Nucleotide Sequences at the N^6-Methyladenosine Sites of HeLa Cell Messenger Ribonucleic Acid†

Cha-Mer Wei and Bernard Moss*

ABSTRACT: Borate gel chromatography was used to separate internal oligonucleotides containing N^6-methyladenosine (m^6A) from methylated 5′-terminal oligonucleotides of HeLa cell polyadenylylated mRNA. N^6-Methyladenosine occurs primarily in two sequences, -G-m^6A-C- (70%) and -A-m^6A-C- (30%). The nucleoside immediately following cytidine may be uridine, cytidine, or adenosine, while guanosine as well as other nucleosides occupy subsequent positions. Each of the four positions preceding the -(G or A)-m^6A-C- sequence may be occupied by a pyrimidine or a purine ribonucleoside. Since on a random basis all possible sequences containing -(G or A)-A-C-(U or C or A)- could occur once per 43 nucleotides whereas there is only one m^6A residue per thousand nucleotides, then either (1) not all potential sites are methylated, (2) there are multiple unique sequences perhaps methylated by several different enzymes, or (3) there are other unrecognized discriminating factors. The possibility that methylation of adenosine occurs exclusively in the region close to the 5′ terminus of the mRNA was considered. However, such a localization was excluded since the majority of m^6A residues were not found in 4 to 6S 5′-terminal fragments isolated by borate gel chromatography.

In addition to a characteristic 5′-terminal sequence, consisting of a m^7G residue connected from the 5′ position through a triphosphate bridge to the 5′ position of an adjacent 2′-*O*-methylribonucleoside (Wei et al., 1975; Perry et al., 1975a,b; Adams and Cory, 1975; Furuichi et al., 1975a), the polyadenylylated mRNAs of cultured animal cells possess a limited number of internal methylated nucleosides. In rat (Desrosiers et al., 1974), human (Wei et al., 1975; Furuichi et al., 1975a), and mouse (Perry et al., 1975a; Adams and Cory, 1975) cells, approximately one m^6A residue was found per thousand nucleotides. In polyadenylylated mRNA from cultured hamster cells, smaller amounts of m^5C as well as m^6A were identified (Dubin and Taylor, 1975). Nevertheless, internal methylated ribonucleosides appear to be absent from both globin mRNA (Perry and Scherrer, 1975; Heckle et al., 1976; Cheng and Kazazian, 1976) and from histone mRNA (Moss et al., 1977), the only defined cellular mRNAs examined thus far. The presence of m^6A in heterogeneous nuclear RNA (Perry et al., 1975b; Salditt-Georgieff et al., 1976) suggests that methylation takes place prior to transport of mRNA to the cytoplasm. In HeLa cell mRNA, the m^6A residues are found predominantly in two sequences -G-m^6A-C- and -A-m^6A-C- (Wei et al., 1976). Although m^6A residues are not adjacent to the 3′-poly(A) segment (Furuichi et al., 1975a,b), further information regarding their location within HeLa cell mRNA is not known. The present report is concerned with two questions: are m^6A residues located within unique sequences that are long enough to account for the low frequency of methylated adenosine residues and are they located within a region of the mRNA adjacent to the 5′ terminus?

Materials and Methods

Isolation of Polyadenylylated mRNA. [^{3}H]Methyl- and [^{14}C]uridine-labeled HeLa polyadenylylated mRNA was isolated by poly(U)[1]-Sepharose chromatography as described previously (Wei et al., 1976), except for the following modification. The polyadenylylated RNA eluted from the first poly(U)-Sepharose column with 90% formamide was heated in formamide at 60 °C for 2 min. After diluting with 4 volumes of buffer containing 0.7 M NaCl, 10 mM Tris-HCl (pH 7.5), 10 mM EDTA, and 0.2% sodium dodecyl sulfate, the sample was rechromatographed on a poly(U)-Sepharose column.

