JOURNAL OF VIROLOGY, Aug. 1977, p. 234-239
Vol. 23, No. 2

Printed in U.S.A.

5'-Terminal and Internal Methylated Nucleosides in Herpes Simplex Virus Type 1 mRNA

BERNARD MOSS,* ALAN GERSHOWITZ, JAMES R. STRINGER, LOUIS E. HOLLAND, AND EDWARD K. WAGNER

Laboratory of Biology of Viruses, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20014, and Department of Molecular Biology and Biochemistry, University of California, Irvine, California 92717*

Received for publication 15 February 1977

RNA labeled with [*methyl*-^{3}H]methionine and/or [^{32}P]orthophosphate was isolated from the polyribosomes of herpes simplex virus (HSV) type 1-infected cells and separated into polyadenylylated [poly(A+)] and non-polyadenylylated [poly(A−)] fractions. Virus-specific RNA was obtained by hybridization in liquid to either excess HSV DNA or filters containing immobilized HSV DNA. Analysis in denaturing sucrose gradients indicated that HSV-specific poly(A+) RNA sedimented in a broad peak, with a modal S value of 20. The ratio of [^{3}H]methyl to ^{32}P decreased with increasing size of RNA, suggesting that each RNA chain contains a similar number of methyl groups. Further analysis indicated an average of one RNase-resistant structure of the type m^{7}G(5')pppN^{m}pNp or m^{7}G(5')pppN^{m}pN^{m}pNp per 2,780 nucleotides. The following components were identified in the 5'-terminal oligonucleotides of polyribosome-associated HSV-specific poly(A+) and poly(A−) RNA: 7-methylguanosine, N^6,2'-*O*-dimethyladenosine, and the 2'-*O*-methyl derivatives of guanosine, adenosine, uridine, and cytidine. The most common 5'-terminal sequences were m^{7}G(5')pppm^{6}A^m and m^{7}G(5')pppG^m. An additional modified nucleoside, N^6-methyladenosine, was present in an internal position of HSV-specific RNA.

Herpes simplex virus (HSV) is a large DNA virus that replicates within the nucleus of infected human cells. Previous studies have shown that HSV-specific polyribosomal RNA shares certain characteristics with that of its host cell, including a polyadenylic acid [poly(A)] tract at its 3' terminal (1, 16, 18) and a smaller average size than total nuclear HSV RNA (16, 18, 22). A further similarity was suggested by a recent report that HSV-specific RNA is methylated at terminal and internal positions (2). In the present study the 5'-terminal structures and the internal methylated nucleosides have been identified.

MATERIALS AND METHODS

Cells and virus. HeLa cells were grown in monolayer culture, using Eagle minimal essential medium (MEM [5]) with Earle salts, 10% calf serum, and no antibiotics. HSV infection was at a multiplicity of 30 PFU/cell. Properties and methods of assay of our L strain of HSV type 1 (HSV-1) have been described (18, 20).

Radioactive RNA. Monolayer cultures of 4×10^7 cells were labeled from 2.5 to 6 h after infection. RNA was methyl labeled by first starving cells for methionine for 15 min in 10 ml of methionine-free MEM with 5% dialyzed fetal calf serum and then adding the above medium supplemented with 20 mM sodium formate-20 μM adenosine-20 μM guanosine-20 μM L-[*methyl*-^{3}H]methionine (5 Ci/mmol). RNA was phosphate labeled by incubation of 4×10^7 cells with 1.25 mCi of [^{32}P]orthophosphate in 12 ml of MEM containing 10% of the usual amount of phosphate and 5% calf serum that had been dialyzed against 0.15 M NaCl. RNA labeled with both ^{32}P and [*methyl*-^{3}H]methionine was prepared by the incubation of cells with 1 mCi of [^{32}P]orthophosphate and 1 mCi of [*methyl*-^{3}H]methionine in methionine-free MEM containing 20 mM sodium formate, 20 μM adenosine, 20 μM guanosine, 10% of the normal phosphate concentration, and 5% dialyzed calf serum.

Isolation of HSV RNA. The fractionation of cells into nuclei and cytoplasm, isolation of polyribosomes, RNA purification, and separation of RNA-containing poly(A), referred to as poly(A+), and RNA-lacking poly(A), referred to as poly(A−), were as described previously (18). Cells were fractionated into nuclei and cytoplasm, and polyribosomes were isolated by the method of Palmiter (13). RNA was extracted from polyribosomes by proteinase K digestion followed by warm phenol-sodium dodecyl sulfate extraction. Poly(A+) and poly(A−) RNA were fractionated by chromatography on polyuridylic acid [poly(U)] bound to glass-fiber filters. Preparative amounts of HSV-specific RNA were obtained either

