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The methylation of adenovirus-specific nuclear and cytoplasmic RNA

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ABSTRACT

Each poly(A) containing cytoplasmic AD-2 mRNA contains at its 5' terminus the general structure $m^7GpppN_1{}^mpN_2p$ or $m^7GpppN_1{}^mpN_2{}^mpNp$ as well as an average of 4 m^6A and 0.5-1 m^5C residues per molecule. Almost all of the $N_1{}^m$ residues are adenine derivatives including A^m, m^6A^m and probably $m_2{}^{6,6}A^m$. The $N_2{}^m$ is mostly C^m 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 m^6A appears to be greater in larger mRNA than in smaller mRNA. Nuclear Ad-2 specific RNA also contains caps, m^6A, and m^5C with about twice as much m^6A 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[2]. Recently a variety of mRNA's of viruses which replicate in the cytoplasm like reo[3], cytoplasmic polyhedrosis virus[4], vaccinia[5], and vesicular stomatitis virus[6], have been shown to contain a blocked and methylated 5'-terminal "cap" structure (m^7GpppN^mpNp). 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[7,8]. 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[19]. 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 μM adenine, 20 μM guanosine and 10 mM NaCOOH and 1-1.5 mC ^{3}H methyl methionine (4-6μM 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/μmole).

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 (RNAse A and T2 purchased from Calbiochem) 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 pyrophosphatase[3,10].

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 ^{3}H 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 infection[14,17,21], 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

Sample	Label	Total CPM	Hybridized to AD-2	Blank	% Hybridized
AD-2 cytoplasmic poly(A) containing	^{32}P	2.4×10^{6}	1.3×10^{6}	2100	54
AD-2 cytoplasmic poly(A) containing	$^{3}H\text{-}CH_3$	2.0×10^{4}	1.5×10^{4}	184	75
AD-2 Nuclear >45S	$^{3}H\text{-}CH_3$	8.2×10^{5}	6.0×10^{4}	500	7.3
AD-2 Nuclear 30-45S	$^{3}H\text{-}CH_3$	7.2×10^{5}	9.4×10^{4}	600	13.0
AD-2 Nuclear 30-60S	^{3}H-uridine	4.0×10^{6}	3.4×10^{5}	9000	8.5
Uninfected Nuclear 30-60S	^{3}H-uridine	5.0×10^{6}	1.0×10^{4}	6000	0.1

RNA labeled with ^{32}P or ^{3}H-methyl-methionine was hybridized to and eluted from 300-500mg AD-2 DNA without RNAse treatment as described (Methods and Ref. 14). The last two lines in the table were taken from earlier work[14] and are entered here for comparison between ^{3}H-uridine labeled RNA and ^{3}H-methyl-methionine labeled RNA.

Complete pancreatic and T2 RNAse digestion of the adenovirus-specific cytoplasmic ^{3}H-methyl-labeled mRNA followed by analysis of the digest by DEAE-cellulose chromatography demonstrated ^{3}H-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 ^{3}H-methyl-label in mononucleotides and 70% in caps in HeLa cell mRNA[8] (Table 2).

