

Multiple Methylated Cap Sequences in Adenovirus Type 2 Early mRNA

SHU-ICHI HASHIMOTO AND MAURICE GREEN*

Institute for Molecular Virology, Saint Louis University School of Medicine, St. Louis, Missouri 63110

Received for publication 26 April 1976

The methylated constituents of early adenovirus 2 mRNA were studied. RNA was isolated from polyribosomes of cells double labeled with [*methyl-³H*]methionine and ³²PO₄ from 2 to 7 h postinfection in the presence of cycloheximide. Cycloheximide ensures that methylation and processing are performed by preexisting host cell enzymes. RNA was fractionated into polyadenylic [poly(A)]⁺ and poly(A)⁻ molecules using poly(U)-Sepharose, and undegraded virus-specific RNA was isolated by hybridization to viral DNA in 50% formamide at 37°C. Viral mRNA was digested with RNase T2 and chromatographed on DEAE-Sephadex in 7 M urea. Two ³H-labeled RNase T2-resistant oligonucleotide fractions with charges between -5 and -6 were obtained, consistent with two classes of 5' terminal methyl "cap" structures, m⁷G(5')ppp(5')N^mpNp (cap 1) and m⁷G(5')ppp(5')N^mN^mpNp (cap 2) (N^m is a ribose 2'-O-methylation). The putative cap 1 contains m⁷G, A^m, G^m, U^m, and m⁶A^m. Cap 2 contains all the methylated constituents of cap 1 plus C^m. The molar ratios of m⁷G to 2'-O-methylnucleosides is about 1.0 for cap 1 and 0.5 for cap 2, consistent with the proposed cap structures. Most significant, compositional analysis indicates four different cap 1 structures and at least three different cap 2 structures. Thus there is a minimum of seven early viral mRNA species with different cap structures, unless each type of mRNA can have more than one 5' terminus. In addition to methylated caps, early mRNA contains internal base methylations, exclusively as m⁶A, as shown by analyses of the mononucleotide (-2 charge) fraction. m⁶A was present in the ratio of 1 mol of m⁶Ap per 450 nucleotides. Thus viral mRNA molecules contain two to three internal m⁶A residues per methyl cap, since there is on the average 1 cap per 1,250 nucleotides.

The transcription of DNA tumor virus genes in infected and transformed cells provides a model for analyzing mRNA biogenesis and posttranscriptional regulation in eukaryotic cells. The human adenoviruses, in particular the well studied adenovirus 2 (Ad2), are especially useful for such studies. Ad2 productively infects cultured human cells (KB or HeLa) and expresses its genome in two stages, "early" (before viral DNA synthesis at 6 to 7 h postinfection) and "late" (14, 32). Early Ad2 mRNA is transcribed in the cell nucleus and processed in the absence of protein synthesis, so host cell enzymes must perform these functions (although the possibility of a virion methylase or RNA polymerase has not been excluded). Therefore, by studying early Ad2 mRNA synthesis we can learn about the cellular mechanisms that regulate both viral and cell gene expression.

In this connection, the possible role of methylation is most intriguing. It has recently been demonstrated that mRNA from animal cells

(26) and viruses that replicate in eukaryotic cells (10) is methylated. With RNA viruses that multiply in the cytoplasm, methylation is exclusively at the 5' terminus in two classes of "capped" oligonucleotides, m⁷G(5')ppp(5')N^m and m⁷G(5')ppp(5')N^mpN^m (N^m is a ribose 2'-O-methylation), respectively, termed cap 1 and cap 2. The base penultimate to m⁷G is A^m (or m⁶A^m) in vesicular stomatitis virus mRNA (in vitro and in vivo) (1, 22, 29), and cytoplasmic polyhedrosis virus mRNA (in vitro) (13), whereas it is G^m in reovirus mRNA (in vitro) (11). The mRNA generated in vitro by cores of vaccinia virus, a cytoplasmic virus with a double-stranded DNA genome, contains two caps with penultimate A^m and G^m (34). All of these in vitro synthesized viral mRNA's but vesicular stomatitis virus contain only cap 1 structures.

The methylation of mRNA synthesized during late stages of infection by two DNA tumor viruses that replicate in the cell nucleus, simian virus 40 (SV40) (3, 18) and Ad2 (9, 21, 36), have been studied. Presumptive evidence for

SV40 mRNA cap 1 structures with penultimate A^m and G^m was reported (18). Late Ad2 mRNA has both cap 1 and cap 2 structures (21, 36). The cap 1 fraction was found to consist of 80% m⁷Gpppm⁶A^m and 20% m⁷GpppA^m (21); the cap 2 fraction was not fully characterized and appears to be more complex. Unlike cytoplasmic viruses, Ad2 (21) and SV40 (18) mRNA, as well as cell RNA (33), contain internal methylations, exclusively m⁶A. In contrast to the restricted composition of viral caps, cell mRNA contains a wide variety of methyl caps, of both type 1 and type 2: 27 different caps were isolated from mRNA of mouse myeloma cells (6), and multiple caps were found in mRNA of HeLa cells (12, 33) and mouse L cells (27).

Several functions of the 5' terminal methylation of cell and viral mRNA have been suggested, including: (i) ribosome binding for the translation of mRNA (4, 23), (ii) recognition signals for processing of nuclear precursors to mRNA (30), and (iii) protection against exonucleases. Conceivably, different cap sequences affect translation control, or reflect different recognition signals for putative cleavage enzymes or initiating sequences for RNA polymerase. Unfortunately, the large number of mRNA species in eukaryotic cells limits experimental approaches to functional analysis of cap structures.

The study of Ad2 early mRNA methylation offers an opportunity to investigate the possible functions of different caps. Unlike late viral mRNA methylation which may involve the functioning of viral gene products, early mRNA methylation probably involves only host cell enzymes. The genes encoding early mRNA have been mapped (28, 31), individual mRNA sequences from each gene have been identified (5, 7, 8; W. Büttner, Z. Veres-Molnar, and M. Green, *J. Mol. Biol.*, in press), and procedures have been developed to preparatively isolate discrete viral mRNA species (Büttner et al., *J. Mol. Biol.*, in press; 5a). Early Ad2 mRNA has been translated *in vitro* into at least six polypeptides whose genes have been located (19). From six (Chin and Maizel, in press; 14a) to as many as 11 (W. S. M. Wold, M. Green, and W. Büttner, in press) early proteins (not necessarily viral coded) have been detected *in vivo*. Thus it is possible to correlate the cap structure of each early mRNA with their position on the viral genome and the synthesis of specific viral proteins. Such information can help elucidate possible functional roles for individual caps. We have analyzed the methylated components of early Ad2 mRNA, and find that it is methylated internally as

m⁶A, as are other mRNA molecules transcribed in the nucleus. But unlike previously reported viral mRNA species, Ad2 early mRNA has a variety of different methyl caps, both type 1 and type 2. Compositional analysis indicates four type 1 caps and at least three type 2 caps. Both types of caps contain m⁶A^m. These data are consistent with the possibility that each Ad2 mRNA species has a different cap structure.

