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Identification of Methylated Nucleosides in Messenger RNA from Novikoff Hepatoma Cells

(RNA methylation/RNA processing/methylnucleoside composition)

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ABSTRACT **The poly(A) tract found in eukaryotic mRNA was used to study methylation in mRNA obtained from Novikoff hepatoma cells. Methyl labeling of RNA was achieved with L-[*methyl*-^{3}H]methionine under conditions that suppress radioactive incorporation into the purine ring. RNA that contains a poly(A) segment was obtained from polysomal RNA by chromatography on oligo(dT)-cellulose. Sucrose density gradient centrifugation of this RNA revealed a pattern expected for mRNA. The composition of the methyl-labeled nucleosides in the RNA was analyzed after complete enzymatic degradation to nucleosides. By use of DEAE-cellulose (borate) chromatography, which separates 2′-*O*-methylnucleosides from normal and base-methylated nucleosides, about 50% of the radioactivity was recovered in the 2′-*O*-methylnucleoside fraction and 50% in the base-methylnucleoside fraction. High-speed liquid chromatography (Aminex A-5) of the 2′-*O*-methylnucleoside fraction produced four peaks coincident with the four 2′-*O*-methylnucleoside standards. Analysis of the base-methylnucleoside fraction revealed a unique pattern. While ribosomal RNA and tRNA possessed complex base-methylnucleoside patterns, the distribution in mRNA was quite simple, consisting predominantly of N^6-methyladenosine. These results demonstrate a unique distribution of methylated nucleosides in mRNA. By analogy to ribosomal RNA synthesis, the presence of methylnucleosides in mRNA may reflect a cellular mechanism for the selective processing of certain mRNA sequences.**

The important role of post-transcriptional modification of RNA in the synthesis of eukaryotic RNA has become increasingly apparent. One of the most striking features of ribosomal RNA (rRNA) synthesis is the specific methylation that occurs on the 45S precursor RNA molecule (1). Although the maturation process is nonconservative (about 50% of the original molecule is lost through degradation), the conserved regions present in the final 28S and 18S products retain all of the methylated sequences (2). Methylnucleosides, containing both base-methyl and 2′-*O*-methylnucleosides, account for 1.5% of the total nucleosides in the mature RNA (3). Methylation of 45S RNA does not occur in the absence of methionine, and mature ribosomal 28S and 18S RNA is not formed (4). Methylation is thus required for proper rRNA processing.

More recently, mRNA of eukaryotic cells has been found to undergo an unusual form of post-transcriptional modification, involving the addition of a sequence of about 200 adenosine residues to the 3′-end (5). Studies on its function are still inconclusive, but several findings can be cited outlining its importance (5–7). Like methylation in rRNA, adenylation seems to play an essential role in the metabolism of mRNA molecules.

Since post-transcriptional events may represent an important control mechanism in the regulation of genetic expression, we have investigated the possibility of methylation as an additional post-transcriptional modification of mRNA. Earlier work with bacterial and phage mRNA produced strong evidence for the essential absence of methylation in these systems, being no higher than one per 3500 nucleotides (8). Other early studies with mammalian heterogeneous nuclear RNA (HnRNA) indicated that methylation was either nonexistent or very low (9, 10). The discovery of poly(A) has now made it possible to obtain pure mRNA fractions through affinity chromatography and, therefore, to search for low levels of methylation in mRNA without interference from rRNA contamination. Recently Perry and Kelley reported the existence of methylation in mouse L cell mRNA at about one-sixth the level found in rRNA, and both base and ribose methylations were found (11). This paper reports the existence of methylated nucleosides in the mRNA of Novikoff hepatoma cells and identifies the unique distribution of methylated moieties. A preliminary report of these results has appeared elsewhere (12).

METHODS

Cell Culture and Labeling. The N1S1 strain of Novikoff hepatoma cells was grown in culture in Swimm's S-77 medium supplemented with 4 mM glutamine and 10% (v/v) dialyzed calf serum (13). The cells were grown in an atmosphere of 5% CO_2 in air in sealed, screw-cap Erlenmeyer flasks with a doubling time of about 12 hr at 37°.

