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Existence of Methylated Messenger RNA in Mouse L Cells

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Summary

Messenger RNA of mouse L cells is methylated in both base and ribose moieties. On the average there are about 2.2 methyl groups per 1000 nucleotides in mRNA, a proportion which is about one-sixth that of mammalian ribosomal RNA. The variety of methylated bases in mRNA is more limited than in ribosomal RNA. A very low level of methylation is detected in heterogeneous nuclear RNA, suggesting that methylation, like polyadenylation, may constitute a post-transcriptional modification of messenger RNA precursor in eucaryotic cells.

Introduction

It has in general been thought that methylation of specific nucleotides in RNA is a phenomenon peculiar to the ribosomal and transfer RNA species (Srinivasan and Borek, 1966; Starr and Sells, 1969). The notion that messenger RNA is not methylated stems principally from two sources: (1) a rigorous demonstration by Moore (1966) that both phage-directed and bacterial mRNAs contain less than one methyl group per 3500 nucleotides; and (2) an observation made with several types of eucaryotic cells that incubation with methyl-labeled methionine results in heavy labeling of precursors of rRNA [pre-rRNA] and little or undetectable labeling of the heterogeneous nuclear RNA species which presumably contain the precursor to mRNA (Brown and Attardi, 1965; Greenberg and Penman, 1966; Perry et al., 1970; Johnson, 1970).

Until recently, it has been very difficult to isolate eucaryotic mRNA sufficiently free of rRNA contamination to determine whether it might contain some methylated nucleotides, albeit in smaller proportion than that characterizing rRNA and tRNA. However, the development of isolation methods based on the poly A content of eucaryotic mRNA (Nakazato and Edmonds, 1972; Brawerman, Mendecki, and Lee, 1972; Sheldon, Jurale, and Kates, 1972) now allows us to obtain mRNA of sufficient purity to reexamine this question. We have found that the mRNA of mouse L cells is indeed methylated in both base and ribose moieties to the extent of about 2 to 3 methyl groups per 1000 nucleotides. This provides evidence for another post-transcriptional modification of eucaryotic mRNA in addition to polyadenylation.

Results

Polyribosomal RNA was extracted from various samples of cells labeled for 4 hr with ^{3}H methyl methionine and ^{14}C-uridine, and resolved into a messenger fraction containing polyadenylate [poly A(+)mRNA] and a nonadenylated fraction (predominantly ribosomal RNA) by chromatography on poly T-cellulose. The $^{3}H/^{14}C$ ratio of the poly A(+)mRNA (Table 1) was measured directly (experiments 1 and 2), and also after a further step of purification by adsorption to glass fiber filters impregnated with polyuridylate [poly U-GFC filters] (experiment 3). These ratios were compared with those of the nonadenylated RNA or of ribosomal RNA further purified by sucrose gradient sedimen-

Table 1. Relative Incorporation of ^{3}H-methyl Methionine and ^{14}C-uridine into Ribosomal and Poly A(+)mRNA

RNA species	^{3}H cpm	^{14}C cpm	$^{3}H/^{14}C$
Experiment 1			
ribosomal RNA	26,626	12,993	2.06
poly A(+)mRNA	888	2,374	0.37
Experiment 2			
ribosomal RNA	37,249	6,524	5.7
poly A(+)mRNA	891	961	0.93
poly A(+)mRNA (low actinomycin)	237	131	1.8
Experiment 3			
poly A(−)RNA (mainly rRNA)	1,547	1,376	1.12
poly A(+)mRNA (1X dT cellulose)	36	204	0.18
poly A(+)mRNA (2X dT cellulose)	45	250	0.18

Experiments 1 and 2: Polyribosomal RNA was separated on a poly T-cellulose column into poly A(+) and poly A(−) fractions. The poly A(+) fraction was concentrated by ethanol precipitation, and designated "poly A(+)mRNA." The poly A(−) fraction was run on a 5–25% sucrose gradient, and the fractions containing 28S and 18S rRNA pooled, concentrated by ethanol precipitation, and designated "ribosomal RNA." Radioactivity was measured in a scintillant containing 3% Protosol. In experiment 2, the sample labeled "low actinomycin" was from cells incubated with 0.08 μg actinomycin D per ml for 1 hr prior to labeling, and during the 4 hr labeling period.

