ble methanol after acid hydrolysis (by an α,β elimination mechanism), are *O*-methylserine and *O*-methylthreonine. Therefore, these two *O*-methylated amino acids may account for one-third of the amino acid ethers which are enzymatically synthesized in these experiments. Confirmation of these data awaits further resolution and analysis of sufficient quantities of the O-methylated amino acids.

It is reasonable to assume that mammalian semen requires an efficient biological mechanism to prevent the integration of foreign DNA into the spermatozoa. Since the modification-restriction mechanism of bacteria to rapidly destroy exogenous DNA (Meselson et al., 1972) has never been demonstrated in any mammalian tissue, it becomes tempting to speculate at this juncture that specific semen proteins bind to exogenous DNA via their basic amino acids and, in so doing, alter their conformation so as to expose amino acid residues which can subsequently be O-methylated. The O-methylated amino acids may then function as recognition sites for restriction nucleases which inactivate the foreign DNA.

References

Alix, J. H., and Hayes, D. (1974), *J. Mol. Biol. 86,* 139.
Burdon, R. H. (1971), *Biochim. Biophys. Acta 232,* 359.
Burdon, R. H., Martin, B. T., and Lal, B. M. (1967), *J. Mol. Biol. 28,* 357.
Garrett, C. T., Katz, C., Moore, R. E., and Pitot, H. C. (1973), *Cancer Res. 33,* 1662.
Haverberg, L. N., Munro, H. N., and Young, V. R. (1974), *Biochim. Biophys. Acta 371,* 226.
Kakimoto, Y., and Akazawa, S. (1970), *J. Biol. Chem. 245,* 5751.
Kalousek, F., and Morris, N. R. (1968), *J. Biol. Chem. 243,* 2440.
Kim, S. (1974), *Arch. Biochem. Biophys. 161,* 652.
Kim, S., and Paik, W. K. (1971), *Anal. Biochem. 42,* 255.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.
Marmur, J. A. (1961), *J. Mol. Biol. 3,* 208.
Meselson, M., Yuan, R., and Heywood, J. (1972), *Annu. Rev. Biochem. 41,* 447.
Okuhara, E. (1970), *Anal. Biochem. 37,* 175.
Paik, W. K., and Kim, S. (1971), *Science 174,* 1114.
Roberts, T. K., Rao, K. S. P., Masson, P. L., and Heremans, J. F. (1972), *Biochim. Biophys. Acta 278,* 219.
Sheid, B., Srinivasan, P. R., and Borek, E. (1968), *Biochemistry 7,* 280.

Methylated Messenger RNA in Mouse Kidney†

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ABSTRACT: Polyadenylated messenger RNA from mouse kidney labeled in vivo exhibited a pattern of methylation distinct from that of rRNA and tRNA. After mice were given L-[*methyl*-^{3}H]methionine, 4% of the polyribosomal RNA label was bound to oligo(dT)-cellulose; 20–24% of orotate- or adenine-labeled polyribosomal RNA eluted in the poly(A)$^+$ RNA fraction under similar conditions. [^{3}H]Methyl radioactivity was not incorporated into low molecular weight (5–5.8 S) rRNA, indicating the extent of nonmethylpurine ring labeling was negligible. [^{3}H]Methyl-labeled poly(A)$^+$ RNA sedimented heterogeneously in sodium dodecyl sulfate containing gradients similarly to poly(A)$^+$ mRNA labeled with [^{3}H]orotic acid. Based on an average molecular length of 2970 nucleotides, renal mRNA was estimated to contain 8.6 methyl moieties per molecule. Analysis of alkaline-hydrolyzed RNA samples by DEAE-Sephadex-urea chromatography provided estimates of the relative amounts of base and ribose methylation. Although 83% of the [^{3}H]methyl radioactivity in rRNA was in the 2′-*O*-methylnucleotide fraction, no methylated dinucleotides were found in mRNA. In poly(A)$^+$ mRNA 60% of the [^{3}H]methyl label was in the mononucleotide fraction; the remainder eluted between the trinucleotide and tetranucleotide markers and had a net negative charge between −4 and −5. The larger structure, not yet characterized, could result from two or three consecutive 2′-*O*-ribose methylations, and is estimated to contain 2.6 methyl residues. Alternatively, the oligonucleotide could be a 5′-terminal methylated nucleotide species containing 5′-phosphate(s) in addition to the 3′-phosphate moiety resulting from alkaline hydrolysis. Either structure could have a role in the processing or translation of mRNA in mammalian cells.

Stable rRNA and tRNA in eukaryotic cells originate in the nucleus as larger precursor molecules that must be modified before appearing in the cytoplasm (Darnell, 1968; Weinberg and Penman, 1970; Burdon and Clason, 1969; Bernhardt and Darnell, 1969). Maturation of both rRNA and tRNA involves post-transcriptional methylation of specific nucleotides in the nuclear precursor molecule (Greenberg and Penman, 1966; Vaughan et al., 1967; Choe and Taylor, 1972), with the methylated regions being conserved in the mature cytoplasmic species (Weinberg et al., 1967; Vaughan et al., 1967; Maden et al., 1972; Maden and Salim, 1974).

Although the mRNAs of certain viruses contain methylated sequences (Wei and Moss, 1974; Shatkin, 1974; Furuichi, 1974; Rhodes et al., 1974), mammalian mRNAs have generally been considered nonmethylated (Greenberg and Penman, 1966; Perry and Kelley, 1970). The presence of

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poly(A) at the 3′-OH terminus of most eukaryotic mRNAs (for reviews see Darnell et al., 1973; Brawerman, 1974), however, has recently provided a marker for the isolation of poly(A)$^+$ mRNA[1] and the means for directly reexamining the methylation of mammalian mRNA. Perry and Kelley (1974) demonstrated that mRNA from mouse L-cells contained about 2.2 methyl residues per 1000 nucleotides. In examining methylation of poly(A)$^+$ mRNA from cultured Novikoff hepatoma, Desrosiers et al. (1974) found methyl-labeled radioactivity was incorporated evenly between the base-methylated nucleosides and the 2′-*O*-methylnucleoside fraction and that the distribution of base methylnucleosides in mRNA was unique (consisting predominantly of N^6-methyladenosine). Here we report that mouse kidney poly(A)$^+$ mRNA is methylated and that a substantial fraction of the [^{3}H]methyl radioactivity incorporated in vivo is found in a short, alkaline-resistant oligonucleotide fraction.

