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5′-Terminal Cap Structures of Oligo(uridylic acid)-Containing Messenger Ribonucleic Acid from HeLa Cells: Comparison with Other Ribonucleic Acid Subpopulations†

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ABSTRACT: The 5′-terminal cap structures of ^{32}P-labeled oligo(uridylic acid)-containing messenger ribonucleic acid [oligo(U+) mRNA] isolated from HeLa cell polyadenylated [poly(A+)] mRNA were analyzed and compared to those of the poly(A+) mRNA. A method employing P1 nuclease, alkaline phosphatase, and adsorption to activated charcoal showed that the types of cap core (m^7 GpppXm) in oligo(U+) mRNA were essentially identical with those in poly(A+) mRNA. Analysis of RNase T_2 digestion products of oligo(U+) mRNA demonstrated the presence of both cap 1 (m^7GpppXmpYp) and cap 2 (m^7GpppXmpYmpZp) in this subpopulation, confirming its cytoplasmic location. The base compositions of these two types of caps were different from each other and nonrandom but did not differ significantly between oligo(U+) and poly(A+) mRNA. The only observed difference between the mRNA populations was a higher ratio of cap 1 to cap 2 in the former. Possible implications of these findings for the relationship between oligo(U+) mRNA and poly(A+) mRNA are discussed.

Some of the poly(A)-containing messenger ribonucleic acid [poly(A+) mRNA][1] molecules in the cytoplasm of HeLa cells also contain an oligo(U) sequence (Korwek et al., 1976; Molloy, 1980). A procedure developed in this laboratory for the isolation of this subset of mRNA molecules (Wood & Edmonds, 1981) has allowed us to examine other structural features of oligo(U)-containing molecules that may differentiate them from mRNA molecules that do not contain oligo(U). One structure, the modified 5′ terminus of mRNA molecules designated as the cap [m^7GpppXmpY(m)p], appears to be present in most of the poly(A)-containing mRNA molecules of HeLa cells (Salditt-Georgieff et al., 1976). It could not be assumed, however, that oligo(U)-containing mRNAs were capped. An uncapped subpopulation of this size

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[1] Abbreviations used: RNA, ribonucleic acid; oligo(U), oligo(uridylic acid); poly(A), poly(adenylic acid); poly(A+) mRNA, poly(A)-containing messenger RNA; oligo(U+) mRNA, oligo(U)-containing mRNA; oligo(U−) mRNA, oligo(U)-lacking mRNA; hnRNA, heterogeneous nuclear RNA; rRNA, ribosomal RNA; DNA, deoxyribonucleic acid; pC, cytidine 5′-monophosphate; pA, adenosine 5′-monophosphate; pG, guanosine 5′-monophosphate; pU, uridine 5′-monophosphate; m^7pG, 7-methylguanosine 5′-monophosphate; pNp, 3′,5′-nucleoside diphosphate; RNase H, ribonuclease H; DBAE, (dihydroxyboryl)aminoethyl; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; RSA, relative specific activity; cpm, counts per minute.

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within the total mRNA pool could easily escape detection because of the inaccuracy inherent in calculations of numbers of caps per mole of RNA for such heterogeneous populations. It seemed that a detailed analysis comparing structures at the 5′ ends of oligo(U)-containing mRNA [oligo(U+) mRNA] with those in oligo(U)-lacking mRNA [oligo(U−) mRNA] and in poly(A)-containing nuclear RNA could provide insights into the origin and function of oligo(U)-containing mRNA. We report here the results of such analyses.

Experimental Procedures

Labeling, Isolation, and Fractionation of RNA. Labeling of HeLa cells with [^{32}P]phosphate, cell fractionation, RNA isolation, and fractionation into polyadenylated and non-polyadenylated species as well as the isolation of oligo(U)-containing mRNA are described in the preceding paper (Wood & Edmonds, 1981). In all experiments described here polyadenylated RNA was obtained by three cycles of binding to and elution from oligo(dT)–cellulose.

