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Biochemistry

Short-lived methylated messenger RNA in mouse kidney

[methylation/mRNA containing poly(A)/decay kinetics/$MgCl_2$ precipitation]

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ABSTRACT In experiments originally designed to examine selective turnover of methylated "caps" in renal mRNA, we observed that [^{3}H]methyl label decayed from mRNA containing poly(A) with a half-life of 1–2 hr. (Caps are blocked, methylated mRNA sequences of the general structure $m^7GpppN^m_{p(1 \text{ or } 2)}Np$.) To distinguish between metabolism of short-lived mRNA and discriminate turnover of "caps", we compared residual [^{3}H]methyl label in 5′ and 3′ mRNA fragments prepared from mRNA isolated during the decay period. Hydrolysis of mRNA at 0° with dilute KOH before oligo(dT)-cellulose selection produced 5′ mRNA fragments enriched with an alkali-resistant oligonucleotide with a −5 charge; the 3′ mRNA fraction was correspondingly reduced in oligonucleotide content. Since methyl label disappeared at the same rate from both fractions, we conclude that mouse kidney contains short-lived mRNA and that the "caps" of these labile mRNAs turn over with the rest of the mRNA molecule.

To examine a possible regulatory function of methylated sequences in mRNA, we compared the relative stability of [^{3}H]methyl and [^{14}C]orotate label in renal poly(A)-containing [poly(A)$^+$] mRNA as a test for selective turnover of methylated "caps" (1–10). (Caps are blocked, methylated mRNA sequences of the general structure $m^7GpppN^m_{p(1 \text{ or } 2)}Np$, where N^m is any 2′-*O*-methylnucleoside.) Because the renal S-[^{3}H]adenosylmethionine pool is depleted rapidly relative to the pyrimidine pools (11), [*methyl*-^{3}H]methionine could selectively label a short-lived class of mRNA not detected by labeling with orotic acid. Although [^{14}C]orotate accumulated in mRNA as expected for its slow chase kinetics (12), most of [^{3}H]methyl label in mRNA decayed with a half-life of 1–2 hr. To determine whether this rapid decay resulted from metabolism of short-lived mRNA or discriminate "cap" turnover, we compared residual [^{3}H]methyl label in 5′ and 3′ mRNA fragments prepared from mRNA isolated during the decay period. The results show that mouse kidney contains rapidly turning-over mRNA and that the "caps" of these labile mRNAs are metabolized synchronously with the rest of the mRNA molecule.

MATERIALS AND METHODS

Animals. Young adult male Charles River mice (40–50 days, 30–35 g, Charles River Laboratories, N. Wilmington, Mass.) were fed freely and kept on alternating 12-hr cycles of light and dark.

Preparation of Polyribosomes by Precipitation with $MgCl_2$. Decapsulated kidneys were disrupted by Dounce homogenization in ice-cold buffer containing 10 mM Tris·HCl (pH 7.4), 0.15 M NaCl, 0.25 M sucrose, 3 mM $MgCl_2$. Postmitochondrial supernatants prepared from homogenates were adjusted to 70 mM $MgCl_2$ with 0.7 M $MgCl_2$, then diluted to 10 ml by addition of ice-cold 70 mM $MgCl_2$. After incubation at 0° for 45 min, precipitated material was deposited by centrifugation at 13,000 rpm for 45 min in the Sorvall SS-34 rotor. RNA was deproteinized at pH 9.0 as described (13, 14). Precipitated ribonucleoprotein, dissolved in 10 ml of 10 mM Tris·HCl (pH 9.0), 0.10 M NaCl, 10 mM EDTA, and 0.5% sodium dodecyl sulfate, was alternately extracted with phenol: chloroform:isoamyl alcohol (50:48:2) and chloroform:isoamyl alcohol (96:4) until the interfacial layer was clear of denatured protein.