For labeling with L-[*methyl*-^{3}H]methionine, cells in midlogarithmic growth phase were pelleted aseptically and resuspended in fresh warm medium containing 0.02 mM methionine (one-fifth the normal concentration) at a cell concentration of about 1.5×10^6/ml. Labeling was performed for 3 hr in the presence of 20 mM sodium formate and 20 μM each of adenosine and guanosine; these conditions have been shown to effectively suppress nonmethyl purine ring labeling via the H_4-folate pathway (14).

Cell Fractionation and RNA Preparation. Cells were poured over frozen crushed saline solution and harvested by centrifugation. The cells were washed once and disrupted by Dounce homogenization in 10 mM Tris·HCl (pH 7.4), 10 mM NaCl,

Abbreviations: Poly(A)(+)RNA, RNA that contains a poly(A) segment; poly(A)(−)RNA, RNA that does not contain a poly(A) segment: HnRNA, heterogeneous nuclear RNA; N_m, 2′-*O*-methylnucleoside; rRNA, ribosomal RNA.

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1.5 mM $MgCl_2$. Nuclei were removed by centrifugation at 800 × *g* for 2 min and mitochondria at 10,000 × *g* for 7 min. The cytoplasmic supernatant was centrifuged through a 10.5-ml, 15–45% sucrose gradient in Tris·HCl–NaCl–$MgCl_2$ for isolation of polysomes. The polysome fraction was made 0.1 M in NaCl, 0.01 M in EDTA, and 0.5% in sodium dodecyl sulfate. Carrier rRNA was added (0.25 mg per ml of solution), and the RNA was collected by ethanol precipitation.

After centrifugation, the RNA pellet was taken up in dodecyl sulfate buffer [10 mM Tris·HCl (pH 7.4), 0.1 M NaCl, 10 mM EDTA, and 0.5% sodium dodecyl sulfate] and incubated at room temperature for 10 min after addition of a small crystal of proteinase K (about 0.3 mg per 10^8 cells) (15). From this point, the RNA extraction procedure followed the technique described by Singer and Penman (16). The final aqueous phase was removed, and the RNA was precipitated with 2 volumes of absolute ethanol.

Oligo(dT)–Cellulose Chromatography. RNA not containing a poly(A) segment [poly(A)(−)RNA] and RNA containing a poly(A) segment [poly(A)(+)RNA] were isolated by oligo-(dT)–cellulose chromatography. The procedure used was essentially that described by Aviv and Leder (17), except that nonadsorbed material was eluted by continued washing with 0.12 M NaCl, 10 mM Tris·HCl (pH 7.4), 1 mM EDTA, and 0.2% sodium dodecyl sulfate. The material retained was then eluted by the same buffer lacking NaCl. Carrier rRNA was used for ethanol precipitation, and the poly(A)(+)RNA was further purified by a second passage through oligo(dT)–cellulose. Over 90% of the radioactivity in the second application was retained by the column.

Poly(A)(−)RNA Fractionation. The poly(A)(−)RNA was fractionated into a 4S fraction and a 28S + 18S fraction by sedimentation through sucrose gradients. Further analysis of the 4S RNA fraction required deacylation of [^{3}H]peptidyl and [^{3}H]methionyl tRNA by incubation in 1.8 M Tris·HCl (pH 8.1) at 37° for 100 min.

Enzymatic Degradation of RNA and Resolution of Nucleosides. The detailed method for degradation of RNA to nucleosides and the subsequent quantitative analysis of methylnucleosides is described elsewhere (18). In brief, RNA samples were completely hydrolyzed to the nucleoside level by simultaneous treatment with alkaline phosphatase, pancreatic ribonuclease A, and phosphodiesterase I. Completeness of the reaction was monitored by paper electrophoresis.