Experiment 3: Polyribosomal RNA was fractionated on a poly T-cellulose column as in experiment 1. The poly A(−) fraction was concentrated by ethanol precipitation, precipitated with trichloroacetic acid and plated on a GFC filter. Over 95% of the ^{14}C and 82% of the ^{3}H radioactivity in this fraction is in ribosomal RNA, as verified by sucrose gradient analysis. An aliquot of the poly A(+) fraction was adsorbed to a poly U-GFC filter ("1X dT-cellulose" assay) and the remainder rechromatographed on a second dT cellulose column. The rechromatographed poly A(+) fraction was concentrated by ethanol precipitation and adsorbed to a poly U-GFC filter ("2X dT-cellulose" assay). The filter pads were counted for 200 min in a toluene based scintillant.

The absolute $^{3}H/^{14}C$ ratios in the three experiments are not the same because of a difference in the specific activity of the ^{14}C uridine used for labeling and a 60% higher ^{3}H counting efficiency in the Protosol system.

tation. The $^{3}H/^{14}C$ ratios (Table 1) indicate a measurable level of ^{3}H methyl incorporation in the poly A(+)mRNA, amounting to about one-sixth that of rRNA.

Two kinds of evidence indicate that the ^{3}H counts in the mRNA fraction are not due to a contaminant of rRNA. First, a sample of poly A(+)mRNA from cells labeled in the presence of a low dose of actinomycin D, which selectively suppresses rRNA synthesis (Perry and Kelley, 1970), did not show a reduced ratio of $^{3}H/^{14}C$. In fact, this mRNA had a somewhat increased $^{3}H/^{14}C$ ratio, possibly due to the increased availability of labeled methyl groups in the actinomycin-treated cells or to a greater effect of the drug on transcription than on methylation. Second, poly A(+)mRNA purified by both poly T-cellulose chromatography and adsorption to poly U-GFC filters (experiment 3) gave essentially the same ratio relative to rRNA as that found with samples purified by poly T-cellulose alone. Moreover, after rechromatography on a second poly T-cellulose column the samples displayed a $^{3}H/^{14}C$ ratio identical to that of the original poly A(+)mRNA sample.

Further evidence indicating that the ^{3}H activity is indeed associated with the poly A(+)mRNA comes from an electrophoretic analysis of the RNA on polyacrylamide gels (Figure 1). The ^{3}H to ^{14}C ratio is relatively constant over the entire range of mRNA mobilities and there are no prominent ^{3}H peaks in the region of the 28S and 18S rRNA components. The relative similarity in ^{3}H and ^{14}C profiles suggests a rather uniform degree of methylation in the various mRNA species.

A similar gel-electrophoretic analysis of the low molecular weight RNA components (Figure 2) revealed substantial methylation of tRNA, but no detectable methylation of 5S RNA. Since the 5S component is known not to contain any methyl groups (Forget and Weissman, 1967), this latter negative finding provides good evidence that under our labeling conditions there is no incorporation of the methionine methyl group into the purine rings.