Oligo(dT)-Cellulose Chromatography. Samples containing 300–500 μg of RNA dissolved in 2.5 ml of 10 mM Tris·HCl (pH 7.4), 0.45 M NaCl, 0.1% Sarkosyl (Ciba-Geigy Corp., Ardsley, N.Y.) were applied to 6 × 0.5 cm columns of oligo(dT)-cellulose (Type T-2, Collaborative Research, Waltham, Mass.) equilibrated with the same starting buffer. Poly(A)$^+$ mRNA, initially bound to the column, was eluted with 10 mM Tris·HCl (pH 7.4), 0.1% Sarkosyl, adjusted to 0.45 M NaCl, and chromatographed again on a column re-equilibrated with high salt buffer. Poly(A)$^+$ mRNA and combined RNA fractions without poly(A) [poly(A)$^-$ RNA] were assayed for radioactivity by measuring aliquots in a gel containing 3 ml of sample and 10 ml of scintillation fluid containing 1 part Triton-X 100 (Rohm & Haas Co., Philadelphia, Pa.), 2 parts xylene, and 8 g of Omnifluor (New England Nuclear Corp., Boston, Mass.) per liter.

DEAE-Sephadex Chromatography. RNA samples dissolved in 3 ml of 0.3 M KOH were hydrolyzed at 37° for 18 hr. Chilled hydrolysates were neutralized by addition of 3 ml of ice-cold perchloric acid, and the potassium perchlorate precipitate was removed by low-speed centrifugation. Neutralized hydrolysates were applied to 20 × 1.2 cm columns of DEAE-Sephadex (A-25, Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) that had been equilibrated with 20 mM Tris·HCl (pH 7.4), 0.10 M NaCl, and 7 M urea (14, 15). Columns were eluted with linear 200-ml gradients of 0.10–0.50 M NaCl in 20 mM Tris·HCl (pH 7.4) and 7 M urea.

Radiochemicals. [5-^{3}H]Orotic acid (11.1 Ci/mmol), [6-^{14}C]orotic acid hydrate (55.2 mCi/mmol), and L-[*methyl*-^{3}H]methionine (10 Ci/mmol) were purchased from New England Nuclear Corporation, Boston, Mass.

RESULTS

Precipitation of polyribosomes with $MgCl_2$

A reliable technique simple enough to permit replicate analysis was essential for preparing undegraded cytoplasmic poly(A)$^+$ mRNA, since the average kidney poly(A)$^+$ mRNA molecule contains only two to three methylated residues per 1000 nucleotides (14). Precipitation of polyribosomes and cytoplasmic ribonucleoproteins with 70 mM $MgCl_2$ (16–18) satisfied this requirement (Fig. 1). As shown in Fig. 1c, precipitation was complete; poly(A)$^+$ mRNA and 28S and 18S rRNAs were absent from the $MgCl_2$-soluble fraction. Separation of the particulate

Abbreviations: poly(A)$^+$ mRNA, mRNA containing poly(A); poly(A)$^-$ RNA, RNA lacking poly(A); caps, blocked, methylated, 5′-terminal mRNA sequences of the general structure $m^7G^{5'}pppN^m_{p(1 \text{ or } 2)}N_p$, where N^m is any 2′-*O*-methylnucleoside.

and soluble cytoplasmic fractions was better than obtained by sedimentation of polysomes in sucrose density gradients, since the gradient region sedimenting slower than nominal 40 S was contaminated with small ribosomal subunits not found in the $MgCl_2$-soluble fraction (Fig. 1). In addition to having a similar sedimentation pattern (Fig. 1), poly(A)$^+$ RNA from $MgCl_2$-precipitated ribonucleoprotein had poly(A) content, methyl content, and labeling characteristics identical to poly(A)$^+$ mRNA prepared from sedimented polyribosomes (14, 19). Since 97% of labeled polysomal poly(A)$^+$ RNA is released from the polyribosomes with EDTA (19), and since 70 mM $MgCl_2$ quantitatively precipitates polyribosomal ribonucleoprotein (Fig. 1), we conclude that the poly(A)$^+$ mRNA from $MgCl_2$-precipitated ribonucleoprotein is the same population as that derived from polyribosomes.