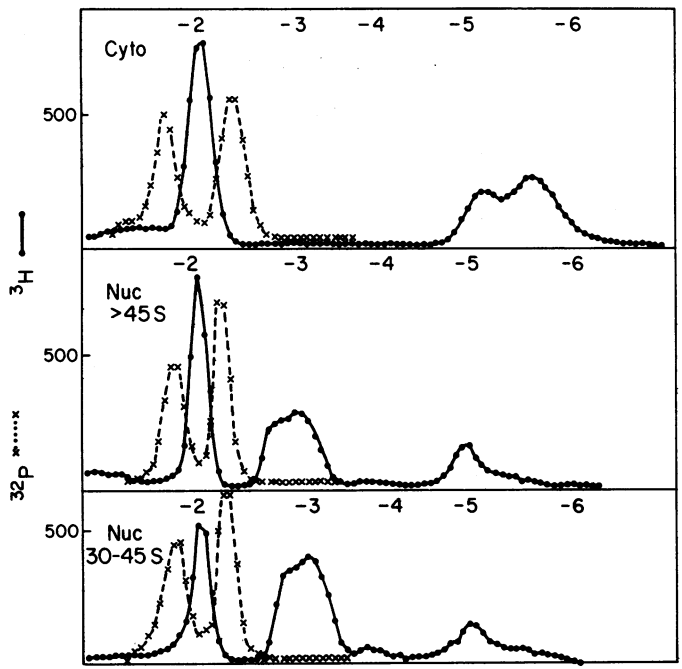

FIGURE 1.
DEAE-Cellulose Chromatography of Methylated Oligonucleotides in AD-2 RNA.
Nuclear and cytoplasmic AD-2 specific RNAs from cells labeled with ^{3}H-methyl-methionine were digested by pancreatic and T_2 RNAse. ^{32}P RNA was added to monitor completeness of digestion. The digests were chromatographed 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

Sample	% Radioactivity as Mononucleotide	Di- or Trinucleotide (-3)	(-5)	m^6Ap/cap
	(-2)			
Cytoplasmic poly(A)-containing				
Exp. 1. AD-2	61	-	39	1.8
2. AD-2	64	-	36	1.6
3. AD-2	55	-	45	1.25
Uninfected	26	-	74	0.35
Nuclear				
2. AD-2 28S	32	58	10	3.2
AD-2 >45S	41	40	19	2.2
AD-2 30-45S	32	53	15	2.1

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^6A as had been found in cellular mRNA[10]. The remainder was m^5C (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 ---$m^7GpppN_1^m$/pN/p or $m^7GpppN_1^m$/pN_2^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'-O methylnucleosides (Fig. 3A),

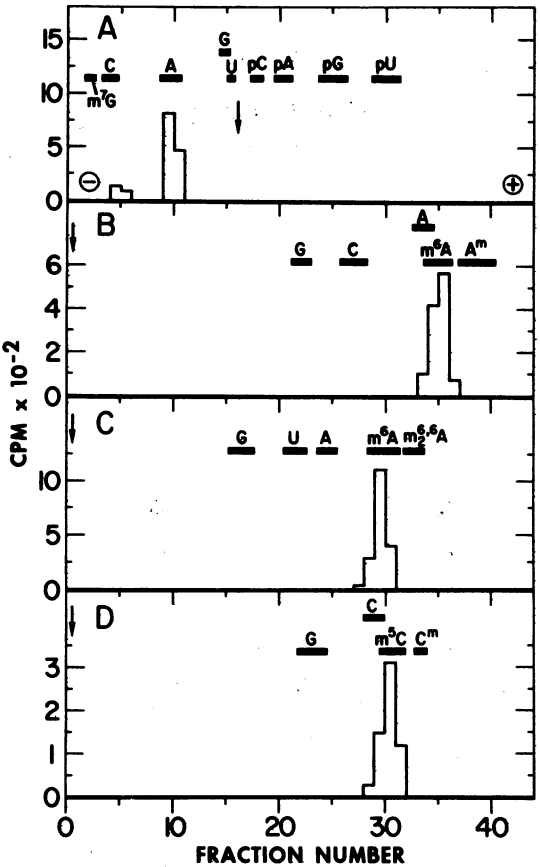

FIGURE 2.
Methylated Mononucleotides in AD-2 poly(A)+ Cytoplasmic RNA

Cytoplasmic poly(U) bound AD-2 specific RNA was digested with pancreatic and T_2 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_4OH (10:6 v/v) and (C) isopropanol: H_2O:NH_4OH (7:2:1 v/v). The C derivative in panel A was analyzed in isobutyric acid: NH_4OH (D).