DEAE–cellulose in the borate form was used to chromatographically separate those nucleosides blocked at the 2′-*O*-position with a methyl group, from the remainder of the normal and base-substituted nucleosides. The 2′-*O*-methylnucleosides are not retained and elute with 0.15 M boric acid just after the void volume. The base-methyl radioactivity is recovered with the normal ribonucleosides by elution with 0.7 M boric acid. Each nucleoside fraction was taken to dryness by flash evaporation, and the boric acid was removed as its methyl ester by successive flash evaporations with absolute methanol (18).

The 2′-*O*-methylnucleosides present in the first DEAE–cellulose (borate) fraction were separated by high-speed liquid chromatography (18). The base-methylnucleoside distribution was determined with the same resin, but elution was with the buffer described for normal nucleoside resolution (18). In this case, the UV absorbance peaks corresponded to the four normal ribonucleosides plus any base-methyl standards added for reference.

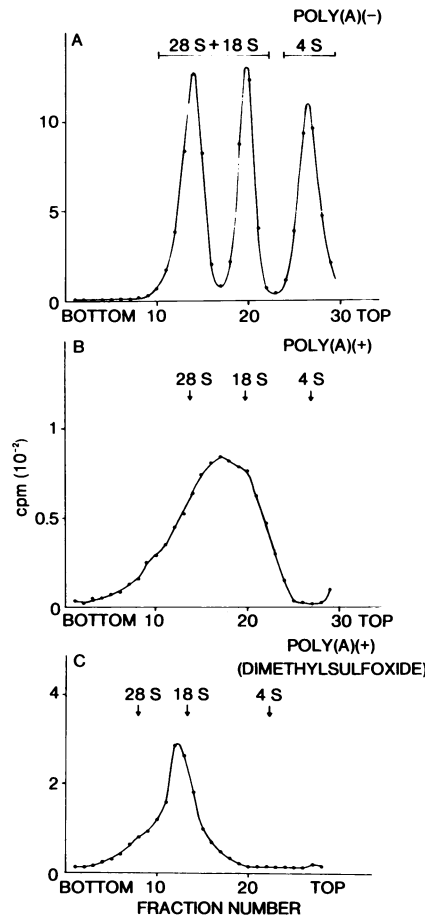

FIG. 1. Sucrose density gradient sedimentation of methyl-labeled polysomal RNA. (A) An aliquot of poly(A)(−)RNA obtained from oligo(dT)–cellulose was layered over a 4.8-ml 5–20% sucrose gradient [10 mM Tris·HCl (pH 7.4) and 5 mM EDTA] and centrifuged at 4° for 160 min at 45,000 rpm in a SW 50.1 rotor. The fractions containing 28S + 18S RNA (indicated by the horizontal bar) were pooled for ethanol precipitation. The fractions containing 4S RNA were also pooled. (B) An aliquot of poly(A)(+)RNA was sedimented as described above. (C) Dimethylsulfoxide–sucrose gradient sedimentation of poly(A)(+)-RNA. Poly(A)(+)RNA (5 μl) was mixed with 100 μl of 99% dimethylsulfoxide (10 mM LiCl, 1 mM EDTA) and heated to 60° for 2 min. All of this material was layered over 4.8 ml of a 5–20% sucrose gradient in 99% dimethylsulfoxide (10 mM LiCl, 1 mM EDTA) (19). Centrifugation was for 15 hr at 27° and 45,000 rpm in a SW 50.1 rotor.