We also examined the incorporation of ^{3}H into polyadenylated and nonadenylated HnRNA. Mea-

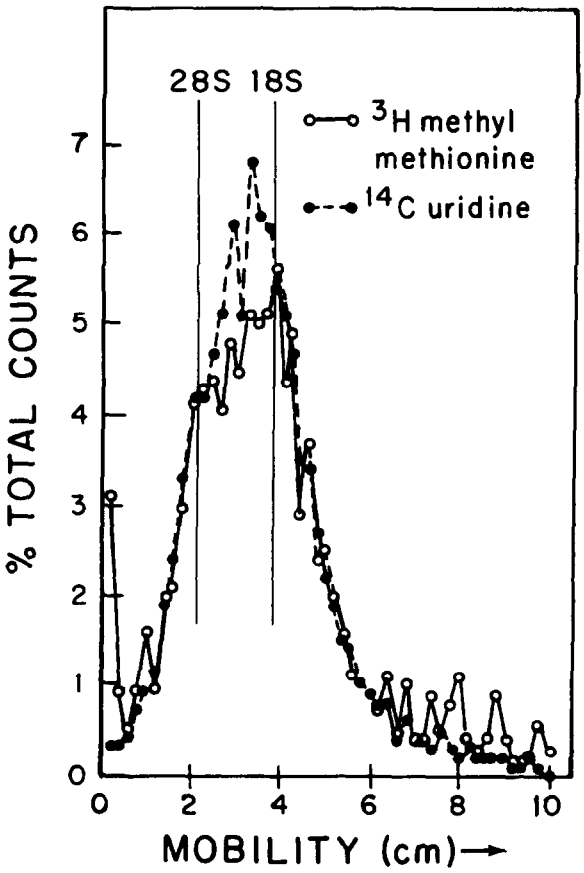

Figure 1. Analysis by Polyacrylamide-Gel Electrophoresis of Poly A(+)mRNA from Cells Labeled with ^{3}H Methyl Methionine and ^{14}C Uridine

Poly A(+)mRNA was purified from polyribosomal RNA by dT-cellulose chromatography, concentrated by ethanol precipitation, and submitted to electrophoresis for 2.5 hr on 2.7% polyacrylamide at 5 mA/gel.

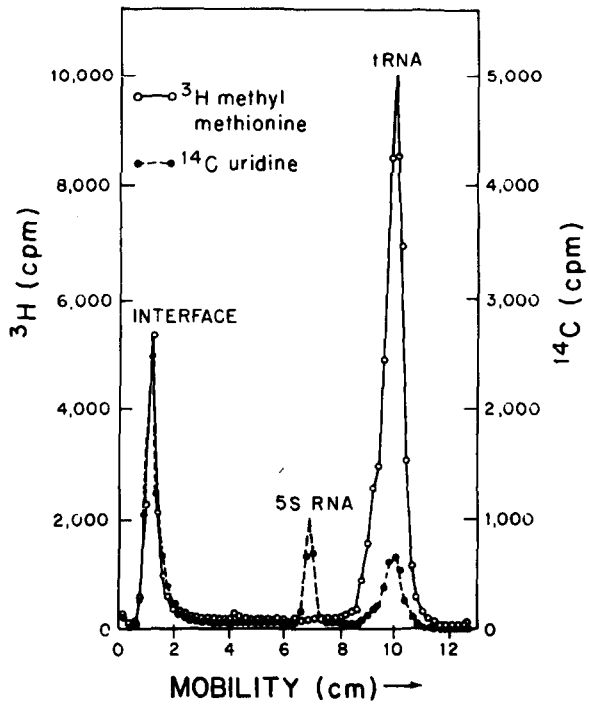

Figure 2. Analysis by Polyacrylamide-Gel Electrophoresis of Low Molecular Weight RNA from Cells Labeled with ^{3}H Methyl Methionine and ^{14}C Uridine.

Polyribosomal RNA was chromatographed on dT-cellulose, and the poly A(−) fraction sedimented on a 5–25% (w/w) sucrose gradient. The low molecular weight fraction (4–8S) was pooled, concentrated by ethanol precipitation and submitted to electrophoresis for 7.5 hr at 5 mA/gel on a 14% polyacrylamide gel overlaid with a 2.7% gel. The electrophoresis buffer was changed after 4 hr to preclude a pH front.

surements of components with estimated molecular weights between 5 and 10 million, that is those which migrate slower than the 45S pre-rRNA component on polyacrylamide gels, indicated a very small, but still detectable level of methylation, the nonadenylated HnRNA possibly having an even lower proportion of methyl label than the poly A(+)HnRNA [the heterogeneous nuclear RNA containing poly A].

