Volume 4 Number 8 August 1977 Nucleic Acids Research

Methylated constituents of Aedes albopictus poly (A)-containing messenger RNA

Chuen-Chin HsuChen and Donald T. Dubin

Department of Microbiology, CMDNJ-Rutgers Medical School, Piscataway, NJ 08854, USA

Received 27 April 1977

ABSTRACT

Poly (A)-containing mRNA prepared from cultured mosquito (Aedes albopictus) cells was found to contain methylated 5'-terminal "caps" as well as internal m^6A residues. Both type I [m^7G(5')ppp(5')Xmp] and type II [m^7G(5')ppp(5')XmpYmp] caps were present, at molar ratio of ca five to one. All four common RNA bases were represented in the second position (Xm) of the caps, adenine being the most abundant and N^6-methyladenine being absent. The four bases were also represented in the third position (Ym), but here uracil was the predominant base. There was approximately one internal m^6A residue for every three caps. These studies demonstrate that mRNA from an invertebrate source can have a methylation pattern comparable with that of mammalian cells in its complexity.

INTRODUCTION

The presence of methylated 5'-terminal "caps" has recently been recognized as a feature of most eukaryotic cellular mRNA (see ref. 1 for review). Mammalian poly A-containing ["poly A(+)"] mRNA populations have been found to contain a complex mixture of such methylated caps, of the form m^7GpppXmp ("Type I") and m^7GpppXmpYmp ("Type II"), where Xm and Ym can be derivatives of any of the four major RNA bases.[2-7] Messenger RNA from lower organisms (yeast,[8,9] slime mold,[10] brine shrimp[11] and sea urchin[12,13]) appears, on the other hand, to have simpler cap patterns (cf Discussion). Two reports on mRNA from insect sources have also indicated rather simple patterns: silk fibroin mRNA terminates exclusively in m^7GpppAmpUmp (ref. 14), and tobacco hornworm oocyte mRNA appears to terminate in GpppGmp (ref. 15). We now present studies on cultured mosquito cells showing that the mixed poly A(+) mRNA of insects can in fact be comparable to that of mammals in the complexity of its 5'-terminal caps. Internal m^6A, another characteristic component of mammalian mRNA (refs. 2-7), was also found in the mosquito mRNA, but at considerably lower levels than in mammalian mRNA.

METHODS

Growth and Labeling of Cells. A cloned strain ("AIS C-3"; ref. 16) of

Aedes albopictus cells (Singh[17]), kindly provided by Mr. Steven Mento, was grown in a medium consisting of Eagle's MEM supplemented with non-essential amino acids,[18] plus 10% heat-inactivated fetal calf serum[16] and adenosine and guanosine, 2 x 10^{-5}M. Cells were seeded at 2-3 x 10^{6} per 10 cm plate and incubated in 10 ml of medium at 28°C for 3 days prior to labeling. For labeling in the presence of 5-fluorouridine (FUrd) cells were pre-incubated for one hr with 5 µg/ml of the drug in methionine-free growth medium. Ethidium bromide, 1 µg/ml, was present during and 10 minutes prior to labeling, to inhibit synthesis of mitochondrial RNA.[19]

Processing of Cells and Preparation of RNA. At the time of harvesting, medium was removed and monolayers were washed with iced phosphate buffered saline[20], scraped into centrifuge tubes and pelleted (2,000 rpm, 5 min, 4°C). The pellets were resuspended in 2 ml of 10 mM Tris-HCl, pH 8.0, containing 1mM $MgCl_2$, 0.3M sucrose, 0.5% Triton X-100, 25 µg/ml polyvinyl sulfate, 35 µg/ml spermine, and 1% diethylpyrocarbonate (modified from ref. 19). After 10 min in ice, the preparations were agitated on a Vortex mixer (2 x 5 sec), and nuclei were removed by centrifugation (2500 rpm, 6 min, 4°C). RNA was prepared from crude cytoplasmic fractions using a slight modification of our earlier phenol-SDS procedure.[21] Samples were brought to 4 mg/ml bentonite, 8mM EDTA, 1.5% SDS and 0.15M sodium acetate, pH 5.2, and held at 37° for 20'. They were then subjected to 3 extractions with equal volumes of phenol and the RNA was precipitated from the aqueous phase with 2 volumes of ethanol. Poly A(+) mRNA was then prepared by two (for cells labeled in the presence of FUrd) or three (for cells labeled in the absence of FUrd) cycles of oligo-T-cellulose chromatography.[6] After re-precipitation with ethanol, samples were dissolved in "Tris-SDS" (0.5% SDS, 0.1N NaCl, in 0.05M Tris HCl, pH 7.4), layered on 5 - 20% (w/v) sucrose gradients in Tris-SDS, and centrifuged in a Spinco SW41 rotor (38,000 rev/min, 17h, 23°C).

