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Sequence of Methylated Nucleotides at the 5'-Terminus of Adenovirus-Specific RNA

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RNA labeled with [*methyl*-^{3}H]methionine and [^{14}C]uridine was isolated from the cytoplasm of adenovirus-infected cells and purified by poly(U)-Sepharose chromatography and hybridization to filters containing immobilized adenovirus DNA. Analysis by dimethyl sulfoxide-sucrose gradient sedimentation suggested that the major mRNA species were methylated. 7-Methylguanosine was identified at the 5'-terminus of the adenovirus-specific RNA and could be removed by periodate oxidation and β-elimination. Structures of the type m^{7}G(5')ppp(5')N^m containing the unusual nucleoside N^6,$O^{2'}$-dimethyladenosine, and smaller amounts of 2'-*O*-methyladenosine were isolated by DEAE-cellulose chromatography after P_1 nuclease digestion of the RNA. Evidence for some 5'-terminal sequences, m^{7}G(5')ppp(5')m^{6}A^{m}pN^m, with additional 2'-*O*-methylribonucleosides was also obtained. A base-methylated nucleoside, N^6-methyladenosine, is located within the RNA chain and is released as a mononucleotide by alkali hydrolysis.

The mRNAs of several cytoplasmic DNA and RNA viruses, including vaccinia virus (29–31), reovirus (15, 27), cytoplasmic polyhedrosis virus (13, 14), and vesicular stomatitis virus (1, 25), are methylated and have the sequences m^{7}G(5')ppp(5')G^m or m^{7}G(5')ppp(5')A^m (abbreviations: m^{7}G, 7-methylguanosine; G^m, 2'-*O*-methylguanosine; A^m, 2'-*O*-methyladenosine) at their 5'-termini. Enzymes used for the modification of the 5'-termini are present within the cores of all of the above viruses and soluble guanylyl- and methyltransferases have been isolated from vaccinia virus (9). Cellular mRNAs are also methylated (8, 22, 23), and those from HeLa cells (16, 32) and mouse myeloma cells (2) have been shown to contain m^{7}G(5')ppp(5')N^m and m^{7}G(5')ppp(5')N^{m}pN^m at their 5'-termini as well as internal N^6-methyladenosine (m^{6}A) residues. Knowledge of the reactions leading to the modification of the cellular mRNAs is of particular interest since it may aid in understanding the relationship between heterogeneous nuclear RNA and mRNA. For example, if mRNA is formed as a 3'-terminal fragment of HnRNA as proposed (20), then the 5'-terminal modifications must occur either during or after the cleavage step. DNA viruses, such as simian virus 40 (SV40) and adenovirus, provide potentially useful model systems for studying the synthesis and processing of RNA in the nucleus of a cell. Recent experiments indicate that SV40 mRNA is methylated (17) and we now demonstrate that adenovirus mRNAs contain the 5-terminal sequences m^{7}G(5')ppp(5')m^{6}A^m (abbreviation: m^{6}A^m; N^6,$O^{2'}$-dimethyladenosine) and m^{7}G(5')ppp(5')A^m as well as internally located m^{6}A residues.

MATERIALS AND METHODS

Isolation of polyadenylated adenovirus RNA. Suspension cultures of HeLa S-3 cells were infected with adenovirus type 2 at a multiplicity of 20 to 25 tissue culture infectious dose units per cell. After 2 h the cells were resuspended at 3×10^5/ml in Eagle medium without serum and containing 10 μM methionine. Fourteen hours later the cells were resuspended in one-tenth of the original volume of medium containing 20 mM sodium formate, 20 μM adenosine, 20 μM guanosine, 10 μM [*methyl*-^{3}H]methionine (Amersham-Searle, 5 Ci/mmol), and 0.45 μM [^{14}C]uridine (New England Nuclear Corp., 57 mCi/mmol). After a 4-h labeling period, RNA was extracted from the cytoplasm with sodium dodecyl sulfate, chloroform, and phenol (24).

Polyadenylated RNA was isolated by poly(U)-Sepharose (Pharmacia Fine Chemicals) chromatography (18). Recovery was 96 to 100% and the poly(A)+ fraction contained 4% of the ^{3}H- and 34% of the ^{14}C-labeled cytoplasmic RNA applied. DNA-RNA hybridization was performed in the presence of formamide at 37 C for 44 to 52 h (6). Nitrocellulose filters containing 20 μg of adenovirus DNA bound approximately 60% of the input RNA. Approximately 77% of the bound RNA was eluted by incubating the filters in

90% formamide, 0.1 mM Tris-hydrochloride (pH 8.1), 0.01 mM EDTA, and 1% sodium dodecyl sulfate for 2.5 h at 37 C followed by 1.5 h at 45 C.