The results of the measurements on the various types of RNA are summarized in Table 2. To allow comparison and averaging among several experiments, we expressed the $^{3}H/^{14}C$ ratios of each RNA component as a multiple of the ratio characteristic of 28S and 18S rRNA from the same experiment, measured under the same conditions. Expressed in this way, the ratio for poly A(+)mRNA exhibited excellent reproducibility among different experiments. From such relative ratios one can make an estimate of the absolute levels of methylation using the known degree of methylation of the rRNA components (see below).

We next investigated the approximate extent of base and ribose methylation in poly A(+)mRNA by submitting the RNA to alkaline hydrolysis, and analyzing the nucleotide product by chromatography on DEAE-Sephadex-urea columns (Tener, 1967). For comparison, a similar analysis was made with rRNA. To a first approximation, the relative proportions of ^{3}H-labeled mononucleotides and alkaline-resistant dinucleotides may be considered as an indicator of the relative amounts of base and ribose methylation, respectively. The error in such determinations, due to the alkaline resistance of phosphodiester bonds adjacent to certain of the methylated bases, leads to an overestimation of the relative proportion of 2′-O methyl groups (Nichols and Lane, 1966). The results, shown in Figure 3, indicate that there is methylation of both base and ribose moieties in mRNA. The relative proportion of base methylation in mRNA appears to be greater than in rRNA. However, whereas essentially all of the rRNA hydrolysis products are eluted from the column between 0 and 0.25 M NaCl, only about half of the mRNA products are eluted in this range of salt concentrations. The remaining radioactive material, which could be eluted by washing the column with 0.3 M NaCl, and which could possibly contain larger oligonucleotides, has not yet been fully characterized.

In order to get an idea of the diversity of methylated bases in mRNA, the mononucleotides were analyzed by a thin layer chromatographic procedure employing polyethyleneimine cellulose (Figure 4). As expected, the ^{14}C radioactivity appears in both uridylic and cytidylic acid, because of the intracellular amination of the ^{14}C labeled uridine precursor. In the rRNA sample the ^{3}H radioactivity has a rather heterogeneous distribution as would be predicted from the large variety of known methylated bases (Iwanami and Brown, 1968; Klagsbrun, 1973). In contrast, the ^{3}H radioactivity of the mRNA sample has a fairly narrow distribution, suggesting that there may be only one or a few species of methylated base in mRNA. Further analysis will be required for a positive identification of this methylated species.

Table 2. Ratio of $^{3}H/^{14}C$ in Various RNA Species Relative to Ribosomal RNA

RNA species	Expt.	$(^{3}H/^{14}C)$ species/ $(^{3}H/^{14}C)$rRNA[a]
poly A(+)mRNA	1	0.180
	2	0.164
	3	0.156
poly A(+)HnRNA (>45S)	1	0.069 } approx.
poly A(−)HnRNA (>45S)	1	0.037 } approx.
tRNA	3	5.39
5S rRNA	3	negligible (<0.002)
18S rRNA	1	1.26
28S rRNA	1	0.81

[a] The rRNA ratio represents a measured value for the combined rRNA components (approximately equimolar amounts of 28S and 18S rRNA).