RESULTS

Preparation of RNA Fractions. Prior to the characterization of methyl-labeled components in mRNA, it is necessary to carefully document that the methylated RNA being examined is mRNA and is not contaminated with other cellular RNA species. The sucrose density gradient sedimentation profiles of the methyl-labeled poly(A)(−) and poly(A)(+)RNA are shown in Fig. 1. The purification of the poly(A)(+)RNA (mRNA) involved repeated binding of the RNA sample to oligo(dT)–cellulose. Fig. 1B shows the characteristic heterogeneous sedimentation profile of mRNA, with the peak of

TABLE 1. *Recovery of labeled methylnucleosides from DEAE-cellulose (borate)**

RNA species	% of total radioactivity	
	Base-methyl-nucleosides	2′-*O*-Methyl-nucleosides
28 S + 18 S	11	89
4 S	55	25
mRNA	50	50

* Each RNA fraction, containing 1.2 mg of carrier RNA, was hydrolyzed at 37° and pH 9.0 for 34 hr with 1 unit of alkaline phosphatase, 10 μg of ribonuclease A, and 0.22 units of phosphodiester I per mg of RNA. The nucleosides produced were then resolved into a 2′-*O*-methylnucleoside fraction and a base-methylnucleoside fraction on a 6 × 120-mm DEAE-cellulose (borate) column.

radioactivity sedimenting slightly faster than 18 S. The mRNA profile obtained with L-[*methyl*-^{3}H]methionine label is virtually identical to that obtained when [^{3}H]uridine is used for the label (data not shown) and is very similar to uridine-labeled mRNA profiles obtained for HeLa and L cells by others (11, 16). The [^{3}H]methyl radioactivity recovered in mRNA generally represented 3.5–4.0% of the radioactivity recovered in 28S + 18S RNA in the 3-hr labeling used here. This level is considerably lower than that obtained when a 3-hr uridine label is used. This result suggests mRNA methylation is considerably less than rRNA methylation (as percent of total nucleotides), in agreement with the more quantitative determinations of Perry and Kelley (11). To insure that the radioactivity seen in mRNA is not due to adventitious binding of smaller, methionine-labeled RNA fragments, the labeled mRNA was analyzed on denaturing dimethylsulfoxide–sucrose gradients (19). As shown in Fig. 1C, most of the RNA remained larger than 18 S.

The region of 28S + 18S rRNA and of 4S RNA indicated in Fig. 1A were pooled for further methylnucleoside analysis to compare with the mRNA. After the 4S region was pooled, the tRNA was deacylated as described in *Materials and Methods*, prior to further analysis.

Separation of Base-Methylnucleosides from 2′-O-Methylnucleosides. To determine the proportion of methyl groups attached to the base moiety as opposed to those attached to the ribose moiety, we completely degraded each RNA fraction enzymatically to the nucleoside level; the nucleosides were then separated by DEAE-cellulose (borate) chromatography into a 2′-*O*-methylnucleoside fraction and a base-methylnucleoside fraction. Table 1 shows the recoveries in the 2′-*O*-methylnucleoside and the base-methylnucleoside fractions as a percent of the radioactivity present in the original RNA sample. While rRNA methylation occurs predominantly on the ribose moiety, and 4S RNA methylation occurs predominantly on the bases, the mRNA is intermediate, with about 50% being 2′-*O*-methylation. Note that essentially complete recovery of radioactivity was obtained for the labeled mRNA and rRNA. Only about 80% of the 4S RNA radioactivity could be recovered, however, from the DEAE-cellulose (borate) column. This incomplete recovery was observed only with 4S RNA and does not interfere with the further characterization of the mRNA, since complete recovery was obtained with this fraction.