Experimental Procedure

Animals. Young adult male Charles River mice (40–50 days, 30–35 g, Charles River Laboratories, North Wilmington, Mass.) were fed ad libitum and kept on an alternating cycle of 12 hr of darkness and 12 hr of light.

Preparation of Polyribosomes. Radiochemicals were administered by intraperitoneal injection. At the end of the labeling period, mice were killed by cervical dislocation, and decapsulated kidneys were placed in ice-cold 10 m*M* Tris-HCl (pH 7.4), 10 m*M* NaCl, and 2 m*M* $MgCl_2$ (RSB buffer). Kidneys were disrupted by Dounce homogenization as described (Priestley and Malt, 1968) at a concentration of four kidneys per ml of RSB buffer. Homogenates were centrifuged at 10,000 rpm for 10 min at 4° in the Sorvall SS-34 rotor. Post-mitochondrial supernatants were centrifuged in 7–47% (w/w) sucrose density gradients in 10 m*M* Tris-HCl (pH 7.4), 0.50 *M* NaCl, and 50 m*M* $MgCl_2$ at 26,500 rpm for 3 hr at 4° in the Beckman SW27 rotor (Kumar and Lindberg, 1972). Gradients were eluted by pumping from the bottom of tubes, and the absorbance at 260 nm was monitored continuously.

Extraction of RNA. Ribonucleoproteins from the polyribosome region and 40–80S regions were concentrated by overnight precipitation at −20° in 2 vol of 95% ethanol. For unknown reasons, failure to separately deproteinize the polyribosome and 40–80S regions resulted in degradation of rRNA and mRNA. RNA was extracted by the procedure of Perry et al. (1972). Ethanol-precipitated polyribosomes dissolved in 10 ml of 10 m*M* Tris-HCl (pH 9.0), 0.10 *M* NaCl, 10 m*M* EDTA, and 0.5% sodium dodecyl sulfate (NETS) were alternately extracted at room temperature with equal volumes of phenol–chloroform–isoamyl alcohol (50:48:2) and chloroform–isoamyl alcohol (96:4) until the interfacial layer was clear of denatured protein. Following deproteinization, RNA samples were combined and stored at −20° as ethanol precipitates. Nominal 4S RNA was prepared from the region of the polyribosome gradients sedimenting slower than the 40S ribosomal subunit. The tops of the gradients were mixed with phenol upon collection, then deproteinized as described. Ethanol-precipitated RNA was centrifuged in 15–30% (w/w) sodium dodecyl sulfate containing sucrose density gradients. RNA sedimenting in the nominal 4S region was pooled and stored at −20° in 2 vol of 95% ethanol.

Oligo(dT)-Cellulose Chromatography. Poly(A)-containing (poly(A)$^+$) RNA was separated from the nonadenylated (poly(A)$^-$) RNA fraction by a modification of the procedure of Aviv and Leder (1972). Samples consisting of less than 1 mg of polyribosomal RNA dissolved in 2–3 ml of 10 m*M* Tris-HCl (pH 7.4)–0.50 *M* NaCl were applied to 15 × 0.8 cm columns of oligo(dT)-cellulose (Type T-2, Collaborative Research, Waltham, Mass.), and poly(A)$^+$ RNA bound to the column was eluted with 10 m*M* Tris-HCl (pH 7.4). Fractions were assayed for radioactivity either by counting 50-μl aliquots in 10 ml of PCS (Amersham/Searle, Arlington Heights, Ill.) or by collecting acid-insoluble material from aliquots on 24-mm glass fiber filters (Whatman GF/A, Reeve Angel Co., Clifton, N.J.) and counting the dried filters in 5 ml of scintillation fluid [4 g of Omnifluor (New England Nuclear Co., Boston, Mass.) per l. of toluene]. Poly(A)$^+$ and poly(A)$^-$ RNA fractions were pooled separately and precipitated with ethanol. No additional poly(A)$^+$ RNA was recovered upon rechromatography of the poly(A)$^-$ RNA fraction; more than 95% of the poly(A)$^+$ radioactivity was bound to the column when chromatographed a second time on oligo(dT)-cellulose.

Sucrose Density Gradient Analysis of RNA. RNA samples dissolved in a total volume of 1.4 ml of NETS buffer (10 m*M* Tris-HCl (pH 7.4), 0.10 *M* NaCl, 10 m*M* EDTA, and 0.5% sodium dodecyl sulfate) were layered onto 34-ml linear 15–30% (w/w) sucrose gradients in NETS buffer. Gradients were centrifuged in the SW27 rotor at 23,000 rpm for 16.5 hr at 23°. Gradients were fractionated as before, and total fractions or aliquot samples were counted as a gel in 3.0 ml of H_2O and 10 ml of PCS solubilizer.