Enzymatic Treatment. Partial digestion with RNase T_1 was performed in a total volume of 0.5 mL containing 10 mM Tris-HCl (pH 7.5)–1 mM EDTA, 100 μg of yeast RNA as carrier, and 1 μg of RNase T_1 at 0 °C for 30 min. For partial digestion with snake venom phosphodiesterase, 0.5 mg of carrier RNA was added and the sample in a total volume of 0.5 mL containing 50 mM Tris-HCl (pH 8.5)–5 mM $MgCl_2$ was treated with 25 μg of *Escherichia coli* alkaline phosphatase at 37 °C for 1 h to remove the 3′-phosphates and was then partially digested with 5 μg of venom phosphodiesterase at 37 °C for 20 min.

Borate Gel Chromatography. The procedure was derived from that described by Rosenberg (1974) and Schott et al. (1973). The dihydroxyboryl-substituted methacrylic polymer (borate gel) was washed sequentially with acetone, water, and elution buffer (0.05 M 2-(*N*-morpholino)ethanesulfonic acid (pH 5.5)–0.1 M $MgCl_2$–1 mM β-mercaptoethanol–1 M sorbitol). The gel was then washed with elution buffer lacking sorbitol and was equilibrated with application buffer (0.05 M morpholine hydrochloride (pH 8.5)–0.1 M $MgCl_2$–1 mM β-mercaptoethanol). The sample was applied in the above buffer to a 0.7 × 10 cm borate gel column. The column was washed with application buffer to remove nonabsorbed oligonucleotides and the oligonucleotides bound by *cis*-hy-

† From the Laboratory of Biology of Viruses, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014. *Received October 20, 1976.*

[1] Abbreviations used: m^7G, 7-methylguanosine; m^6A, N^6-methyladenosine; m^5C, 5-methylcytidine; G^m, 2′-*O*-methylguanosine; A^m, 2′-*O*-methyladenosine; C^m, 2′-*O*-methylcytidine; U^m, 2′-*O*-methyluridine; Py, pyrimidine ribonucleoside; Pu, purine ribonucleoside; poly(U), poly(uridylic acid); poly(A), poly(adenylic acid); Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

droxyls were eluted with the sorbitol-containing elution buffer. For the isolation of large RNA fragments, 5 A_{260nm} units of *E. coli* tRNA and 10 A_{260nm} units of a partial T_1 digest of yeast total RNA were added as carrier to the sample prior to borate gel chromatography.

DEAE-Cellulose Chromatography. DEAE-cellulose chromatography in 7 M urea was carried out as described previously (Wei and Moss, 1974).

Materials. E. coli alkaline phosphatase and snake venom phosphodiesterase were purchased from Worthington Biochemical Corp.; ribonucleases were from Sigma Chemical Corp.; borate gel was from Aldrich Chemical Co.; [*methyl*-^{3}H]methionine and [^{14}C]uridine were from Schwarz/Mann and New England Nuclear Corp., respectively. Trinucleotides were a gift of D. Hatfield.