Release of Caps from Intact RNA. (a) P1 Nuclease plus Phosphatase Treatment for Cap Core Isolation. RNA (50–200 μg including yeast RNA carrier) was suspended in 105 μL of 10 mM sodium acetate, pH 5.3, before the addition of 30 μL of P1 nuclease (1 mg/mL; Sigma Biochemicals). After 1–3 h at 37 °C, the sample was adjusted to 35 mM Tris-HCl (pH 8.2) and 5 mM $MgCl_2$, and 6–14 μg of *Escherichia coli* alkaline phosphatase (Worthington, BAPF) was added. Incubation was continued for at least 8 hrs at 37 °C.

(b) Mixed Ribonuclease Digestion for Cap 1 and Cap 2 Isolation. RNA (50–200 μg) in 200 μL containing 5 mM Tris-HCl, pH 7.4, 5 mM Na EDTA, pH 7.4, 0.3 μg of RNase A (Sigma Type XII-A), 22.5 units of RNase T_2 (Sigma), and 50 units RNase T_1 (Calbiochem) was incubated for at least 4 h at 37 °C before reducing the pH by the addition of sodium acetate, pH 4.5, to a final concentration of 20 mM. An additional 22.5 units of RNase T_2 was added, and incubation was continued for 4 h.

Binding and Release of Caps from Charcoal. Acid-washed activated charcoal (10 mg; Norite A, Fisher; suspended as a 100 mg/mL slurry in 10 mM EDTA) was added to the P1 nuclease plus alkaline phosphatase digest obtained as described in (a) above. After the mixture was filtered through a 0.2-μm polycarbonate filter (Nuclepore), the filter was rinsed several times with water before adsorbed material was eluted with 5–10 mL of 60% ethanol containing 5 mM NH_4OH. Solvent was removed from the eluted fractions by flash evaporation.

Anion-Exchange Chromatography of Caps. Two milliliters of 7 M urea containing 25 mM Tris-HCl, pH 7.4, and 0.05 M NaCl was added to the mixed RNase digest obtained as described in (b) above. ^{3}H-Labeled oligonucleotide charge markers were added before passing it over a 28 × 0.7 cm DEAE-Sephacel (Pharmacia) column equilibrated with the same 7 M urea buffer. After ~40 2-mL fractions were collected, a 200-mL linear gradient of 0.05–0.225 M NaCl in the 7 M urea buffer was passed over the column. The 2-mL fractions were counted for Cerenkov radiation before removing 0.2-mL aliquots for assay of the ^{3}H-Labeled oligonucleotide markers. Urea and salt were removed as described (Tener, 1968).

Analysis of Purified 5′ End Components. P1 nuclease digestion of purified 5′ end groups was carried out in 20 μL containing 8 mM sodium acetate, pH 5.3, and 5 μg of P1 nuclease at 37 °C for 1 h. For subsequent phosphatase digestion, the reaction was made 45 mM with Tris-HCl pH 8.2, and 7.5 mM with $MgCl_2$, 1–2 μg of alkaline phosphatase was added, and incubation was continued for 1 h at 37 °C. If subsequent treatment involved nucleotide pyrophosphatase, potassium phosphate, pH 7.4, was added to 50 mM and $MgCl_2$ to 5 mM, and 0.3 unit of nucleotide pyrophosphatase (Sigma Type II; dialyzed overnight against H_2O to inactivate phosphomonoesterases) was added before continuing the incubation for 1 h at 37 °C. Unlabeled cap core (10 μg; m^{7}GpppXm, P-L Biochemicals) was included in the latter digestion as a control for complete enzymatic hydrolysis; the products are visible as ultraviolet absorbing spots after high-voltage paper electrophoresis carried out as previously described (Salzman & Sebring, 1964).

Table I: Formulas Used To Calculate Relative Specific Activity (RSA)[a]

substrate	enzyme treatment: P1 + nucleotide pyrophosphatase	enzyme treatment: P1 + alkaline phosphatase
cap 1	$RSA = \frac{4P - 100}{100 - P}$	$RSA = \frac{200 - 4P}{P}$
cap 2	$RSA = \frac{5P - 100}{100 - P}$	$RSA = \frac{300 - 5P}{P}$

[a] Tabulation of the results from several experiments yielded an RSA of 1.82 ± 0.42.