Polyacrylamide Gel Electrophoresis. RNA samples were dissolved in 50 μl of 40 m*M* Tris-acetate (pH 7.4), 2 m*M* EDTA, 15% glycerol, and 0.2% sodium dodecyl sulfate and electrophoresed in a 10% polyacrylamide–0.25% diacrylate gel (Weinberg et al., 1967; Weinberg and Penman, 1968) at 5 mA per gel for 3.0 hr at room temperature. The electrode buffer was 40 m*M* Tris-acetate (pH 7.4), 2 m*M* EDTA, 10% glycerol, and 0.2% sodium dodecyl sulfate. After gels were fractionated by crushing in a Savant Autogeldivider (Hicksville, N.Y.), fractions were counted in 4.5 ml of scintillation fluid containing 2 vol of xylene, 1 vol of Triton-X 100 (Rohm & Haas Company, Philadelphia, Pa.), and 8 g of Omnifluor per liter.

DEAE-Sephadex–Urea Chromatography. Samples of poly(A)$^+$ and poly(A)$^-$ polyribosomal RNA and of 4S RNA, each previously rechromatographed on oligo(dT)-cellulose, were dissolved in 3 ml of 0.3 *N* KOH and hydrolyzed for 24 hr at 37°. Chilled samples were neutralized by the addition of 3 ml of cold 0.3 *N* perchloric acid; the resulting precipitate was removed by centrifugation at 4000 rpm for 10 min. Neutralized alkaline hydrolysates were applied to 19 × 1.2 cm columns of DEAE-Sephadex (A-25, Pharmacia Fine Chemicals, Inc., Piscataway, N.J.), which had been equilibrated with 20 m*M* Tris-HCl (pH 7.4), 0.10 *M* NaCl, and 7 *M* urea (Tener, 1967; Perry and Kelley, 1974). Columns were washed with 1.5 vol of 20 m*M* Tris-HCl (pH 7.4), 0.10 *M* NaCl, and 7 *M* urea and eluted with linear 200-ml gradients of 0.10 to 0.40 *M* NaCl in 20 m*M* Tris-HCl (pH 7.4) and 7 *M* urea. Fractions of 1.85 ml were assayed for radioactivity by direct counting in a gel of 1.0 ml of H_2O plus 10 ml of PCS solubilizer.

[1] Abbreviations used are: poly(A)$^+$ mRNA, mRNA containing poly(A); poly(A)$^-$ RNA, RNA lacking poly(A); RSB buffer, 10 m*M* Tris-HCl (pH 7.4), 10 m*M* NaCl, and 2 m*M* $MgCl_2$; NETS buffer, 10 m*M* Tris-HCl (pH 7.4), 0.10 *M* NaCl, 10 m*M* EDTA, and 0.5% sodium dodecyl sulfate.

Table I: Oligo(dT)-Cellulose Chromatography of Renal Polyribosomal RNA.[a]

Expt	Radioisotope	Labeling Period (hr)	Radioactivity (cpm)		
			Poly(A)⁻ RNA	Poly(A)⁺ RNA	Poly(A)⁺/ Polyribosomal RNA (%)
1	L-[*methyl*-^{3}H] Methionine	1	12,340	580	4
2	L-[*methyl*-^{3}H] Methionine	2	52,740	2340	4
3	[^{3}H] Adenine	2.5	4,168	1300	24
4	[^{3}H] Orotic acid	1	13,415	3901	23
5	[^{3}H] Orotic acid	2	19,505	4492	19

[a] Mice were labeled as shown and polyribosomes were prepared as described under Experimental Procedure. Deproteinized polyribosomal RNA was chromatographed on oligo(dT)-cellulose; aliquots were assayed for radioactivity by direct counting or as acid-insoluble material: experiment 1, a total of four mice were labeled with 500 μCi each; experiment 2, a total of 12 mice were labeled with 415 μCi each; experiment 3, a total of 4 mice were labeled with 420 μCi each; experiments 4 and 5, a total of 4 mice were labeled with 100 μCi each.

Radiochemicals. The following radiochemicals were purchased from New England Nuclear Co. (Boston, Mass.): L-[*methyl*-^{3}H]methionine, 190–230 mCi/mmol; [5-^{3}H]orotic acid, 11.1 Ci/mmol; [6-^{14}C]orotic acid hydrate, 55.2 mCi/mmol; [^{3}H]adenine, 8.26 Ci/mmol. L-[*methyl*-^{3}*H*]Methionine was obtained in lyophilized form and was dissolved with sterile 0.15 *M* NaCl to 1 mCi/ml just before injection.

Results

Fractionation of Polyribosomal RNA. Radioactivity in RNA labeled with L-[*methyl*-^{3}H]methionine may result from actual methylation of RNA or from transfer of the terminal methionine methyl group into purine rings via the one-carbon folic acid pool. In cultured cells, labeling of purine rings via the tetrahydrofolate pool (nonmethyl labeling) may be eliminated by labeling cells in the presence of formate, adenosine, and guanosine to suppress de novo purine biosynthesis (Maden et al., 1972). Since manipulations of this type are not possible in vivo, the extent of nonmethyl purine ring labeling had to be estimated by analysis of labeled products.

Poly(A)⁺ mRNA was separated from polyribosomal poly(A)⁻ RNA by selection on oligo(dT)-cellulose. If the majority of the methyl radioactivity incorporated into polyribosomal RNA entered through de novo synthesis of purines, then the percentage of methyl label in poly(A)⁺ RNA should be similar to that found when labeled adenine or orotic acid was used as the precursor. As shown in Table I, although the relative incorporation of labeled purine and pyrimidine precursors into mRNA and rRNA was similar, it was substantially different when L-[*methyl*-^{3}H]methionine was the precursor. The value of 4% of the total methyl radioactivity in polyribosomal RNA in the poly(A)⁺ RNA fraction agrees with the 2–3% value obtained by Perry and Kelley (1974) for cultured mouse L-cell polyribosomal RNA labeled 4 hr under conditions that suppress de novo purine biosynthesis.