Results

Nucleotide Sequences on the 5′ Side of m^{6}A. Our previous studies (Wei et al., 1976) indicated that, in HeLa cell polyadenylylated mRNA, m^{6}A occurs predominantly or exclusively in two internal sequences, -G-m^{6}A-C- and -A-m^{6}A-C-. Efforts to extend these sequences by the isolation of larger oligonucleotides were hindered by the presence of methylated 5′-terminal oligonucleotides. To remove the 5′-terminal oligonucleotides, RNase digests of [*methyl*-^{3}H]methionine-labeled polyadenylylated mRNA were passed through a column of dihydroxyboryl-substituted methacrylic polymer (Schott et al., 1973). The method is based on the fact that a 5′-terminal oligonucleotide with a m^{7}G residue containing free 2′,3′-hydroxyls can complex with borate and will be retained by the column whereas internal oligonucleotides including those containing m^{6}A will pass through. A similar approach using a borate-substituted cellulose derivative (Rosenberg, 1974) was described by Furuichi et al. (1975b). Analysis of the effluent from the borate gel column indicated that virtually all of the m^{7}G-containing oligonucleotides had been removed. The methylated internal oligonucleotides were then treated with *E. coli* alkaline phosphatase to remove their 3′-phosphates and chromatographed on a DEAE-cellulose column in 7 M urea at pH 7.6. Under these chromatography conditions, oligonucleotides are separated on the basis of the number of negatively charged phosphates (Tener, 1967). When the mRNA was digested with RNase A and T_1 and then subjected to all of the above procedures, two methyl-labeled peaks that eluted with charges of −1 and −2 were detected (Figure 1A). They were identified as m^{6}ApC and Apm^{6}ApC by cochromatography and coelectrophoresis with ApC and ApApC markers as previously described (Wei et al., 1976). The m^{6}ApC (−1 peak) obtained by digestion of the mRNA with a combination of RNase A and T_1 must have been derived from -G-m^{6}A-C- as deduced previously (Wei et al., 1976) since very little material with a charge of −1 was obtained by these procedures when RNase A was used without RNase T_1 (Figure 1B). Accordingly, the amounts of radioactive material in the −1 and −2 peaks of Figure 1A indicated that approximately 70% of m^{6}A is in -G-m^{6}A-C- and the remainder is in -A-m^{6}A-C-. In addition, the very low amounts of radioactive material in peaks more negatively charged than −2 when RNase T_1 and A were used (Figure 1A) indicated that -A-A-m^{6}A-C- or other possible sequences occur infrequently. Additional information obtained from the RNase A digestion was the existence of a series of -Py-(Pu-)$_n$m^{6}A-C- sequences giving rise upon RNase A digestion to the −2, −3, −4, and −5 peaks (Figure 1B). Of these, -Py-Pu-Pu-m^{6}A-C- is common since the −3 peak (Figure 1B) was prominent.

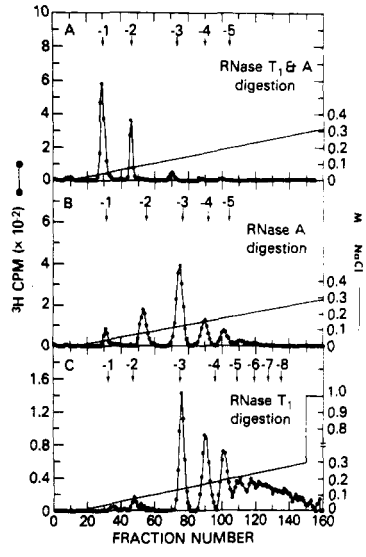

FIGURE 1: DEAE-cellulose chromatography of oligonucleotides containing m^{6}A. [^{3}H]Methyl-labeled HeLa cell polyadenylylated mRNA and 0.5 mg of yeast tRNA carrier in 0.5 mL of 10 mM Tris-HCl (pH 7.5)-1 mM EDTA were treated with 25 μg of RNase T_1 and/or 12.5 μg of RNase A at 37 °C for 1 h. The digests were chromatographed on borate gel columns as described in Materials and Methods. The materials not bound to the borate gel were collected and desalted with Sephadex G-10 columns. The lyophilized materials were treated with 0.1 mg of alkaline phosphatase per mL in 50 mM Tris-HCl (pH 8.5) and 5 mM $MgCl_2$ and chromatographed on a DEAE-cellulose column. A portion of each fraction was taken for scintillation counting. The numbered arrows indicate the elution positions and net negative charges of oligonucleotide markers.

Nucleotide Sequences on the 3′ Side of m^{6}A-C. Since most m^{6}A residues are preceded by guanosine and virtually all are followed by cytidine, hydrolysis of methyl-labeled mRNA with RNase T_1 followed by passage through a borate gel column, treatment with alkaline phosphatase, and DEAE-cellulose column chromatography could give rise to the following series of oligonucleotides: m^{6}ApCpG (−2), m^{6}ApCpNpG (−3), m^{6}ApCpNpNpG (−4), etc., where N is A, U, or C. Since about 30% of the m^{6}A residues are preceded by adenosine, other digestion products are also anticipated but in lower amounts than the previous series. The results of such an experiment appear in Figure 1C. The virtual absence of a −1 charge peak was anticipated since m^{6}A is followed by cytidine. The very small −2 peak generated by RNase T_1 digestion further indicated that -G-m^{6}A-C-G- is rare. The prominence of the −3 peak, which could be m^{6}ApCpNpG or Apm^{6}ApCpG, suggested, however, that either -G-m^{6}A-C-N-G in which N is not G or -G-A-m^{6}A-C-G- is common. To distinguish between these possibilities, the fractions comprising the −3 peak were pooled and then treated with RNase A and alkaline phosphatase. Since m^{6}ApC but no Apm^{6}ApC was found by thin-layer chromatography (not shown), the sequence -G-A-m^{6}A-C-G- was excluded.

