The methylation of adenovirus-specific nuclear and cytoplasmic RNA

S. Sommer*, M. Salditt-Georgieff*, S. Bachenheimer*, J. E. Darnell*, Y. Furuichi†, M. Morgan* and A. J. Shatkin†

*Rockefeller University, New York, NY 10021 and †Roche Institute of Molecular Biology, Nutley, NJ 08110, USA

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ABSTRACT

Each poly(A) containing cytoplasmic AD-2 mRNA contains at its 5' terminus the general structure m7GpppNmpN2p or m7GpppNmpN2pNpP as well as an average of 4 m6A and 0.5-1 m5C residues per molecule. Almost all of the m6A residues are adenine derivatives including m2A, m3A and probably m2,6A. The m5C is mostly Cm but small amounts of the other three methylated bases are also present. All the methylated constituents of mRNA are distant from the 3' terminal poly(A). The amount of m6A appears to be greater in larger mRNA than in smaller mRNA. Nuclear Ad-2 specific RNA also contains caps, m6A, and m5C with about twice as much m6A relative to caps as cytoplasmic mRNA. The similarity of Ad-2 nuclear and mRNA to HeLa hnRNA and mRNA suggests that adenovirus mRNA production is a good model for eukaryotic mRNA production.

INTRODUCTION

In mammalian cells both ends of mRNA molecules are modified post-transcriptionally. It was first found that most mRNA molecules have a 3' terminal 200 nucleotide poly(A) segment (See Darnell, 1973 for review). Recent work has shown another post-transcriptional step in mRNA manufacture, methyl group additions, to occur in the 5' portion of various cell and virus mRNA molecules. Methyl groups in mRNA were first found by Perry and Kelley in L cells. Recently a variety of mRNA's of viruses which replicate in the cytoplasm like reo, cytoplasmic polyhedrosis virus, vaccinia, and vesicular stomatitis virus, have been shown to contain a blocked and methylated 5'-terminal "cap" structure (m7GpppNmpP). The same type of cap structure has now been identified in a variety of cultured cells and can be synthesized in vitro in isolated nuclei. In addition to the cap structure, some cellular mRNAs...
contain internal $N^6$-methyladenosine.$^9,10,11,12$

The formation of adenovirus mRNA is similar to cellular mRNA formation in many ways; the virus DNA is transcribed in the cell nucleus$^{13,14,15,16}$, probably exclusively in the form of RNA molecules much longer than the final size of mRNA$^{15,16}$. The large molecules are modified after transcription by cleavage and addition at the 3' OH terminus of a 200 nucleotide segment of polyadenylic acid.$^{17}$ Finally, the mRNA emerges into the infected cell cytoplasm to be translated into virus specific proteins.$^{18}$

To determine whether adenovirus and HeLa were similar in yet another parameter, the methylation pattern of AD-2 nuclear and cytoplasmic RNA was examined.

**METHODS AND MATERIALS**

The growth of HeLa cells, infection with AD-2, labeling of infected cells late in infection (14-18 hrs.), extraction of nuclear and cytoplasmic RNA, and hybridization of RNA to AD-2 DNA have all been described.$^{14,17}$ Poly(U) sepharose selection of poly(A) terminated RNA was carried out by formamide gradient elution.$^{18}$ For labeling AD-2 RNA with $^3$H-methyl methionine, cells were collected 14 hours after infection, and resuspended in methionine-free Eagle's medium supplemented with 5% dialyzed serum, 20 uM adenine, 20 uM guanosine and 10 mM NaCOOH and 1-1.5 mC $^3$H methyl methionine (4-GuM methionine)/20 ml of infected cell culture (1-2 x 10$^6$ cells/ml); $^3$H-methyl methionine was purchased from New England Nuclear (10 mC/umole).

Alcohol precipitated nuclear RNA samples were dissolved in 90% DMSO (dimethyl sulfoxide), 5% DMF (dimethyl formamide), 5% TES (0.04 M Tris buffer, pH 7.4 containing 0.01 M EDTA and 0.2% SDS) followed by sedimentation analysis in sucrose gradients.$^{20}$

Analysis of complete enzymatic (RNAsase A and T2 purchased from Cal-biochem) digests of RNA was carried out by a variety of techniques includ-
ing DEAE-cellulose chromatography, paper electrophoresis and paper chromatography. Enzymatic degradation of the cap structure to allow further analysis was carried out with bacterial alkaline phosphatase (BAP), Penicillium nuclease (P1), and nucleotide pyrophosphatase3,10.