Half-life of methyl-labeled mRNA

In earlier experiments using [^{3}H]orotate for labeling of RNA, renal poly(A)$^+$ mRNA was observed to decay with half-lives of 6 hr and 24 hr (12). In the present study, since the extent of mRNA methylation was low, the decay kinetics of methyl-labeled mRNA were determined using double-isotope analysis with [^{14}C]orotate and [*methyl*-^{3}H]methionine to provide an internal mRNA marker with known kinetics of decay. Kidneys were analyzed for both ^{3}H and ^{14}C radioactivity in poly(A)$^+$ mRNA and rRNA at various times after injection of label (Fig. 2). Since the half-life of rRNA in mouse kidney is approximately 5 days (20) and since RNA content of kidneys from mice older than 34 days is stable (21), the time when rRNA reaches constant specific activity shows when the chase becomes effective. Decay of labile molecules can then be measured. The [*methyl*-^{3}H]methionine pool decreased more rapidly than [^{14}C]orotate since the specific activity of methyl label in rRNA was constant from 1 to 2 hr after labeling, but incorporation of [^{14}C]orotate into rRNA continued for at least 6 hr (Fig. 2a) and is known to increase for 8–12 hr (12).

[*methyl*-^{3}H]Methionine labeled mRNA to maximal activity by the first hour, then underwent rapid exponential decay. The slope of the decay curve for methylated poly(A)$^+$ mRNA, drawn by single linear regression, gave a half-life of 2.25 hr (Fig. 2b), but because stable mRNA molecules, not detectable as distinct decay components, would also have been labeled in the pulse, the observed half-life could be an overestimate. If the changed slope of the [^{3}H]methyl decay curve seen between 6 and 18 hr is indicative of methyl decay in stable mRNA and is extrapolated to zero time, radioactivity in this component can be subtracted from the early timepoints providing a corrected half-life of 1 hr. However, because of the low level of radioactivity at the later timepoints, the actual half-life is most likely between 1 and 2 hr.

Preparation of 3′ and 5′ mRNA fractions and distribution of alkali-resistant methylated oligonucleotides

The decay kinetics of methyl-labeled mRNA could be interpreted in two ways. First, since the radioactive S-adenosylmethionine pool is rapidly depleted, a labile class of mRNA could have been selectively labeled during the brief pulse. Alternatively, the labeled 5′ termini of stable mRNAs could have turned over while the rest of the molecule remained intact. The most rigorous test of these possibilities would have been a direct comparison on DEAE-Sephadex columns (14, 15) of methyl label remaining in "caps" (−5 to −6 charge) with label remaining in N^6-methyladenosine (−2 charge). Lack of sufficient [^{3}H]methyl radioactivity after 3 hr of labeling, however, precluded analysis on DEAE-Sephadex columns. As an alternative, we prepared an mRNA fraction enriched in "caps" by limited alkaline hydrolysis of poly(A)$^+$ mRNA at 0° (1) followed by separation of poly(A)$^+$ and poly(A)$^-$ fragments of the hydrolyzed molecules by oligo(dT)-cellulose chromatography. For a native poly(A)$^+$ mRNA population of uniform size, production of one single-strand break per molecule will result in only 50% of the label binding to oligo(dT)-cellulose upon reselection (22). In our experiments, 52% of orotate label in mRNA was bound to oligo(dT)-cellulose after cold alkaline hydrolysis (see legend to Fig. 3), and the poly(A)$^+$ and poly(A)$^-$ fragments had equivalent sedimentation properties (Fig. 3), suggesting native molecules were left with one single-strand break by the procedure. However, since renal mRNA has a heterogeneous size distribution (19) and since every phosphodiester bond has an equal chance of being hydrolyzed, it is likely that some large mRNAs may have received two single-strand breaks and that small mRNAs may have totally escaped hydrolysis (1).