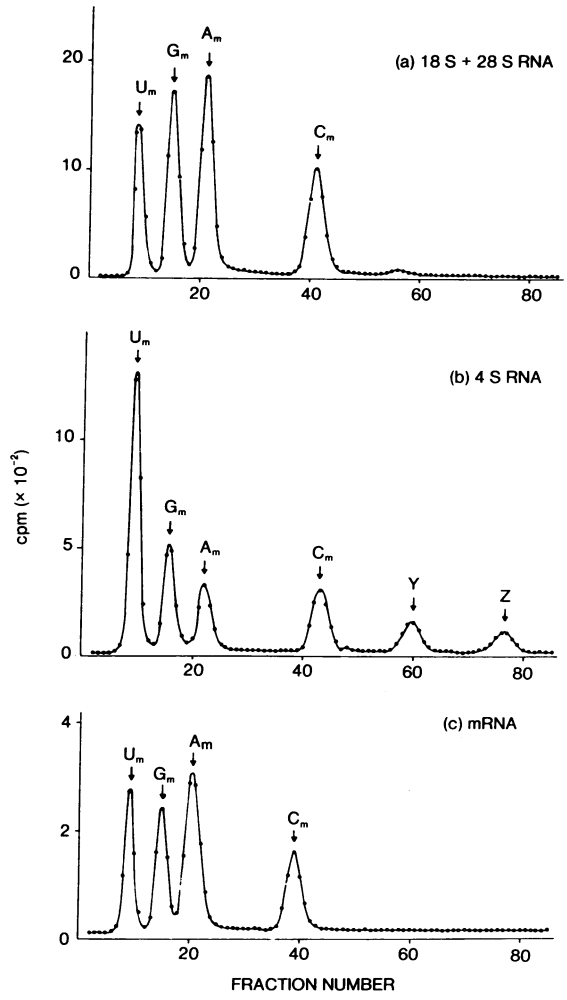

FIG. 2. High-speed liquid chromatography of 2′-*O*-methylnucleoside fraction. Twenty microliters of each 2′-*O*-methylnucleoside fraction, dissolved in 0.4 M ammonium formate in 40% ethylene glycol adjusted to pH 4.25 with formic acid, were injected onto a high-speed liquid chromatography column. The column was developed at 31.5° and 2500 lbs./inch^2. The flow rate was 0.4 ml/min, and 0.5-ml fractions were collected. (a) 28S + 18S RNA; (b) 4S RNA; (c) mRNA.

2′-O-Methylnucleoside Distribution. The ribose methylated fraction for each of the RNA classes studied was further analyzed by high-speed liquid chromatography (Fig. 2). The separation of 2′-*O*-methylnucleosides derived from mRNA (Fig. 2*c*) revealed the presence of all four 2′-*O*-methylnucleosides also found in rRNA (Fig. 2*a*). The tRNA 2′-*O*-methylnucleoside distribution depicted in Fig. 2*b* contains two additional peaks. These additional peaks represent 2′-*O*-methylnucleosides that contain some other modification.

This radioactivity present in each particular 2′-*O*-methylnucleoside peak may be used to determine the percent of each labeled 2′-*O*-methylnucleoside in each class of RNA. As shown in Table 2, the 2′-*O*-methylnucleoside distribution of mRNA differed considerably from that found for 4S RNA. The 2′-*O*-methylnucleoside distribution of mRNA was quite similar, however, to that found for rRNA.

This similarity in distribution contrasts with the different sizes of oligonucleotides produced by alkaline digestion. Alkaline digestion of rRNA produces primarily mono-

TABLE 2. *Radioactive 2'-O-methylnucleoside composition**

	% of total radioactivity		
	28S + 18S RNA	mRNA	4S RNA
U_m	18.0	21.9	40.0
A_m	33.4	35.0	14.0
G_m	26.8	23.2	19.2
C_m	21.5	19.9	11.9
Y	0	0	8.9
Z	0	0	6.1

* The radioactivity present in each of the peaks shown in Fig. 2 is presented as percent of the total radioactivity recovered from high-speed liquid chromatography.

nucleotides and small amounts of dinucleotides, due to the alkaline stability of phosphodiester bonds adjacent to 2'-*O*-methyl groups. In studies not shown, reversed phase chromatography was used to compare the sizes of alkaline stable oligonucleotides produced by NaOH digestion of methyl-labeled mRNA and rRNA. While almost all the alkaline-stable radioactivity of rRNA eluted in the dinucleotide region, a large portion of the mRNA alkaline-stable radioactivity eluted considerably past the dinucleotide region. This finding indicates that some of the methyl groups occur as adjacent 2'-*O*-methylnucleotides or in some species containing multiple phosphates.