The above experiments indicated that guanosine does not or only rarely occurs on the 3′ side of -G-m^{6}A-C- or -G-A-m^{6}A-C-. To determine which nucleosides follow cytidine, it was necessary to produce trinucleoside diphosphates of the type m^{6}ApCpN which have a charge of −2. Formation of m^{6}ApCpN from -G-m^{6}A-C-(N-)$_n$G- sequences was accomplished by complete RNase T_1 and alkaline phosphatase digestion of mRNA followed by partial snake venom phosphodiesterase digestion. Unavoidably, some Apm^{6}ApC, which also has a charge of −2, was formed. As seen in Figure 1C, a significant amount of material eluting with a charge of −2 was

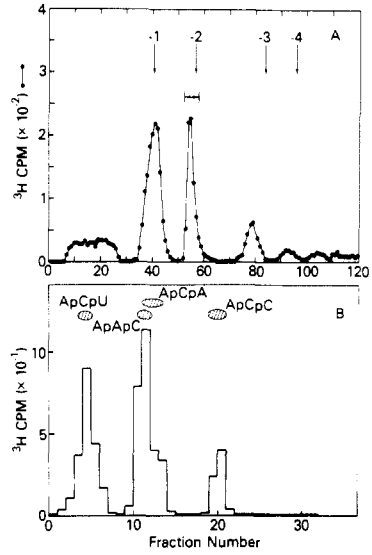

FIGURE 2: Identification of the nucleoside following m^{6}ApC. (A) [^{3}H]Methyl-labeled polyadenylylated mRNA was digested with RNase T_1 and the 5′-terminal oligonucleotide was removed by borate gel column chromatography. The material not bound to the borate gel was desalted, dephosphorylated, and partially digested with snake venom phosphodiesterase as in Materials and Methods. The partial digest was chromatographed on a DEAE-cellulose column as in Figure 1 and a portion of each fraction was used for scintillation counting. (B) The −2 charge peak isolated from panel A was desalted with a small DEAE-cellulose column and chromatographed on a 20 × 20 cm thin-layer PEI-cellulose sheet with 0.15 M acetic acid. The sheet was cut into 0.5-cm strips for scintillation counting. ApCpA, ApCpC, ApCpU, and ApApC were used as markers.

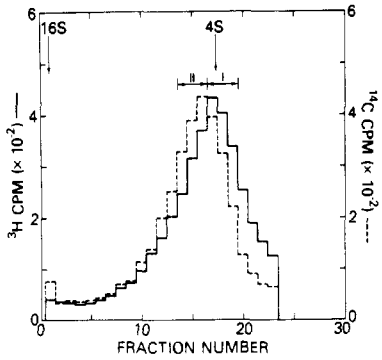

FIGURE 3: Isolation of RNase T_1 fragments of polyadenylylated mRNA by sucrose gradient sedimentation. [^{3}H]Methyl- and [^{14}C]uridine-labeled polyadenylylated mRNA was partially digested by RNase T_1 as described in Materials and Methods. The digest was extracted with 50 mM Tris-HCl (pH 8.0)-saturated phenol, following which the phenol was removed by filtration through a Sephadex G-15 column. The sample was then sedimented on a 5–20% sucrose gradient containing 10 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 10 mM EDTA, and 0.2% sodium dodecyl sulfate in an SW-41 rotor at 40 000 rpm for 10 h. *E. coli* tRNA and rRNA were used for 4S and 16S markers. A portion of each fraction was removed for scintillation counting and then the fractions corresponding to I and II were pooled and the RNA was recovered by ethanol precipitation with carrier tRNA.