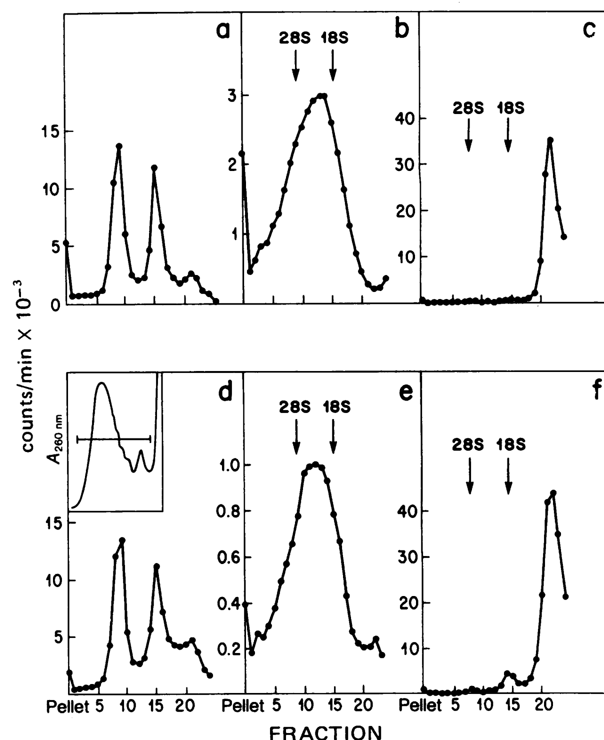

FIG. 1. Sedimentation properties of RNA from polyribosomes precipitated with $MgCl_2$. Two mice were labeled by subcutaneous injection with 200 μCi each of [5-^{3}H]orotic acid for 2 hr. Kidneys were disrupted in 10 mM Tris·HCl (pH 7.4), 0.25 M sucrose, 0.15 M NaCl, 3 mM $MgCl_2$ by Dounce homogenization at 0°. One-half of the postmitochondrial supernatant was centrifuged in a 36-ml 7–47% sucrose density gradient in 10 mM Tris·HCl (pH 7.4), 0.5 M NaCl, 50 mM $MgCl_2$ at 26,500 rpm in the SW27 rotor for 3 hr at 4°. RNA was prepared from structures sedimenting faster than nominal 40 S (inset, brackets) and from the <40S region (inset, right of brackets). Polyribosomes were precipitated from the remaining postmitochondrial supernatant with $MgCl_2$ (*Materials and Methods*). Samples of less than 500 μg of RNA were chromatographed on oligo(dT)-cellulose columns, and poly(A)$^-$ and poly(A)$^+$ RNAs were precipitated with ethanol. RNA dissolved in 1.0 ml of buffer containing 10 mM Tris·HCl (pH 7.4), 0.1 M NaCl, 10 mM EDTA, 0.2% sodium dodecyl sulfate was centrifuged in 34-ml 15–30% sucrose density gradients in the same buffer at 23,000 rpm for 16.5 hr at 23° in the SW27 rotor. (a) $MgCl_2$-precipitated, poly(A)$^-$ RNA; (b) $MgCl_2$-precipitated, poly(A)$^+$ RNA; (c) $MgCl_2$-soluble RNA [not chromatographed on oligo(dT)-cellulose]; (d) poly(A)$^-$ RNA from ≥40S structures; (e) poly(A)$^+$ RNA from ≥40S structures (only ⅓ of total sample analyzed); (f) RNA from <40S region [not chromatographed on oligo(dT)-cellulose].