Base-Methylnucleoside Distribution. The base-methylnucleoside distribution in mRNA is distinctly different from that of either 4S RNA or rRNA. The separation of 28S + 18S RNA base-methylnucleosides by Aminex A-5 high-speed liquid chromatography is shown in Fig. 3*a*. It is significant that essentially no radioactivity appears coincident with the unmodified nucleosides, guanosine and adenosine (Fig. 3*a*, markers F and G, respectively). This result indicates that non-methyl purine labeling has indeed been effectively suppressed and all the radioactivity seen in RNA is, in fact, due to methylation. The primary peaks of 28S and 18S base-methylnucleosides correspond to N^6-methyladenosine, N^6-dimethyladenosine, 5-methylcytidine, and material that migrates near the solvent front (primarily modified uridine). This finding corresponds well with observations made by others with HeLa cell RNA using acid hydrolysis techniques, except for the absence of 1-methyladenosine and 7-methylguanosine (20, 21). 1-Methyladenosine is relatively unstable at neutral and alkaline pH (22) and is readily converted, under the incubation conditions used here to generate nucleosides, to N^6-methyladenosine. Thus, the peak of N^6-methyladenosine radioactivity contains an unknown contribution from 1-methyladenosine. 7-Methylguanosine is also relatively unstable (22) and is converted under our hydrolysis conditions to a product that migrates near the solvent front on high-speed liquid chromatography.

Also in agreement with studies using HeLa cells (20, 21), the 4S RNA does not contain much N^6-dimethyladenosine but does contain the unique nucleosides, 1-methylguanosine, N^2-methylguanosine, and N^2-dimethylguanosine (Fig. 3*b*). Peaks of radioactivity also occur coincident with 5-methylcytidine, with N^6-methyladenosine, and with the methylated uridines. Klagsbrun has reported no N^6-methyladenosine for 4S RNA in HeLa cells (20), so in this case the peak at N^6-methyladenosine may be due entirely to 1-methyladenosine.

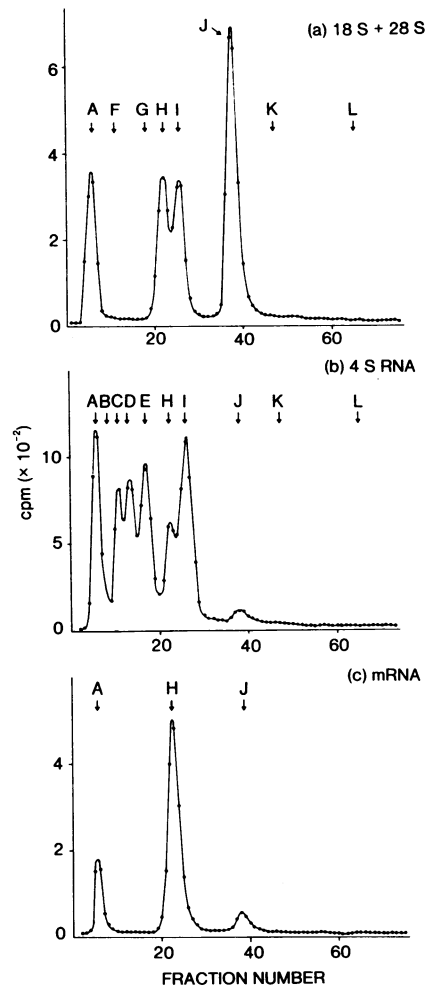

FIG. 3. High-speed liquid chromatography of base-methylnucleoside fraction. Two hundred fifty microliters of each base-methylnucleoside fraction, dissolved in H_2O, were injected onto a high-speed liquid chromatography column. The column was developed at 2500 lbs./inch² at 31.5°. The flow rate was 0.8 ml/min, and 0.67-ml fractions were collected. At fraction 35, the volume collected per fraction was changed to 1.35 ml. The letters correspond to the location of the following standards: A, 3-methyluridine, thymine riboside, and uridine; B, 1-methylinosine; C, 1-methylguanosine; D, N^2-dimethylguanosine; E, N^2-methylguanosine; F, guanosine; G, adenosine and N^4-methylcytidine; H, N^6-methyladenosine; I, 5-methylcytidine; J, N^6-dimethyladenosine; K, 1-methyladenosine; L, 7-methylguanosine. (a) 28S + 18S RNA; (b) 4S RNA; (c) mRNA.