MATERIALS AND METHODS

Cell culture and virus infection. Exponentially growing KB cells in suspension were cultured in Eagle minimum essential medium (MEM) containing 5% horse serum. Cells were concentrated to 3×10^6 to 5×10^6 cells/ml by brief centrifugation and infected in MEM with 100 PFU/cell of Ad2 (plaque 4, strain 38-2, free of adenovirus-associated viruses). After 1 h of adsorption, cells were diluted with 10 volumes of MEM (with horse serum) containing 25 μ g of cycloheximide per ml. At 2 h postinfection cells were concentrated 10-fold, resuspended in phosphate-free MEM containing one-tenth the concentration of methionine and 25 μ g of cycloheximide per ml, and labeled from 2 to 7 h postinfection with 1 mCi of ³²P₄ per ml and 200 μ Ci of [*methyl*-³H]methionine per ml (13 Ci/mmol, Schwarz/Mann).

Cell fractionation and isolation of Ad2-specific RNA. RNA was isolated from the polyribosomes of infected cells as previously described (5). Polyribosomal RNA was fractionated on polyuridylic acid [poly(U)]-Sepharose columns into polyadenylic acid [poly(A)]⁺ and poly(A)⁻ RNA fractions (20) and annealed in 50% formamide at 37°C with Ad2 DNA immobilized on membrane filters (5). In some experiments, hybridization-purified RNA was further purified by rehybridization to Ad2 DNA. Poly(A)⁺ [³H]RNA used in control experiments was isolated from polyribosomes of uninfected KB cells labeled for 15 h in the presence of 20 μ Ci of [³H]uridine per ml (39 Ci/mmol, New England Nuclear). In additional control experiments, [*methyl*-³H]-labeled poly(A)⁺ RNA was isolated from polyribosomes of uninfected KB cells labeled for 12 h in the presence of 50 μ Ci of [*methyl*-³H]methionine (13 Ci/mmol, New England Nuclear). Ribosomal RNA used as markers for gel electrophoresis was isolated as described by Hashimoto and Muramatsu (15).

Polyacrylamide gel electrophoresis of viral mRNA. Hybridization-purified viral mRNA was analyzed by electrophoresis on gels (0.6 by 20 cm) containing 2.5% polyacrylamide, 0.5% agarose, and 0.2% sodium-*N*-lauroylsarcosinate (ICN Pharmaceuticals) (5). Electrophoresis was performed for 6 h with constant voltage (150 V) at 5°C with recirculation of buffer. Ribosomal [³H]RNA markers were coelectrophoresed with Ad2 mRNA. Gels were fractionated into 2-mm portions with a Gilson gel fractionator (Gilson Medical Electronic Co.) and counted in Aquasol (New England Nuclear) using a scintillation counter.

RNase T2 digestion and DEAE-Sephadex column chromatography. ^{32}P - and [*methyl*- ^3H]-labeled virus-specific RNA together with 0.5 to 1 mg of carrier yeast tRNA were digested with 2 units of RNase T2 (Sankyo Co., Ltd) in 0.5 ml of 0.05 M sodium acetate (pH 4.5) at 37°C for 15 h (15). One unit of RNase T2 was added, and incubation was continued for an additional 5 h. The digest was treated with 0.1 N HCl at 0°C for 5 h to cleave cyclic phosphodiester linkages. After neutralization, the solution was diluted with 5 ml of 7 M urea-0.03 M Tris-hydrochloride (pH 7.6) and loaded onto a DEAE-Sephadex A-25 column (0.4 by 30 cm). Five optical density units at 260 nm (OD_{260}) of a pancreatic RNase digest of yeast tRNA was added in the application buffer. Elution was performed with a linear gradient of 0 to 0.4 M NaCl (70 ml of each) in application buffer. In several experiments, elution with 200 ml of eluant (100 ml each) was utilized (16). The OD_{260} of each fraction (1.4 ml) was measured, and 100 to 200 μl was analyzed for ^{32}P and ^3H counts per minute.

Compositional analysis of methylated cap structures isolated from Ad2 mRNA. Cap 1 (-5 charge) and cap 2 (-6 charge) fractions resolved by DEAE-Sephadex chromatography were pooled, desalted by elution from DEAE-Sephadex (0.3 by 2.5 cm) with 2 M triethylamine bicarbonate, dried, and dissolved in 20 μl of 50 mM Tris-hydrochloride (pH 8.5) -5 mM MgCl_2 containing nucleoside markers (A^m , U^m , C^m , and m^7G , approximately 1 OD_{260} unit of each). The mixture was incubated at 37°C for 4 h with 5 μg each of venom phosphodiesterase and *Escherichia coli* alkaline phosphatase (Worthington Corp.). The mixture was reincubated at 37°C for 1 h after the addition of an additional 5 μg of each enzyme. Nucleosides were separated by ascending paper chromatography on Whatman no. 3MM paper strips (1.5 by 46 cm) using 1-butanol-0.8 M boric acid-concentrated ammonia (2,000:270:8, vol/vol/vol) (2). The chromatogram was developed at room temperature until the solvent had ascended 30 to 35 cm. The paper was dried, cut into 0.5-cm strips, and eluted with 0.5 ml of 0.01 N HCl at 37°C for 15 h. The four marker peaks which were detected and identified by absorbance at 260 nm and UV spectrum at pH 2 migrated in the order A^m , U^m , C^m , m^7G . The absorbance at 260 nm showed that elution of nucleosides was quantitative. G^m was identified from parallel chromatograms of phosphodiesterase, alkaline phosphatase digests of *methyl*- ^3H -labeled RNase T2 stable dinucleotides, isolated from KB cells ribosomal RNA (16). Samples of each eluant (100 to 200 μl) were dissolved in 5 ml of Biofluor (New England Nuclear) and counted in a scintillation spectrophotometer. In some experiments (after samples for counting were taken), fractions of m^7G and m^6A^m peaks were pooled and analyzed on Dowex-50 columns as described below.

Dowex-1 and Dowex-50 chromatography. Analysis of mononucleotide and cap fractions and identification of m^7G , m^6A , and m^6A^m . The mononucleotide fractions (-2 charge) obtained by DEAE-Sephadex column chromatography were pooled, diluted fivefold with water, and loaded on a Dowex-1 column (0.3 by 20 cm). The column was equilibrated with

0.004 N HCl and developed with a concave gradient formed with a 40-ml mixing chamber (0.004 N HCl) and a 20-ml reservoir (0.1 M NaCl and 0.01 N HCl) (15). Constituents eluted in the order Cp, Ap, m^6Ap , inorganic phosphate, Up, and Gp. One-milliliter fractions were collected and suitable samples were counted. m^6Ap -containing fractions were pooled, rechromatographed on Dowex-1, depurinated, and chromatographed on Dowex-50 as described below.