(Dihydroxyboryl)aminoethylcellulose Chromatography. Poly(A)-containing nuclear RNA sedimenting more rapidly than 10 S in sucrose gradients carried out as described (Wood & Edmonds, 1981) was digested with the mixed RNases as described above. The sample was adjusted to 0.5 M NaCl, 0.1 M $MgCl_2$, 0.05 M morpholine hydrochloride, pH 7.8, and 20% ethanol (binding buffer) and then passed over a 6 × 0.7 cm DBAE-cellulose (Collaborative Research) column equilibrated with binding buffer. After 60–80 mL of binding buffer had been passed over the column, bound material was eluted with 0.05 M ammonium acetate, pH 5.5. The above procedure was carried out at 4 °C. This eluate, dried down several times by flash evaporation, was suspended in 25 mL of 7 M urea buffer and run on an anion-exchange column as described above.

Relative Specific Activity Calculations. The relative specific activity of the β-phosphate in caps was calculated by using the percent (P) of the total cpm in caps converted to P_i by different enzymatic treatments as shown in Table I.

Results

Detection of Caps in Oligo(U+) mRNA. Caps were first detected in oligo(U)-containing mRNA digested with P1 nuclease. After release of monoesterified phosphate in the digest with alkaline phosphatase, a resistant ^{32}P-labeled cap core structure, m^{7}GpppXm, was isolated by adsorption to charcoal. Treatment of the charcoal-bound material with a nucleotide pyrophosphatase released m^{7}pG and four nucleoside monophosphates. Figure 1 shows the separation and migration of these products relative to authentic nucleotides during high-voltage electrophoresis. As would be expected for this structure, there is a rough equivalence of ^{32}P in m^{7}pG with that of the sum of the four mononucleotides. A reduction in the recovery of m^{7}pG was occasionally observed (Figure 1) that may have resulted from the opening of the guanine imidazole ring during the release of this cap from charcoal with slightly alkaline buffers (J. Wallace, unpublished observations). The insert table of Figure 1 showing the distribution of nucleotides at the X site of the cap core suggests few differences exist between oligo(U+) and oligo(U−) mRNA molecules.

The amount of ^{32}P in the cap relative to that in the total RNA in this and similar experiments indicated that about half

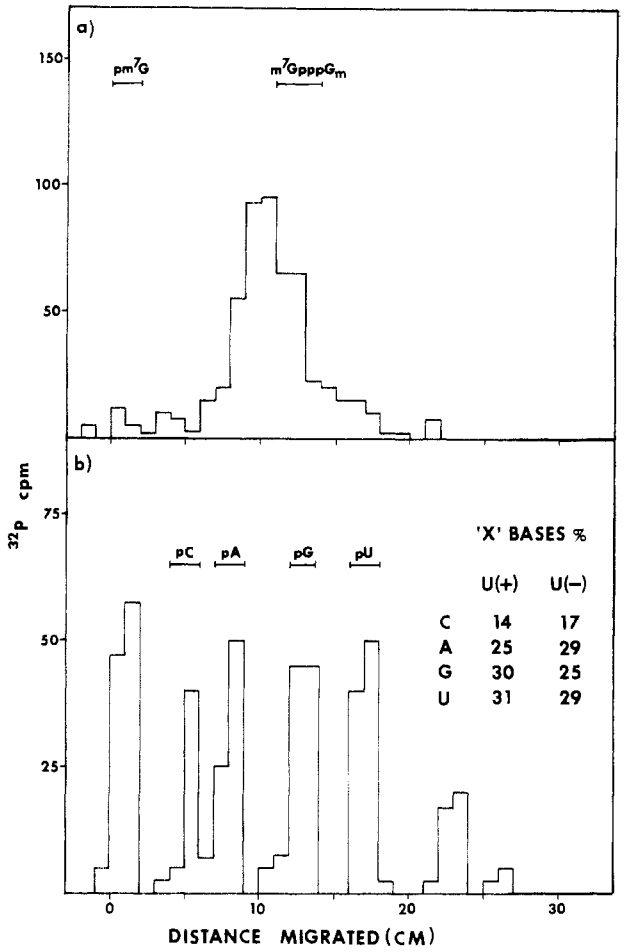

FIGURE 1: Cap core structures were isolated from oligo(U+) RNA by digestion with P1 nuclease and alkaline phosphatase as described under Experimental Procedures and then analyzed by paper electrophoresis at pH 3.5. (a) Control cap core; (b) cap core treated with nucleotide pyrophosphatase. The insert table shows the base compositions of the X bases of oligo(U+) and oligo(U−) RNAs. The 2′-O-methylation of the ribose of the X nucleotides does not significantly affect their electrophoretic mobility.