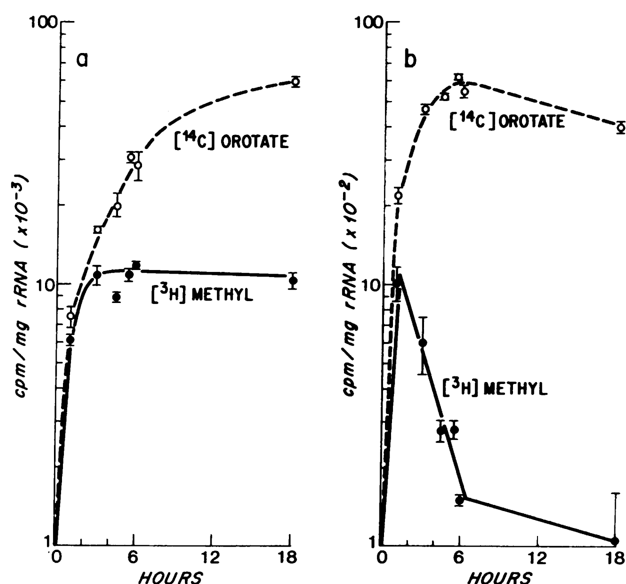

FIG. 2. Accumulation and decay of [^{3}H]methyl label in mRNA. $MgCl_2$-insoluble ribonucleoprotein was prepared from kidneys of mice labeled subcutaneously with 2.5 μCi of [6-^{14}C]orotic acid and 500 μCi of L-[*methyl*-^{3}H]methionine for the times indicated. RNA was fractionated by double passage on oligo(dT)-cellulose (*Materials and Methods*). Aliquots of poly(A)$^+$ and poly(A)$^-$ RNA were assayed for radioactivity by counting in a gel of 3 ml of H_2O + 10 ml of xylene-based scintillation fluid. Points are mean values of three replicate samples ±SEM; each replicate consists of both kidneys from two labeled animals. All timepoints were done on separate days. The slope of the methyl-labeled mRNA decay curve was determined by single linear regression. (a) poly(A)$^-$ mRNA; (b) poly(A)$^+$ RNA.

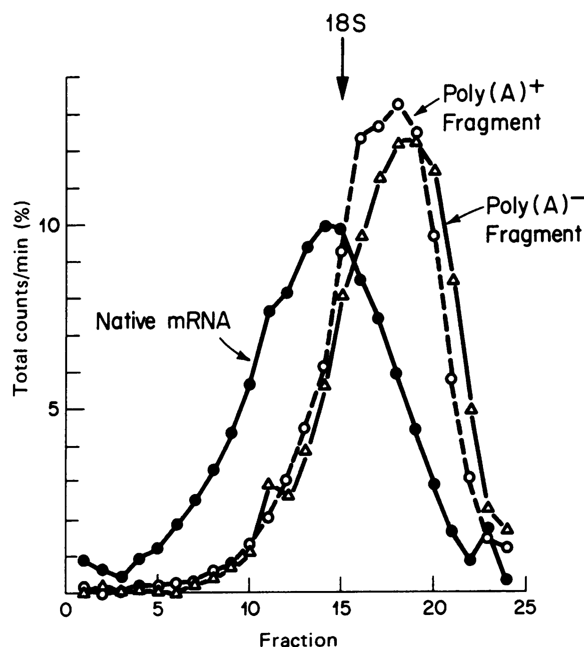

FIG. 3. Alkaline hydrolysis of kidney mRNA at 0°. Poly(A)$^+$ mRNA from [^{3}H]orotate-labeled mouse kidney isolated by double selection on oligo(dT)-cellulose was dissolved in 3 ml of ice-cold 10 mM NaCl. One milliliter of mRNA solution was precipitated immediately with added cold 18S rRNA in 2 volumes of 95% ethanol. The remaining mRNA was adjusted to 0.1 M NaOH at 0° and incubated with shaking at 0° for 3 min. After the hydrolysate was neutralized with an equal volume of ice-cold 1 M HCl, it was diluted with an equal volume of 100 mM Tris-HCl (pH 7.4), 1.0 M NaCl, and 0.2% Sarkosyl. This preparation had a final pH of 7.4. Poly(A)$^+$ and poly(A)$^-$ fragments of the digested mRNA were separated by single chromatography on oligo(dT)-cellulose and precipitated with cold 18S rRNA in 2 volumes of ethanol. Of the original radioactivity in mRNA, 51.7% remained in the poly(A)$^+$ RNA fragment after hydrolysis. Native mRNA and poly(A)$^+$ and poly(A)$^-$ RNA fragments were sedimented as in Fig. 1. Total radioactivity recovered from each gradient: native mRNA, 17,500 cpm; poly(A)$^-$ RNA fragment, 16,948 cpm; poly(A)$^+$ RNA fragment, 18,136 cpm.