not obtained by digestion with RNase T_1 and alkaline phosphatase alone. Preliminary experiments were carried out to maximize the amount of −2 charge material produced with snake venom phosphodiesterase. A typical result is shown in Figure 2A. When the oligonucleotides in the −2 charge peak were analyzed by thin-layer chromatography (Figure 2B), three methyl-labeled peaks corresponding to marker ApCpU (37%), ApCpA + ApApC (50%), and ApCpC (13%) were detected. Similar results were obtained by paper electrophoresis at pH 3.5 except that ApCpA (10%) and ApApC (40%) were separated. We concluded that -G-m^{6}A-C- may be followed by U, C, or A residues.

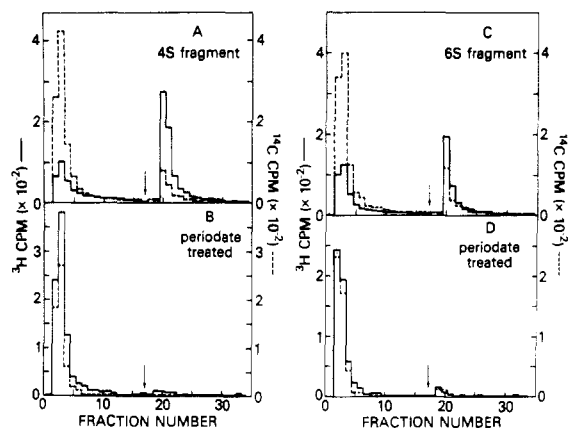

FIGURE 4: Separation of 5′-terminal fragments from internal fragments by borate gel chromatography. After sucrose gradient sedimentation, the [^{3}H]methyl- and [^{14}C]uridine-labeled fragments of a partial RNase T_1 digest of mRNA were passed through a poly(U)-Sepharose column to remove 3′-terminal poly(A) fragments. Carrier RNA was added and the RNA fragments were then chromatographed on a borate gel column as described in Materials and Methods or were first treated with 10 μL of 0.1 M sodium periodate in 0.25 mL of 0.15 M sodium acetate buffer (pH 5.3) in the dark at 0 °C for 1 h before applying to borate gel column. Fractions of 1.1 mL were collected. The arrow indicates the start of the sorbitol elution. (A) Pooled I (4S) fractions from Figure 3; (B) pooled I fractions treated with periodate; (C) pooled II (6S) fractions from Figure 3; (D) pooled II fractions treated with periodate.

Proximity of m^{6}A to the 5′ Terminus of mRNA. Although the above experiments indicated some sequence specificity surrounding the m^{6}A residues in HeLa cell mRNA, the absence of a long unique sequence suggested that additional factors might be necessary to account for the relatively low number of methylated adenosine residues. One possibility considered was that the m^{6}A residues are located close to one of the ends of the mRNA. Such a situation would not only provide additional specificity but would place the m^{6}A residues outside of the coding region where they might interfere with base pairing. Moreover, a location near the 5′ terminus would place the m^{6}A residues close to the other methylated nucleosides which enhance binding of mRNA to ribosomes (Both et al., 1975). To test the possibility of a 5′-terminal location, we prepared a partial RNase T_1 digest of [*methyl*-^{3}H]methionine and [^{14}C]uridine-labeled polyadenylylated mRNA and isolated fragments approximately 4S and 6S in size by sucrose gradient sedimentation (Figure 3). Under these conditions, digestion does not seem to be entirely random since the smaller size fragments had a higher ^{3}H/^{14}C ratio. Next, we passed the fragments through a poly(U)-Sepharose column to remove the 3′-terminal poly(A) segments and then through a borate gel column to separate 5′-terminal from internal fragments. After extensively washing the column, the bound material was eluted with sorbitol. With the 4S fragments, approximately 63% of the methyl-label but only 14% of the uridine-label were bound (Figure 4A). With the 6S fragments, which had a lower ^{3}H/^{14}C ratio, approximately 48% of the methyl-label and 16% of the uridine-label were bound (Figure 4C). The low level of nonspecific binding to the borate gel under our conditions was demonstrated by treating the RNA fragments with periodate to destroy *cis*-hydroxyls prior to chromatography (Figures 4B and 4D).