Enzymatic digestions and chemical treatments. Digestions were carried out with 125 μg of P_1 nuclease (Yamasa Shoyu Co.) in 0.4 ml of 10 mM sodium acetate (pH 6) for 2 h at 37 C. The solution was then adjusted to contain 50 mM Tris-hydrochloride (pH 8.5) and 5 mM $MgCl_2$ and incubated with 40 μg of alkaline phosphatase (Worthington Biochemical Corp.) for an additional hour. Combined digestion with snake venom phosphodiesterase (25 μg, Worthington Biochemical Corp.) and alkaline phosphatase (10 μg) was in 50 μl of 50 mM Tris-hydrochloride (pH 8.5), 5 mM $MgCl_2$ for 4 h at 37 C. Alkali hydrolysis in 0.3 N KOH was for 30 h at 37 C. Nucleotides were depurinated in 1 N HCl at 100 C for 30 min.

Periodate oxidation and β-elimination were performed essentially as described by Fraenkel-Conrat and Steinschneider (11). Adenovirus RNA was incubated in the dark at 0 C for 30 min in 0.12 ml of a solution containing 0.9 mM sodium periodate and 0.15 M sodium acetate (pH 5.3). The RNA was precipitated two times with ethanol and dissolved in 0.1 ml of a solution containing 0.3 M aniline and 0.01 M acetic acid adjusted to pH 5 with concentrated HCl. After 3 h at room temperature, the RNA was precipitated with ethanol. The supernatant was dried under nitrogen, and the residue was dissolved in 1 N HCl and heated at 100 C in a sealed capillary tube for 30 min. The eliminated base was then analyzed by paper chromatography.

Chromatography. DEAE-cellulose (Whatman DE-52) chromatography was carried out with columns (0.9 by 15 cm) equilibrated with 10 mM Tris-hydrochloride (pH 7.6) in 7 M urea (30). Gradients (200 ml) were from 0 to 0.2 or 0.3 M NaCl in 7 M urea. Fractions were desalted using DEAE-cellulose columns (0.7 by 5 cm) equilibrated with triethylammonium bicarbonate (pH 8.0). After extensive washing, the oligonucleotides were eluted with 2 M triethylammonium bicarbonate and repeatedly dried under a stream of nitrogen.

RESULTS

Methylated nucleosides in adenovirus-specific RNA. At late times after adenovirus infection, polyadenylated RNA isolated from polyribosomes sediments in dimethyl sulfoxide-sucrose gradients primarily as a major 26*S* peak with a 22*S* shoulder and minor species on either side (19). To determine whether adenovirus-specific RNA is methylated, cell cultures were labeled with [*methyl*-^{3}H]methionine and [^{14}C]uridine from 16 to 20 h after infection and the cytoplasmic polyadenylated RNA was isolated by poly(U)-Sepharose chromatography. Results obtained by dimethyl sulfoxide-sucrose gradient sedimentation analysis suggested that the major RNA species were methylated (Fig. 1A). Proof that the methyl-labeled RNA was virus specific was obtained by hybridization to filters containing immobilized adenovirus DNA (Fig. 1B). Except for the removal of very small amounts of residual highly methylated rRNA and tRNA, the profiles before and after hybridization appeared virtually identical. One striking observation was the increase in ratio of ^{3}H to ^{14}C with fraction number. When corrected for differences in the size of the RNA, however, the ratios become more constant (Fig. 1), suggesting that different length RNAs contained similar numbers of methyl groups. An estimate of that number may be obtained by comparing the ^{3}H/^{14}C ratios with that of rRNAs for which the degree of methylation is known (22, 32). This calculation involves a number of assumptions including the synthesis of all types of RNA from equivalent nucleotide and *S*-adenosylmethionine pools. A ^{3}H/^{14}C ratio of 11.3 for 18*S* rRNA was obtained by gradient sedimentation of the RNA fraction, from the cytoplasm of adenovirus-infected cells, that did not adsorb to poly(U)-Sepharose. Hybridization experiments indicated a negligible amount of adenovirus-specific RNA in the peak fraction. Using the value 1 methyl group per 48 nucleotides in HeLa cell 18*S* rRNA (4), average ^{3}H/^{14}C ratios of 1.3 to 1.5 for the peak fractions of polyadenylated adenovirus-specific RNA (Fig. 1), and the estimated molecular weights of the latter, about five methyl residues per RNA chain was calculated. An entirely different method of estimation, described below, involving none of the previous assumptions and based on an analysis of the methylated nucleosides, predicts about six methyl groups per RNA chain if all the cytoplasmic, polyadenylated, adenovirus-specific RNA is methylated. Taken together, the two sets of results would indicate that about 80% of the isolated RNA is methylated. It should be realized that these calculations have been made to obtain a preliminary estimate of what proportion of RNA molecules are methylated, and that the existence of a nonmethylated fraction of cytoplasmic, polyadenylated, adenovirus-specific RNA has not been demonstrated. Based on the cytoplasmic location, poly(A) content, and similarity in sedimentation to RNA isolated from polyribosomes (19) and demonstrated to code for adenovirus proteins in a cell-free system (3, 10), it is our working assumption that at least a portion of the methylated adenovirus-specific RNA is mRNA and this term has been used throughout the text.