RESULTS

Methylation in AD-2 mRNA

In order to determine whether virus-specific cytoplasmic RNA contained methyl groups, AD-2 infected Hela cells were labeled with 3H methyl methionine from 14-18 hrs. after infection. The poly(A)-containing cytoplasmic RNA fraction, which is largely virus-specific mRNA at this time after infection14,17,21, contained radioactivity. To purify further the virus-specific sequences, the methylated RNA was hybridized to and eluted from AD-2 DNA on nitrocellulose filters; 50-75% of the poly(A) terminated methylated RNA bound to the filters (Table 1).

Table 1
Hybridization of RNA to AD-2 DNA

<table>
<thead>
<tr>
<th>Sample</th>
<th>Label</th>
<th>Total CPM</th>
<th>Hybridized to AD-2</th>
<th>Blank</th>
<th>% Hybridized</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD-2 cytoplasmic poly(A) containing</td>
<td>32p</td>
<td>2.4x10^6</td>
<td>1.3x10^6</td>
<td>2100</td>
<td>54</td>
</tr>
<tr>
<td>AD-2 cytoplasmic poly(A) containing</td>
<td>3H-CH₃</td>
<td>2.0x10⁴</td>
<td>1.5x10⁴</td>
<td>184</td>
<td>75</td>
</tr>
<tr>
<td>AD-2 Nuclear &gt;45S</td>
<td>3H-CH₃</td>
<td>8.2x10⁵</td>
<td>6.0x10⁴</td>
<td>500</td>
<td>7.3</td>
</tr>
<tr>
<td>AD-2 Nuclear 30-45S</td>
<td>3H-CH₃</td>
<td>7.2x10⁵</td>
<td>9.4x10⁴</td>
<td>600</td>
<td>13.0</td>
</tr>
<tr>
<td>AD-2 Nuclear 30-60S</td>
<td>3H-uridine</td>
<td>4.0x10⁶</td>
<td>3.4x10⁵</td>
<td>9000</td>
<td>8.5</td>
</tr>
<tr>
<td>Uninfected Nuclear 30-60S</td>
<td>3H-uridine</td>
<td>5.0x10⁶</td>
<td>1.0x10⁴</td>
<td>6000</td>
<td>0.1</td>
</tr>
</tbody>
</table>

RNA labeled with 32P or 3H-methyl-methionine was hybridized to and eluted from 300-500μg AD-2 DNA without RNAse treatment as described (Methods and Ref. 14). The last two lines in the table were taken from earlier work14 and are entered here for comparison between 3H-uridine labeled RNA and 3H-methyl-methionine labeled RNA.
Complete pancreatic and T2 RNAse digestion of the adenovirus-specific cytoplasmic 3H-methyl-labeled mRNA followed by analysis of the digest by DEAE-cellulose chromatography demonstrated 3H-labeled mononucleotides (eluting with -2 net charge) and oligonucleotides (eluting in the region of caps, i.e., at -5 to -6) (Fig. 1). In these experiments 55-64% of the radioactivity was recovered in mononucleotides and the remainder in caps. This contrasts with only about 30% of the 3H-methyl-label in mononucleotides and 70% in caps in HeLa cell mRNAB (Table 2).

**FIGURE 1.**
DEAE-Cellulose Chromatography of Methylated Oligonucleotides in AD-2 RNA. Nuclear and cytoplasmic AD-2 specific RNAs from cells labeled with 3H-methyl-methionine were digested by pancreatic and T2 RNAse. 32P RNA was added to monitor completeness of digestion. The digests were chromatographed on a DEAE-cellulose column (See Methods). The cytoplasmic RNA was the total poly(A)-containing AD-2 DNA-selected sample; the nuclear samples were first separated on sucrose gradients as in Fig. 5 and then selected on AD-2 DNA.
Table 2