To compare the m^{6}A content of the fragments that were and

TABLE I: N^6-Methyladenosine Content of 5′-Terminal Fragments.[a]

RNA Fragment	% Bound to Borate Gel				Molar Ratio, $m^6A/5'$ Terminal
		[^{3}H]Methyl			
	[^{14}C]Uridine	Total	5′ Terminal	m^6A	
4S	14.0	63.0	59.3	3.7	0.18
6S	15.5	48.4	42.6	5.8	0.41
Intact mRNA			59.8	40.2	2.01

[a] Molar ratios are based on an average of 3 methyls per 5′ terminal and were calculated by dividing the methyl counts in m^6A by one-third of the methyl counts in the 5′-terminal oligonucleotides.

were not bound to the borate gel, the fractions were digested with RNase T_2 and alkaline phosphatase and the products were analyzed by paper chromatography. Since the methylated nucleotides at the 5′-terminus are resistant to RNase digestion, they remained near the origin while m^6A cochromatographed with internal marker (Figure 5). That the digestion was complete was demonstrated by recovery of virtually all of the ^{14}C-label as nucleosides (Figure 5). Evidently, small amounts of methyl-label entered the purine ring structures since the two small peaks on either side of the uridine marker (Figures 5C and 5D) correspond to the positions of guanosine and adenosine. Clearly, most of the 5′-terminal methylated nucleosides were associated with RNA fragments that were retained by the borate gel (Figures 5A and 5B), while most of the m^6A was in RNA fragments that were not retained by the column (Figures 5C and 5D). Quantitation of the results indicates that the molar ratio of m^6A to 5′-terminal methylated nucleosides is one-tenth that of whole mRNA in 4S 5′-terminal fragments and one-fifth in 6S 5′-terminal fragments (Table I).

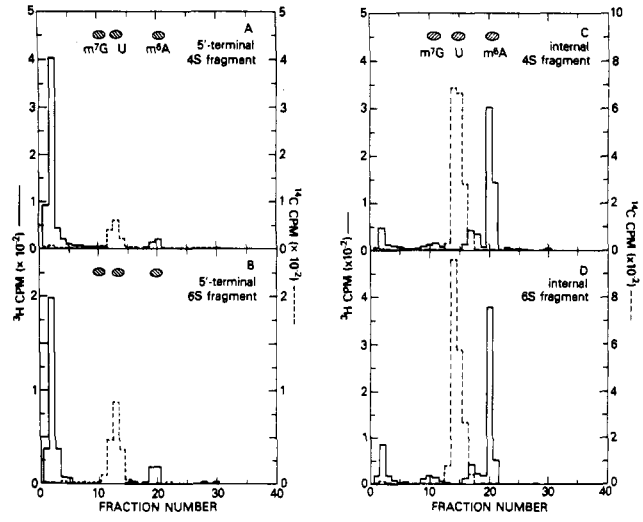

FIGURE 5: Analysis of m^6A in 5′-terminal fragments. The [^{3}H]methyl- and [^{14}C]uridine-labeled fragments isolated from Figure 4 were desalted with Sephadex G-15 columns and digested with 5 units of RNase T_2 in 25 μL of 10 mM sodium acetate (pH 4.5) at 37 °C for 2 h and then treated with 5 μg of alkaline phosphatase in 50 μL of 50 mM Tris-HCl (pH 8.5) and 5 mM $MgCl_2$ at 37 °C for 1.5 h. The digests were analyzed by ascending paper chromatography in solvent containing 2-propanol–concentrated NH_3–H_2O (7:1:2) for 20 h. (A) Borate gel bound 4S fragment; (B) borate gel bound 6S fragment; (C) not bound 4S fragment; (D) not bound 6S fragment.

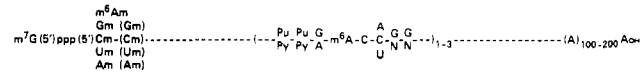

FIGURE 6: Representation of HeLa cell polyadenylylated mRNA.