Identification of $m^7G(5')ppp(5')m^6A^m$ and $m^7G(5')ppp(5')A^m$. All further analyses were carried out on [*methyl*-^{3}H]methionine- and

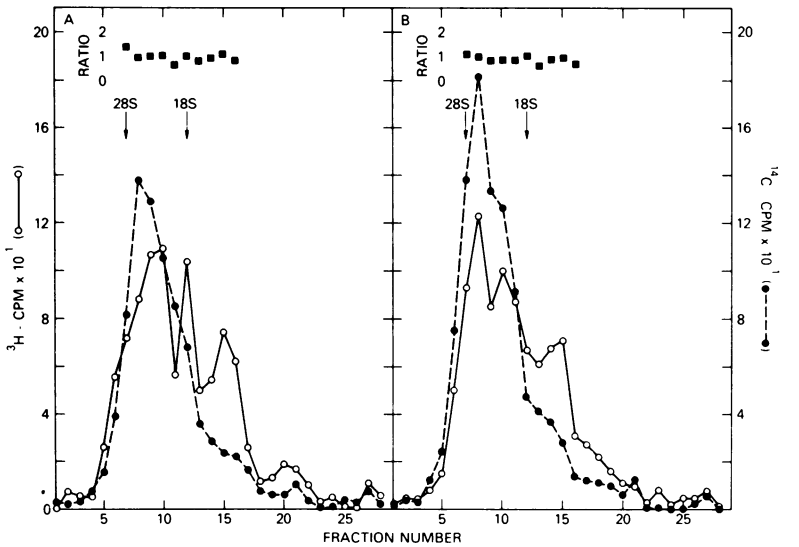

FIG. 1. *Dimethyl sulfoxide-sucrose gradient analysis of polyadenylated RNA from adenovirus-infected cells. [Methyl-^{3}H]methionine- and [^{14}C]uridine-labeled RNA from adenovirus-infected cells was selected by adsorption to a poly(U)-Sepharose column and then sedimented through a 0 to 15% sucrose gradient containing 99% dimethyl sulfoxide, 10 mM LiCl, and 1 mM EDTA (pH 7.1) for 20 h at 165,000 × g at 25 C in a Spinco SW50.1 rotor. Fractions of 0.15 ml were collected from the bottom of the tubes and 25-μl portions were counted directly to give the sedimentation profile in (A). The remainder was hybridized in dimethyl sulfoxide (7) to filters containing 2 μg of immobilized adenovirus DNA (B). Filters containing 2 μg of Escherichia coli DNA were included in each hybridization reaction to correct for nonspecific binding of RNA. The ratios were obtained from the formula $^3H/^{14}C$ × molecular weight of RNA/10^6. The positions of ribosomal RNA markers used to calculate molecular weights (7) are indicated by arrows.*

[^{14}C]uridine-labeled cytoplasmic RNA selected by both poly(U)-Sepharose chromatography and hybridization to adenovirus DNA.

To determine whether nuclease-resistant structures of the type m^{7}G(5')ppp(5')N^m are present in adenovirus mRNA, an enzymatic digestion was carried out with P_1 nuclease (which degrades RNA to 5'-nucleotides regardless of whether 2'-*O*-methylribonucleotides are present [12]) and alkaline phosphatase (which removes terminal phosphates). The products of digestion were then chromatographed on DEAE-cellulose in 7 M urea. All of the [^{14}C]uridine-labeled nucleotides and more than half of the methyl-labeled nucleotides were converted to nucleosides and did not adsorb to the column. However, 44% of the methyl-labeled material eluted between oligonucleotide markers with charges of −2 and −3 (Fig. 2). This is precisely the elution position expected for structures of the type m^{7}G(5')ppp(5')N^m (15).

Although resistant to P_1 nuclease, m^{7}G(5')ppp(5')N^m can be cleaved by snake venom phosphodiesterase and then converted to nucleosides with alkaline phosphatase (31). This procedure, followed by paper chromatography, was used to determine the nucleoside composition of the putative m^{7}G(5')ppp(5')N^m structure isolated from adenovirus mRNA. Approximately 34% of the labeled nucleosides cochromatographed with m^{7}G, 6% did so with A^m, and 55% migrated ahead of A^m (Fig. 3). Only barely detectable amounts were in the position of other 2'-*O*-methylribonucleosides. According to the structure proposed above, however, there should be equal amounts of radioactivity in m^{7}G and the sum of all the 2'-*O*-methylribonucleosides. The relatively low proportion of radioactivity associated with m^{7}G suggested that either some of the guanosine was not methylated or that the material migrating ahead of A^m contained two methyl groups. The possibility of a mixture of structures, some beginning with guanosine and others with m^{7}G, seemed unlikely since the latter methylated nucleoside has an additional positive charge and no evidence of heterogeneity was seen on chromatography (Fig. 2). To test the second possibility and identify the presumptive dimethylated nucleoside, the P_1nuclease-resistant material was depurinated with 1 N HCl. Three labeled products were identified by thin-