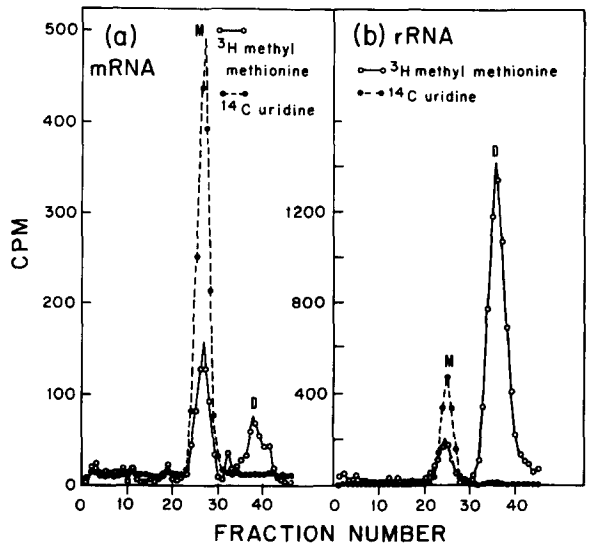

Figure 3. Chromatography on DEAE-Sephadex-Urea Columns of Alkaline Hydrolysates of Poly A(+)mRNA and rRNA from Cells Labeled with ^{3}H Methyl Methionine and ^{14}C Uridine

Columns of DEAE-Sephadex (Pharmacia), 0.6 × 7.5 cm, were equilibrated with 20 mM Tris buffer, pH 7.6, containing 7 M urea. Alkaline hydrolysates of poly A(+) mRNA (a) and rRNA (b) were applied to the columns in this buffer and eluted in 0.7 ml fractions with 30 ml linear gradients of 0–0.25 M NaCl in the same buffer. Mononucleotides/(M) and dinucleotides/(D) eluted at about 0.12 and 0.18 M NaCl, respectively. At the end of the elution the columns were washed with 5 ml of Tris-urea buffer containing 0.3 M NaCl. Negligible amounts of ^{14}C and ^{3}H radioactivity appeared in the wash of the rRNA sample. The wash of the mRNA sample contained a negligible amount of ^{14}C radioactivity and about 50% of the input ^{3}H radioactivity.

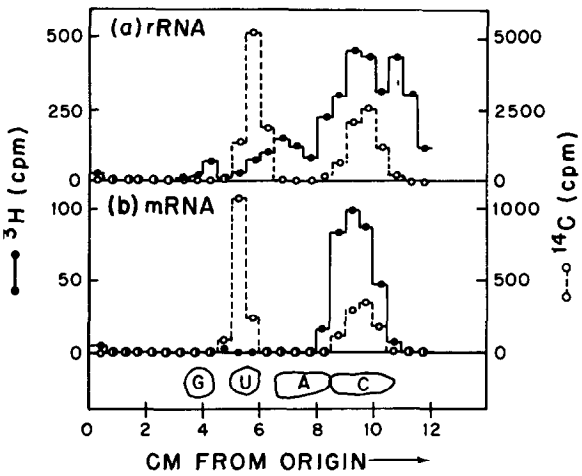

Figure 4. Chromatography on PEI Cellulose Strips of Mononucleotides Produced by Alkaline Hydrolysis of rRNA (a), and Poly A(+)mRNA (b) from Cells Labeled with ^{3}H Methyl Methionine and ^{14}C Uridine

Mononucleotide fractions from columns such as those shown in Figure 3 were desalted, concentrated, mixed with an unlabeled alkaline hydrolysate of sRNA which provided $A_{260\ nm}$ markers, applied to a strip of polyethyleneimine cellulose, and submitted to ascending chromatography with 1 N acetic acid and 0.3 M LiCl. After drying and sectioning the strips into 5 mm-wide pieces, the PEI cellulose was scraped from the plastic backing and counted in a scintillant containing 3% Protosol and 1% H_2O. The spots diagrammed below the abscissa show the position of the $A_{260\ nm}$ markers.