Further identification of m^7G and m^6A^m from caps and m^6Ap from the -2 fraction was performed by Dowex-50 column chromatography using a micro modification of the procedure of Iwanami and Brown (17). The nucleoside or nucleotide fraction was depurinated by treatment with 1 N HCl for 60 min at 100°C. The sample was diluted to 0.02 N HCl, applied onto a Dowex-50 column (0.2 by 20 cm), washed with 0.02 N HCl, and eluted with 0.5 N HCl. After 20 fractions (0.6 ml each) were collected, elution was performed by 0.7 N HCl. The elution sequence of purines was Gua, m^7Gua , m^1Ade , Ade, m^6Ade .

RESULTS

Isolation from polyribosomes of poly(A) terminated early Ad2 mRNA double labeled with [*methyl*- ^3H]methionine and $^{32}\text{PO}_4$. To obtain early Ad2 mRNA for analysis of methylated constituents, cells were labeled with high concentrations of $^{32}\text{PO}_4$ (1 mCi/ml) and [*methyl*- ^3H]methionine (200 $\mu\text{Ci}/\text{ml}$) from 2 to 7 h post-infection in the presence of cycloheximide. Cycloheximide was used because it increases the yield of early viral mRNA (5, 25); it also blocks late viral mRNA synthesis, and prevents the synthesis of early viral-coded proteins, thus assuring that host mechanisms were utilized to process (methylate) early viral RNA transcripts (unless there is a viral methylase). From 2 to 3 mg of polyribosomal RNA with ^{32}P specific activity of about 2×10^5 cpm/ μg was isolated from 1 liter of infected KB cells (3×10^5 to 5×10^5 cells/ml). Polyribosomal RNA was fractionated into poly(A)⁺ and poly(A)⁻ material using poly(U)-Sephacrose; analysis of five preparations showed that about 40% of [^{32}P]RNA was poly(A)⁺ and 60% was poly(A)⁻. Viral mRNA was selected by hybridization to Ad2 DNA in 50% formamide at 37°C. In a representative analysis (Table 1), 15.6% of poly(A)⁺ RNA and 2.0% of poly(A)⁻ RNA formed hybrids. In control experiments, generally less than 0.1% of poly(A)⁺ RNA isolated from uninfected cells hybridized to Ad2 DNA (e.g., Table 1). This indicates that contamination of early poly(A)⁺ viral mRNA with cell mRNA after hybridization should be less than 1%.

The integrity of hybridization-purified viral RNA was routinely checked by polyacrylamide gel electrophoresis. The typical gel pattern shown in Fig. 1A is similar to that reported pre-

TABLE 1. Selection of poly(A)⁺ and poly(A)⁻ Ad2 mRNA by hybridization to viral DNA

Labeled RNA	Input (cpm)	RNA hybridized	
		cpm	%
poly(A) ⁺ (³² P, [methyl- ³ H]) ^a Ad2-infected KB cells	9,644,000 (³² P)	1,515,000 (³² P)	15.6
poly(A) ⁻ (³² P, [methyl- ³ H]) ^a Ad2-infected KB cells	73,670,000 (³² P)	1,529,000 (³² P)	2.0
poly(A) ⁺ (³ H)uridine) ^b uninfected KB cells	5,516,000 (³ H)	3,000 (³ H)	0.05
poly(A) ⁺ ([methyl- ³ H]) ^c uninfected KB cells	43,000 (³ H)	50 (³ H)	0.11

^a Polyribosomal RNA (2 to 3 mg) isolated from KB cells labeled with [methyl-³H]methionine and ³²PO₄ from 2 to 7 h postinfection with Ad2 was fractionated into poly(A)⁺ and poly(A)⁻ RNA on a poly(U)-Sephacose column. One-tenth of poly(A)⁺ RNA and one-half of poly(A)⁻ RNA were annealed to Ad2 DNA (50 μg) immobilized on nitrocellulose filters in 1 ml of hybridization buffer at 37°C for 15 h.

^b Polyribosomal RNA (2 to 3 mg) was isolated from uninfected KB cells labeled with [³H]uridine and fractionated into poly(A)⁺ and poly(A)⁻ RNA. One tenth of the poly(A)⁺ RNA was annealed with 50 μg of Ad2 DNA as described above.

^c Polyribosomal RNA (2 to 3 mg) was isolated from uninfected KB cells labeled with [methyl-³H]methionine and fractionated into poly(A)⁺ and poly(A)⁻ RNA. One-tenth of the poly(A)⁺ RNA was annealed with 50 μg of Ad2 DNA as described above.

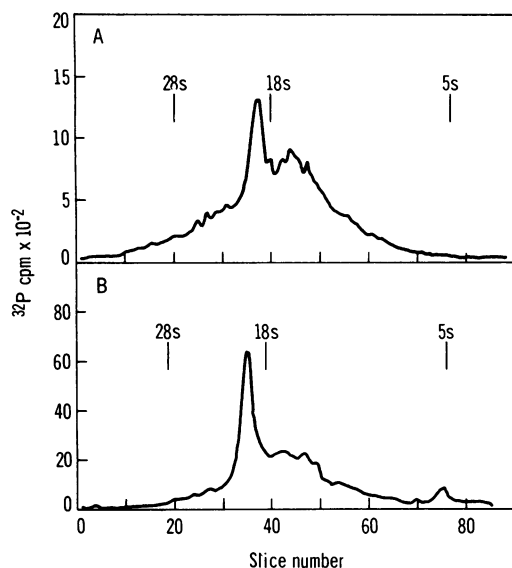


FIG. 1. Polyacrylamide gel electrophoresis of poly(A)⁺(A) and poly(A)⁻(B) Ad2-specific early mRNA. Early polyribosomal RNA labeled with ³²PO₄ and [methyl-³H]methionine was fractionated into poly(A)⁺ and poly(A)⁻ RNA by chromatography on poly(U)-Sephacose, and each fraction was annealed to membrane filters containing Ad2 DNA. Viral RNA was eluted from the hybrid and electrophoresed on polyacrylamide agarose gels together with KB cell ribosomal [³H]RNA marker.

viously (8, 20, 25). Several small peaks were reproducibly found between the major 20–21S peak and 13–14S RNA, and a broad shoulder was found between 21S and 28S. This complex pattern includes at least five to seven viral mRNA molecules. Craig et al. (7, 8) have detected at least seven early mRNA species by an analytical mapping procedure (20S, 19S, three

13S, and two 11S); Büttner et al. (J. Mol. Biol., in press) have preparatively isolated and mapped five major early mRNA species (24S, 20.5S, two 20S, and one or two 14S).