RNA analysis. Our procedures for enzymatic and acid hydrolysis of RNA and nucleotides, and for chromatographic and electrophoretic separation of products, have been described in detail,[22] except as follows.

For combined digestion with Penicillium nuclease ("P1") and alkaline phosphatase, samples were dissolved in 75 µl of 10 mM sodium acetate pH 6.0, containing 0.5 mg P1/ml, and incubated at 37° for 45 min. 5 µl of Tris-HCl (1M, pH 8.5), 1 µl of $MgCl_2$ (0.5M) and 20 µl of alkaline phosphatase (0.5 mg/ml in 0.05M Tris-HCl buffer, pH 8.5) were then added and incubation was continued for 2h.

For combined digestion with T2 ribonuclease and alkaline phosphatase, the reaction mixture after T2 digestion[6] was brought to 50 mM Tris-HCl (pH 8.5), 5 mM $MgCl_2$ and 0.1 µg/ml alkaline phosphatase (as above) and incubated at 37°C for an additional 2 h.

Combined digestion with phosphodiesterase and alkaline phosphatase was accomplished by incorporating venom phosphodiesterase (0.2 mg/ml) into the alkaline phosphatase mix described previously.[6]

Thin layer chromatography was performed following the procedure of Wei and Moss,[23] using 20 x 20 cm cellulose plates, and developing for 2h with ethyl acetate : isopropanol : conc. NH_4OH : n-butanol (3 : 2 : 2 : 1). Borate chromatography was performed using n-butanol : 0.8M boric acid : conc. NH_4OH (200 : 27 : 0.8) essentially as described by Al-Arif and Sporn.[24]

Materials. Ribonuclease T2 was obtained from Sigma Chemical Co., E. coli alkaline phosphatase and rattlesnake venom phosphodiesterase from Worthington Biochemical Corp., and Penicillium nuclease P1 from Yamasa Shoyu Co. Markers for chromatography and electrophoresis were obtained as previously described,[6] except that Gm was a product of P-L Biochemicals, Inc.; and m^6Am was isolated (as a methyl-labeled compound) from hamster cell mRNA (ref. 6). [Methyl-^{3}H]methionine was obtained from Schwarz Biochemical Corp. or Amersham-Searle Co., and [2-^{14}C]uridine from New England Nuc. Corp.

RESULTS

An initial experiment was patterned after earlier studies by Spradling et al[19] designed to obtain intact poly A(+) mRNA from uridine-labeled A. albopictus cells. In particular, synthesis of cytoplasmic rRNA and mitochondrial RNA were preferentially suppressed with FUrd and ethidium bromide, respectively; and poly A(+) RNA was obtained from a crude cytoplasmic fraction after disrupting cells with detergent in the presence of ribonuclease inhibitors. In the experiments described here, cells were labeled for 5h with both [methyl-^{3}H]methionine and ^{14}C-uridine, and adenosine and guanosine were added to dampen "leakage" of ^{3}H into purine rings. The Poly A(+) RNA yielded density gradient patterns (not shown) essentially the same as the "5h" pattern of Spradling et al[19]: the ^{14}C-labeled RNA sedimented heterogeneously, with a mode at approx. 12S. The pattern for *methyl* label in RNA, as detected by the ^{3}H, roughly paralleled the ^{14}C pattern (cf ref. 6), and relatively broad (8-30S) cuts were used for the analyses below.