Distribution of $^3$H-Methyl in AD-2 Specific RNA

<table>
<thead>
<tr>
<th>Sample</th>
<th>% Radioactivity as Mononucleotide</th>
<th>Di- or Trinucleotide</th>
<th>$m^6$Ap/cap</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytoplasmic poly(A)-containing</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exp. 1. AD-2</td>
<td>61</td>
<td>-</td>
<td>39</td>
</tr>
<tr>
<td>2. AD-2</td>
<td>64</td>
<td>-</td>
<td>36</td>
</tr>
<tr>
<td>3. AD-2</td>
<td>55</td>
<td>-</td>
<td>45</td>
</tr>
<tr>
<td>Uninfected</td>
<td>26</td>
<td>-</td>
<td>74</td>
</tr>
<tr>
<td>Nuclear</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. AD-2 28S</td>
<td>32</td>
<td>58</td>
<td>10</td>
</tr>
<tr>
<td>AD-2 &gt;45S</td>
<td>41</td>
<td>40</td>
<td>19</td>
</tr>
<tr>
<td>AD-2 30-45S</td>
<td>32</td>
<td>53</td>
<td>15</td>
</tr>
</tbody>
</table>

Preparations of methyl-labeled AD-2 specific RNA from three separate experiments were digested and subjected to DEAE chromatography as in Fig. 1. In Experiment 3 a culture of uninfected cells was also labeled and cytoplasmic poly(A)-containing RNA analyzed.

The $^3$H methyl-labeled mononucleotides from AD-2 mRNA were converted to nucleosides by digestion with alkaline phosphatase (BAP) and examined by paper electrophoresis (Fig. 2A) and chromatography (2B,C); 85% of the radioactivity was recovered as $m^6$A as had been found in cellular mRNA$^9$. The remainder was $m^5$C (Fig. 2A, 2D) which was also found previously in nuclear RNA but not in cytoplasmic poly(A)-containing RNA of HeLa cells$^8$.

Oligonucleotides eluting from DEAE-cellulose in the -5 to -6 region were analyzed after digestion with Penicillium nuclease (P$_1$) plus BAP. P$_1$ cleaves nucleotides from the 3' portion of the cap structure as indicated $--\text{m}^7\text{GpppN}_1\text{m/pN/p}$ or $\text{m}^7\text{GpppN}_1\text{m/pN}_2\text{m/pN/p}^{10}$, and BAP removes all phosphates from the released mononucleotides. A P$_1$ and BAP-resistant fraction characteristic of cap structures was observed after paper electrophoresis with about 25% of the released radioactivity as 2'-0 methyl nucleosides (Fig. 3A).
Methylated Mononucleotides in AD-2 poly(A)+ Cytoplasmic RNA

Cytoplasmic poly(U) bound AD-2 specific RNA was digested with pancreatic and T<sub>2</sub> RNase and the mononucleotides (-2) were separated by DEAE-cellulose column chromatography as in Fig. 1. The desalted mononucleotides were subjected to paper electrophoresis at pH 3.5. The radioactivity in panel A corresponding to A derivatives was eluted and analyzed by descending paper chromatography with marker compounds in (B) isobutyric acid: 0.5 N NH<sub>4</sub>OH (10:6 v/v) and (C) isopropanol: H<sub>2</sub>O:NH<sub>4</sub>OH (7:2:1 v/v). The C derivative in panel A was analyzed in isobutyric acid: NH<sub>4</sub>OH (D).

Further characterization of the 3H-methyl cap structures by paper electrophoresis after digestion with nucleotide pyrophosphatase and BAP revealed positively charged m<sup>7</sup>G and a smaller amount of the neutral ring-opened derivative of m<sup>7</sup>G remaining at the origin (Fig. 3B). Most of the radioactivity released from caps migrated as methylated A (Fig. 3B) which was
3H-methyl-labeled cap material (-5 to -6) was isolated by DEAE-cellulose column chromatography from RNAse digests of AD-2 cytoplasmic RNA as in Fig. 1. The -5 to -6 material was desalted, digested with P1 nuclease and BAP, and analyzed by paper electrophoresis at pH 3.5 as described previously3 (A). The negatively charged material (fractions 19-22 in A) was eluted, treated with nucleotide pyrophosphatase and BAP and reanalyzed by electrophoresis (B). The radioactive A derivatives were eluted and identified by paper chromatography in isopropanol: H2O- NH4OH (7:2:1 v/v) (C). The major constituent which migrated similar to the marker m7Gm7A was eluted (fractions 32-34), depurinated in 1 N HCl at 100° for 30 min. and reanalyzed by chromatography in isobutyric acid: 0.5 M NH4OH (10:6 v/v) (D). The C derivative in panel A was identified as CmA in the same solvent system (E).