Discussion

The results presented here establish that the poly A(+)mRNA of mouse L cells is methylated both in the ribose and base moieties. Since the bulk of the mRNA in these cells is polyadenylated (Greenberg and Perry, 1972), methylation may be considered as a general property of mouse mRNA. Indeed, it seems likely that methylation, like polyadenylation (Perry, Kelly, and LaTorre, 1972), may be a distinctive feature of eucaryotic mRNA. The overall extent of methylation can be roughly estimated from the comparisons with rRNA given in Table 2. If we neglect base compositional differences and the possibility of different *ratios* of ^{3}H methyl/^{14}C uridine specific activity in the different RNA species, and if we assume a value of 1.3% for the proportion of methylated nucleotides in an equimolar mixture of 28S and 18S rRNA (Brown and Attardi, 1965; Wagner, Penman, and Ingram, 1967), we can estimate that the proportion of methyl groups in mRNA is about $0.167 \times 1.3\% = 0.22\%$. Using the data of Table 2, similar estimates can be made of the relative extent of methylation of the individual rRNA components (28S versus 18S) and of tRNA. The estimated values (1.5 for 18S:28S methylation and 7% methylated nucleotides for tRNA) are in excellent agreement with those calculated by detailed chemical analysis (Brown and Attardi, 1965; Wagner, Penman, and Ingram, 1967; Dayhoff, 1972), thus attesting to the general validity of this type of calculation.

Several observations indicated that the methyl groups were indeed incorporated into mRNA and not into another contaminating RNA species. These were: (1) a failure to detect a reduced ratio of ^{3}H methyl/^{14}C uridine incorporation in the presence of low doses of actinomycin D which completely suppressed synthesis of rRNA; (2) the detection of the same ^{3}H/^{14}C ratio after repeated purification of the mRNA; (3) the demonstration that incorporated ^{3}H methyl radioactivity has the same gel-electrophoretic profile as that of nucleoside-labeled mRNA; (4) the finding of a distribution of alkaline-resistant nucleotides in mRNA which is markedly different than that characteristic of rRNA; and (5) the observation that there is a more limited variety of methylated bases in mRNA than is found in rRNA. The possibility of internal incorporation of the methyl group into the purine ring system was negated by the absence of ^{3}H activity in 5S RNA, a species conclusively known to be nonmethylated, and by the lack of ^{3}H activity in the guanylic and adenylic acid regions of the thin layer chromatograms.

Given an average length of mRNA of 3000 nucleotides, one may calculate that there are about 6 or 7 methylated nucleotides per mRNA molecule. The fact that a fairly large proportion of the methyl label is in alkaline-resistant material which is elutable from DEAE-Sephadex-urea columns only at salt concentrations higher than 0.25 M suggests that some of the methyl groups may be in adjacent nucleotides or possibly at the 5′ termini of the mRNA molecules. It would seem reasonable to expect that the methylated bases would be in a region of the mRNA molecule which does not perform a coding function (McCarthy, Holland and Buck, 1966), although a limited amount of ribose methylation might not interfere with template activity if present in the coding region (Dunlap, Friderici and Rottman, 1971). The gel-electrophoretic profile of the double-labeled mRNA (Figure 1) does not reveal any large differences in the degree of methylation of different size classes of mRNA, suggesting that the large mRNA molecules have proportionately more methyl groups per molecule than the small mRNA molecules. However, the extent of variability in methyl content among different mRNA species cannot be specified without further fractionation of the mRNA population.

The proportion of methylated nucleotides in HnRNA is appreciably lower than that found in mRNA, and in fact is near the limit of detectability. If, analogous to the situation with pre-rRNA and rRNA (Greenberg and Penman, 1966), the methylated nucleotides were predominantly located in the portions of the HnRNA which are conserved in the

processing to mRNA, one might expect, on the basis of relative molecular weights, that HnRNA would have roughly a five-fold lower $^{3}H/^{14}C$ ratio than mRNA. This is within the accuracy of our present determinations, The indication that poly A (−)HnRNA may have a lower methyl content than poly A(+)HnRNA is consistent with the methylation being a post-transcriptional event, occurring perhaps concurrently with polyadenylation. Given the very low level of methylation of HnRNA (less than one in 2500 nucleotides on the average) it is not surprising that it has been overlooked in earlier investigations.