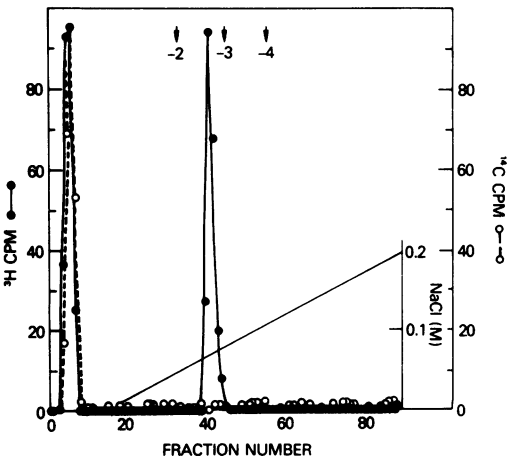

FIG. 2. *DEAE-cellulose column chromatography of P_1 nuclease and alkaline phosphatase-resistant product. [^{14}C]uridine- and [methyl-^{3}H]methionine-labeled adenovirus mRNA was digested with P_1 nuclease and alkaline phosphatase and chromatographed on DEAE-cellulose in 7 M urea at pH 7.6. The arrows indicate the positions and negative charges of oligonucleotide markers.*

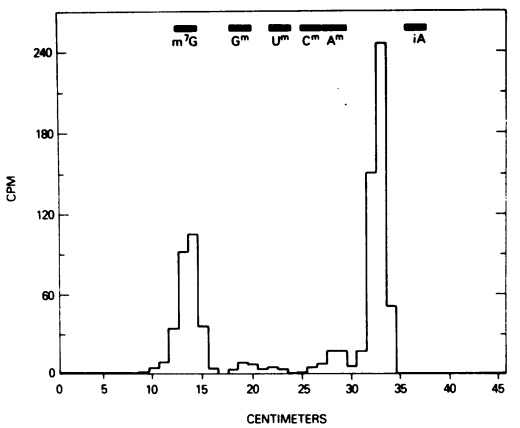

FIG. 3. *Paper chromatography of nucleosides derived from P_1 nuclease and alkaline phosphatase-resistant product. The nuclease-resistant material obtained by column chromatography (Fig. 2) was desalted and then digested with venom phosphodiesterase and alkaline phosphatase. The nucleosides were analyzed by descending chromatography in isopropanol-water-NH_3 (7:2:1), and 1-cm strips were counted. Abbreviation: iA, isopentenyladenosine.*

layer chromatography (Fig. 4A). The one nearest the origin (Fig. 4A) is in the position expected for the acid degradation product of 2'-*O*-methylribose, the next co-chromatographed with 7-methylguanine (m^{7}Gua), and the third co-chromatographed with N^6-methyladenine (m^{6}Ade). These results suggested that the penultimate nucleoside was the dimethylated derivative of adenosine m^{6}A^m. As a control, m^{7}G(5')ppp(5')A^m isolated from vaccinia virus mRNA was analyzed in a similar manner (Fig. 4B). As expected, approximately half of the label was in the acid degradation product of 2'-*O*-methylribose and the remainder was in m^{7}Gua. No m^{6}Ade was recovered. A more detailed identification of m^{6}A^m from HeLa cell mRNA, which chromatographs with the nucleoside isolated from adenovirus mRNA, is presented elsewhere (33). Taking into account the presence of two labeled methyl groups in m^{6}A^m and only one in A^m, we deduced from the nucleoside composition that about 80% of the P_1 nuclease-resistant 5'-termini were m^{7}G(5')ppp(5')m^{6}A^m and 20% were m^{7}G(5')ppp(5')A^m. Since 44% of the methyl was in the P_1

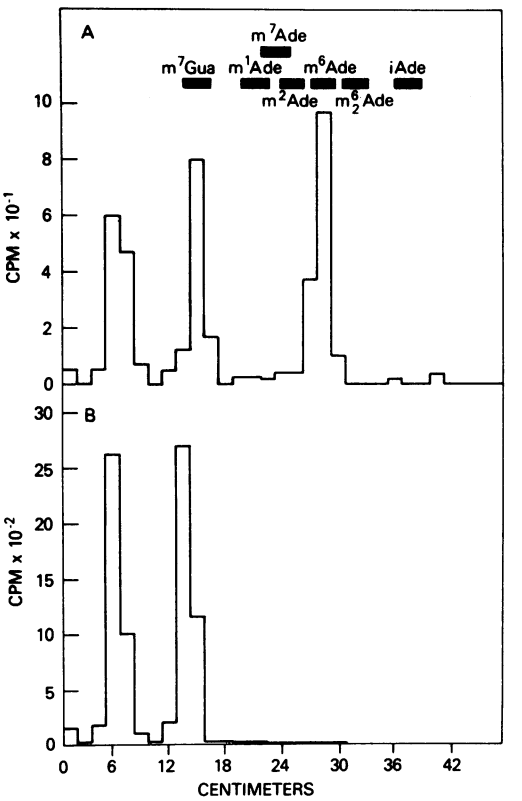

FIG. 4. *Paper chromatography of products obtained by HCl treatment of nuclease-resistant material. (A) The nuclease-resistant material obtained by column chromatography (Fig. 2) was desalted and then heated at 100 C for 30 min in 1 N HCl. (B) m^{7}G(5')ppp(5')A^m isolated from vaccinia virus mRNA was treated in an identical fashion. Descending chromatography was carried out in isopropanol-H_2O-NH_3 (7:2:1); 1.5-cm strips were counted.*

nuclease-resistant structure which contains an average of 2.8 methyls, there should be a little more than six methyl groups per RNA chain.

m^7G is at the 5'-terminus of adenovirus mRNA. Nucleosides that have free 2',3'-hydroxyls and that are located at the end of an RNA chain may be removed by periodate oxidation and β-elimination (11). This procedure was previously used to prove that m^7G is at the 5'-terminus of vaccinia virus (31) and HeLa cell (32) mRNAs. m^7G was released from adenovirus mRNA by periodate oxidation and β-elimination and identified by paper chromatography, indicating that it also has free 2',3'-hydroxyls and is located at the 5'-terminus (Fig. 5).