In order to discriminate between internal, and 5'-terminal, methylated residues, samples were digested with T2 ribonuclease followed by DEAE-cellulose column chromatography. As shown in Fig. 1, approx. 50% of the ^{3}H-labeled nucleotides eluted with the mononucleotide ("-2") peak. When this fraction was recovered and subjected to acid hydrolysis followed by paper chromatography in a system[25] that separates adenine and the commonly occurring methylated adenines ($m_1$ade, $m_2$ade, m^6ade and m_2^6ade), 80-85% of the ^{3}H was found to be in adenine, presumably representing "leakage" into purine skeleton. However, a portion, amounting to 10-15% of the mono-nucleotide ^{3}H, was found to be in m^6ade.

The remaining ^{3}H-labeled nucleotides eluted as a major peak in the region of the "-5" marker (as expected [3,4,7] for m^7GpppXmpYp), with a minor shoulder extending towards the "-6" region (presumably representing some m^7GpppXmpYmpNp). When labeled material from this fraction was subjected to acid hydrolysis followed by electrophoresis at pH 3.5, approx. 45% ran as a

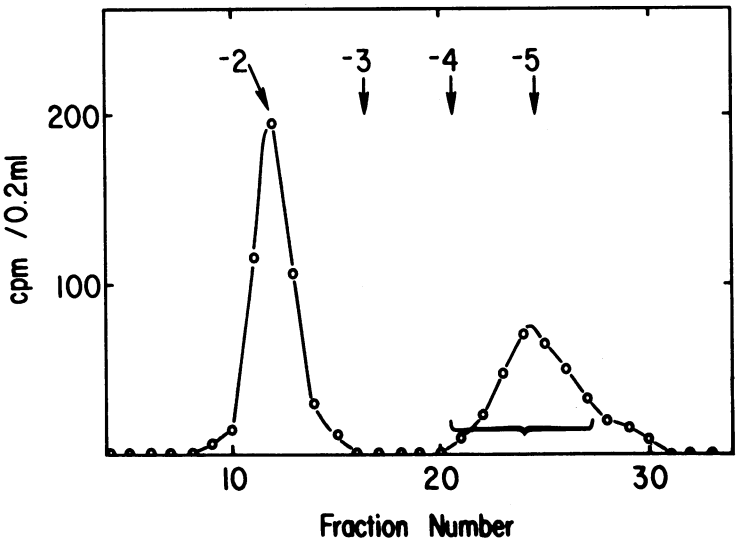

Fig. 1. DEAE-Cellulose Pattern of T2 Ribonuclease-Treated A. albopictus Poly A(+) mRNA. Five plates were labeled in the presence of FUrd, as described in Methods, with [Methyl-^{3}H]methionine (10 mci at 4.7 mci/μmole; 15μg/ml in 20 ml total) and 2-^{14}C-uridine (20 μci at 60 mci/mmole; 1.7×10^{-5}M). The poly A(+) cytoplasmic RNA was obtained as described in the text and a portion (15% of total) was digested with T2 ribonuclease followed by DEAE cellulose chromatography as described in Methods (230 ml total gradient volume, 0.05-0.5N NaCl; 2 ml fractions). The positions and net charges of pancreatic RNase digest markers are shown by arrows. Only the ^{3}H is plotted. The ^{14}C, as expected, was detectable only in the mononucleotide (-2) region.

Table 1. General Distribution of Methylated Residues in A. albopictus Poly A(+) mRNA.

	Residues Per m^7Gua	
Experiment	m^6Ade	5'-Terminal Methyl Ribose
1 (+FUrd)	0.36	1.21
2 (-FUrd)	0.32	1.35

For experiment 1 cells were labeled in the presence of FUrd and for experiment 2 cells were labeled in its absence, as described in the legends to Figs. 1 and 3. T2 ribonuclease-released "-2" (mononucleotide) and "-5 to -6" (terminal oligonucleotide) pools were recovered from DEAE separations as for Fig. 1, and portions of these were subjected to acid hydrolysis followed by electrophoresis at pH 3.5. ^{3}H running in the adenine region from "-2" pools was eluted from parallel electropherograms for chromatographic analysis, as described in the text. ^{3}H running on electropherograms heterogeneously towards the anode (beyond Cp) was scored as methyl ribose (cf. ref. 6).