predominantly C^m. Further characterization of the ^3H-methyl cap structures by paper electrophoresis after digestion with nucleotide pyrophosphatase and BAP revealed positively charged m^7G and a smaller amount of the neutral ring-opened derivative of m^7G remaining at the origin (Fig. 3B). Most of the radioactivity released from caps migrated as methylated A (Fig. 3B) which was

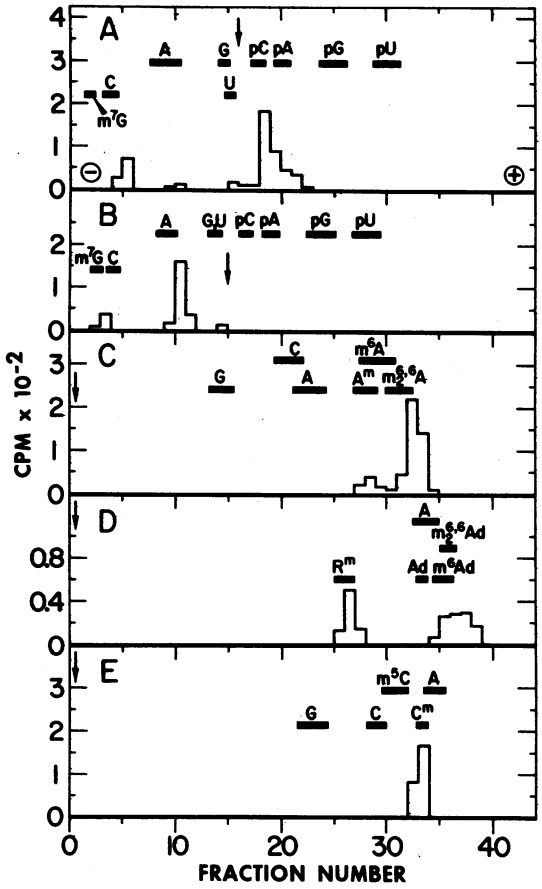

FIGURE 3
Distribution of Methylated Constituents in Caps of Poly(A)+ Cytoplasmic AD-2 RNA

^{3}H-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 P_1 nuclease and BAP, and analyzed by paper electrophoresis at pH 3.5 as described previously[3] (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: H_2O NH_4OH (7:2:1 v/v) (C). The major constituent which migrated similar to the marker $m_2^{6,6}A$ 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 NH_4OH (10:6 v/v) (D). The C derivative in panel A was identified as C^m in the same solvent system (E).

resolved by paper chromatography into two components: one migrating slightly faster than the position of N_1^6-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 (R^m) and the remainder with N^6 mono- and dimethyladenine (Fig. 3D). Since depurination of A^m would yield 13% of the radioactivity from 2'-O-methyladenosine as R^m, 31% (44%-13%) of the total ^{3}H-methyl in the R^m portion is derived from the other major peak of methylated adenosine, and the ratio of methylated adenine to R^m is 56/31 = 1.8. N^6mA^m and N^6, $^6m_2A^m$ would yield ratios of 1 and 2, respectively. Thus the results are consistent with the presence of a mixture of trimethyl A ($N^6,^6m_2A^m$ = 70%), dimethyl A (N^6mA^m = 17%), and monomethyl A (A^m = 13%) in the cap structures of AD-2 mRNA. (Similar results were obtained by Moss, pers. comm.) In addition to cap 1 structures ($m^7GpppN_1{}^mpNp$) AD-2 mRNA also contains some cap 2 structures ($m^7GpppN_1{}^mpN_2{}^mpNp$) as shown by the release of mononucleotides by P_1 nuclease and BAP treatment (Fig. 3A). The predominant $N_2{}^m$ in the cap structures is apparently C^m (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 mRNA[8,9,10,11], but there is about three times as much methyl-labeled m^6A 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 ^{32}P 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 long[17]. 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 ^{3}H-methyl radioactivity in mononucleotides is consistent with the presence of about 4 m^6A