Analysis of methylated nucleotides released from adenovirus mRNA by alkali. Thus far we have demonstrated that the sequences $m^7G(5')ppp(5')m^6A^m$ and $m^7G(5')ppp(5')A^m$ are located at the 5'-termini of adenovirus mRNAs. Further studies were carried out to identify internally located methylated nucleosides. [*methyl*-^{3}H]- and [^{14}C]uridine-labeled adenovirus mRNA was hydrolyzed with alkali (which in contrast to P_1 nuclease degrades RNA to 3'-nucleotides and also does not cleave phosphodiester bonds of 2'-*O*-methylribonucleosides) and chromatographed on DEAE-cellulose in 7 M urea. Two major methyl-labeled peaks were obtained (Fig. 6A). The first peak, with a charge of -2, which is

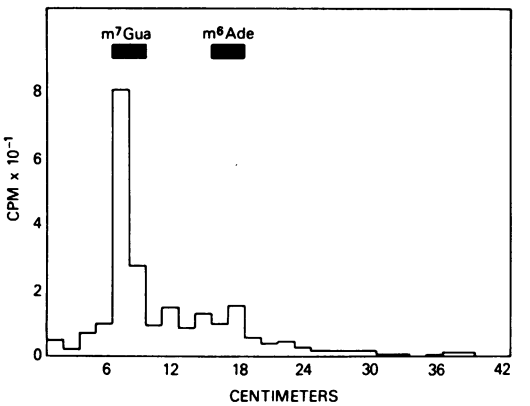

FIG. 5. *Identification of 5'-terminal nucleoside. [Methyl-^{3}H]methionine-labeled adenovirus mRNA was treated with periodate and aniline. The eliminated base was chromatographed in isopropanol-HCl-water (680:170:140); 1.5-cm strips were counted.*

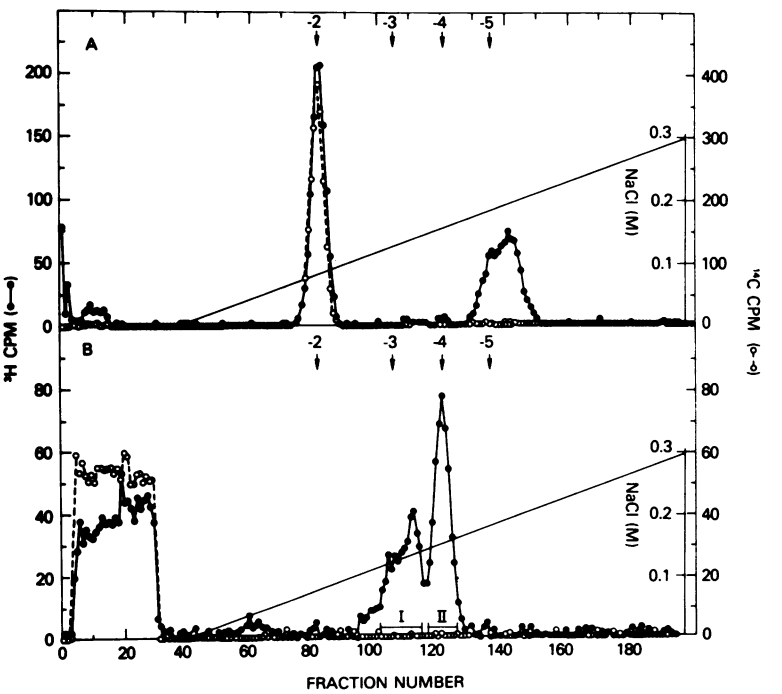

FIG. 6. *DEAE-cellulose column chromatography of alkali digest of adenovirus mRNA. (A) Polyadenylated mRNA labeled with [methyl-^{3}H]methionine and [^{14}C]uridine was hybridized at 65 C in the absence of formamide to filters containing adenovirus DNA (26) and then hydrolyzed with 0.3 N KOH, neutralized with AG-50[H^+] resin, and chromatographed on a DEAE-cellulose column in 7 M urea at pH 7.6. (B) The alkali hydrolysate was treated with alkaline phosphatase and chromatographed in a similar fashion.*