Although chemical analysis of "caps" in renal mRNA is not complete, methyl-labeled mRNA from kidney contains an alkaline-resistant oligonucleotide that has two to three methyl residues and a charge of −4 to −5 (14). The distribution of this oligonucleotide in poly(A)$^-$ and poly(A)$^+$ fractions (Fig. 3) of methyl-labeled mRNA containing single-strand breaks was examined by DEAE-Sephadex chromatography of completely hydrolyzed fragments (Table 1). For intact mRNA and both fragments of the molecules with single-strand breaks, only two peaks of methyl radioactivity were evident: a mononucleotide peak with a charge of −2 and a peak cochromatographing with the tetranucleotide marker bearing a −5 charge (data not shown). The ratio of labeled oligonucleotide to labeled mononucleotide showed the poly(A)$^-$ fragments were 4-fold enriched over the poly(A)$^+$ fraction with respect to the −5 charge residue, a characteristic consistent with a 5′ terminal location. The poly(A)$^+$ fraction contains −5 charge structures from mRNA molecules which probably did not receive single-strand breaks during the short hydrolysis, but the enrichment of the poly(A)$^-$ fragments for the oligonucleotide should still permit selective turnover of "caps" to be detected.

Turnover of [^{3}H]methyl label in the 3′ and 5′ mRNA fragments

If the rapid decay of [^{3}H]methyl label resulted from turnover of "cap" structures only, and label was not directly reutilized, the percent of mRNA methyl label in the poly(A)$^+$ fragments after alkaline hydrolysis at 0° should increase rapidly once chase

Table 1. Distribution of alkali-resistant methylated oligonucleotide in renal mRNA*

Sample	Radioactivity (cpm) Mononucleotides (−2)	Radioactivity (cpm) Oligonucleotide (−5)	Oligonucleotide/ Mononucleotide
Total mRNA	913	258	0.28
Poly(A)$^-$ mRNA fragment	511	245	0.48
Poly(A)$^+$ mRNA fragment	1108	135	0.12

* Poly(A)$^+$ mRNA, prepared from 10 mice labeled for 2 hr with 500 μCi each of [*methyl*-^{3}H]methionine, was hydrolyzed at 0° as in Fig. 3. Samples hydrolyzed with 0.3 M KOH for 16 hr at 37° were neutralized, combined with 2 mg of renal rRNA that had been digested for 16 hr with pancreatic RNase A, and chromatographed on DEAE-Sephadex. Total radioactivity in mononucleotide and oligonucleotide regions was determined by summing the counts under the respective peaks.

Table 2. Distribution of [^{3}H]methyl and [^{14}C]orotic acid after limited alkaline hydrolysis*

Hours	Radioactivity (cpm)					
	[^{14}C]Orotate			[^{3}H]Methyl		
	Poly(A)$^-$ RNA	Poly(A)$^+$ RNA	% in poly(A)$^+$ RNA	Poly(A)$^-$ RNA	Poly(A)$^+$ RNA	% in poly(A)$^+$ RNA
1	1728	1148	40	530	719	58
3	2862	2162	43	216	300	58
4.5	2960	2636	47	160	197	55
5.5	4749	4501	48	99	175	63
6	3672	3045	45	51	234	82
18	2379	2253	49	72	177	71