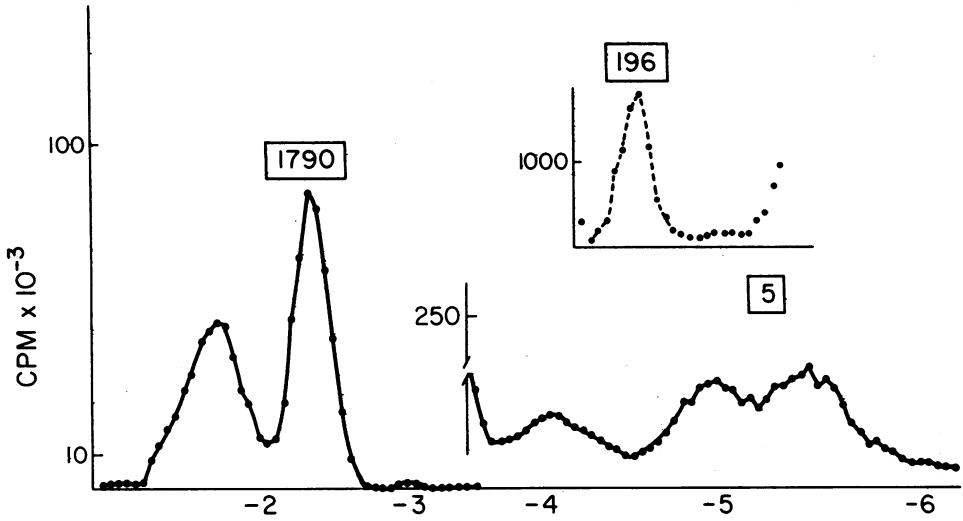

FIGURE 4.
"Cap" Content of ^{32}P Labeled Poly(A)+ Cytoplasmic AD-2 RNA

Cells were labeled with ^{32}P 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 (m^7GpppN^mpNp), 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 ^{32}P results indicating one cap per molecule would then imply an average about 4 m^6A residues per molecule. An additional suggestion about the distribution of the m^6A residues was revealed by co-sedimenting ^{32}P 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 ^{32}P label should have increased in smaller molecules, but this was definitely not the case. The faster sedimenting (26S, 4000-5000 nucleotides) ^{32}P labeled RNA had the same ratio of $^3H/^{32}P$ 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 findings[14,17,21] 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 m^6A residues in long than in short AD-2 mRNA molecules.

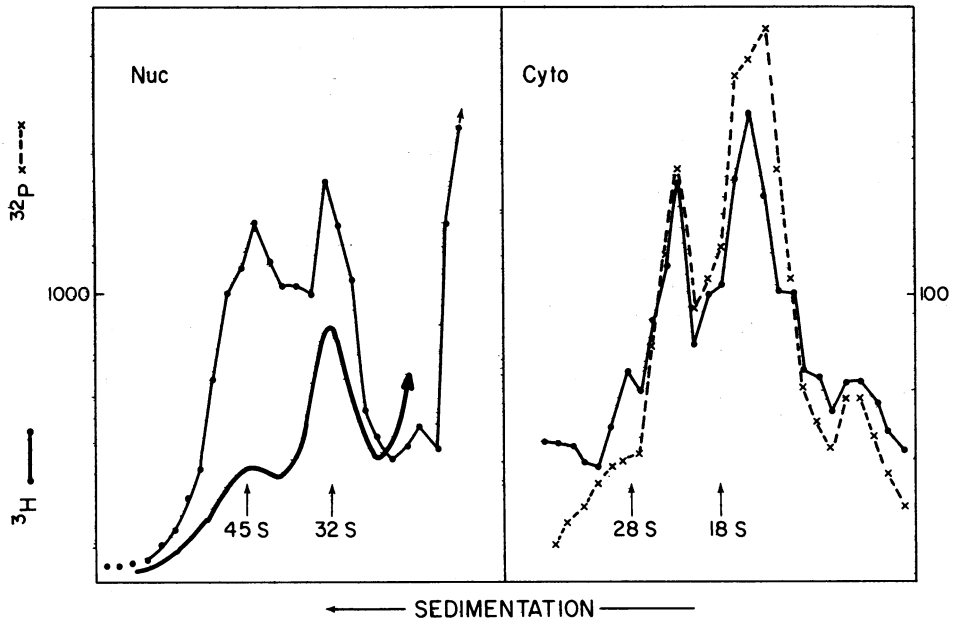

FIGURE 5
Sedimentation of RNA from AD-2 Infected Cells.

