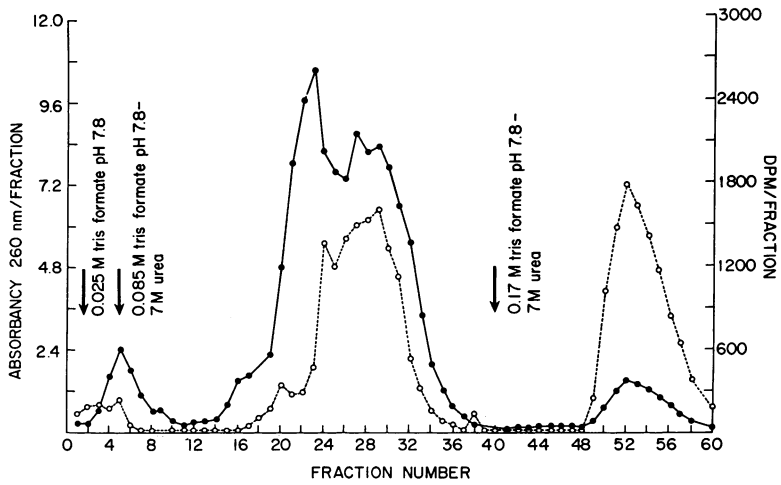

FIG. 1. Incorporation of methyl groups from AdoMet into RNA *in vitro*, and separation of mononucleotides and dinucleotides, produced by alkaline hydrolysis of this RNA, by chromatography on a DEAE-cellulose column. Nuclei (total DNA content, 1.2 mg) were incubated for 10 min with [^{3}H]AdoMet, their RNA was isolated and hydrolyzed with alkali, and the neutralized alkaline hydrolysate was applied to a DEAE-cellulose column (1 cm diam. × 10 cm length). The column was eluted as shown. 3-ml Fractions were collected, and A_{260} (*closed circles*) and radioactivity (*open circles*) were measured. When nuclei that had been treated for 5 min at 100° were used, incorporation into the dinucleotide fraction was only 13% of that observed with unheated nuclei.

TABLE 1. *Identification of 2'-O-methyl ribonucleosides in an alkali-stable dinucleotide fraction by paper chromatography of digests of dinucleotides*

Band	Exp. 1, Method A dpm	Exp. 1, Method A Percent of recovered dpm	Exp. 2, Method B dpm	Exp. 2, Method B Percent of recovered dpm
Am	1735	(16.3)	470	(15.2)
Um	[4220	(39.6)]	675	(21.8)
Cm			400	(12.9)
Gm	1430	(13.4)	410	(13.2)
Origin of chromatogram	3115	(29.2)	720	(23.2)

In two separate incubations with different batches of nuclei, methyl groups from AdoMet were incorporated into nuclear RNA *in vitro*, and the RNA was treated as described in Fig. 1 to yield an alkali-stable dinucleotide fraction, which was then desalted and degraded to sugar-methylated ribonucleosides, base-methylated ribonucleosides, and nonmethylated ribonucleosides, by the combined action of phosphodiesterase and alkaline phosphatase. The digests from Exp. 1 were applied to Whatman 1 paper (not impregnated with ammonium borate, Method *A*), and the digests from Exp. 2 were applied to Whatman 3MM paper (which had been impregnated with ammonium borate, Method *B*). All papers were developed with butanol–boric acid–ammonia. The fast-running bands, containing Am, Um, Cm, and Gm, as well as a band of material remaining at the origin, were cut out and their radioactivity was determined. In Exp. 1, 93% of the total dpm applied were recovered from the paper; in Exp. 2, 88% were recovered. Method *A* does not resolve Um from Cm, while Method *B* does.

tion of the components of the methyl-labeled alkali-stable dinucleotide fraction is thus required to confirm that sugar-methylation has occurred *in vitro*.