indicative of a mononucleotide derived from an internal location within the RNA, was m^6Ap since it was converted to m^6A with alkaline phosphatase and identified by paper chromatography (Fig. 7). The absence of material with a charge of −3 (Fig. 6A) indicated that adenovirus-specific RNA, unlike tRNA and rRNA, has no single 2′-*O*-methylribonucleosides that would be released as dinucleotides by alkali treatment. The second major peak eluted in the position expected for alkali-resistant 5′-terminal oligonucleotides (Fig. 6A; reference 31). When the alkali digest was also treated with alkaline phosphatase, the 5′-terminal oligonucleotide peak eluted earlier, as expected if the 3′-terminal phosphate were removed (Fig. 6B). In addition, the peak was now clearly separated into two components. The components (I and II) were digested with venom phophodiesterase and alkaline phosphatase and analyzed by paper chromatography. Peak I had a nucleoside composition (Fig. 8) similar to that of the P_1 nuclease-resistant material (Fig. 2). Approximately 33% of the label was recovered in m^7G, 54% was recovered in m^6A^m, and 7% was recovered in A^m, again consistent with 80% $m^7G(5')ppp(5')m^6A^m$ and 20% $m^7G(5')ppp(5')A^m$. A similar nucleoside analysis of peak II indicated only 9% m^7G and 22% m^6A^m (Fig. 8). However, 41% of the material migrated with C^m and 23% migrated with G^m and/or U^m (Fig. 8). We considered that component

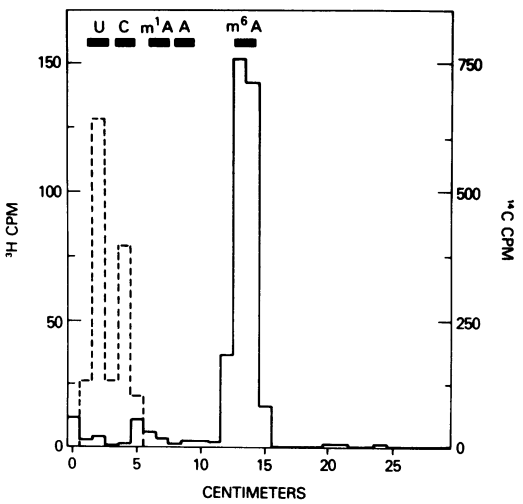

FIG. 7. *Identification of m^6A. Radioactively labeled material with a net charge of −2 was isolated as in Fig. 6A, desalted, treated with alkaline phosphatase, and analyzed by ascending chromatography in butanol-NH_3-water (86:5:14); 1-cm strips were counted. Symbols:* ——, *^3H;* -----, *^{14}C.*

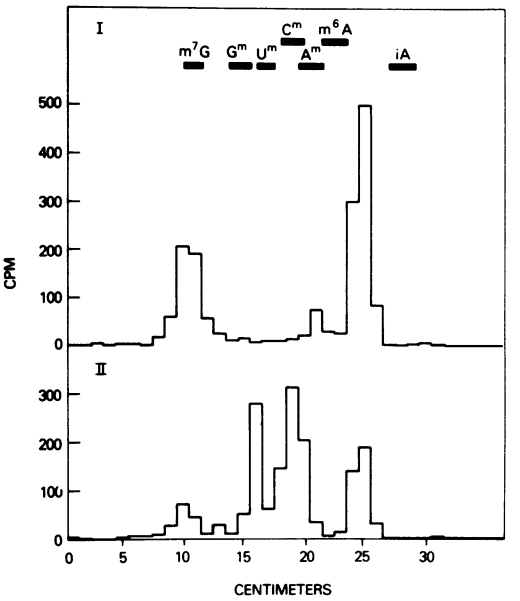

FIG. 8. *Nucleoside analysis of alkali and alkaline phosphatase-resistant oligonucleotides. Components I and II (Fig. 6B) were desalted, digested with snake venom phosphodiesterase and alkaline phosphatase, and then analyzed by ascending chromatography in isopropanol-water-NH_3 (7:2:1); 1-cm strips were counted.*

II might be $m^7G(5')ppp(5')m^6A^mpN^mpN$, in which C^m, G^m, or U^m is in the third position, since such a structure would elute from DEAE-cellulose columns after $m^7G(5')ppp(5')m^6A^m$. If this were true, however, the relatively large percentage of radioactive C^m, G^m, or U^m would indicate that the methyl groups in these nucleosides have a higher specific radioactivity than the methyl groups in m^7G and m^6A^m and hence were derived from different *S*-adenosylmethionine pools. A second alternative is that the nucleosides in peak II have additional methyl groups on the base which could account for some of the apparent discrepancy. Still another possibility is that peak II represents an internal sequence of five or six consecutive 2′-*O*-methylribonucleosides which would yield an alkali-resistant oligonucleotide of the appropriate charge.