Analysis of 5.8S rRNA. rRNA (5.8S) is associated with 28S rRNA by noncovalent bonding. This low molecular weight RNA is substantially undermethylated relative to 28S, 18S, and tRNA, containing one methyl residue per molecule (Maden and Salim, 1974). Analysis of 5.8S RNA, therefore, provides an assay for nonmethyl purine ring labeling. During a 17-hr labeling period, no detectable L-[*methyl*-^{3}H]methionine was incorporated into 5.8S RNA, although tRNA was extensively labeled (Figure 1). When [^{14}C]orotate was the precursor, labeled 5.8S and 4S RNA were both evident. The data in Table I and Figure 1 show the extent of nonmethyl purine ring labeling was negligible and that the measurable [^{3}H]methyl radioactivity in RNA resulted from methylation.

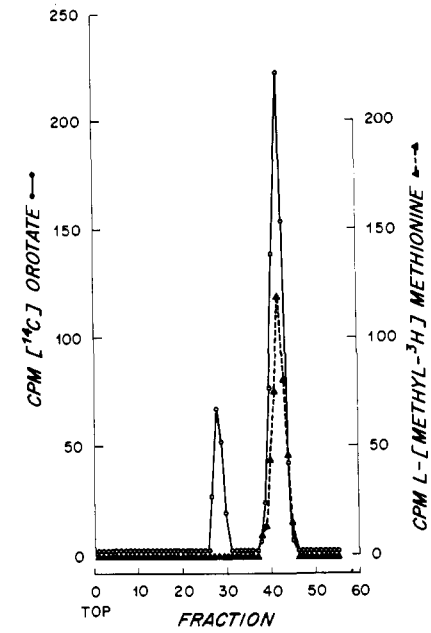

FIGURE 1: Each of 4 mice was labeled for 17 hr with 250 μCi of L-[*methyl*-^{3}H]methionine and 2 μCi of [^{14}C]orotic acid. Poly(A)⁻ polyribosomal RNA was prepared and sedimented in a 34-ml 15–30% sodium dodecyl sulfate containing sucrose density gradient at 23,000 rpm for 16.5 hr at 23° in the SW27 rotor (Experimental Procedure). The 4–10S region of the gradient was ethanol precipitated, redissolved in 50 μl of 40 m*M* Tris-acetate (pH 7.4), 2 m*M* EDTA, 15% glycerol, and 0.2% sodium dodecyl sulfate and electrophoresed in a 10% polyacrylamide gel as described under Experimental Procedure.

Sedimentation Properties of [^{3}H]Methyl-Labeled Poly(A)⁺ mRNA. Poly(A)⁺ mRNA from mammalian cells sediments in aqueous sodium dodecyl sulfate containing sucrose density gradients as a heterogeneous population of RNA molecules with a mean sedimentation coefficient of approximately 22 S (Penman et al., 1968; Singer and Penman, 1973). Poly(A)⁺ mRNA from mouse kidney sediments with a broad peak in the 20–24 S range (A. J. Ouellette et al., submitted for publication). To examine the size distribution of L-[*methyl*-^{3}H]methionine-labeled cytoplasmic RNA species, methyl-labeled RNA fractions were compared with corresponding RNA samples labeled with [^{3}H]orotic acid by sedimentation in sodium dodecyl sulfate

Table II: Relative Extent of Poly(A)$^-$ and Poly(A)$^+$ RNA Methylation.[a]

Precursor	Radioactivity (cpm)			$^3H/^{14}C$		
	Poly(A)$^-$ RNA	Poly(A)$^+$ RNA	Poly(A)$^+$ Polyribosomal RNA (%)	Poly(A)$^-$ RNA	Poly(A)$^+$ RNA	(Poly(A)$^+$ $^3H/^{14}C$)/(Poly(A)$^-$ $^3H/^{14}C$)
L-[*methyl*-^{3}H] Methionine	6,301	316	5	0.066	0.011	0.17
[^{14}C] Orotic acid	95,001	29,797	23			

[a] Each of 4 mice was injected with both 250 μCi of L-[*methyl*-^{3}H] methionine and 5 μCi of [^{14}C] orotic acid. Polyribosomal RNA was prepared as described under Experimental Procedure and fractionated on oligo(dT)-cellulose.