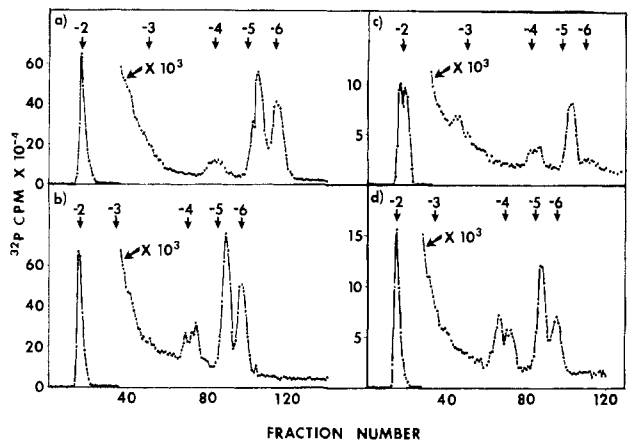

FIGURE 2: DEAE-cellulose profiles of RNases T_1, T_2, and A digested [^{32}p]RNA. (a) Poly(A+) mRNA; (b) depolyadenylated mRNA; (c) poly(A+) nuclear RNA; (d) depolyadenylated oligo(U+) mRNA. Arrows indicate the positions of elution of ^{3}H charge markers, produced by RNase T_1 digestion of [^{3}H]rRNA.

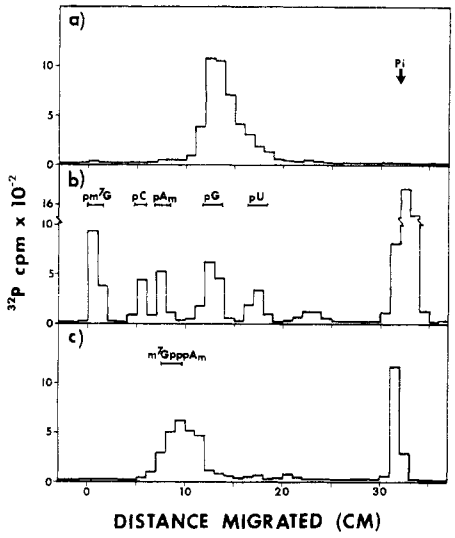

FIGURE 3: Paper electrophoretic analysis of a cap 1 structure from poly(A+) mRNA. The cap 1 peak from a DEAE-cellulose column such as that in Figure 2a was pooled, desalted, and analyzed. (a) Control; (b) P1 nuclease plus nucleotide pyrophosphatase; (c) P1 nuclease plus alkaline phosphatase.

of the oligo(U+) mRNA was capped. Due to uncertainty about the recovery of caps by this procedure, however, further quantitation and characterization of 5′ termini by another method was desirable.

Characterization of Cap Structures in Oligo(U+) mRNA. The resistance of phosphodiester bonds of 2′-O-methylated nucleosides to attack by cyclizing ribonucleases allows the isolation of the more complete cap structures present in the mRNAs of higher eukaryotes. Exhaustive digestion of total poly(A+) mRNA with a combination of such ribonucleases resulted in several components that separated on the basis of charge during anion-exchange chromatography (Figure 2a). The component eluting shortly after a marker with a net charge of −5 and that which followed it with a charge of approximately −6 were characterized as cap 1 (m^7GpppXmpYp) and cap 2 (m^7GpppXmpYmpZp) structures, respectively, by the two types of specific enzymatic analyses described for Figure 3. In this identification, materials eluting from DEAE-cellulose with negative charges characteristic of cap 1 or cap 2 were each converted to a core structure (m^7GpppXm) by digestion with P1 nuclease. When alkaline phosphatase was included in the digest, monoesterified phosphate produced by P1 nuclease digestion was recovered as inorganic phosphate (Figure 3c). When nucleotide pyrophosphatase was included instead, the material was degraded to m^7pG, inorganic phosphate, and a mixture of 5′-nucleotides that occupy the X and Y sites of cap 1 and the X, Y, and Z sites of cap 2 (Figure 3b). The relative amount of ^{32}P recovered in each of these components is that expected if essentially all ^{32}P recovered in the DEAE fractions was present as caps.