DISCUSSION

A large proportion of the cytoplasmic, polyadenylated, adenovirus-specific RNA made at late times after infection of HeLa cells is specifically methylated. Analysis on dimethyl sulfoxide-sucrose gradients suggested that different size mRNA species contain similar numbers of

methyl groups. m^7G was shown to be at the 5'-terminus and to have free 2',3'-hydroxyls since it was removed by periodate oxidation and β-elimination. After P_1 nuclease and alkaline phosphatase digestion, structures of the type $m^7G(5')ppp(5')N^m$ were isolated. Approximately 80% of such sequences contained the novel nucleoside m^6A^m and 20% contained A^m in the penultimate position. These structures containing an additional unmethylated nucleoside, $m^7G(5')ppp(5')N^mpN$, were isolated after treatment of adenovirus mRNA with alkali and alkaline phosphatase. A methylated oligonucleotide fraction with a greater negative charge and containing additional methylated ribonucleosides tentatively identified as C^m, G^m, and/or U^m was also isolated after the latter treatment. This material may consist of sequences of the type $m^7G(5')ppp(5')m^6A^m$-pN^mpN, although alternate possibilities were considered. Another methylated nucleoside, m^6A, is present in adenovirus mRNA and is released as a mononucleotide after alkali hydrolysis, indicating that it has an internal position and is not adjacent to a 2'-*O*-methylribonucleoside.

It is of particular interest to compare the results reported here for adenovirus mRNA with those previously obtained for the mRNA of the uninfected host cell. HeLa cell mRNA contains 5'-terminal sequences of the general type $m^7G(5')ppp(5')N^m$ and $m^7G(5')ppp(5')N^mpN^m$, with all four 2'-*O*-methylribonucleosides in the penultimate position (16, 32). The unusual nucleoside m^6A^m is also found next to the 5'-terminal nucleotide of HeLa cell and L-cell mRNA but is present in no more than 25 to 30% of such terminal sequences (33). m^6A^m is not present in tRNA or rRNA but recently has been identified in an internal position of a low-molecular-weight nuclear RNA (U-2) of hepatoma cells (28). An unusual nucleoside was found next to the 5'-terminus of vesicular stomatitis virus mRNA made in vivo (21) and is believed to be m^6A^m (S. A. Moyer and A. K. Banerjee, personal communication). HeLa cell mRNA also contains m^6A in the internal position (16, 32) as does adenovirus mRNA. Thus far, methylated nucleosides have not been found in internal positions in mRNAs of any cytoplasmic virus, suggesting that this modification has a nuclear function. A report (17) of the methylated nucleosides of SV40, the only other nuclear virus examined, also indicated the presence of internal m^6A as well as presumptive 5'-terminal sequences containing m^7G, A^m, and G^m.

Most of the polyadenylated mRNA made at late times after infection is adenovirus specific, making this system extremely useful for studying the relationship between methylation and processing of mRNA. Since methylation appears to be necessary for efficient translation of mRNAs in vitro (5), it will also be of interest to determine whether any modification of this process is involved in the turn-off of host protein synthesis and the shift from early to late viral gene expression during adenovirus replication.

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LITERATURE CITED

1. Abraham, G., D. P. Rhodes, and A. K. Banerjee. 1975. The 5'-terminal structure of the methylated mRNA synthesized *in vitro* by vesicular stomatitis virus. Cell **5**:51-58.
2. Adams, J. M., and S. Cory. 1975. Modified nucleosides and bizarre 5'-termini in mouse myeloma mRNA. Nature (London) **255**:28-33.
3. Anderson, C. W., J. B. Lewis, J. F. Atkins, and R. F. Gesteland. 1974. Cell-free synthesis of adenovirus 2 proteins programmed by fractionated messenger RNA: a comparison of polypeptide products and messenger RNA lengths. Proc. Natl. Acad. Sci. U.S.A. **71**: 2756-2760.
4. Brown, G. M., and G. Attardi. 1965. Methylation of nucleic acids in HeLa cells. Biochem. Biophys. Res. Commun. **20**:298-302.
5. Both, G. W., A. K. Banerjee, and A. J. Shatkin. 1975. Methylation-dependent translation of viral messenger RNAs *in vitro*. Proc. Natl. Acad. Sci. U.S.A. **72**:1189-1193.
6. Büttner, W., Z. Veres-Molnár, and M. Green. 1974. Isolation of DNA strand-specific early messenger RNA species in cells infected by human adenovirus 2. Proc. Natl. Acad. Sci. U.S.A. **71**:2951-2955.
7. Carter, B., and J. Rose. 1975. Transcription *in vivo* of a defective parvovirus: sedimentation and electrophoretic analysis of RNA synthesized by adenovirus-associated virus and its helper adenovirus. Virology **61**:182-199.
8. Desrosiers, R., K. Friderici, and F. Rottman. 1974. Identification of methylated nucleosides in messenger RNA from Novikoff hepatoma cells. Proc. Natl. Acad. Sci. U.S.A. **71**:3971-3975.
9. Ensinger, M. J., S. A. Martin, E. Paoletti, and B. Moss. 1975. Modification of the 5'-terminus of mRNA by soluble guanylyl and methyl transferases from vaccinia virus. Proc. Natl. Acad. Sci. U.S.A. **72**:3385-3389.
10. Eron, L., and H. Westphal. 1974. Cell-free translation of highly purified adenovirus messenger RNA. Proc. Natl. Acad. Sci. U.S.A. **71**:3385-3389.
11. Fraenkel-Conrat, H., and A. Steinschneider. 1967. Stepwise degradation of RNA:periodate followed by aniline cleavage, p. 243-246. *In* L. Grossman and K. Moldave (ed.), Methods in enzymology, vol. 12B. Academic Press Inc., New York.
12. Fujimoto, M., A. Kuninaka, and H. Yoshino. 1974. Substrate specificity of nuclease P_1. Agric. Biol. Chem. **38**:1555-1561.
13. Furuichi, Y. 1974. Methylation-coupled transcription by virus-associated transcriptase of cytoplasmic polyhedrosis virus containing double-stranded RNA. Nucleic Acids Res. **1**:809-822.