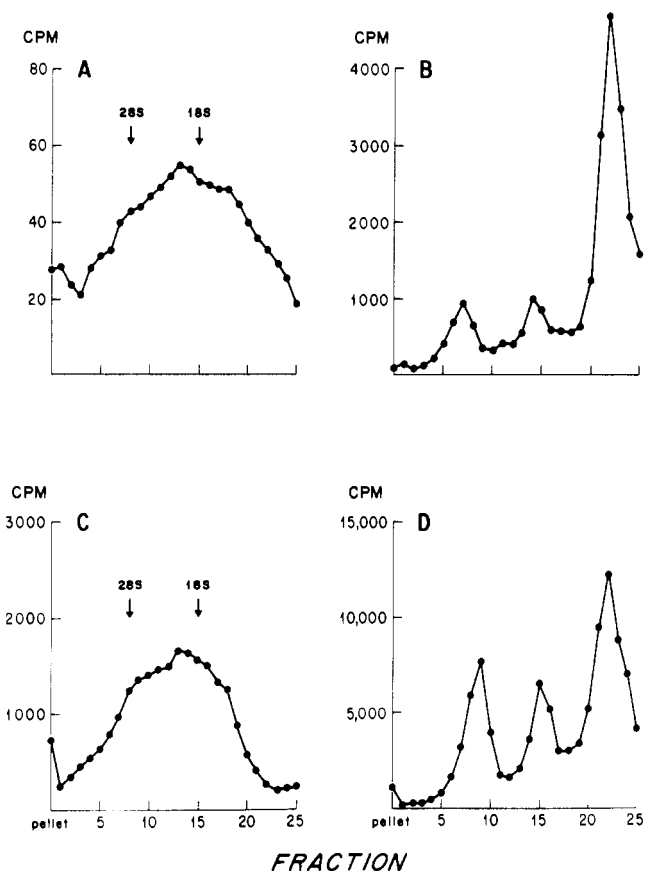

FIGURE 2: Sedimentation properties of methyl- and orotate-labeled polyribosomal RNA. Mice (4) were labeled for 2 hr with 100 μCi of [^{3}H]orotate or 500 μCi of L-[*methyl*-^{3}H]methionine. Cytoplasmic RNA prepared by combining separately deproteinized polyribosomal and 4S RNA was chromatographed on oligo(dT)-cellulose, and the poly(A)$^+$ RNA fraction was again passed through oligo(dT). Poly(A)$^-$ and poly(A)$^+$ cytoplasmic RNA fractions were concentrated by ethanol precipitation, dissolved in 1.4 ml of NETS buffer, and sedimented in 34 ml of 15–30% sodium dodecyl sulfate containing sucrose density gradients in the SW27 rotor at 23,000 rpm for 16.5 hr at 23°. Total fractions were counted in a gel of 3 ml of H_2O and 10 ml of PCS. Values represent net counts per minute: panel A, methyl-labeled poly(A)$^+$ mRNA; panel B, methyl-labeled poly(A)$^-$ cytoplasmic RNA; panel C, orotate-labeled poly(A)$^+$ mRNA; panel D, orotate-labeled poly(A)$^-$ cytoplasmic RNA.

containing sucrose density gradients. Methyl-labeled poly(A)$^+$ mRNA (Figure 2A), although markedly lower in total radioactivity, sedimented as typical renal mRNA (Figure 2C) with a mean sedimentation coefficient of 22 S. There was no apparent contamination of the poly(A)$^+$ RNA fraction with rRNA or tRNA. The sedimentation patterns of the methyl or orotic acid labeled poly(A)$^-$ cytoplasmic RNAs showed the 28S and 18S rRNAs and tRNA (Figures 2B and 2D).

Specific activities of rRNAs were determined by pooling appropriate fractions from the gradients and measuring radioactivity per absorbance unit. The measured specific activities of methyl-labeled 28S and 18S rRNA were 149 cpm/A_{260} and 186 cpm/A_{260}, respectively, which gives an 18 S/28 S specific activity ratio of 1.25. This value is in reasonable agreement with data from HeLa cells (Brown and Attardi, 1965; Wagner et al., 1967; Maden and Salim, 1974) and mouse L-cells (Perry and Kelley, 1974); in both those systems the 18 S/28 S ratio is 1.5.

Estimation of Number of Methylated Nucleotides per mRNA Molecule. Like [^{3}H]orotate-labeled mRNA, methylated renal poly(A)$^+$ mRNA sediments heterogeneously with a mean sedimentation coefficient of 22 S (Figure 2A). Assuming that sedimentation of single-stranded RNA is similar to denatured DNA, it is possible to calculate a mean molecular weight for the poly(A)$^+$ mRNA population using the equation of Studier (1965). The calculated average molecular weight of kidney mRNA is 1.01×10^6. From the molecular weight, an average molecular length of 2970 nucleotides was computed by assuming an equal distribution of the four nucleotides in renal mRNA and an average ribonucleoside monophosphate mol wt of 340.

By double labeling mice with L-[*methyl*-^{3}H]methionine and [^{14}C]orotate, the relative extents of methylation in renal poly(A)$^+$ RNA and poly(A)$^-$ RNA were estimated from the ratios of the two isotopes in each RNA fraction (Table II). The degree of mRNA methylation was 17% of that of rRNA. Assuming kidney rRNA has the same base composition as rRNA from HeLa cells, 1.69% (117 methyl moieties/6911 nucleotides) of the nucleotides in renal rRNA should contain methyl residues (Maden and Salim, 1974). From this, $1.69 \times 0.17 = 0.29\%$ methylated bases in renal mRNA. Therefore, 2970 nucleotides/mRNA molecule $\times$ 0.0029 = 8.6 methylated bases per mRNA molecule or 2.9 methylated bases/1000 nucleotides. This value is slightly higher than the 2.2 methyl moieties/1000 nucleotides reported by Perry and Kelley (1974) for L-cell mRNA. Since kidney and L-cell mRNAs are both methylated to 17% of the extent of the respective rRNAs, the difference in methyl content is due to the fact that Perry and Kelley based their calculations on a methyl content of 1.3% for HeLa rRNA, while our values were calculated using an rRNA methyl content of 1.69% derived from the more recent data of Maden and Salim (1974).

DEAE-Sephadex-Urea Chromatography of Hydrolyzed Methyl-Labeled RNA Fractions. The methyl residues associated with renal mRNA could result from methylation of the bases or of the 2′-OH of the ribose moiety. Since methylation of the 2′-OH group protects the adjacent 3′,5′-phos-