resolved by paper chromatography into two components: one migrating slightly faster than the position of N1-6-dimethyladenosine (87%) and the other with
2'-O-methyladenosine (13%) (Fig. 3C). After depurination of the methylated A mixture, 44% of the radioactivity migrated with 2'-O-methylribose (Rm) and the remainder with N6 mono- and dimethyladenine (Fig. 3D). Since depurination of A would yield 13% of the radioactivity from 2'-O-methyladenosine as Rm, 31% (44%-13%) of the total 3H-methyl in the Rm portion is derived from the other major peak of methylated adenosine, and the ratio of methylated adenine to Rm is 56/31 = 1.8. N6mA and N6,6mA would yield ratios of 1 and 2, respectively. Thus the results are consistent with the presence of a mixture of trimethyl A (N6,6mA = 70%), dimethyl A (N6mA = 17%), and monomethyl A (A = 13%) in the cap structures of AD-2 mRNA. (Similar results were obtained by Moss, pers. comm.) In addition to cap 1 structures (m7GpppNmmpNp) AD-2 mRNA also contains some cap 2 structures (m7GpppNmnpNmnpNp) as shown by the release of mononucleotides by P1 nuclease and BAP treatment (Fig. 3A). The predominant N2m in the cap structures is apparently Cm (Fig. 3E) suggesting that the first two nucleotides in many AD-2 mRNAs are A and C.

AD-2 mRNA contains the same mononucleotide and oligonucleotide components as those found in HeLa cells mRNA8,9,10,11, but there is about three times as much methyl-labeled m6A relative to caps as in cellular mRNA.

To quantitate the number of molecules containing caps, a preparation of poly(A) containing cytoplasmic virus-specific RNA was prepared from cells labeled with 32P from 14-18 hours after infection. Total radioactivity, label in cap structures (-5 to -6 on DEAE-cellulose), and label in poly(A) were all measured. There were 5 CPM in caps/196 CPM for poly(A)/1970 CPM in mRNA (Fig. 4). The number average size of AD-2 late mRNA falls into the range of about 2,000 nucleotides (Fig. 5 and Ref. 21) and the poly(A) in AD-2 mRNA is about 200 nucleotides long17. These results suggest the presence of one cap in every mRNA of 1790 nucleotides containing a poly(A) unit which is 200 nucleotides long. The finding (Fig. 1) of 55-64% of the 3H-methyl radioactivity in mononucleotides is consistent with the presence of about 4 m6A.
FIGURE 4. "Cap" Content of 32P Labeled Poly(A)+ Cytoplasmic AD-2 RNA

Cells were labeled with 32P in AD-2 infection and the cytoplasmic poly(A)-containing RNA selected on AD-2 DNA. The total radioactivity, the radioactivity in poly(A) (inset), and the radioactivity in caps were assayed. Assuming that each cap contains 5 phosphates (m7GpppNmmpNp), the ratios of CPM in caps to poly(A) and to total chains were calculated and appear in boxes.

per cap since most caps contain 3-4 methyl groups. The 32P results indicating one cap per molecule would then imply an average about 4 m6A residues per molecule. An additional suggestion about the distribution of the m6A residues was revealed by co-sedimenting 32P and 3H-methyl-labeled mRNA (Fig. 5). If each mRNA molecule had an equal number of methyl groups, the ratio of 3H-methyl-label to 32P label should have increased in smaller molecules, but this was definitely not the case. The faster sedimenting (26S, 4000-5000 nucleotides) 32P labeled RNA had the same ratio of 3H/32P as did the more slowly sedimenting molecules (15S, 1500 nucleotides and 10S, 750 nucleotides). Almost all of the labeled poly(A)-containing cytoplasmic RNA is AD-2 specific at 14-18 hrs after infection according to previous findings14,17,21 and as suggested by the presence of distinct peaks characteristic of AD-2 mRNA (Fig. 5 and 21). These results indicate that there are more m6A residues in long than in short AD-2 mRNA molecules.
Sedimentation of RNA from AD-2 Infected Cells.

Left Panel: Nuclear RNA from AD-2 infected cells (labeled with $^{3}$H-methyl methionine 14-18 hrs. after infection) was treated with DMSO (Derman and Darnell, 1974) and sedimented through a sucrose gradient. Total acid-precipitable radioactivity and O.D. at 260 are given.

Right Panel: $^{32}$P and $^{3}$H-methyl methionine-labeled cytoplasmic poly(A) containing RNA from late in AD-2 infection was mixed and sedimented through a sucrose gradient. Total acid precipitable radioactivity is presented. 28S and 18S markers were sedimented in a parallel gradient.