Only about 20% of total viral RNA was poly(A)⁻ (Table 1). Rechromatography on poly(U)-Sephacose showed that about 40% of hybridization-purified poly(A)⁻ viral RNA eluted in the poly(A)⁺ fraction; therefore, 10 to 20% of poly(A)⁺ viral RNA was not retained on the first poly(U)-Sephacose column. The gel pattern of poly(A)⁻ early viral mRNA included the major 20–21S peak (Fig. 1B) and decreased levels of smaller viral mRNA species. The methylated components of poly(A)⁻ mRNA were analyzed in parallel with poly(A)⁺ viral mRNA, as described below.

Resolution of methylated components released from Ad2 mRNA by RNase T2. To characterize the methylated nucleotides of early Ad2 mRNA, RNase T2 digests were prepared and fractionated on DEAE-Sephadex. RNase T2 hydrolyzes phosphodiester bonds through a 2',3'-cyclic intermediate so that phosphodiester bonds in caps (pyrophosphate) and those linking the 3' hydroxyl of internal 2'-O-methylated nucleotides are stable. Chromatography on DEAE-Sephadex in 7 M urea at neutral pH resolves oligonucleotides on the basis of charge. Figure 2 shows the DEAE-Sephadex chromatograms of T2 digests prepared from ³²PO₄ plus [methyl-³H]methionine-labeled poly(A)⁻-selected Ad2 mRNA, once (Fig. 2A) and twice (Fig. 2B) hybridization purified. Over 98% of the ³²P radioactivity applied to the column was recovered, and no further radioactivity was eluted with 1 M NaCl after completion of the gradient. The mononucleotide peak (-2 charge) contained 99.6% of the total ³²P-labeled

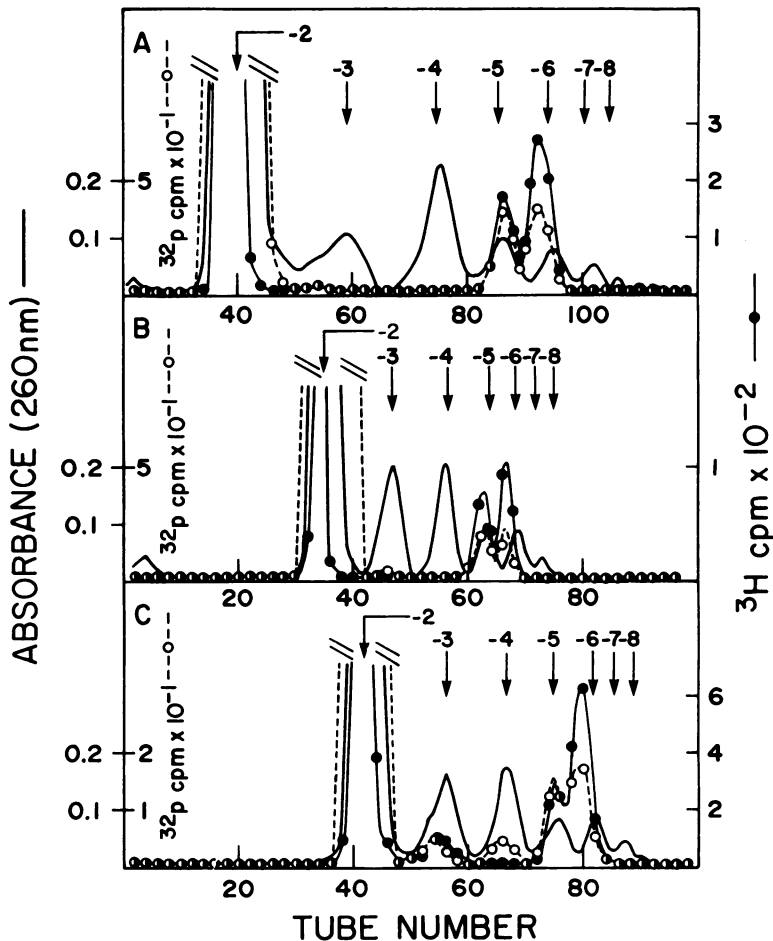


FIG. 2. Chromatography of RNase T2 digest of ^{32}P -, [methyl- ^3H]labeled Ad2 early mRNA on a DEAE-Sephadex column in 7 M urea at pH 7.6. Each enzyme digest was co-chromatographed with 5 OD_{260} units of pancreatic RNase digest of yeast tRNA to provide oligonucleotide markers as described in Materials and Methods. ^3H cpm due to ^{32}P spillover counts (about 2%) in mononucleotides fraction were not subtracted. (A) Poly(A) $^+$ RNA (once hybridized); (B) poly(A) $^+$ RNA (twice hybridized); (C) poly(A) $^-$ RNA (once hybridized).

material eluted from digests of Ad2 poly(A) $^+$ mRNA, the dinucleotide (-3 charge), and trinucleotide (-4 charge) peaks contained no counts, and oligonucleotide peaks with approximate charges -5 and -6, respectively, contained about 0.20% and 0.25% of the ^{32}P radioactivity (Fig. 2A and 2B). From further analysis of the -5 and -6 charged fractions (see below), and from the positions of elution from the column, we tentatively conclude that the -5 and -6 fractions, respectively, represent 5' terminal structures of the general type $\text{m}^7\text{GpppN}^{\text{m}}\text{pNp}$ (cap 1) and $\text{m}^7\text{GpppN}^{\text{m}}\text{pN}^{\text{m}}\text{pNp}$ (cap 2), derived from Ad2 early mRNA. We calculate 1 cap per 1,250 nucleotides, based on the ratio of the radioactivity in ^{32}P -labeled caps

(-5 plus -6 fractions) to that of total Ad2 [^{32}P]mRNA.

Analysis of Ad2 poly(A) $^-$ early mRNA (Fig. 2C) showed a slightly higher content of fractions with approximate charges of -5 (0.25%) and -6 (0.38%), probably because the poly(A) $^-$ fraction contained some viral mRNA lacking 3' sequences, perhaps due to degradation. About 0.1% of the ^{32}P radioactivity was present in the dinucleotide fraction and coeluted with a peak of ^3H radioactivity. This peak was probably derived from contaminating rRNA; e.g., 3% contamination with rRNA would produce 0.1% RNase-resistant dinucleotides (16).