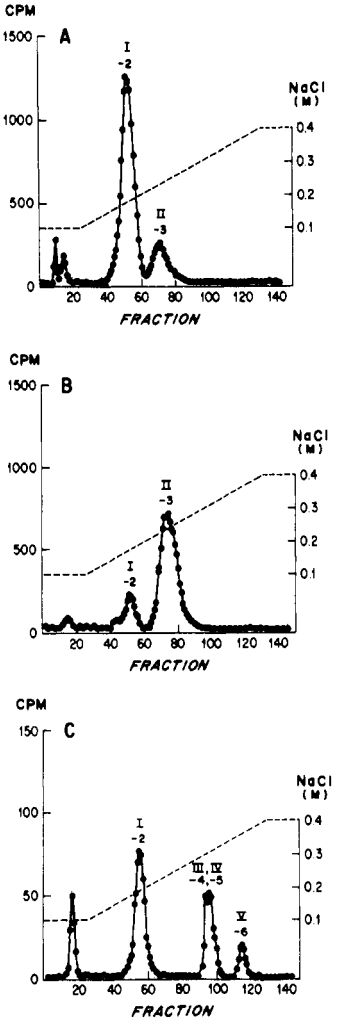

FIGURE 3: DEAE-Sephadex-urea chromatography of alkaline hydrolysates of methylated cytoplasmic RNA fractions. Neutralized alkaline hydrolysates of poly(A)$^+$ and poly(A)$^-$ polyribosomal RNA and 4S RNA were chromatographed by DEAE-Sephadex-urea chromatography. The elution pattern of oligonucleotide standards was determined by similar chromatography of 1 mg of renal rRNA digested for 18 hr with 100 μg/ml of pancreatic RNase A (Worthington Biochemical Corp., Freehold, N.J.). Fractions of 1.85 ml were counted directly in a gel of 1 ml of H_2O and 10 ml of PCS solubilizer: panel A, 4S RNA; panel B, rRNA; panel C, poly(A)$^+$ mRNA.

phodiester bond from alkaline hydrolysis, anion exchange chromatography of neutralized alkaline hydrolysates in the presence of 7 *M* urea provides a means for distinguishing between these two possible types of RNA modification (Tener, 1967).

The relative extent of base and 2′-OH ribose methylation in renal tRNA, rRNA, and poly(A)$^+$ mRNA was determined by chromatography of alkaline hydrolysates of these methylated RNA fractions on DEAE-Sephadex-urea columns (Tener, 1967; Perry and Kelley, 1974). The elution patterns of the alkali-digested products of these RNAs were distinct (Figure 3). The tRNA (Figure 3A) and rRNA (Figure 3B) fractions contained methyl radioactivity exclusively in the mononucleotide (I) and dinucleotide (II) peaks, indicating the presence of base and ribose methylations in both classes of RNA. Messenger RNA, on the other hand, contained a methylated mononucleotide fraction, but in contrast to L-cell mRNA (Perry and Kelley, 1974), had no methyl label in the dinucleotide region (Figure 3C). Instead, a substantial amount of radioactivity eluted between the trinucleotide and tetranucleotide standards (Figure 3C).

Table III: Relative Distribution of Methyl Radioactivity in Mononucleotide, Dinucleotide, and Oligonucleotide Fractions.[a]

RNA Fractions	Radioactivity (% of Total)			
	I	II	III, IV	V
rRNA	17	83		
tRNA	82	18		
mRNA	60		28	12

[a] Total radioactivity recovered in each of the oligonucleotide peaks by DEAE-Sephadex–urea chromatography of [^{3}H]methyl-labeled rRNA, tRNA, and mRNA (Figures 2A–2C) was determined and is expressed as percent of the total radioactivity in that RNA fraction.

The total radioactivity was determined for each of the peaks in Figure 3; the distribution of methyl label in the different nucleotide fractions for each of these RNAs is shown in Table III. For tRNA, 82% of the methyl label was incorporated by base methylation. The reverse was true for rRNA, in which 83% of the [^{3}H]methyl residues were associated with the dinucleotide fraction, presumably from 2′-OH ribose methylation. These values are in close agreement with data from other systems (Brown and Attardi, 1965; Wagner et al., 1967). In the mRNA fractions, 60% of the methyl radioactivity was in the mononucleotide fraction, approximately 30% of the label in a structure eluting between the trinucleotide and tetranucleotide. Since there are 8.6 methyl moieties per mRNA molecule, 8.6 × 0.30 = 2.6; therefore, the alkali-stable oligonucleotide contains 2–3 methyl residues per mRNA molecule. The importance of the small peak in the pentanucleotide region is not clear because of insufficient radioactivity in that peak. Preliminary observations (unpublished results) suggest these oligonucleotides may be located at or near the 5′-termini of renal mRNA molecules since the −5 charge structure is absent from partially degraded poly(A)$^+$ mRNA samples. These data agree with the observation that approximately half of the alkaline hydrolysis products of methylated L-cell mRNA elute from similar DEAE-Sephadex columns at an ionic strength in excess of 0.25 *M* NaCl (Perry and Kelley, 1974). Desrosiers et al. (1974) reported, in addition, that a large portion of alkali-stable [^{3}H]methyl radioactivity from Novikoff hepatoma mRNA eluted considerably past the dinucleotide region.

Discussion

Mouse kidney polysomes incorporate [^{3}H]methyl-labeled methionine into poly(A)$^+$ mRNA in vivo. The sedimentation properties of the methyl-labeled poly(A)$^+$ RNA are similar to those of poly(A)$^+$ mRNA labeled with orotic acid. Since the centrifugation data and the results of DEAE-Sephadex chromatography would seem to eliminate contamination of the poly(A)$^+$ mRNA by rRNA or tRNA, we conclude that mRNA from the polyribosomes of intact mouse kidney is methylated and that the pattern of methylation is unique to that class of RNA.

Until base composition analyses are performed on the methylated mononucleotide region from alkaline-hydrolyzed mRNA, it will not be clear whether the labeling resulted from post-transcriptional methylation or from non-methyl purine ring labeling. However, since [^{3}H]methyl label was not detectable in 5.8S rRNA, the amount of L-

[*methyl*-^{3}H]methionine radioactivity entering RNA via the tetrahydrofolate pathway is likely to have been negligible relative to that catalyzed by RNA methylases. Desrosiers et al. (1974) originally identified the base methylnucleosides of Novikoff mRNA largely as N^6-methyladenosine or N^1-methyladenosine or both, and in every system examined thus far (Wei et al., 1975; Perry et al., 1975; Adams and Cory, 1975) N^6-methyladenosine has been the predominant base-methyl derivative. Therefore, at least some of the label incorporated into the mononucleotide fraction during the in vivo labeling of mouse kidney probably represents specific methylation of certain bases.