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 $O.D._{260}$ 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[8,10]. 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.

Location of Methyl Radioactivity in AD-2 mRNA

Sample	Total CPM	Hybridized to AD-2
Alkali broken poly(U) bound	9,600	4,000
Alkali broken poly(U) flow through	90,000	44,000

^3H-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 .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

^3H-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 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 ^3H-uridine labeled AD-2 nuclear RNA[17]. The selected RNA was digested with pancreatic and T_1 ribonucleases and analyzed by DEAE-cellulose chromatography. Three types of ^3H-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^6A (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 proportion of ^3H-methyl eluting at -6 than at -5 (Fig. 1B,C). These results suggest that "cap 1" structures in (m^7GpppN^m) 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 $m_2^{6,6}A$ which has essentially the same Rf (.85) as m^6A^m, 2'-O-N^6-dimethyl adenosine, (0.82) in this solvent system (Fig. 6D). After depurination, 42% of the ^3H-methyl radioactivity migrated with 2'-O-methyl ribose and 58% with $m_2^{6,6}$ adenine (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^6A^m and $m_2^{6,6}A^m$.

The T_2-resistant dinucleotides from the nuclear RNA (-3) were completely sensitive to P_1 nuclease digestion, indicating that they arose from internal 2'-O-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 extent 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

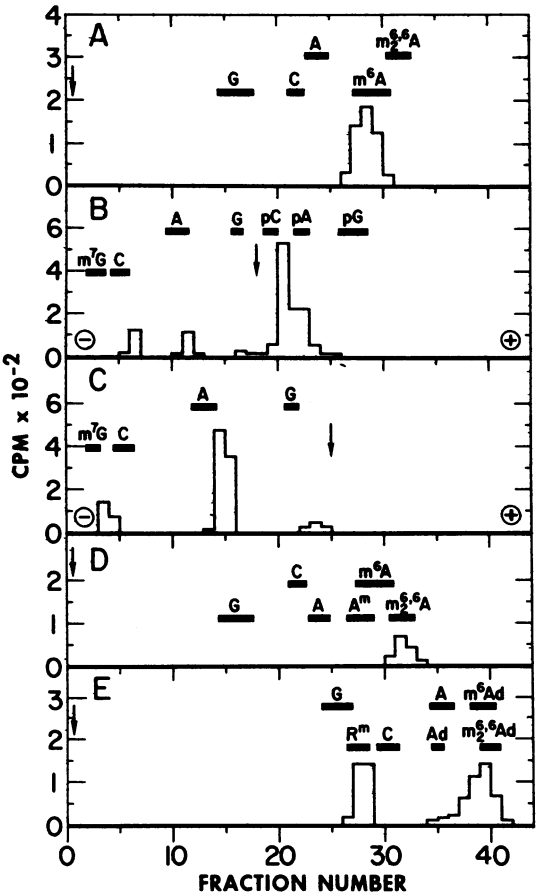

FIGURE 6.
Methylated Constitutents in Nuclear AD-2 RNA

The ^{3}H-methyl-labeled mononucleotides obtained by RNAse digestion and DEAE-cellulose column chromatography were desalted and analyzed by paper chromatography in isopropanol: H_2O: NH_4OH (7:2:1 v/v) (A). The material eluting from DEAE-cellulose at -5 to -6 was desalted, digested with P_1 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: NH_4OH (D) and isobutyric acid: 0.5 N NH_4OH 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 iodoacetate^{14} washes. A third aliquot was eluted, boiled, and rehybridized. All three were digested with RNAse A and T_2 and analyzed by DEAE chromatography. The hybridized, RNAse-treated sample still