Methylated constituents of cap 1 and cap 2 fractions in Ad2 mRNA. The ^3H -labeled nu-

cleoside components of cap 1 and cap 2 isolated from hybridization-purified Ad2 mRNA were analyzed. Each cap fraction was digested with bacterial alkaline phosphatase and venom phosphodiesterase, and the resulting ^3H -methylated nucleosides were resolved by paper chromatography. Figures 3A-D show the chromatograms of poly(A)⁺ cap 1, poly(A)⁺ cap 2, poly(A)⁻ cap 1, and poly(A)⁻ cap 2, respectively. Peaks 1, 4, 5, 6, and 7 are m⁷G, G^m, C^m, U^m, and A^m, respectively, as shown by co-chromatography with marker nucleosides. Peak 1 was eluted from paper chromatograms and also identified as m⁷G by depurination and chromatography on Dowex-50. The elution position was identical to m⁷Gua prepared by depurination of authentic m⁷G (Fig. 4A). Usually some m⁷Gua in isolated caps was partially converted to the ring-opened form (m⁷Gua*, 2-amino-4-hydroxyl-5-(*N*-methyl)formamide-6-ribosylamino-pyrimidine). This occurs during isolation and analysis of caps under slightly alkaline pH conditions (pH 8 to 9). m⁷Gua* was identified from the elution profile of m⁷Gua* produced by alkaline treatment (1 N NH₄OH, 37°C, 6 h) and depurination of pm⁷G. m⁷Gua* was eluted from Dowex-50 in fraction 2 to 4 after application of 0.5 N HCl. m⁷Gua* was obtained by depurination of alkaline treated mRNA, whereas only m⁷Gua was obtained from untreated mRNA. Peak 8 was expected to be a 2'-*O*-methylated adenosine derivative, because only 2'-*O*-methylated nucleosides (or 2'-deoxynucleosides) would move with this solvent system and *N*⁶-methyl-2-deoxyadenosine moves the furthest among 2'-*O*-blocked nucleosides (2). To characterize peak 8, the chromatogram spot was eluted, depurinated, and chromatographed on Dowex-50 (Fig. 4b). Equimolar amounts of ^3H -labeled m⁶Ade and ^3H -labeled noncharged material, 2'-*O*-methylribose were found, as shown by co-chromatography with authentic m⁶Ade. Therefore, peak 8 is *N*⁶, 2'-*O*-dimethyladenosine. Peaks 2 and 3 were present in small and variable amounts and were absent from some preparations; chromatography on Dowex-50 indicated that peaks 2 and 3 are m⁶Ade and 2'-*O*-methylribose, presumably degradation products of *N*⁶, 2'-*O*-dimethyladenosine.

Table 2 gives the radioactivity and molar percent of each methylated nucleoside in cap 1 and cap 2 fractions from two different preparations of poly(A)⁺ and one preparation of poly(A)⁻ Ad2 mRNA. The molar ratio of m⁷G to 2'-*O*-methylnucleosides was approximately 1.0 for the cap 1 fractions and 0.5 for the cap 2 fractions. Analyses of three additional preparations of early Ad2 poly(A)⁺ mRNA (not shown)

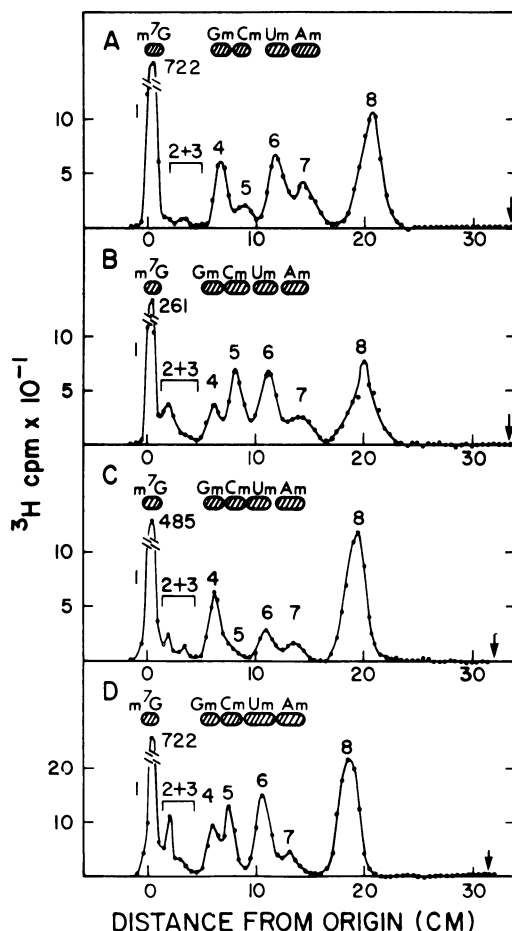


FIG. 3. Paper chromatography of methyl- ^3H -labeled cap 1 and cap 2 fractions of early Ad2 mRNA after digestion with venom phosphodiesterase and alkaline phosphatase. Enzyme digestion and paper chromatography were performed as described in Materials and Methods. The shadowed circles indicate nucleoside markers, the numbers correspond to those in Table 2, and the arrows indicate the solvent front. The paper was cut into 0.5-cm pieces, and nucleosides were eluted in 0.01 N HCl and counted in a scintillator. (A) Cap 1 of poly(A)⁺ Ad2 mRNA; (B) cap 2 of poly(A)⁺ Ad2 mRNA; (C) cap 1 of poly(A)⁻ Ad2 mRNA; (D) cap 2 of poly(A)⁻ Ad2 mRNA.

gave similar ratios with some variation in the molar percent of individual ^3H -methylated nucleosides but no differences in the specific methylated components detected. This variation is expected if the relative amounts of different viral mRNA molecules fluctuated in different preparations and if each mRNA has a different cap. Although trace amounts of C^m (less than 3% of total ^3H cpm) were found in some cap 1

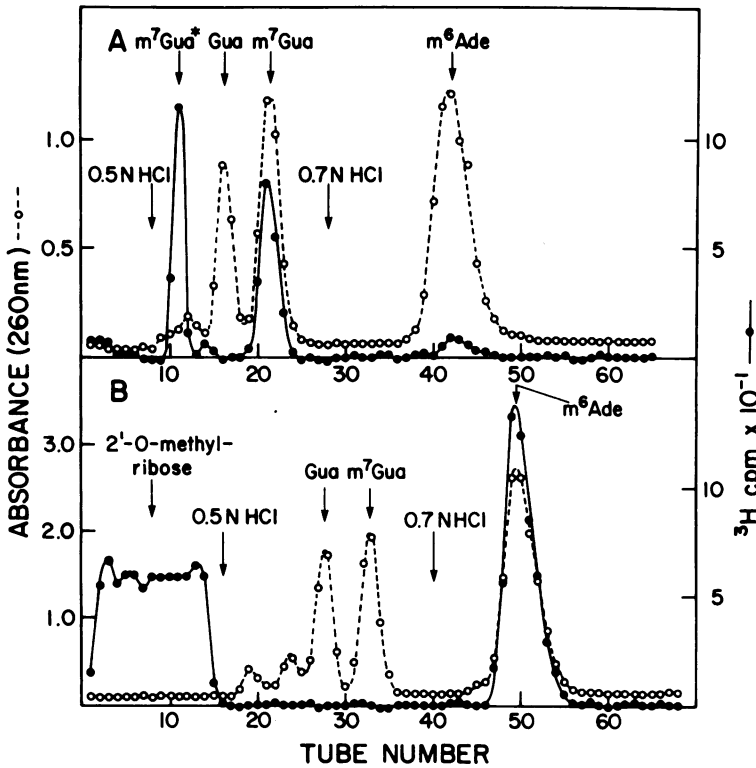


FIG. 4. Identification of m^7G and m^6A by Dowex-50 column chromatography. Peaks 1 and 8 in Fig. 3 were eluted with 0.01 N HCl, dried, and dissolved in 0.1 (A) or (B) 0.2 ml of 1 N HCl containing 1 to 3 OD_{260} units each of pG, pm^7G , and m^6A markers. The sample was depurinated, diluted to 0.02 N HCl, and loaded on a Dowex-50 column. The column was washed with 1 ml of 0.02 N HCl and eluted with 0.5 N HCl and 0.7 N HCl. Fractions (0.6 ml) were collected, OD_{260} was measured, and each fraction was dissolved in 10 ml of Biofluor and counted. The position of each base was established by its UV spectrum. (A) Analysis of m^7G ; (B) analysis of m^6A .