Identification of 2'-*O*-methyl ribonucleosides in dinucleotides

More conclusive identification of products of sugar-methylation of RNA *in vitro* was made by degradation of the labeled alkali-stable dinucleotides to component nucleosides by digestion with venom phosphodiesterase and *E. coli* alkaline phosphatase, followed by paper chromatographic analysis of the digests. As shown in Table 1, the majority of the radioactivity in such digests was recovered in bands corresponding to known Am, Um, Cm, and Gm markers. About 25–30% of the recovered counts remained at the origin, and presumably result from transfer of methyl groups from AdoMet to purines or pyrimidines. Only minimal radioactivity was found on the chromatograms in the zones immediately in front of Am, between Am and Um, between Um and Cm, between Cm and Gm, and between Gm and the origin.

Linearity of reaction

The transfer of methyl groups from AdoMet to nuclear RNA during a (20 min) standard incubation was proportional to the amount of nuclei, when nuclei containing from 0.34 mg of DNA to 1.36 mg of DNA were used. The reaction was linear both for transfer of methyl groups to macromolecular RNA that yielded dinucleotides upon alkaline hydrolysis, as well as to macromolecular RNA that yielded mononucleotides upon alkaline hydrolysis.

The transfer of methyl groups from AdoMet to nuclear RNA was also linear with time up to 40 min when nuclei containing 1.2 mg of DNA were incubated and assayed under standard conditions, as shown in Fig. 2. The reaction was linear for incorporation into both the mononucleotide and dinucleotide fractions derived from alkaline hydrolysis of the nuclear RNA.

Effect of addition of RNA on incorporation of methyl groups

Addition of wheat-germ ribosomal RNA caused a greater than 4-fold increase in transfer of methyl groups from AdoMet to macromolecular RNA that yielded dinucleotides upon alkaline hydrolysis, as shown in Table 2, Exp. 2. Neither undermethylated tRNA from *E. coli*, nor poly(A), nor poly(I)·(C) caused such an increase in activity (Table 2, Exps. 3, 4, and 5), although addition of the *E. coli* tRNA did yield a marked increase in presumed base-methylated products. The component 2'-*O*-methyl ribonucleosides in the alkali-stable dinucleotides produced in the reaction stimulated by wheat-germ RNA (Exp. 2, Table 2) were identified; Table 3 shows that the addition of wheat-germ RNA markedly changes the relative incorporation of methyl groups into the four nucleosides, Am, Gm, Um, and Cm (as compared to relative incorporation of methyl groups into these same four nucleosides in the absence of wheat-germ RNA, see Table 1). There was much more sugar-methylation of the purine ribonucleosides, relative to the pyrimidine ribonucleosides, in the reaction stimulated by wheat-germ RNA. Although the use of 1 M NaOH at 37° during alkaline hydrolysis of RNA does deaminate a small fraction of Cm to Um (14, 15), the observed differences in the extent of sugar-methylation of purine ribonucleosides, as compared to pyrimidine ribonucleosides, cannot be explained on this basis. The above data suggest, but in no way prove, that sugar-methylation of the wheat-germ RNA may have occurred *in vitro*.

DISCUSSION

The data presented above indicate that isolated rat liver nuclei have the enzymatic capacity to methylate the 2'-oxygen of adenosine, guanosine, uridine, and cytidine in macromolecular RNA. In spite of the fact that the *in vitro*

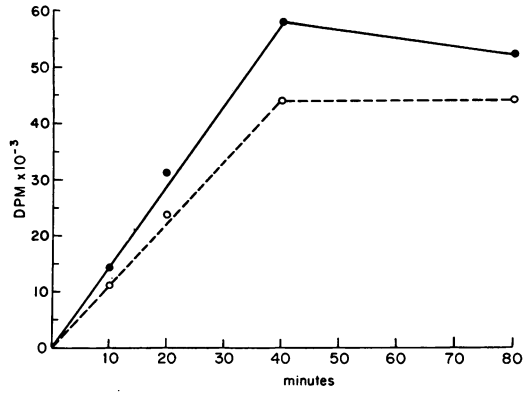

FIG. 2. Rate of incorporation of methyl groups from AdoMet into nuclear RNA *in vitro*. Nuclei (total DNA content 1.2 mg) were incubated with [^{3}H]AdoMet under standard assay conditions for various times, and the RNA was isolated, hydrolyzed, and chromatographed on DEAE-cellulose as described in Fig. 1. Total radioactivity in the mononucleotide fraction (*closed circles*) and dinucleotide fraction (*open circles*) was measured.