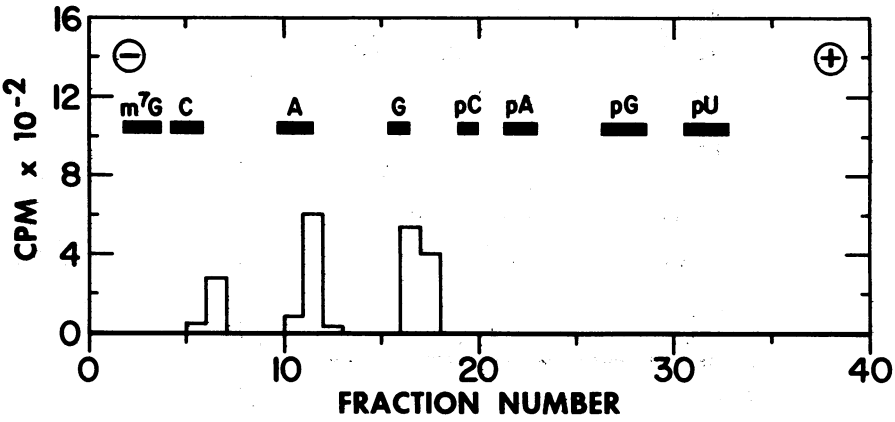

FIGURE 7
Separation of 2'-O-Methylated Nucleosides from Nuclear Di/Trinucleotides (-3)

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 P_1 nuclease and BAP and analyzed by paper electrophoresis[10].

TABLE 4

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

Sample	% Methyl Label As		
	Mono (-2)	Di (-3)	Cap (-5)
First hybrid	22	68	10
RNAse resistant	34	41	25
Second hybrid	80	6	14

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 (1X hybrid), 2) by treatment of filters bearing AD-2 DNA with 2.5 µg/ml RNAse A and 5 units of RNAse T1[14] 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 $MgCl_2$, 0.0015 M $CaCl_2$, 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)[14], boiled for 3 min. and hybridized and 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.

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 $m^7GpppN_1{}^mpNp$ and $m^7GpppN_1{}^mpN_2{}^mpNp$, and the internal methylation is m^6A (and possibly m^5C). 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 m^6A per molecule than HeLa mRNA. AD-2 nuclear RNA and HeLa HnRNA both contain a higher number of m^6A 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 m^6A is found in cellular mRNAs[8,9,10,11,12] SV40 mRNA[24], avian sarcoma virus RNA[25], and now AD-2 mRNA. All of these mRNAs proabably derive from the nuclear transcription of DNA followed by post-transcriptional modification. Several viruses[3], cytoplasmic polyhedrosis virus[4], vaccinia[5,26], Newcastle disease virus[27], and vesicular stomatitis virus[6] 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 m^6A 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[15,16]. Previous experiments have shown poly(A) to be present in the high molecular weight AD-2 specific nuclear RNA[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[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^m, 2'-O-methyladenosine; m^6A, N^6-methyladenosine; $m_2^6,^6A$, $N^6,6$-dimethyladenosine; m^6Ad, N^6-methyladenine; $m_2^6,^6Ad$, N^6-dimethyladenine; m^6A^m, N^6-methyl-2'-O-methyladenosine; $m_2^6,^6A^m$, N^6-dimethyl-2'-O-methyladenosine; G, guanosine; m^7G, 7-methylguanosine; pU, 5'-uridylic acid; U, uridine; pC, 5'-cytidylic acid; C, cytidine; C^m, 2'-O-methyl cytosine; m^5C, 5-methylcytidine; R^m, 2'-O-methylribose; AD-2, adenovirus 2.

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