by hybridization to 20 to 30 μg of HSV DNA bound to nitrocellulose filters (23) or by DNA excess hybridization (18). Filter disk hybridization was for 24 h at 50°C in 50% formamide–0.5 M NaCl–0.1 M Tris-hydrochloride (pH 7.5)–10 mM EDTA. After hybridization, the filters were rinsed with several changes of 2× SSC (SSC is 0.15 M NaCl plus 0.015 M Na_3 citrate) at 55°C for a period of 4 h and then rinsed for an additional 12 h in 2× SSC at 37°C. Such washing eliminated adventitiously bound radioactive RNA from control filter disks. Viral RNA was eluted from the filters by incubation for 10 min at 72°C in 90% formamide–0.15 M NaCl–0.1 M Tris-hydrochloride (pH 7.5)–0.01 M EDTA–20 μg of carrier RNA per ml. DNA excess hybridization was carried out with 25 to 50 μg of denatured HSV DNA per ml fragmented by boiling in alkali. Hybridization was in 0.5 M NaCl–0.1 M Tris-hydrochloride–10 mM EDTA (pH 7.5)–50% formamide for 24 h at 46°C. HSV-specific RNA was obtained by hydroxyapatite chromatography in 8 M urea as described previously (11, 18).

RNA sedimentation in sucrose-formaldehyde gradients. HSV-specific RNA was mixed with unlabeled HeLa cell rRNA as a size marker. The RNA was denatured by incubating at 72°C for 10 min in a buffer of 9 mM Na_2HPO_4 and 1 mM NaH_2PO_4 containing 3% formaldehyde. Denatured RNA was fractionated by sedimentation on 18 ml of 5 to 20% sucrose gradients containing 0.1 M NaCl, 10 mM NaH_2PO_4, 10 mM Na_2HPO_4, and 1% formaldehyde. Sedimentation was for 20 h at 4°C and 24,000 rpm in a Spinco SW27 rotor. This procedure has been described in detail previously (18).

Enzyme digestions. The procedures for enzyme digestions were essentially those used elsewhere (24). Purified HSV RNA was digested with a mixture of RNases T_2 (10 U/ml), T_1 (20 μg/ml), and A (100 μg/ml) in 0.1 M ammonium acetate (pH 4.5) for 7 h. Nuclease P_1 was used at 0.5 mg/ml in 10 mM ammonium acetate (pH 6.0) for 2 h. Oligonucleotides were treated with snake venom phosphodiesterase (0.2 mg/ml) and/or bacterial alkaline phosphatase (0.2 mg/ml) in 50 mM Tris-hydrochloride (pH 8.5) and 5 mM $MgCl_2$ for 2 h. All digestions were at 37°C.

Chromatography. DEAE-cellulose column chromatography was carried out at pH 7.6 in 7 M urea with markers prepared by limited alkali digestion of poly(A) (21, 25). Appropriate fractions were desalted by readsorption to small DEAE-cellulose columns and elution with 2 M triethylammonium bicarbonate (12). Chromatography on Whatman no. 1 paper employed solvent A (isopropanol-water-NH_3 [7:2:1]) or solvent B (isopropanol-concentrated HCl-water [680:170:144]). Electrophoresis at 3,000 V on Whatman 3MM paper was in 5% acetic acid-0.5% pyridine-1 mM EDTA.

Materials. DEAE-cellulose and Whatman paper were purchased from Whatman, Inc. Enzymes were supplied by Worthington Biochemicals Corp., except for RNase T_2, nuclease P_1, and proteinase K, which came from Sigma Chemical Co., Yamasa Shoyu Co., and Merck Pharmaceuticals, respectively. [*methyl*-^{3}H]methionine was purchased from Schwarz/Mann, and $Na^{32}PO_4$ was from Amersham/Searle.

RESULTS

Size distribution of ^{32}P and methyl-labeled HSV-specific poly(A+) RNA. HeLa cells were labeled with [^{32}P]orthophosphate and L-[*methyl*-^{3}H]methionine from 2.5 to 6 h after infection. The polyribosomal RNA was isolated, separated from the bulk of highly methyl-labeled tRNA and rRNA by poly(U)-filter chromatography and from HeLa cell mRNA by hybridization to excess HSV DNA. The HSV-specific poly(A+) RNA, so obtained, was denatured with formaldehyde and fractionated on 5 to 20% sucrose gradients containing 1% formaldehyde. The doubly labeled RNA sedimented as a broad peak, with a modal S value of 20 (Fig. 1). The ratio of ^{3}H to ^{32}P decreased with increasing molecular weight, suggesting a similar number of methyl groups per RNA chain (12, 26). Methyl-labeled poly(A+) HSV RNA obtained from the nuclear fraction had a modal S value similar to that of the poly(A+) polyribosomal RNA shown in Fig. 1. When polyribosomal poly(A−) HSV RNA was analyzed on su-