Discussion

It has been possible to separate the methylated 5′-terminal oligonucleotides from methylated internal oligonucleotides by taking advantage of the binding of the free 2′,3′-hydroxyls of the former to a previously described (Schott et al., 1973) borate gel. This procedure has allowed us to investigate two questions regarding the sequence and location of the internal m^6A residues of polyadenylylated HeLa cell mRNA. First, in agreement with previous results (Wei et al., 1976), m^6A was found to occur in two sequences, -G-m^6A-C- (70%) and -A-m^6A-C- (30%). Extension of this sequence determination toward the 5′ end of the RNA indicated further variability with either purines or pyrimidines occurring in the next three positions. Extension of the -G-m^6A-C- and -A-m^6A-C- sequences in the 3′ direction demonstrated the presence of U, C, or A but not G following the C residue. Guanosine or other nucleosides, however, may be present in succeeding positions. Since on a random basis all of the possible sequences containing -(G or A)-A-C-(U, C or A)- may occur once per 43 nucleotides ($\frac{1}{2} \times \frac{1}{4} \times \frac{1}{4} \times \frac{3}{4}$) whereas there is only one m^6A residue per thousand nucleotides then either (1) not all potential sites are methylated, (2) there are multiple unique sequences each recognized by a separate enzyme, or (3) there are other unrecognized discriminating factors. Some possibilities for the latter include secondary structure or proximity to one end of the mRNA. Using oligo(dT)-cellulose to bind poly(A)-containing fragments, Adams and Cory (1975) reported that m^6A is present in both 5′ and 3′ "halves" of mouse myeloma cell mRNA. Using similar procedures, Furuichi et al. (1975a) found that m^6A is not located close to the 3′-poly(A) segment of HeLa cell mRNA. Our present work indicates that, although some m^6A residues are contained within 4 to 6S size 5′-terminal fragments isolated by borate gel chromatography, the majority are located more internally, possibly in translatable regions. A representation of HeLa cell mRNA indicating the methylated 5′-terminal sequences and the internal location and predominant nucleosides around the m^6A residues is shown in Figure 6.

Investigations regarding the location of m^6A residues have also been carried out with the 10 000 nucleotide genome of Rous sarcoma virus (Beemon and Keith, 1976). Approximately 10 of 11 to 12 m^6A residues are located between 500 and 4000 nucleotides from the 3′-poly(A) end. The *src* gene, corresponding to a deletion site of transformation defective mutants, and part of the envelope glycoprotein gene map in this region. Additional experiments with Rous sarcoma virus mRNA as opposed to virion RNA have not yet been carried out.

The function of m^6A in mRNA is unknown. Prior to its finding in eukaryotic mRNA, hnRNA, and viral RNA, naturally occurring m^6A had only been described in *E. coli* valine tRNA 1, where it occurs on the 3′ side of the anticodon (Harada et al., 1969; Yaniv and Barrell, 1969). Studies of Griffin et al. (1964) and Engel and Von Hippel (1974) indicate that a methyl group at the N-6 position destabilizes Watson-Crick base pairing with uridine. In accord with this, substitution of m^6A in poly(A) interferes with translation in in vitro systems (McCarthy et al., 1966; Michelson and Grunberg-Manago, 1974). Possibly, the putative methyltransferase primarily methylates -(G or A)-A-C- sequences in regions of base pairing and the m^6A then serves to weaken this secondary structure. The frequency of G or C residues, capable of strong base pairing, adjacent to m^6A might be significant in this regard. Additionally, the strong base pairing of a G or C residue adjacent to m^6A may be necessary for it to still function as an effective codon if m^6A lies in the translated region of mRNA.

Note Added in Proof

The sequences G-m^6A-C and A-m^6A-C have also been found in avian sarcoma virus RNA (Dimock and Stoltzfus, 1977) and mouse L cell mRNA and hnRNA (R. P. Perry, personal communication).

Acknowledgments

We thank W. Hatfield for oligonucleotide markers and M. Rosenberg and J. Keith for helpful discussions.

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