peak corresponding to m^7Gua; its identity was verified by paper chromatography. The remainder ran heterogeneously towards the anode, as expected for ribose-methylated derivatives, while none (<2%) ran with adenine, as m^6ade residues would.[6] No DEAE peak corresponded to the dinucleotide ("-3") region, indicating the virtual absence of label from contaminating rRNA (over 80% of whose methyl groups are on internal ribose residues that are released as dinucleotides by alkali). These results are summarized in quantitative form in Table 1, together with results from a similar analysis of an RNA sample from cells labeled in the absence of FUrd (v.i.). There were 0.32-0.36 internal m^6ade residues per cap, and in caps there were 1.2 to 1.3 putative ribose-methylated residues per m^7Gua residue.

The presence of ribose-methylated residues in the 5'-termini was verified, and their nature was examined, by subjecting samples from "-5" peaks to mixed phosphodiesterase-alkaline phosphatase digestion followed by thin layer and borate chromatography. As illustrated in Fig. 2 and summarized in Table 2, significant amounts of ribose-methylated derivatives of each of the 4 major bases occurred. No m^6Am was detected, in accord with our failure to detect m^6ade in acid hydrolysates of such samples.

A second set of experiments was performed with two considerations in mind: (1) it was possible that FUrd was artifactually affecting methylation patterns. Poly A(+) mRNA was therefore obtained from cells labeled in the same manner but in the absence of FUrd. These preparations con-