* The remainder of replicate poly(A)+ mRNA samples from each timepoint prepared in Fig. 2 were pooled and rechromatographed on oligo-(dT)-cellulose. Poly(A)+ mRNA was precipitated in ethanol with 100 μg of unlabeled rRNA. RNA dissolved in 2.0 ml of ice-cold 10 mM NaCl was hydrolyzed and chromatographed on oligo(dT)-cellulose as in Fig. 3. Total radioactivity in poly(A)$^-$ and poly(A)+ mRNA fragments was assayed as in Fig. 2.

conditions were in effect. Between 1 and 5.5 hr after injection of label, the distribution of methyl radioactivity in the 3′ and 5′ fractions was essentially constant, demonstrating that methyl label decayed at the same rate from both mRNA segments. Thus, methionine selectively labels a population of short-lived mRNA, and turnover of "caps" in this mRNA class seems synchronous with turnover of whole mRNA molecules. Although the percent of methyl label in poly(A)$^+$ mRNA increased in the 6- and 18-hr timepoints, nearly all of the short-lived mRNA had already decayed, and the level of radioactivity in the samples was perhaps too low for confidence.

DISCUSSION

Analysis of mRNA stability in most mammalian systems has revealed only stable classes of mRNA with half-lives in excess of 7 hr (23–26). Poly(A)$^+$ mRNA from mouse kidney labeled with [^{3}H]orotate has biphasic decay kinetics with half-lives of 6 hr and 24 hr (12), but chase conditions are not effective using [^{3}H]orotate until 8–12 hr after administration of label. When [^{3}H]uridine-labeled cells in culture were washed and chased with excess unlabeled uridine, 6–8 hr were also required before incorporation of label into RNA was sufficiently reduced to permit analysis of decay of labile molecules (25, 26). Such necessarily long chase periods after labeling with pyrimidine precursors probably prevented detection of rapidly turning-over mRNAs, although Berger and Cooper (28), labeling human lymphocytes with uridine, described cytoplasmic poly(A)$^+$ RNA with a 17-min half-life. In our experiments, short-lived renal mRNA, not detectable after orotate labeling, was observed with methionine labeling, because the radioactive methionine pool is depleted rapidly after injection of label (11). Similar rapid chase kinetics of the [^{3}H]guanosine pool allowed Puckett *et al.* (27) to demonstrate mRNA with a 1- to 2-hr half-life in HeLa cells.

Studies of nuclear and cytoplasmic poly(A) labeling have implied that a simple precursor–product relationship could not exist for these cellular RNAs (29). Kinetic models constructed on the assumption that cytoplasmic mRNA is long-lived require nuclear poly(A) turnover and/or cytoplasmic turnover of poly(A) to resolve the data (29). It has been argued that a kinetic model with quantitative transfer of nuclear poly(A) to the cytoplasm can be constructed if short-lived mRNA exists (27, 28). To the extent that our results describe rapidly metabolized mRNA, they suggest that explanations of the kinetics of cytoplasmic poly(A) accumulation should be re-evaluated.

Recent data have shown that 5′ "caps" in eukaryotic mRNA are heterogeneous with a general structure $m^7G^{5'}ppp^{5'}N^m{}_{p(1-2)}N_p$, where N is any nucleoside (1–10). Perry *et al.* (9) reported the distribution of $m^7G^{5'}ppp^{5'}N^m{}_pNp$ and $m^7G^{5'}ppp^{5'}N^m{}_pN^m{}_pNp$ varied with the labeling period since the larger "cap" predominated early, but decreased in relative amount as the labeling time was extended. These data imply that short-lived mRNAs may have 5′ terminal sequences different from more stable mRNAs and suggest a possible correlation between mRNA stability and "cap" structure. It seems clear that the "caps" in short-lived renal mRNA are not degraded or metabolized independently of whole mRNA molecules, but these results do not exclude the possibility of separate "cap" turnover in more stable mRNAs. The last two timepoints in Table 2 suggest both of these events may be taking place. Changing the labeling conditions *in vivo* should permit selective methylation of stable mRNAs and further studies of the relationship between "cap" structure and stability of mRNA sequences in the cytoplasm.

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