The base methyl distribution for mRNA is strikingly simple (Fig. 3*c*). About 80% of the radioactivity elutes with N^6-methyladenosine, indicating that base methylation of mRNA is primarily N^6-methyladenosine and/or 1-methyladenosine. Small peaks of radioactivity also occur near the solvent front (about 15%) and coincident with N^6-dimethyladenosine (about 5%).

DISCUSSION

Several lines of evidence presented in this paper demonstrate that the radioactivity recovered in the mRNA fraction is indeed due to methylation of mRNA. The RNA has been passed twice through oligo(dT)–cellulose columns and is apparently free of rRNA and tRNA contamination. The incorporated

radioactivity is only in methylated species, and the amount of radioactivity found in normal adenosine and guanosine is below detectable levels. The distribution of radioactive methyl groups between base and ribose moieties and the base-methylnucleoside distribution are distinctly different for mRNA when compared to rRNA or tRNA labeled under identical conditions. Furthermore, the pattern of alkaline-stable oligonucleotides is different in mRNA. Thus, not only does mRNA contain methylated species, but the distribution of the methylated species is unique.

The distribution of radioactivity between base and ribose groups for rRNA determined here agrees well with values obtained for HeLa cell RNA by others, using alkaline digestion (23, 24). Using acid hydrolysis techniques, the percentage of ribose methylation observed by others is considerably less (40–50%) (20, 21). It is most likely that acid treatment resulted in this lower estimate of 2′-*O*-methylation due to release of 2′-*O*-methyl groups as methanol (25, 26). Complete recovery of the rRNA radioactivity applied to the DEAE–cellulose (borate) column strengthens the value for ribose methylation reported here (89%). Since 100% of the mRNA radioactivity was recovered, we probably have observed all the methylated mRNA nucleosides labeled under these conditions.

Although the role of 2′-*O*-methylation in rRNA processing is not clearly understood, its presence does seem necessary for the proper processing of the large 45S precursor (4). 2′-*O*-Methyl groups are known to alter the secondary structure of RNA (27) and, thus, may be involved in conferring the proper structure for the precise cleavages that occur. The presence of 2′-*O*-methylnucleotides stabilizes synthetic RNA molecules against hydrolysis by a 3′-OH-exoribonuclease thought to be involved with rRNA processing (28). As suggested by these studies *in vitro*, a 2′-*O*-methylnucleotide conceivably could function as a stop signal for a processing exonuclease. A 2′-*O*-methylnucleotide conceivably could also function as a recognition site for an endonuclease. Similar mechanisms may be involved in mRNA precursor processing, if indeed methylation occurs at the precursor stage.

The processing of mRNA found in eukaryotes has the additional interesting feature of being a potentially important control point in gene expression. If such control does in fact occur, as some hybridization studies indicate (29), we are faced with the problem of identifying the means by which certain messenger sequences are selected. The addition of poly(A) may not be sufficient to define which mRNA sequences are to be transported, since all poly(A) synthesized in the nucleus does not exit to the cytoplasm as part of functional messenger RNA (30). Methylation could function in such a selection process.

The labeled mRNA observed here most probably represents many different species of messenger. It is interesting, then, that the base methylation should occur primarily with adenine. Calculations based on the level of methylation determined by Perry and Kelley (11) and the distribution of methylation described in these studies, as well as the average size of mRNA, indicate that there is sufficient methylated adenosine for each messenger molecule to contain this modified nucleoside at least once. The presence of this base-methylnucleoside in all mRNA molecules would suggest some essential function for the modification. The existence of a poly(A) tract in mRNA molecules raises the possibility that the base modification occurs in this segment.

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