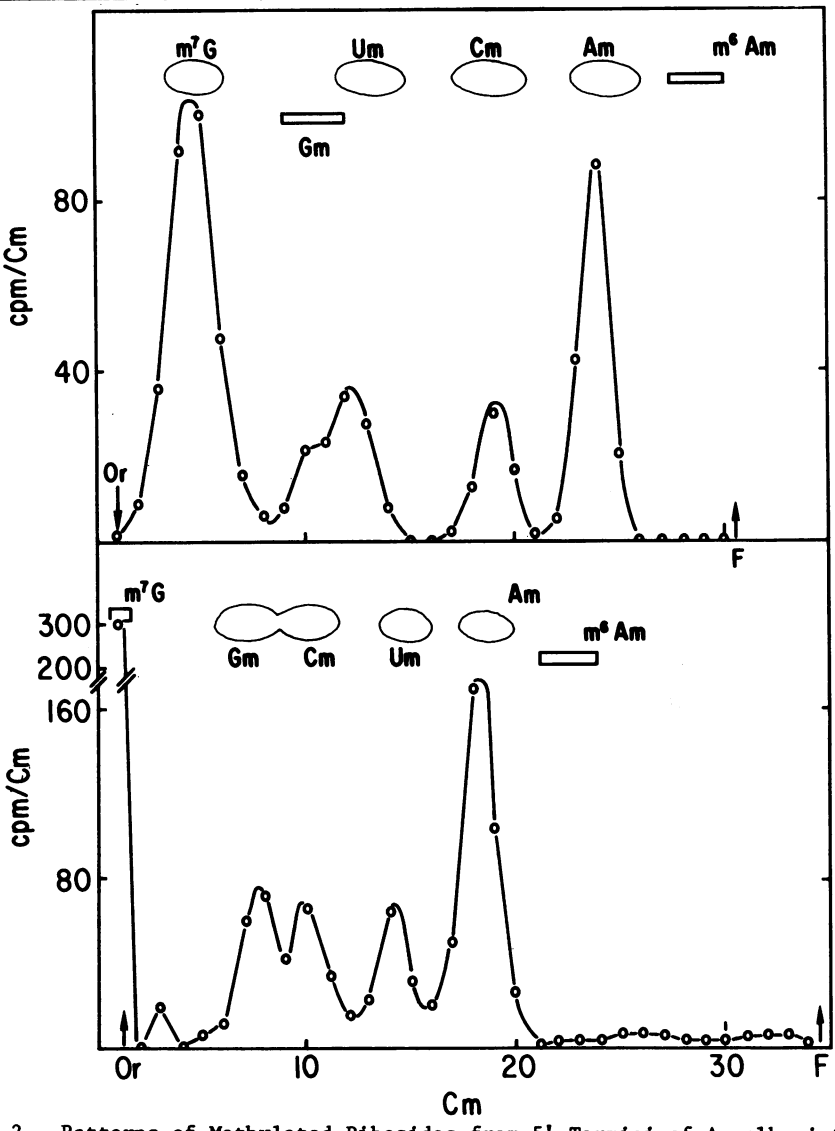

Fig. 2. Patterns of Methylated Ribosides from 5'-Termini of A. albopictus Poly A(+) mRNA. The "-5" pool from the separation of Fig. 1 (bracket) was recovered and treated with venom phosphodiesterase and alkaline phosphatase, and portions of the digest were subjected to thin layer chromatography (top) and borate paper chromatography (bottom), all as described in Methods. Ovals represent markers run with the labeled samples and bars represent markers run in parallel.

tained a small amount (ca 10%) of methyl label as internal Nm residues, presumably from traces of labeled rRNA. They were otherwise essentially the same in sedimentation and methylation properties as the preparation described above. (2) In earlier studies on invertebrate mRNA little or no

Table 2. Percentages of Methylated Ribosides in the "X" and "Y" Positions of A. albopictus mRNA caps

Experiment	Peak	X				Y			
		Cm	Am	Gm	Um	Cm	Am	Gm	Um
1	-5	21	43	19	18				
2	-3	19	45	19	18				
	-2.5	17	55	17	11				
	-4					5	21	14	60
3	-2.5	22	42	27	10				
	-4					10	15	11	64

Peak designations refer to the apparent net charge corresponding to DEAE-cellulose elution positions of particular sample analyzed. "-5" refers to a T2-released terminus as described for Figs. 1 and 2; "-3" and "-4" refer to T2-ribonuclease plus phosphatase-released termini designated by brackets A and B respectively in Fig. 3; and "-2.5" refers to P1 plus phosphatase-released termini as described for Fig. 4. Experiments 1 and 2 are those described for Table 1, and 3 was similar to 2 (cf legend to Fig. 4). Methylated riboside analyses were performed as described for Fig. 2, except for the second Xm analysis listed for Expt. 2; these data arise from the pattern of Fig. 4.

methylated pyrimidine was found in the Xm position of 5'-terminal caps (v. Discussion). It seemed important, therefore, to verify that the substantial amounts of Um and Cm in our preparations indeed arose from Xm residues rather than the Ym residues of contaminating Type II caps. To this end we applied the following strategy (patterned after Perry and Kelly[3]). A sample was subjected to T2 ribonuclease plus alkaline phosphatase, followed by DEAE chromatography. As shown in Fig. 3, the dephosphorylated Type I termini (m^7GpppXmpN) eluted, as expected (cf ref. 3), near the -3 marker, and were well resolved from the small proportion (15-20% of the cap methyl label) of type II caps (m^7GpppXmpYmpN), that eluted in the -4 region. A portion of the -3 peak was processed as described for Fig. 2, and yielded a distribution of ribose-methylated components similar to that obtained earlier (Table 2, expt. 2). A second portion was treated with P1 ribonuclease (which cleaves XmpN phosphodiester bonds) plus alkaline phosphatase, yielding termini with the presumed structure m^7GpppXm; on DEAE chromatography the product yielded a single methyl-labeled ("-2.5") peak between the -2 and -3 markers, also as expected.[3] The methylated riboside distribution of the "-2.5" peak was, again, similar to those ob-