Oligo(U+) mRNA contains both cap 1 and cap 2 structures, since the materials eluting with the appropriate charges from DEAE-cellulose (Figure 2d) were identified as caps by the procedures described above. Thus oligo(U+) mRNA resembles cytoplasmic mRNA in this property rather than nuclear RNA which contained little if any cap 2 material (Figure 2c). The absence of cap 2 structures from hnRNA has been reported for mouse L cells (Perry ' Kelly, 1976) and CHO cells (Salditt-Georgieff et al., 1980).

Other 5′ Termini in Messenger RNA. Close examination of each of the elution profiles of Figure 2 shows that some ^{32}P-labeled material was eluted with a net negative charge of about −4. This material was identified as a mixture of 3′,5′-nucleoside diphosphates (pNp) since all of the ^{32}P was released as inorganic phosphate after treatment with alkaline phosphatase but only 50% was released by P1 nuclease, which has a 3′-phosphatase activity. In the latter case, the remaining 50% was recovered in the four 5′-mononucleotides separated

Table II: 5'-End Composition of RNA Fractions

RNA	expt	5'-end composition (mol %)[a]			cap 1/ cap 2[a]	RNA size based on[b]	
		pNp	cap 1	cap 2		5' ends	caps
poly(A+) mRNA	1	32	42	27	1.56	2096	3076
	2	37	29	34	1.43	2334	2817
	6	24	44	32	1.38	1819	2405
depolyadenylated mRNA	2	39	36	25	1.45	1595	2633
	6	29	42	28	1.49	1681	2381
depolyadenylated oligo(U–) mRNA	1	53	28	19	1.45	1356	2904
	3	61	24	15	1.54	920	2351
	4	45	32	23	1.35		
depolyadenylated oligo(U+) mRNA[c]	2	61 (412)	25 (500)	13 (308)	1.91	1445	3716
	3	61 (468)	26 (570)	13 (338)	1.96	1992	5152
	4	61 (399)	26 (488)	13 (281)	2.05	1913	4966
	6[d]	43 (302)	35 (708)	23 (544)	1.53	1209	2115
poly(A+) nuclear RNA	6[e]	49	44	8[e]	5.64[e]	4068	7920
>10S poly(A+) nuclear RNA	6[f]				9.42		
>10S poly(A–) nuclear RNA	1[f]				24.7		

[a] The cpm contained in each of the peaks from DEAE-cellulose columns (Figure 2) corresponding to charges of −4.2, −5.3, and −6 were quantitated by subtracting out appropriate backgrounds and converted to relative numbers of 5' ends (the relative specific activity of the β-phosphate of caps were calculated to be 1.82 by the formulas described in Table I). The type of end, the structure, and the relative number of ends, respectively, are as follows: for peak −4.2, monophosphate, pNp, and cpm/2; for peak −5.3, cap 1, m^7GpppXmpYp, and cpm/5.82; for peak −6, cap 2, m^7GpppXmpYmpZp, and cpm/6.82. These numbers were then used to calculate the percent of each type of 5' end and the cap 1/cap 2 ratio for a given experiment. [b] The RNA size calculations are based on the ratio of cpm eluting at a charge of −2 (Np's) either to the total relative number of 5' ends or to the relative number of capped ends. [c] Numbers in parentheses indicate the actual cpm used to calculate the relative number of ends. [d] This oligo(U+) RNA was isolated by using poly(A)-Sepharose instead of poly(A)-agarose. [e] Analysis of total nuclear poly(A+) RNA. Much of the material which elutes at a charge of −6 (Figure 2c) is not cap 2, since it failed to bind to DBAE-cellulose. [f] Analysis of the RNase T_2 digestion products of >10S nuclear poly(A+) or poly(A–) RNA which bound to DBAE-cellulose.

by high-voltage electrophoresis (data not shown).