An alkaline-resistant, methylated oligonucleotide with a charge of −5 has been reported in 28S rRNA of HeLa cells (Maden and Salim, 1974) and *Xenopus laevis* kidney cells (Slack and Loening, 1974) after equilibrium labeling, but oligonucleotides larger than dimers were not detectable in mouse kidney rRNA labeled 2 hr with L-[*methyl*-^{3}H]methionine. Therefore, under our labeling conditions the methylated oligonucleotide fraction eluting with 0.32 *M* NaCl from DEAE-Sephadex may be considered mRNA specific.

Preliminary reports of methylated mRNA from cultured cells have described a large portion of mRNA methyl radioactivity eluting from DEAE-Sephadex (Perry and Kelley, 1974) or RPC-5 (Desrosiers et al., 1974) at an ionic strength above that required to elute dinucleotides. Both studies estimated approximately half the mRNA methyl radioactivity to be in this oligonucleotide fraction. By our measurements, 40% of the methyl label was found in structures eluting from DEAE-Sephadex after the dinucleotide peak. In mouse kidney mRNA, the major peak in this fraction elutes between the trinucleotide and tetranucleotide markers, bearing charges between −4 and −5, respectively, and is estimated to contain 2–3 methyl groups.

The chemical structure of the mRNA methylated oligonucleotide has not been determined. It could be either a tri- or a tetranucleotide containing consecutive 2′-*O*-methyl residues or, possibly, a smaller 5′-terminal oligonucleotide containing additional phosphate moieties. Messenger RNAs of several viruses are methylated (Wei and Moss, 1974; Shatkin, 1974; Furuichi, 1974; Rhodes et al., 1974), and contain unusual 5′ termini of the general structure $X^mppp(Y^mp)_{1-2}Zp$ or $X^mpp(Y^mp)_{1-2}Zp$ where X is *N*-methylguanosine, nucleoside Y contains 2′-*O*-methyl residues, and Z may or may not be base methylated (Furuichi et al., 1975a,b; Wei and Moss, 1975; Abraham et al., 1975; Moyer et al., 1975). These methylated, alkali-resistant products elute from anion exchange resins with a charge of −5 to −6. Recent data indicate mRNA from mouse L-cells (Perry et al., 1975), HeLa cells (Wei et al., 1975), cultured Novikoff hepatoma (Desrosiers et al., 1975), reticulocytes (Muthukrishnan et al., 1975), and mouse myeloma cells (Adams and Cory, 1975) contain similarly modified 5′ termini, all terminating in $m^7G^{5'}ppp^{5'}N^mpNp$ or $m^7G^{5'}ppp^{5'}N^mpN^mpNp$, where N is any nucleoside and N^m is a methylated derivative. From the diversity of these cell lines, it would appear that blocked, methylated 5′-terminal structures may be a common feature of eukaryotic mRNAs. Whether the 5′ ends of renal mRNAs are similarly terminated is not clear, but it seems likely since the major methylated, alkali-resistant product in kidney mRNA also bears a charge of −5 and appears to be near the 5′ terminus, since it is not found in slightly degraded poly(A)$^+$ mRNA (unpublished results).

From the current understanding of mammalian mRNA biogenesis (Darnell et al., 1973; Brawerman, 1974), the blocking of the 5′ terminus would necessarily occur after the final hnRNA processing event. HeLa cells starved for methionine synthesize undermethylated 45S ribosomal precursor RNA which is degraded rather than exported to the cytoplasm (Vaughan et al., 1967); normal methylation, therefore, would appear to be required for normal maturation of rRNA. The interior base methyl groups or 2′-*O*-methyl residues in mRNA could play a similar role in determining the secondary structure of nuclear pre-mRNA, and, in so doing, place the mRNA precursor in the appropriate conformation for the specific processing steps or provide specific recognition sites for processing enzymes. In the cytoplasm, a methylated 5′ terminus, whether blocked or not, could affect translation at the level of initiation. This seems possible, since the in vitro translation of methylated reovirus and vesicular stomatitis virus mRNA was elevated when compared with the corresponding nonmethylated mRNAs (Both et al., 1975). Although the positions of methylated nucleotides within the renal mRNA molecule are not known, their presence in mRNA may yield additional information in an in vivo model regarding the processing or translation of mRNA transcripts.

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References

Abraham, G., Rhodes, D. P., and Banerjee, A. K. (1975), *Cell 5,* 51.

Adams, J. M., and Cory, S. (1975), *Nature (London) 255,* 28.

Aviv, H., and Leder, P. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 1408.

Bernhardt, D., and Darnell, J. E. (1969), *J. Mol. Biol. 42,* 43.

Both, G. W., Banerjee, A. K., and Shatkin, A. J. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 1189.

Brawerman, G. (1974), *Annu. Rev. Biochem. 43,* 621.

Brown, G. M., and Attardi, G. (1965), *Biochem. Biophys. Res. Commun. 20,* 298.

Burdon, R. H., and Clason, A. E. (1969), *J. Mol. Biol. 39,* 113.

Choe, B. K., and Taylor, M. W. (1972), *Biochim. Biophys. Acta 272,* 275.

Darnell, J. E. (1968), *Bacteriol. Rev. 32,* 262.

Darnell, J. E., Jelinek, W. R., and Molloy, G. R. (1973), *Science 181,* 1215.

Desrosiers, R., Friderici, K., and Rottman, F. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 3971.

Desrosiers, R., Friderici, K., and Rottman, F. (1975), *Fed. Proc., Fed Am. Soc. Exp. Biol. 34,* 628.

Furuichi, Y. (1974), *Nucleic Acid Res. 1,* 809.

Furuichi, Y., Morgan, M., Muthukrishnan, S., and Shatkin, A. J. (1975a), *Proc. Natl. Acad. Sci. U.S.A. 72,* 362.

Furuichi, Y., Muthukrishnan, S., and Shatkin, A. J. (1975b), *Proc. Natl. Acad. Sci. U.S.A. 72,* 742.