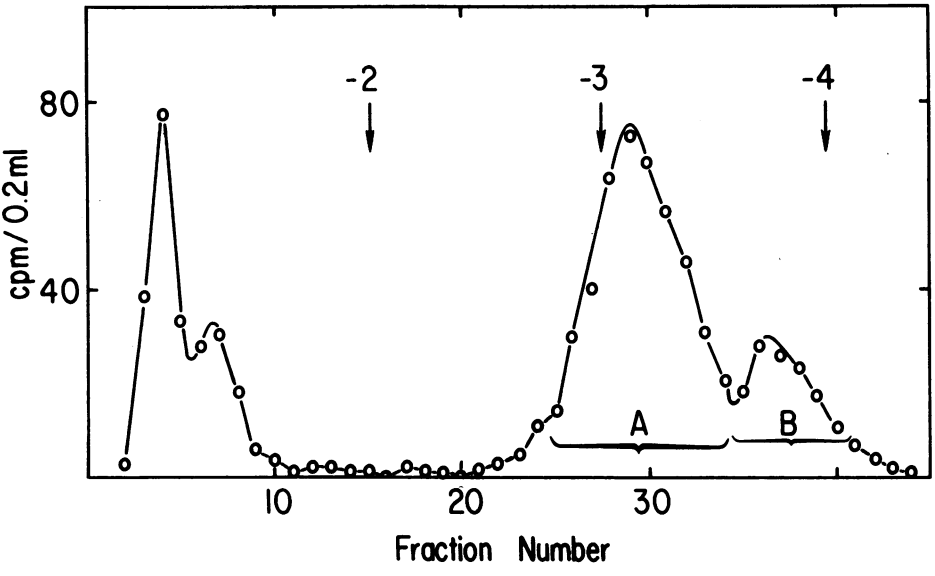

Fig. 3. DEAE Cellulose Pattern of Combined T2 Ribonuclease-Alkaline Phosphatase Digest of A. albopictus Poly A(+) mRNA. Poly A(+) RNA was obtained as for Fig. 1, except that cells were labeled in the absence of FUrd, with [Methyl-^{3}H]methionine (10 mci at 14 mci/mmole, 5.3µg/ml in 20 ml) and 2-^{14}C-uridine (14 µci at 60 mci/mmole; 1.2 x 10^{-5}M). A portion (1/3) of the preparation was subjected to combined digestion with T2 ribonuclease and alkaline phosphatase, followed by DEAE cellulose chromatography (230 ml gradient, 0.05 to 0.275 N NaCl; 2 ml fractions) as described in Methods. Symbols are as for Fig. 1.

tained earlier (Table 2). A somewhat different sort of analysis of a "-2.5" peak from a third preparation also yielded concordant results (Fig. 4; experiment 3 of Table 2). We conclude that the Xm residues of Aedes mRNA caps do indeed contain a substantial representation of each of the 4 major ribosides.

We also examined Ym residues by treating T2 ribonuclease-released termini with P1 ribonuclease plus alkaline phosphatase, and subjecting digests directly to thin layer and borate paper chromatography. Methyl-labeled ribosides released thereby from T2-derived termini [m^{7}GpppXmpYmpN(p)] must be Ym residues; residual phosphorylated compounds remain at the origin in both chromatographic systems, and do not distort the riboside patterns. As summarized in Table 2, the Ym residues also included derivatives of each of the major bases, but there was a striking enrichment for Um.