TABLE 2. *Effects of addition of different RNAs on incorporation of methyl groups from AdoMet into RNA*

Exp.	RNA added	Relative incorporation into mononucleotide fraction	Relative incorporation into dinucleotide fraction
1	None	100	100
2	Ribosomal, from wheat germ	280	450
3	Transfer, undermethylated, from *E. coli* (Borek strain)	470	150
4	Poly(A)	60	105
5	Poly(I)·(C)	125	109

Identical nuclear assay mixtures (total DNA content, 1.2 mg) were incubated with [^{3}H]AdoMet and 10 mg of the above RNAs under standard conditions for 20 min. The total RNA was isolated, hydrolyzed, and chromatographed on DEAE-cellulose, as described in Fig. 1. Total radioactivity in the mononucleotide and dinucleotide fractions was measured. Data have been normalized by use of a value of 100 for Exp. 1, in which no exogenous RNA was added to the incubation; in this experiment, 31,000 dpm was recovered in the mononucleotides, and 23,500 dpm in the dinucleotides. When nuclei that had been treated for 5 min at 100° were used in Exp. 2, incorporation into the dinucleotide fraction was only 2% of that observed with unheated nuclei.

incubations were performed under conditions in which various nuclear ribonucleases are active (16, 17), the sugar-methylation of endogenous nuclear RNA proceeded in a linear fashion for as long as 40 min. These data suggest that the RNA molecules that are being methylated may be quite inaccessible to the action of nucleases, as might occur if they were complexed with either proteins or DNA. Further studies with a more purified extract, in which the methylating enzymes and the endogenous RNA substrate have been separated from each other, will be necessary to clarify this point.

The number and specificity of the enzymes that sugar-methylate nuclear RNA are not known at present. Such enzymes offer obvious potential for "post-transcriptional" control of nuclear RNA metabolism. Since the nucleolus is believed to be the intranuclear site of sugar-methylation of RNA (2, 3, 18), and inasmuch as many potent carcinogens, as well as several steroid hormones, cause significant changes in nucleolar structure or function (18), it will be of importance to determine whether these agents significantly affect the sugar-methylation of RNA. We have suggested (19) that certain chemical carcinogens may interfere with the normal mechanisms for processing newly synthesized nuclear RNA. The present assay should allow direct measurement of sugar methylation of nuclear RNA *in vitro*, under various physiological conditions.

TABLE 3. *Identification of 2'-O-methyl ribonucleosides in an alkali-stable dinucleotide fraction produced in a reaction stimulated by addition of wheat-germ RNA*

	Exp. 1, Method *A*		Exp. 2, Method *B*	
Band	dpm	Percent of recovered dpm	dpm	Percent of recovered dpm
Am	8330	(30.8)	1775	(29.8)
Um	[7305	(25.5)]	885	(14.9)
Cm			560	(9.4)
Gm	9530	(33.2)	1585	(26.6)
Origin of chromatogram	2100	(7.8)	485	(8.1)

Dinucleotides (Table 2, Exp. 2) from two separate incubations with different batches of nuclei were degraded to component nucleosides, and chromatographed as described in Table 1. In Exp. 1, 93% of the total dpm applied were recovered from the paper; in Exp. 2, 85% were recovered.

We thank Mr. Joseph M. Smith for expert assistance with preparation of the nuclei, and Dr. Philip Leder for helpful comments on the manuscript.

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