The presence of a small, but significant, quantity of 3',5'-nucleoside diphosphates in a total RNase digest of poly(A)-containing mRNA has also been reported by others (Schibler & Perry, 1976; Levis & Penman, 1978). The origin of such 5' termini is not known. In several cases the RNA preparations from which poly(A) had been removed with RNase H (Wood & Edmonds, 1981) appeared to contain more than twice as many 5'-phosphorylated ends as untreated mRNA (Figure 2a,b and Table II), suggesting that some nicking may have resulted from this treatment. The fact that we seldom observed reduction in the average size of mRNAs following such treatment [Wood & Edmonds (1981) and Table II] suggests that endonucleolytic activity associated with RNase H was confined to sites close to the ends of molecules. One experiment (number 6, Table II) carried out with a reduced level of RNase H that still removed poly(A) effectively resulted in a significant reduction in the generation of 5'-phosphorylated ends. This suggests that it is possible to eliminate this feature of poly(A) removal.

Quantitation of 5' Termini in Messenger RNA. Molar frequencies of the three types of 5' termini detected in mRNA preparations obtained from several 4-h labelings of HeLa cells with $^{32}PO_4$ are tabulated in Table II. The number of RNA molecules bearing cap 1 relative to those with cap 2 is remarkably constant for these preparations. The ratio is unaffected by removal of poly(A) with RNase H or by removal of the small fraction of oligo(U)-containing mRNA molecules from the total mRNA population (Wood & Edmonds, 1981). However, this ratio was different in the oligo(U)-containing mRNA where a significantly higher value of 2.0 was found in three of four experiments. This result is difficult to evaluate for a single labeling period since it has been reported that the value of this ratio declines until cytoplasmic 2'-O-methylation at the Y site reaches a steady state where label in cap 2 exceeds that in cap 1 (Perry & Kelley, 1976; Friderici et al., 1976). A kinetic analysis of caps might establish whether oligo(U+) mRNA is in fact metabolically distinct from the bulk of the mRNA. Other possible explanations for this increased ratio would include contamination of the oligo(U+) mRNA with oligo(U)-containing nuclear RNA since hnRNA contains very little, if any, cap 2. Our own estimates of this ratio for total poly(A)-containing nuclear RNA (Table II, experiment 6[e]) are an underestimate since much of the material recovered from DEAE-cellulose columns with a negative charge of 6 (Figure 2c) is not a cap structure: ~50% of it failed to bind to DBAE-cellulose as expected for caps (Schibler & Perry, 1976). The small amount of this material that is cap 2 may also come from cytoplasm since the nuclear fraction was not detergent washed (Penman, 1966).

The average lengths of the molecules in these mRNA preparations, calculated from the moles of 5' ends relative to the moles of mononucleotides as described in the legend to Table II, are compatible with the distribution of lengths obtained from the electrophoretic mobilities and sedimentation velocities of these mRNA preparations (Wood & Edmonds, 1981). The average lengths of 1200–2300 nucleotides calculated from 5' end group analysis would be compatible with the observed comigration in denaturing gels of the peak fraction of this mRNA with HeLa 18S ribosomal RNA, estimated to be 2000 ± 60 nucleotides (McMaster & Carmichael, 1977), although, of course, the mRNA profile is much more heterogeneous. It is evident that the average lengths of oligo(U+) mRNA molecules fall within this size range, and in two experiments they were at the upper end of the range.

If only capped termini are used to calculate average lengths, large overestimates are seen in the case of oligo(U+) mRNA. This would be expected if some capped fragments have been lost by RNase H induced nicks between the cap and the oligo(U) sequence. Although the increased number of 5'-phosphorylated ends suggests that similar nicking occurs in oligo(U–) mRNA (Table II), the discrepancy in lengths observed for the two methods was much less in this case because the capped fragments were not lost as was the case for oligo(U)-containing mRNA, which had been further selected on poly(A)–agarose.