An experiment to locate the position of m$^{6}$A within AD-2 mRNA was then carried out. In HeLa cell mRNA broken to approximately 500 nucleotides by limited T$_{1}$ RNase digestion or by brief alkali treatment almost all (>90%) of the m$^{6}$A is released from association with the 3' poly(A) containing segments. A similar experiment employing breakage and poly(U) sepharose reselection of the 3' poly(A) portion was performed on poly(A) terminated RNA prepared from adenovirus infected, $^{3}$H-methyl-labeled cells. After alkali breakage, the poly(U) sepharose bound and unbound fractions were hybridized to AD-2 DNA (Table 3). Over 90% of the hybridizable $^{3}$H-methyl-labeled RNA was in the RNA which did not rebind to poly(U) sepharose (i.e., segments of...
mRNA distant from poly(A)). The ratio of m^6A to cap in the bound fraction was similar to the unbound fraction indicating that the bound molecules were unbroken mRNA. Although there are an average of 4 m^6A residues per molecule, the m^6A, like in cellular mRNA, seems not to be in the 3' terminal third of AD-2 mRNA.

Table 3.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total CPM</th>
<th>Hybridized to AD-2 CPM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkali broken poly(U) bound</td>
<td>9,600</td>
<td>4,000</td>
</tr>
<tr>
<td>Alkali broken poly(U) flow through</td>
<td>90,000</td>
<td>44,000</td>
</tr>
</tbody>
</table>

\(^3\text{H}-\text{methyl-labeled poly(A)}\) containing RNA from the cytoplasm of AD-2 infected cells was treated with alkali (0.2 N NaOH at 0° for 20 min. in 0.1 M NaCl, 0.01 M EDTA, 0.01 M tris pH 7.4 and 0.2% SDS; see Ref. 30), to reduce the size of RNA to approximately 500 nucleotides. The RNA was then reselected by poly(U) sepharose and the bound and unbound samples hybridized to AD-2 DNA.

METHYLATION IN AD-2 NUCLEAR RNA

\(^3\text{H}-\text{methyl-labeled nuclear RNA from infected cells was DMSO treated and separated by sucrose gradient sedimentation (Fig. 5). Virus-specific RNA was selected from the 30-45S and } >45S \text{ regions of the sucrose gradient by hybridization to and elution from AD-2 DNA without RNAse treatment as had previously been done to purify cytoplasmic AD-2 methyl-labeled RNA (Fig. 1) and } ^3\text{H-uridine labeled AD-2 nuclear RNA}^{17}. \text{ The selected RNA was digested with pancreatic and T} _1 \text{ ribonucleases and analyzed by DEAE-cellulose chromatography. Three types of } ^3\text{H-methyl-labeled components were found: 1) mononucleotides eluting at -2; 2) presumptive cap structures eluting at -5, both of which had been seen in the cytoplasmic virus-specific RNA; and 3) material eluting at -3 which was not observed in the cytoplasmic RNA (Fig. 1A). While the ratio of radioactivity in the cytoplasmic virus-specific...
RNA was about 1.5 for mononucleotide/cap, the same ratio in nuclear high-
molecular weight virus-specific RNA was about 2.5 (Table 3). Like the
cytoplasmic RNA, the mononucleotide from the nuclear RNA was found to be
\( m^5A \) (Fig. 6A). (In another preparation where more radioactivity was
available 6% of the radioactivity was present as \( m^5C \).) The cap structures
behaved similarly to the cytoplasmic caps but with perhaps a lower propor-
tion of \( {}^3H \)-methyl eluting at -6 than at -5 (Fig. 1B,C). These results
suggest that "cap 1" structures in (\( m^7GpppNm \)) rather than "cap 2" structures
predominate in nuclear AD-2 RNA. However, after digestion with \( P_1 \) nuclease
and BAP followed by paper electrophoresis (Fig. 6B), 20% of the radioactivity
was present as mononucleosides, suggesting that cap 2 structures predominate.
Digestion of the \( P_1 \) and BAP-resistant portion of the caps with pyrophosphatase
and BAP released \( m^7G \) and methylated A (Fig. 6C). The methylated A derivative
migrated during paper chromatography in the position of the marker \( m_2^6,6'6A \)
which has essentially the same Rf (.85) as \( m^6Am, 2'\text{-}0\text{-H}^6\text{-dimethyl adenosine},
(0.82) in this solvent system (Fig. 6D). After depurination, 42% of the \( {}^3H \)-
methyl radioactivity migrated with 2'-0-methyl ribose and 58% with \( m_2^6,6'6A \)
adine (Fig. 6E). As described above for the cytoplasmic caps, the deviation
from the expected values of 50% suggests that the cap structures in
nuclear AD-2 RNA may contain di- and trimethylated derivatives of A, i.e.,
\( m^6Am \) and \( m_2^6,6'6A \).