Experimental Procedures

Mouse L cells, cultivated as described previously (Perry and Kelley, 1968), were suspended at 1.5 to 2.5 × 10^{6} cells/ml in growth medium containing 20μM methionine (1/5 the normal methionine concentration), 20 mM sodium formate, 20 μM each of adenosine and guanosine, 0 to 4 μM unlabeled uridine, 18–20 μCi ^{3}H methyl methionine (New England Nuclear Corp. 6.3 Ci/mmole)/ml, and 0.1 μCi 2-(^{14}C) uridine (New England Nuclear Corp. 57 mCi/mmole)/ml. The inclusion of sodium formate in the labeling medium has been shown by Maden, Salim, and Summers (1972) to inhibit effectively the incorporation of methionine methyl groups into the purine rings via the H_4-folate pathway. After 4 hr incubation at 37°C the cells were harvested, washed in balanced saline solution, and frozen as a pellet at −70°C.

Upon thawing the cells were lysed with 0.05% Triton X-100 and separated into nuclear and cytoplasmic fractions (Perry and Kelley, 1968). Polyribosomes were isolated on 15–45% sucrose gradients, and polyribosomal RNA extracted as previously described (Perry and Kelley, 1973). Nucleoplasm was isolated by the method of Penman (1966), and nucleoplasmic RNA extracted with 0.5% sodium dodecyl-sulfate-chloroform-phenol (Perry et al., 1972).

The polyribosomal and nucleoplasmic RNA was fractionated into polyadenylated and nonadenylated components by chromatography on poly T-cellulose columns (Nakazato and Edmonds, 1972). Assays of polyadenylated RNA were also made using GFC filters impregnated with poly U (Sheldon, Jurale, and Kates, 1972). Ribosomal and low molecular weight (5S and 4S) RNAs were further purified from the nonadenylated polyribosomal RNA fraction by sedimentation on 5–25% sucrose gradients in 0.1% Sarkosyl (Geigy), and electrophoresis of the peak fractions on polyacrylamide gels (Perry and Kelley, 1973). Polyadenylated mRNA and HnRNA were also analyzed by electrophoresis on 2.7% and 2.2% polyacrylamide gels, respectively.

Alkaline hydrolysis of poly A(+)mRNA and rRNA was achieved by incubation for 18–22 hr at 37°C in 0.3 N KOH. The alkaline hydrolysates were neutralized with perchloric acid, and after removal of the insoluble potassium perchlorate by centrifugation, the nucleotide products were analyzed by chromatography on DEAE Sephadex columns equilibrated with 20 mM Tris, pH 7.6, and 7 M urea (Tener, 1967). The columns were eluted with linear gradients of 0 to 0.25 M NaCl in the Tris-urea buffer and given final washes with 0.3 M NaCl.

The eluent fractions containing mononucleotides were pooled and desalted by rechromatography on small DEAE-Sephadex columns using ammonium carbonate elution and repeated lyophilization to remove most of the volatile salt. The mononucleotides were then mixed with an $A_{260\ nm}$ marker consisting of an alkaline hydrolysate of yeast sRNA (Miles Labs), applied to PEI cellulose strips and chromatographed with acetic acid and LiCl as previously described (Kelley and Perry, 1971).

Radioactive samples were courted in a Beckman Liquid Scintillation Counter with channel and gain settings adjusted for maximum tritium efficiency (∼42%) and minimum ^{14}C crossover (∼18%). Aqueous samples and samples on filter pads were counted in Protosol-Liquifluor-Toluene (3:4:96) and Liquifluor-Toluene (4:96) scintillants, respectively. Backgrounds were determined on appropriate blank samples, and subtracted from all data presented.

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