Table III: Base Composition of Caps[a]

RNA	expt	cap 1 (%) pC	pA	pG	pU	total cpm analyzed	cap 2 (%) pC	pA	pG	pU	total cpm analyzed
poly(A+) mRNA	2	17	22	42	19	2987	18	18	26	38	3435
	6	20	23	38	19	764	18	23	21	38	655
depolyadenylated oligo(U−) mRNA	1	19	22	40	19	4433	17	22	23	38	4064
	3	19	22	38	21	1991	16	20	23	43	1640
	5	22	19	37	22	1571	20	18	23	40	1465
depolyadenylated oligo(U+) mRNA	2	15	20	44	21	199	16	16	29	38	201
	3						18	25	20	37	121
>10S poly(A+) nuclear RNA	6	19	26	34	21	756	16	24	27	34	169
>10S poly(A−) nuclear RNA	1	15	24	40	20	1174					

[a] Cap base compositions were determined by measuring the cpm in each of the four mononucleotides generated by P1 nuclease plus nucleotide pyrophosphatase treatment of isolated caps, separated by paper electrophoresis as in Figure 3b.

Nucleotide Composition of Caps in Oligo(U+) mRNA. Nucleotide analysis of both cap 1 and cap 2 structures was carried out as described under Experimental Procedures and illustrated in Figure 3b to see if the caps of oligo(U+) mRNA molecules differed from those of mRNAs lacking oligo(U). Table III summarized the nucleotide composition of cap 1 (X plus Y bases) and cap 2 (X, Y, and Z bases) structures of the mRNA populations described in Table II (the separation method employed does not distinguish between 5′-nucleotides and their 2′-*O*-methyl derivatives). It is apparent that in each case this distribution is nonrandom; cap 1 has a high percentage of ^{32}P in pG and cap 2 in pU. Although the data are limited and are based on low amounts of label, it is evident that the nucleotide compositions of caps 1 and 2 in oligo(U+) mRNA resemble those of total poly(A+) mRNA. It is not possible from these very limited data to conclude that some specific differences might not exist in the nucleotides of oligo(U+) mRNA cap structures, but their overall similarity in both X base composition (Figure 1) and total base composition (Table III) to oligo(U−) mRNA is sufficiently striking to discourage attempts to detect such differences. It should also be noted that the nucleotide composition of the cap 1 structures of poly(A+) hnRNA resembles that of cap 1 of mRNA (Table III) as expected from previous reports with L cells (Perry et al., 1975).

Discussion

The experiments reported here show that the 5′ termini of the subpopulation of cytoplasmic polyadenylated mRNA that contain a short internal poly(U) sequence are remarkably similar to the 5′ termini of the total polyadenylated mRNA. Both cap 1 and cap 2 structures are present in each, albeit in somewhat different relative amounts, and the base compositions of the two types of caps are different from each other but do not differ significantly between the two RNA populations.

The presence of cap 2 in the oligo(U+) mRNA demonstrates that this RNA is cytoplasmic, since methylation at the Y nucleoside of caps appears to occur exclusively in the cytoplasm (Perry & Kelley, 1976). This confirms a conclusion reached earlier on the basis of several less direct pieces of evidence that cytoplasmic mRNA contains oligo(U) sequences (Korwek et al., 1976).

The fact that the base compositions of cap 1 and cap 2 structures of oligo(U+) mRNAs neatly parallel the compositions of these same structures in the total polyadenylated mRNA raises the possibility that other sequence information in the two RNA populations may be similar. Hybridization experiments using DNA sequences complementary to mRNA sequences could establish such homologies.

One difference that does emerge from the experiments reported here is the higher ratio of cap 1 to cap 2 found in the oligo(U+) molecules. Although this could stem from contamination with nuclear oligo(U)-containing RNA, it might also reflect a more rapid turnover of these molecules, since methylation of the Y nucleoside is a progressive event (Friderici et al., 1976). A more rapid turnover of oligo(U+) mRNA would be consistent with the precursor role suggested for it on the basis of its larger size (Wood & Edmonds, 1981) and the similarities of 5′ termini reported here. Such suggestions must, however, be regarded as speculative until kinetic and sequence information are obtained.

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