Greenberg, H., and Penman, S. (1966), *J. Mol. Biol. 21,* 527.

Kumar, A., and Lindberg, U. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 681.

Maden, B. E. H., and Salim, M. (1974), *J. Mol. Biol. 88,*

133.
Maden, B. E. H., Salim, M., and Summers, D. F. (1972), *Nature (London), New Biol. 237,* 5.
Moyer, S. A., Abraham, G., Adler, R., and Banerjee, A. K. (1975), *Cell 5,* 59.
Muthukrishnan, S., Both, G. W., Furuichi, Y., and Shatkin, A. J. (1975), *Nature (London) 255,* 33.
Penman, S., Vesco, C., and Penman, M. (1968), *J. Mol. Biol. 34,* 49.
Perry, R. P., and Kelley, D. E. (1970), *J. Cell Physiol. 76,* 127.
Perry, R. P., and Kelley, D. E. (1974), *Cell 1,* 37.
Perry, R. P., Kelley, D. E., Friderici, K., and Rottman, F. (1975), *Cell 4,* 387.
Perry, R. P., Latorre, J., Kelley, D. E., and Greenberg, J. R. (1972), *Biochim. Biophys. Acta 262,* 220.
Priestley, G. C., and Malt, R. A. (1968), *J. Cell Biol. 41,* 886.
Rhodes, D. P., Moyer, S. A., and Banerjee, A. K. (1974), *Cell 3,* 327.
Rottman, F., Shatkin, A. J., and Perry, R. P. (1974), *Cell 3,* 197.
Shatkin, A. J. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 3204.
Singer, R. H., and Penman, S. (1973), *J. Mol. Biol. 78,* 321.
Slack, J. M. W., and Loening, U. E. (1974), *Eur. J. Biochem. 43,* 69.
Studier, W. F. (1965), *J. Mol. Biol. 11,* 373.
Tener, G. M. (1967), *Methods Enzymol. 12,* 398.
Vaughan, M. H., Soeiro, R., Warner, J. R., and Darnell, J. E. (1967), *Proc. Natl. Acad. Sci. U.S.A. 58,* 1527.
Wagner, E. K., Penman, S., and Ingram, V. M. (1967), *J. Mol. Biol. 29,* 371.
Wei, C. M., Gershowitz, A., and Moss, B. (1975), *Cell 4,* 379.
Wei, C. M., and Moss, B. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 3014.
Wei, C. M., and Moss, B. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 318.
Weinberg, R. A., Loening, U., Willems, M., and Penman, S. (1967), *Proc. Natl. Acad. Sci. U.S.A. 58,* 1088.
Weinberg, R. A., and Penman, S. (1968), *J. Mol. Biol. 38,* 289.
Weinberg, R. A., and Penman, S. (1970), *J. Mol. Biol. 47,* 169.

Characterization of Novikoff Hepatoma mRNA Methylation and Heterogeneity in the Methylated 5′ Terminus†

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ABSTRACT: KOH digestion of methyl-labeled poly(A)$^+$ mRNA purified by (dT)-cellulose chromatography produced mononucleotide and multiple peaks of a large oligonucleotide (−6 to −8 charge) when separated on the basis of charge by Pellionex-WAX high-speed liquid chromatography in 7 *M* urea. Heat denaturation of the RNA before application to (dT)-cellulose was required to release contaminants (mostly 18S rRNA) that persisted even after repeated binding to (dT)-cellulose at room temperature. Analysis of the purified poly(A)$^+$ mRNA by enzyme digestion, acid hydrolysis, and a variety of chromatographic techniques has shown that the mononucleotide (53%) is due entirely to N^6-methyladenosine. The large oligonucleotides (47%) were found to contain 7-methylguanosine and the 2′-*O*-methyl derivatives of all four nucleosides. No radioactivity was found associated with the poly(A) segment. Periodate oxidation of the mRNA followed by β elimination released only labeled 7-methylguanine consistent with a blocked 5′ terminus containing an unusual 5′–5′ bond. Alkaline phosphatase treatment of intact mRNA had no effect on the migration of the KOH produced oligonucleotides on Pellionex-WAX. When RNA from which 7-methylguanine was removed by β elimination was used for the phosphatase treatment, distinct dinucleotides (NmpNp) and trinucleotides (NmpNmpNp) occurred after KOH hydrolysis and Pellionex-WAX chromatography. Thus Novikoff hepatoma poly(A)$^+$ mRNA molecules can contain either one or two 2′-*O*-methylnucleotides linked by a 5′–5′ bond to a terminal 7-methylguanosine and the 2′-*O*-methylation can occur with any of the four nucleotides. The 5′ terminus may be represented by $m^7G^{5'}ppp^{5'}$ $(Nmp)_{1or2}Np$, a general structure proposed earlier as a possible 5′ terminus for all eucaryotic mRNA molecules (Rottman, F., Shatkin, A., and Perry, R. (1974), *Cell 3,* 197). The composition analyses indicate that there are 3.0 N^6-methyladenosine residues, 1.0 7-methylguanosine residue, and 1.7 2′-*O*-methylnucleoside residues per average mRNA molecule.

Only in recent years has considerable understanding of the structure and composition of eucaryotic mRNA been obtained. The existence of a poly(A)[1] tract not only accounts for a portion of the large untranslated region but also has greatly facilitated the isolation of pure mRNA from cells. More recently, poly(A)$^+$ mRNA has been found

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[1] Abbreviations used are: poly(A), poly(adenylic acid); Am, 2′-*O*-methyladenosine; Gm, 2′-*O*-methylguanosine; m^7G, 7-methylguanosine; m^6A, 6-methyladenosine; poly(A)$^+$ mRNA, mRNA containing poly(A); poly(A)$^-$ RNA, RNA lacking poly(A).