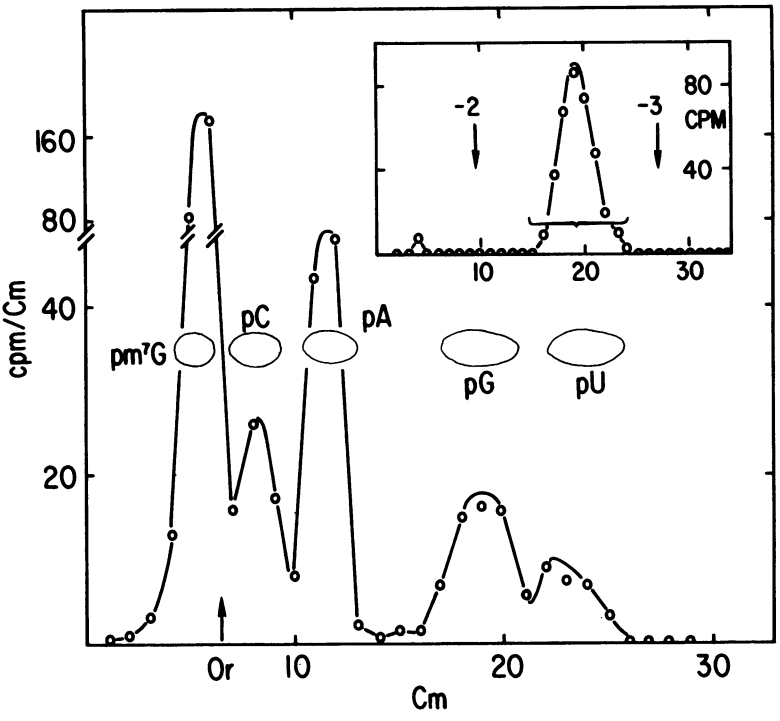

Fig. 4. Pattern of Methylated Nucleotides from type I 5'-Termini of A. albopictus Poly A(+) mRNA. Poly A(+) RNA was obtained from an experiment similar to that described for Fig. 3, and nucleotides from a cut corresponding to the "-3" peak of that Figure were recovered, digested with P1 ribonuclease plus alkaline phosphatase, and re-run on a similar column; 0.2 ml of each fraction was counted (insert). The "-2.5" peak (bracket) was recovered and a portion was digested with venom phosphodiesterase and subjected to electrophoresis at pH 3.5 (3000 V/90 cm, 3.5h, sample spotted 45 cm from cathode edge of 110 cm sheet). The ribose-methylated nucleotides migrate approx. with the marker major nucleotides in this system.

DISCUSSION

Analyses of mRNA caps from several sources have permitted the beginnings of a rudimentary "cap phylogeny."[1] There is evidence that complexity of caps correlates with complexity - and perhaps evolutionary status - of organisms. For example, yeast mRNA appears to contain only type zero ($m^7GpppXp$) caps, with the X position limited to purines[8,9]; ameba (Dictostelium) mRNA is similar, except for the presence of a small proportion of Type I caps[10]; brine shrimp mRNA contains all Type I caps but with still only purines in the X position[11]; sea urchin mRNA contains Type I caps with a predominance of purines but some pyrimidines in the X

position[12,13]; and mammalian[2-7] and chick cell (HsuChen and Dubin, unpublished observations) poly A(+) mRNA contains Types I and II caps with substantial representation of all 4 major bases in the X position. A study on lepidoptera (hornworm oocyte) poly A(+) mRNA can be interpreted to indicate that insect mRNA may be "primitive" with regard to this scheme, since the only type of cap noted had the properties of GpppGmp.[15] The present work, on the other hand, shows that the poly A(+) mRNA of cultured mosquito cells exhibits a cap complexity similar to that of vertebrate mRNA. The similarity between insect and vertebrate mRNA populations may extend also to the nature of the Y residues of Type II caps, which are markedly enriched for U residues in both mouse (ref. 3) and mosquito cells (this work).

If we presume that our present results are representative of at least certain insect systems, the question arises as to whether the complexity of the A. albopictus caps defies the "cap phylogeny" scheme outlined above. We believe it does not. Although insects as individuals may be "simpler" than typical vertebrates, many insect species have extraordinarily complex developmental, behavioral and social characteristics. In addition, the order diptera is considered to be highly evolved and has been eminently successful in diverse natural environments.[26] Thus the complexity of the Aedes caps is in accord with the idea that cap complexity correlates with evolutionary progress and/or organism complexity. Whether the similarity between man and mosquito reflects parallel evolution, or inheritance from a common very primitive ancestor to both vertebrates and invertebrates (cf ref. 26), remains to be seen.