preparations (Fig. 2), the amount was variable, and C^m was absent from several preparations. C^m in cap 1 may represent minor contamination with cell mRNA or more likely cross-contamination on DEAE-Sephadex with cap 2. On the basis of these nucleoside analyses, four cap 1 and at least three cap 2 sequences exist: $m^7G(5')ppp(5')N^mp$ with N^m as A^m , U^m , G^m , or m^6A^m ; and $m^7G(5')ppp(5')N^mpN^mp$ with N^m as pairs of A^m , U^m , C^m , G^m , or m^6A^m .

To rule out possible contamination with cell mRNA, poly(A)⁺ and poly(A)⁻ viral mRNA selected by hybridization to Ad2 DNA was purified by a second hybridization to viral DNA. RNase T2-stable cap 1 and cap 2 fractions were isolated and analyzed. No qualitative differences were found in the 3H -methylated nucleoside components of once and twice hybridized viral mRNA.

Analyses of the mononucleotide fraction. It is not possible to determine directly whether 3H -labeled nucleotides are present in the mon-

onucleotide fraction (-2 charge) eluted from DEAE-Sephadex (Fig. 2) due to the large ^{32}P counts which can mask small amounts of 3H counts. To detect internal base methylations in Ad2 early mRNA, the mononucleotide fraction from DEAE-Sephadex (Fig. 2) was chromatographed on a Dowex-1 column (15).

The four ^{32}P -labeled ribonucleotides eluted in the order Cp, Ap, Up, and Gp and possessed no detectable 3H cpm; thus, the incorporation of methyl- 3H into purine rings is negligible (Fig. 5A). A distinct 3H peak was identified that eluted after Ap and before inorganic phosphate. The peak was further purified by re-chromatography on Dowex-1 (Fig. 5B), depurinated, and chromatographed on Dowex-50 (Fig. 5C). The resulting base was identified as m^6Ade by comparison with standards including m^1Ade and m^6Ade . The molar ratio of m^6Ap to total nucleotides in viral mRNA was 1 in 450, in both poly(A)⁺ and poly(A)⁻ RNA, as measured from the ^{32}P content of m^6Ap elution from Dowex-1.

TABLE 2. Composition of methylated oligonucleotides in Ad 2 early mRNA Cap 1 and Cap 2

Cap	Peak ^a no.	Nucleoside	poly(A) ⁺				poly(A) ⁻	
			Expt 1		Expt 2			
			cpm	Molar % ^b	cpm	Molar %	cpm	Molar %
1	1	m ⁷ G	917	50.5	239	49.7	631	49.6
	2 + 3	(m ⁶ Ade + 2'-O-methylribose) ^c	0	0	25	5.2	66	5.2
	4	G ^m	180	9.9	50	10.4	163	12.8
	5	C ^m	63	3.5	0	0	0	0
	6	U ^m	254	14.0	58	12.1	94	7.4
	7	A ^m	153	8.4	43	8.9	62	4.9
	8	m ⁶ A ^m	497	13.7	132	13.7	514	20.2
	2	1	m ⁷ G	396	27.2	404	29.6	916
2 + 3		(m ⁶ Ade + 2'-O-methylribose) ^c	111	7.6	172	12.6	229	8.0
4		G ^m	107	7.4	100	7.3	276	9.6
5		C ^m	222	15.3	121	8.9	302	10.6
6		U ^m	283	19.5	315	23.1	500	17.5
7		A ^m	142	9.8	89	6.5	153	5.4
8		m ⁶ A ^m	388	13.3	323	11.8	971	17.0

^a Peak numbers correspond to those in Fig. 3.

^b Moles of each methylated nucleoside/100 mol of methylated nucleosides present in the cap. Two moles of methyl residue of peak 8 were calculated as 1 mol of m⁶A^m. Putative degradation products (peak 2 and 3) were not added to m⁶A^m values.

^c Tentative analysis (see text). Molar percent of these peaks was calculated with one mole of methyl residue corresponding to one mole of the constituent.

DISCUSSION

Poly(A)-terminated polyribosomal early mRNA was double labeled with [*methyl*-³H]-methionine and ³²PO₄ in the presence of cycloheximide (to enhance the yield of early mRNA) and Ad2-specific mRNA purified by hybridization to and elution from Ad2 DNA immobilized on nitrocellulose filters. The RNA was digested with RNase T2 and chromatographed on DEAE-Sephadex in 7 M urea at pH 7.6. A radioactive fraction of charge -2 (mononucleotide) was observed, and two additional fractions of charges -5 (cap 1) to -6 (cap 2). These fractions were further characterized by paper and column chromatography. m⁶Ap was the only methylated constituent in the mononucleotide fraction. m⁷G and the 2'-O-methyl derivatives of A, G, U, and m⁶A were found in the cap 1 fraction. The cap 2 fraction contained all the constituents of the cap 1 plus 2'-O-methyl C. The molar ratios of m⁷G to the 2'-O-methylated derivatives were 1.0 and 0.5 for the cap 1 and cap 2 fractions, respectively. Since the cap 1 and cap 2 fractions apparently represent 5' termini, these data indicate that there are at least seven different 5' termini, and therefore at least seven early viral genes (unless a specific mRNA molecule can contain more than one type of 5' terminus). Based upon the analyses of 5' termini of vesicular stomatitis virus and reovirus mRNA's, we conclude that the

general structures of these termini are: m⁷G(5')ppp(5')N^mp(cap 1) and m⁷G(5')ppp(5')-N^mpN^mp(cap 2). Four cap 1 structures exist with N^m = G^m, U^m, A^m, and m⁶A^m. Three is a minimum and 25 is a maximum number for cap 2 structures (25 different pairs of five methylated nucleosides can exist). The existence of at least seven methyl caps is interesting in view of the early Ad2 mRNA mapping studies of Craig et al. (7, 8) and Büttner et al. (J. Mol. Biol., in press) which suggest a minimum of five to eight early mRNA species. In addition, we have recently obtained evidence of 11 Ad2-induced early proteins, eight which were not detected in appreciable quantity in uninfected cells (Wold et al., in press). Thus, more than seven early genes may exist.