The \( T_2 \)-resistant dinucleotides from the nuclear RNA (-3) were com-
pletely sensitive to \( P_1 \) nuclease digestion, indicating that they arose from
internal 2'-0-methylations (Fig. 7A). The dinucleotides could be pre-rRNA
contaminants because even though very little new rRNA reaches the cytoplasm
late in infection\(^{17,22}\), 45 and 32S RNA is still abundantly synthesized (Fig. 5),
and more than 90% of its methylation is on the ribose\(^{23}\). To determine the ex-
tent of pre-rRNA contamination a sample of AD-2 nuclear RNA was hybridized
with extensive pre-elution washings as before. One aliquot was eluted and
FIGURE 6.
Methylated Constituents in Nuclear AD-2 RNA

The 3H-methyl-labeled mononucleotides obtained by RNAse digestion and DEAE-cellulose column chromatography were desalted and analyzed by paper chromatography in isopropanol: H₂O: NH₄OH (7:2:1 v/v) (A). The material eluting from DEAE-cellulose at -5 to -6 was desalted, digested with P₁ nuclease and BAP and analyzed by paper electrophoresis at pH 3.5 (B). The cap material (fractions 20-24) was eluted, digested with nucleotide pyrophosphatase and BAP and analyzed by electrophoresis (C). The A derivatives were further analyzed by paper chromatography in isopropanol: NH₄OH (D) and isobutyric acid: 0.5 N NH₄OH after depurination (1 N HCl, 30 min., 100°C) (E).

saved. A second aliquot was treated with RNAse A which was subsequently inactivated by extensive iodoacetate¹⁴ washes. A third aliquot was eluted, boiled, and rehybridized. All three were digested with RNAse A and T₂ and analyzed by DEAE chromatography. The hybridized, RNAse-treated sample still
Separation of 2'-O-Methylated Nucleosides from Nuclear Di/Trinucleotides (-3)

The RNAse digested nuclear material eluting from DEAE-cellulose column at -3 charge (Fig. 1) was desalted and dissolved in 5 mM Na acetate buffer at pH 6. After heating at 80° for 2 min., the sample was treated with P1 nuclease and BAP and analyzed by paper electrophoresis (10).

<table>
<thead>
<tr>
<th>Table 4</th>
</tr>
</thead>
</table>

Distribution of Methyl Label Constituents of AD-2 Nuclear RNA After Two Cycles of Hybridization

<table>
<thead>
<tr>
<th>Sample</th>
<th>First hybrid</th>
<th>RNAse resistant</th>
<th>Second hybrid</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Methyl Label As</td>
<td>(2)</td>
<td>(3)</td>
<td>(5)</td>
</tr>
<tr>
<td>Mono</td>
<td>22</td>
<td>34</td>
<td>80</td>
</tr>
<tr>
<td>Di</td>
<td>68</td>
<td>41</td>
<td>6</td>
</tr>
<tr>
<td>Cap</td>
<td>10</td>
<td>25</td>
<td>14</td>
</tr>
</tbody>
</table>

Methyl-methionine labeled AD-2 nuclear RNA was purified by hybridization in three ways: 1) elution from AD-2 DNA as described in Table 1 (IX hybrid), 2) by treatment of filters bearing AD-2 DNA with 2.5 μg/ml RNAse A and 5 units of RNAse T1 at 37° in 2 x SSC (standard saline citrate) followed by extensive washes at 55° with 2 x SSC plus 0.15 M iodoacetate, pH 6.5 (RNAse resistant hybrid) prior to elution or 3) elution as in 1) followed by ethanol precipitation, resuspension in 0.01 M MgCl₂, 0.0015 M CaCl₂, 0.05 M NaCl, and 0.01 M tris, pH 7.4 plus 50 μg/ml DNase for 30 min. at 37°. The sample was then extracted 2X with phenol, precipitated with 2 volumes of ethanol redissolved in 2X TESS (the hybridization buffer) for 30 °C. The sample was then eluted again from AD-2 DNA filters. Approximately 55% of the acid precipitable RNA hybridized a second time with no labeled RNA adsorbed on blank filters. All three samples were then analyzed by DEAE chromatography as in Fig. 1 for methylated constituents. At least 20,000 CPM were analyzed in each sample.