Little is known as to the functional significance of the diversity, or the modifications, of the X and Y residues of mRNA caps, but a persuasive argument can be made that the relative abundance of pyrimidines in the X position is correlated with the extent to which the mRNA of a particular organism arises via endonucleolytic processing (see, e.g., refs. 1, 3, 4 and 8). In this regard it is pertinent to note that kinetic and physical properties of A. albopictus hnRNA and mRNA suggest that extensive processing does occur in these cells.[27]

There is a well-defined difference between A. albopictus and mammalian caps, namely the presence of considerable Am and no m^6Am in the former, compared to the preponderance of the base-methylated form in the latter. In this regard the insect mRNA seems to resemble that of yeast and the lower invertebrates mentioned above.[8-13]

In its low content of internal m^6A the insect mRNA also differs from

mammalian mRNA. Compared to values of 1 to 3 residues per molecule for the latter[3-7], the present values (assuming all molecules are capped) amount to only one internal m^6A residue per 3 molecules on the average. This value is close to upper limits of internal m^6A established for yeast[8] and dictostelium[10] (which were considered to be zero). On the other hand, brine shrimp and sea urchin poly A(+) mRNA, although lacking penultimate m^6A, appear to have levels of internal m^6A comparable to those of mammalian mRNA.[11-13] In any event, whatever the function of this residue, it appears to be largely or wholly dispensable in some cell types, including A. albopictus.

This work was supported by Research Grant No. GM-14957 from the National Institute of General Medical Sciences. C-C. H.C. is a post-doctoral trainee under Institutional National Research Award No. CA-09069 from the National Cancer Institute.

REFERENCES

1 Shatkin, A.J. (1976) Cell 9, 645-653.
2 Cory, S. and Adams, J.M. (1975) J. Mol. Biol. 99, 519-547.
3 Perry, R.P. and Kelly, D.E. (1976) Cell 8, 433-442.
4 Salditt-Georgieff, M., Jelinek, W., Darnell, J., Furuichi, Y., Morgan, M., and Shatkin, A. (1976) Cell 7, 227-237.
5 Desrosiers, R.C., Friderici, K.H., and Rottman, F.M. (1975) Biochem. 14, 4367-4374.
6 Dubin, D.T. and Taylor, R.H. (1975) Nuc. Acids Res. 2, 1653-1668.
7 Wei, C.M., Gershowitz, A., and Moss, B. (1976) Biochem. 15, 397-401.
8 Sripati, C.E., Groner, Y., and Warner, J.R. (1976) J. Biol. Chem. 251, 2898-2904.
9 Mager, W.H., Klootwijk, J., and Klein, I. (1976) Mol. Biol. Rep. 3, 9-18.
10 Dottin, R., Weiner, A.M., and Lodish, H.F. (1976) Cell 8, 233-244.
11 Groner, Y., Grosfeld, H., and Littauer, U.Z. (1976) Eur. J. Biochem. 71, 281-293.
12 Surrey, S., and Nemer, M. (1976) Cell 9, 589-595.
13 Faust, M., Millward, S., Duchastel, A., and Fromson, D. (1976) Cell 9, 597-604.
14 Yang, N.S., Manning, R.F. and Gage, L.P. (1976) Cell 7, 339-347.
15 Kastern, W.H., and Berry, S.J. (1976) Biochem. Biophys. Res. Comm. 71, 37-44.
16 Sarver, N. and Stollar, V. (1977) Virology, in press.
17 Singh, K.R.P. (1967) Curr. Sci. 36, 506-508.
18 Eagle, H. (1959) Science 130, 432-437.
19 Spradling, A., Hui, H., and Penman, S. (1975) Cell 4, 131-137.
20 Dulbecco, R. and Vogt, M. (1954) J. Exp. Med. 99, 167-199.
21 Dubin, D.T. (1964) J. Mol. Biol. 8, 749-767.
22 Dubin, D.T., Stollar, V., HsuChen, C.C., Timko, K. and Guild, G.M. (1977) Virology, in press.
23 Wei, C.M. and Moss, B. (1975) Proc. Natl. Acad. Sci., U.S.A. 72, 318-322.

24 Al-Arif, A. and Sporn, M.B. (1972) Anal. Biochem. 48, 386-393.
25 Dubin, D.T. (1974) J. Mol. Biol. 84, 257-273.
26 Gordon, M.S. (1976) Zoology, pp. 327; 391-397. Macmillan Publishing Co., N.Y.
27 Lengyel, J. and Penman, S. (1975) Cell 5, 281-290.