The significance of multiple cap structure in early Ad2 mRNA is unknown. Compositional analysis indicates that different cap structures are not minor components. Analysis of late Ad2 mRNA by Moss and Koczot (21) gave a much simpler pattern of two cap 1 structures (m⁷Gpppm⁶A^m and m⁷GpppA^m); the cap 2 fraction was not fully characterized. The marked differences between early and late Ad2 mRNA methylation patterns may suggest viral induced modification. Two prominent features of late Ad2 gene expression are the switch from early to late viral mRNA synthesis and the block in host cell protein synthesis. Although most, if not all, late Ad2 RNA sequences are

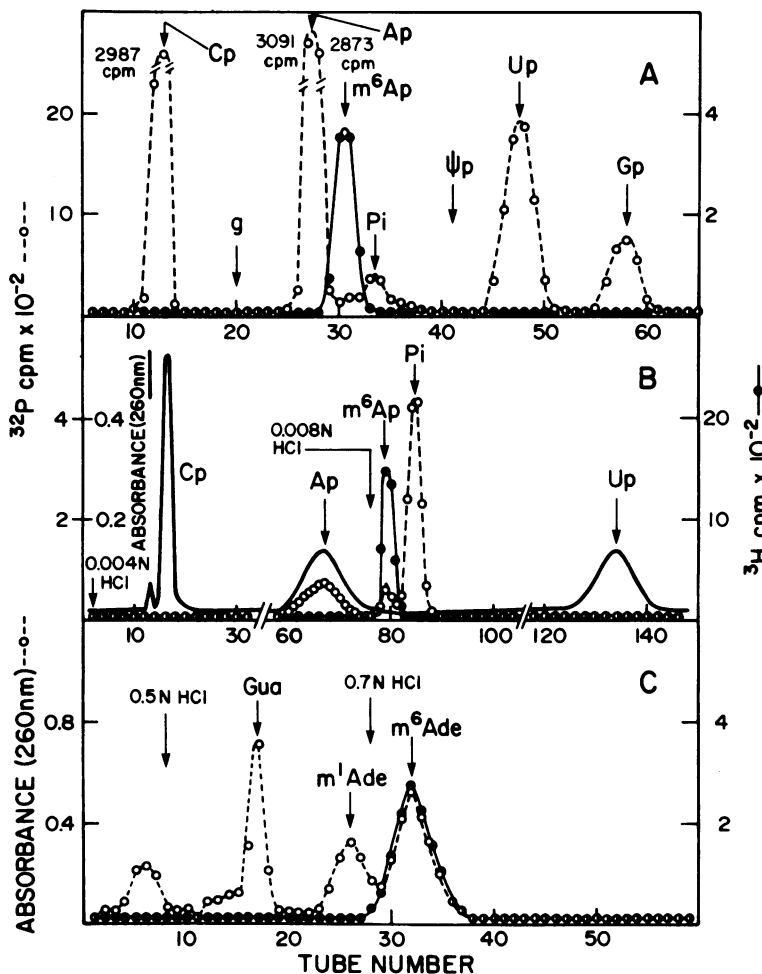


FIG. 5. Analysis of m^6Ap in the mononucleotide fraction. The mononucleotide fraction of Fig. 2A was pooled, diluted fivefold with water, and loaded on a Dowex-1 column (A). The column was equilibrated with 0.004 N HCl and developed starting with fraction 20 with a concave gradient formed with 40 ml of 0.004 N HCl in the mixing chamber and 20 ml of 0.1 M NaCl and 0.01 N HCl in the reservoir. One-milliliter fractions were collected, OD_{260} was measured, and 0.1 ml of each fraction was counted. Each peak was identified by its UV spectrum at pH 2 and 12 (OD_{260} is not shown). Fractions 29 to 33 were pooled, neutralized, diluted fivefold with water containing 1 OD_{260} unit each of Cp, Ap, Up, and Gp as markers, and loaded on a Dowex-1 column (B). The column was equilibrated with 0.004 N HCl and elution was performed with 0.008 N HCl after Ap was eluted. Fractions (1 ml) were collected and 0.3 ml of each was counted. Fractions 77 to 82 were pooled, dried, and dissolved in 0.1 ml of 1 N HCl containing 1 to 2 OD_{260} units of pG, m^1A and m^6A as markers. The sample was depurinated and analyzed on a Dowex-50 column as shown in Fig. 4.

transcribed during early stages of infection, they do not mature to mRNA and are retained (or degraded) in the cell nucleus (31, 35). Late after infection, the same viral RNA sequences are processed to mRNA and transported to polyribosomes. Conceivably this switch is a consequence of an altered methylation pattern. For example, specific methylated bases may be recognition sites for enzymes that transport mRNA out of the nucleus and onto polyribosomes, or for cleavage by putative processing enzymes. Methylation may also "restrict" de-

struction of late viral mRNA precursor molecules by nucleases. In addition, methylation could participate in the block of host cell protein synthesis that occurs late after Ad2 infection. Perhaps cell mRNA is not capped late after infection or caps are removed by specific pyrophosphatases, such as recently described by Nuss et al. (24). Such enzymes may attack cell caps which are similar to some early viral mRNA caps; late mRNA caps may be different. Viral-induced enzymes that affect or modify methylation are an interesting possibility.

The m⁷G portion of the 5' terminal cap has been reported necessary for translation of mRNA (4, 23), but the biological functions of the penultimate 2'-O-methylation and internal m⁶A are not known. Since the methyl caps of each or most early mRNA's are different, perhaps each mRNA is metabolized differently according to its cap structure. A less interesting explanation of multiple caps is that the type of 2'-O-methylated constituents in the cap is irrelevant to the biological function of the cap. That is, the terminal m⁷G is presumably added post-transcriptionally, and the penultimate methylated base may result from nonspecific 2'-O-methylation after the capping reaction. Whether other internal base sequences located close to the 5' terminus serve as signals for capping, cleavage, or methylating enzymes is unknown. Finally, we point out that we have not studied the effect of cell culture conditions on the methylation patterns of early Ad2 mRNA, and it is possible that different results would be obtained under different RNA labeling conditions. However, preliminary analyses of Ad2 mRNA prepared without use of cycloheximide have revealed no substantial differences in methylation patterns from those described here.

ACKNOWLEDGMENTS

We thank W. Büttner for helpful discussion on preparation of Ad2 early mRNA, S. Lowery for technical assistance, and W. S. M. Wold for critical evaluation of the manuscript.