14. Furuichi, Y., and K. Miura. 1975. A blocked structure at the 5'-terminus of mRNA from cytoplasmic polyhedrosis virus. Nature (London) **253**:374-375.
15. Furuichi, Y., M. Morgan, S. Muthukrishnan, and A. J. Shatkin. 1975. Reovirus messenger RNA contains a methylated, blocked 5'-terminal structure: $m^7G(5')ppp(5')G^mpCp$-. Proc. Natl. Acad. Sci. U.S.A. **72**: 362-366.
16. Furuichi, Y., M. Morgan, A. J. Shatkin, W. Jelinek, M. Salditt-Georgieff, and J. E. Darnell. 1975. Methylated, blocked 5'-termini in HeLa cell mRNA. Proc. Natl. Acad. Sci. U.S.A. **72**:1904-1908.
17. Lavi, S., and A. J. Shatkin. 1975. Methylated simian virus 40-specific RNA from nuclei and cytoplasm of infected BSC-1 cells. Proc. Natl. Acad. Sci. U.S.A. **72**: 2012-2016.
18. Lindberg, U., and T. Persson. 1972. Isolation of mRNA from KB-cells by affinity chromatography on polyuridylic acid covalently linked to Sepharose. Eur. J. Biochem. **31**:246-254.
19. Lindberg, U., T. Persson, and L. Philipson. 1972. Isolation and characterization of adenovirus messenger ribonucleic acid in productive infection. J. Virol. **10**:909-919.
20. Molloy, G. R., W. Jelinek, M. Salditt, and J. E. Darnell. 1974. Arrangement of specific oligonucleotides within poly(A) terminated HnRNA molecules. Cell **1**:43-53.
21. Moyer, S. A., G. Abraham, R. Adler, and A. K. Banerjee. 1975. Methylated and blocked 5'-termini in vesicular stomatitis virus *in vivo* mRNAs. Cell **5**:59-67.
22. Perry, R. P., and D. E. Kelley. 1974. Existence of methylated messenger RNA in mouse L cells. Cell **1**:37-42.
23. Perry, R. P., D. E. Kelley, K. Friderici, and F. Rottman. 1975. The methylated constituents of L cell messenger RNA: evidence for an unusual cluster at the 5'-terminus. Cell **4**:387-394.
24. Perry, R. P., J. Latorre, D. E. Kelley, and J. R. Greenberg. 1972. On the lability of poly(A) sequences during extraction of messenger RNA from polyribosomes. Biochim. Biophys. Acta **262**:220-226.
25. Rhodes, D. P., S. A. Moyer, and A. K. Banerjee. 1974. In vitro synthesis of methylated messenger RNA by the virion-associated RNA polymerase of vesicular stomatitis virus. Cell **3**:327-333.
26. Rose, J., and F. Koczot. 1971. Adenovirus-associated virus multiplication. VI. Base composition of the deoxyribonucleic acid strand species and strand-specific in vivo transcription. J. Virol. **8**:771-777.
27. Shatkin, A. J. 1974. Methylated messenger RNA synthesis *in vitro* by purified reovirus. Proc. Natl. Acad. Sci. U.S.A. **71**:3204-3207.
28. Shibata, H., T. S. Ro-Choi, R. Reddy, Y. C. Choi, D. Henning, and H. Busch. 1975. The primary nucleotide sequence of nuclear U-2 ribonucleic acid. The 5'-terminal portion of the molecule. J. Biol. Chem. **250**:3909-3920.
29. Urishibara, T., Y. Furuichi, C. Nishimura, and K. Miura. 1975. A modified structure at the 5'-terminus of mRNA of vaccinia virus. FEBS Lett. **49**:385-389.
30. Wei, C. M., and B. Moss. 1974. Methylation of newly synthesized viral messenger RNA by an enzyme in vaccinia virus. Proc. Natl. Acad. Sci. U.S.A. **71**:3014-3018.
31. Wei, C. M., and B. Moss. 1975. Methylated nucleotides block 5'-terminus of vaccinia virus messenger RNA. Proc. Natl. Acad. Sci. U.S.A. **72**:318-322.
32. Wei, C. M., A. Gershowitz, and B. Moss. 1975. Methylated nucleotides block 5'-terminus of HeLa cell messenger RNA. Cell **4**:379-386.
33. Wei, C. M., A. Gershowitz, and B. Moss. 1975. $N^6,O^{2'}$-dimethyladenosine, a novel methylated ribonucleoside next to the 5'-terminal of animal cell and virus mRNAs. Nature (London) **257**:251-253.