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contained significant amounts of di-nucleotide but the sample which was re-
hybridized a second time had lost 90% of the dinucleotides in spite of the
fact that about 60% of the RNA recovered after one hybridization hybridized
a second time to AD-2 DNA. We conclude that most if not all dinucleotides
derive from contaminating pre-rRNA.

DISCUSSION

The present results demonstrate a great similarity between methylated
structures in adenovirus-specific nuclear and cytoplasmic RNA and the
corresponding fractions from uninfected HeLa cells. In both cases, the mRNA
exhibits two general types of methylated structures, a "cap" and internal
base methylations. The cap is compatible with the structure proven in other
cases to be m7GpppN1NdNp and m7GpppN1NdN2NdNp, and the internal methylation
is m6A (and possibly m5C). In both there is about one cap per molecule and
the internal methylations are all or almost all in the 5' two-thirds of the
molecule. One difference is that adenovirus mRNA contains threefold more
m6A per molecule than HeLa mRNA. AD-2 nuclear RNA and HeLa HnRNA both con-
tain a higher number of m6A per cap than the corresponding cytoplasmic mRNA.
Thus poly(A) addition and methylation during AD-2 mRNA formation are very
similar to HeLa mRNA, indicating that adenovirus is a good model for the
study of cell mRNA formation.

It is interesting that m6A is found in cellular RNAs8,9,10,11,12
SV40 mRNA24, avian sarcoma virus RNA25, and now AD-2 mRNA. All of these
mRNAs probably derive from the nuclear transcription of DNA followed by
post-transcriptional modification. Several viruses3, cytoplasmic poly-
hedrosis virus4, vaccinia5,26, Newcastle disease virus27, and vesicular
stomatitis virus6 which (i) multiply in the cytoplasm, (ii) have mRNA's
that are produced by virion polymerases and virion methylases and (iii)
are possibly used directly without size reduction do not contain the m6A
modification.
Other evidence indicates that the major transcription products from AD-2 DNA are very high molecular weight molecules which must be cleaved to yield mRNA\textsuperscript{15,16}. Previous experiments have shown poly(A) to be present in the high molecular weight AD-2 specific nuclear RNA\textsuperscript{14} and these experiments indicate that some long nuclear virus-specific molecules also contain caps. These findings are consistent with several possible reactions during RNA processing to generate AD-2 mRNA: e.g., 1) capping or poly(A) addition at the RNA polymerase-created 5' or 3' end, respectively, followed by endonucleolytic cleavage to present the other end of the mRNA for final processing or 2) 2 or more endonucleolytic cleavages, to yield both the 5' and 3' ends of the mRNA followed by capping and poly(A) addition. If the many different AD-2 mRNA molecules which exist late in infection\textsuperscript{28,29} are derived from large precursor molecules then cleavage to yield both ends of the mRNA must occur frequently.

ABBREVIATIONS

pA, 5'-adenylic acid; A, adenosine; Ad, adenine; A\textsuperscript{m}, 2'-0-methyladenosine; m\textsuperscript{6}A, N\textsuperscript{6}-methyladenosine; m\textsubscript{2,6}A, N\textsuperscript{6,6}-dimethyladenosine; m\textsuperscript{6}Ad, N\textsuperscript{6}-methyladenine; m\textsubscript{2,6}Ad, N\textsuperscript{6}-dimethyladenine; m\textsuperscript{6}A\textsuperscript{m}, N\textsuperscript{6}-methyl-2'-0-methyladenosine; m\textsubscript{2,6}A\textsuperscript{m}, N\textsuperscript{6}-dimethyl-2'-0-methyladenosine; G, guanosine; m\textsuperscript{7}G, 7-methylguanosine; pU, 5'-uridylic acid; U, uridine; pC, 5'-cytidylic acid; C, cytidine; C\textsuperscript{m}, 2'-0-methyl cytosine; m\textsuperscript{5}C, 5-methylcytidine; R\textsuperscript{m}, 2'-0-methylribose; AD-2, adenovirus 2.

REFERENCES

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