This investigation was supported by Public Health Service grant AI-01725 from the National Institute of Allergy and Infectious Diseases and by contract NO1 CP43359 within the Virus Cancer Program of the National Cancer Program of the National Cancer Institute. S. H. was partly supported by Naito Research grant for travel expenses. M. G. is a Research Career Awardee (5K6-AI-4739) of the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

- 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.
- Ali-Arif, A., and M. B. Sporn. 1972. An analytical method for the separation of sugar-methylated ribonucleosides from base-methylated and nonmethylated ribonucleosides. *Anal. Biochem.* 48:386-393.
- Aloni, Y. 1975. Methylated SV40 mRNAs. *FEBS Lett.* 54:363-367.
- 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.* 71:2951-2955.
- Büttner, W., Z. Veres-Molnar, and M. Green. 1974. Isolation of DNA strand-specific early messenger RNA species in cell infected by human adenovirus 2 infected cells. *Proc. Natl. Acad. Sci. U. S. A.* 71:2951-2955.
- Chinnadurai, G., H. M. Rho, R. B. Horton, and M. Green. 1976. mRNA from the transforming segment of the adenovirus 2 genome in productively infected and transformed cells. *J. Virol.* 20:255-263.
- Cory, S., and J. M. Adams. 1975. The modified 5'-terminal sequences in messenger RNA of mouse myeloma cells. *J. Mol. Biol.* 99:519-547.
- Craig, E. A., M. McGrogan, C. Mulder, and H. J. Raskas. 1975. Identification of early adenovirus type 2 RNA species transcribed from the left-hand end of the genome. *J. Virol.* 16:905-912.
- Craig, E. A., S. Zimmer, and H. J. Raskas. 1975. Analysis of early adenovirus 2 RNA using Eco R·R1 viral DNA fragments. *J. Virol.* 15:1202-1213.
- Fernandez, S. M., and H. J. Raskas. 1975. Methylation of cytoplasmic adenovirus RNA synthesized early and late in productive infection. *Biochem. Biophys. Res. Commun.* 66:67-74.
- Furuichi, Y. 1974. "Methylation-coupled" transcription by virus-associated transcriptase of cytoplasmic polyhedrosis virus containing double-stranded RNA. *Nucleic Acids Res.* 1:809-818.
- Furuichi, Y., M. Morgan, S. Muthukrishnan, and A. J. Shatkin. 1975. Reovirus messenger RNA contains a methylated, blocked 5'-terminal structure: m⁷G(5')ppp(5')G^mpCp-. *Proc. Natl. Acad. Sci. U. S. A.* 72:362-366.
- Furuichi, Y., M. Morgan, A. H. 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.
- Furuichi, Y., and K. I. Miura. 1975. A blocked structure at the 5' terminus of mRNA from cytoplasmic polyhedrosis virus. *Nature (London)* 253:374-375.
- Green, M., J. T. Parsons, M. Pina, K. Fujinaga, H. Caffer, and I. Landgraf-Leurs. 1970. Transcription of adenovirus genes in productively infected and in transformed cells. *Cold Spring Harbor Symp. Quant. Biol.* 35:803-818.
- Harter, M. L., G. Shanmugam, W. S. M. Wold, and M. Green. 1976. Dection of adenovirus type 2-induced early polypeptides using cycloheximide pretreatment to enhance viral protein synthesis. *J. Virol.* 19:232-242.
- Hashimoto, S., and M. Muramatsu. 1973. Differences in nucleotide sequences of ribosomal RNA between the liver and a hepatoma of C3H/He mice. *Eur. J. Biochem.* 33:446-458.
- Hashimoto, S., M. Sakai, and M. Muramatsu. 1975. 2'-O-Methylated oligonucleotides in ribosomal 18S and 28S RNA of a mouse hepatoma, MH134. *Biochemistry* 14:1956-1964.
- Iwanami, Y., and G. M. Brown. 1968. Methylated bases of transfer ribonucleic acid from HeLa and L cells. *Arch. Biochem. Biophys.* 124:472-482.
- Lavi, S., and A. 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.
- Lewis, J. B., J. F. Atkins, P. R. Baum, B. Solem, and R. F. Gesteland. 1976. Location and identification of the genes for adenovirus type 2 early polypeptides. *Cell* 7:141-151.
- Lindberg, U., T. Persson, and L. Philipson. 1972. Isolation and characterization of adenovirus messenger ribonucleic acid in productive infection. *J. Virol.* 10:909-919.
- Moss, B., and F. Kocot. 1976. Sequence of methylated nucleotides at the 5' terminus of adenovirus specific RNA. *J. Virol.* 17:385-392.
- 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.
- Muthukrishnan, S., G. W. Both, Y. Furuichi, and A. J. Shatkin. 1975. 5'-Terminal 7-methylguanosine in eukaryotic mRNA is required for translation. *Nature (London)* 255:22-37.
- Nuss, D. L., Y. Furuichi, G. Koch, and A. J. Shatkin.

1975. Detection of HeLa cell extracts of a 7-methyl guanosine specific enzyme activity that cleaves m^7GpppN^m . *Cell* 6:21-27.
25. **Parsons, J. T., and M. Green.** 1971. Biochemical studies on adenovirus multiplication, XVII. Resolution of early virus specific species in Ad2 infected and transformed cells. *Virology* 45:154-162.
26. **Perry, R. P., and D. A. Kelley.** 1974. Existence of methylated messenger RNA in mouse L cells. *Cell* 1:37-42.
27. **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.
28. **Philipson, L., U. Pettersson, U. Lindberg, C. Tibbetts, B. Vennström, and T. Persson.** 1974. RNA Synthesis and processing in adenovirus infected cells. *Cold Spring Harbor Symp. Quant. Biol.* 39:447-456.
29. **Rose, J. K.** 1975. Heterogeneous 5'-terminal structures occur on vesicular stomatitis virus mRNAs. *J. Biol. Chem.* 250:8098-8104.
30. **Rottman, F., A. Shatkin, and R. P. Perry.** 1974. Sequences containing methylated nucleotides at the 5' termini of messenger RNAs: possible implications for processing. *Cell* 3:197-199.
31. **Sharp, P. A., P. H. Gallimore, and S. J. Flint.** 1974. Mapping of adenovirus 2 RNA sequences in lytically infected cells and transformed cell lines. *Cold Spring Harbor Symp. Quant. Biol.* 39:457-474.
32. **Thomas, D. C., and M. Green.** 1969. Biochemical studies on adenovirus multiplication XV. Transcription of the adenovirus type 2 genome during productive infection. *Virology* 39:205-210.
33. **Wei, C. M., A. Gershowitz, and B. Moss.** 1975. Methylated nucleotides block 5' terminus of HeLa cell messenger RNA. *Cell* 4:379-386.
34. **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.
35. **Wold, W. S. M., M. Green, K. H. Brackman, M. A. Cartas, and C. Devine.** 1976. Genome expression and mRNA maturation at late stages of productive adenovirus type 2 infection. *J. Virol.* 20:465-477.
36. **Wold, W. S. M., M. Green, and T. W. Munns.** 1976. Methylation of late adenovirus 2 nuclear and messenger RNA. *Biochem. Biophys. Res. Commun.* 68:643-649.