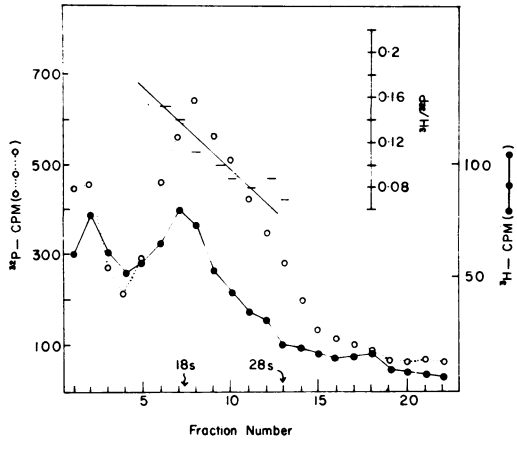

FIG. 1. *Size distribution of HSV-specific [^{3}H]methyl and ^{32}P-labeled poly(A$^+$) polyribosomal RNA. Approximately 4 × 10^7 HeLa cells were incubated with 1 mCi each of* L-*[methyl-^{3}H]methionine and [^{32}P]orthophosphate from 2.5 to 6 h after infection. Polyribosomal poly(A$^+$) RNA was purified by preparative hybridization to excess viral DNA. Unlabeled HeLa cell rRNA was added as an optical density marker, and the hybrids were heat denatured in formaldehyde and layered over an 18-ml gradient of 5 to 20% sucrose in 1% formaldehyde. Centrifugation was at 4°C for 20 h at 24,000 rpm in a Beckman Spinco SW27 rotor. The gradient was pumped through a Gilford recording spectrophotometer, and 0.7-ml fractions were collected. Scintillation counting was done in Aquasol, with windows set to allow no more than 1% of the ^{32}P radioactivity to spill into the ^{3}H window and negligible ^{3}H into the ^{32}P window.*

crose gradients, higher ratios of ^3H to ^{32}P in the positions of 18S and 28S rRNA's were obtained, suggesting residual contamination with highly methyl-labeled rRNA species as discussed below.

Isolation of 5'-terminal methylated oligonucleotides. Methyl-labeled poly(A+) HSV RNA was digested with a combination of RNases (T_2, T_1, and A) to hydrolyze all 3',5'-phosphodiester bonds except those in which the ribonucleoside contains a 2' substitution. The products were then chromatographed on a DEAE-cellulose column in 7 M urea at pH 7.6. Under these conditions, nucleotides or enzyme-resistant oligonucleotides separate on the basis of net negative charge (21). Of major interest to this study was the double peak of methyl-labeled material that eluted with a net negative charge of −5 to −6 (Fig. 2A). In separate experiments, this double peak of poly(A+) HSV RNA varied from 63 to 78% of the total radioactive material recovered. After further treatment with bacterial alkaline phosphatase, the two peaks eluted with net charges of approximately −3 and −4 (data not shown), indicating that only the terminal 3'-phosphate was susceptible to digestion. These properties are consistent with structures of the types $m^7G(5')pppN^mpNp$ (−5 charge) and $m^7G(5')pppN^mpN^mpNp$ (−6 charge) previously described in HeLa cell mRNA (7, 24). Apparently, under the labeling conditions employed, the putative $m^7G(5')pppN^mpNp$ was the major methyl-labeled component in HSV RNA.

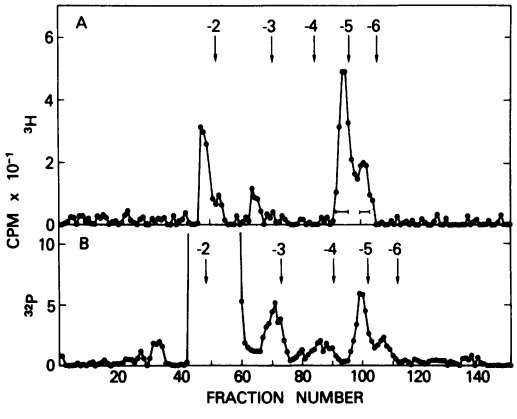

FIG. 2. *DEAE-cellulose column chromatography of RNase T_2, T_1, and A digests of HSV-specific polyribosomal poly(A+) RNA. The arrows indicate the positions and net negative charges of oligoadenylic acid markers. (A) Digest of methyl-labeled HSV RNA. The brackets indicate the fractions pooled for subsequent analysis. (B) Digest of ^{32}P-labeled HSV RNA.*

Assuming that the −5 and −6 peaks represented $m^7G(5')pppN^mpNp$ and $m^7G(5')pppN^mpN^mpNP$, respectively, ^{32}P-labeled polyribosomal poly(A+) HSV RNA was isolated to determine the percentage of radioactive materials in these structures. The results (Fig. 2B) indicated that 0.14% of the ^{32}P was in the position of $m^7G(5')pppN^mpNp$ and 0.054% was in the position of $m^7G(5')pppN^mpN^mpNp$. From the combined values we calculated that there is one terminal oligonucleotide per 2,780 nucleotides.

The demonstration of nuclease P_1 (an enzyme that cleaves all phosphodiester bonds regardless of 2' substitution [6]) and alkaline phosphatase-resistant methyl-labeled oligonucleotides with mobilities similar to those of authentic $m^7G(5')pppA^m$ and $m^7G(5')pppG^m$ (27) provided further evidence for "cap" structures in HSV RNA (Fig. 3).

Analysis of methylated nucleosides in the 5'-terminal structures. The separated peaks of radioactive material with net charges of −5 and −6 were digested with a combination of snake venom phosphodiesterase and bacterial alkaline phosphatase to liberate nucleosides. The principal methyl-labeled nucleosides from the putative $m^7G(5')pppN^mpNp$ (−5) peak co-chro-

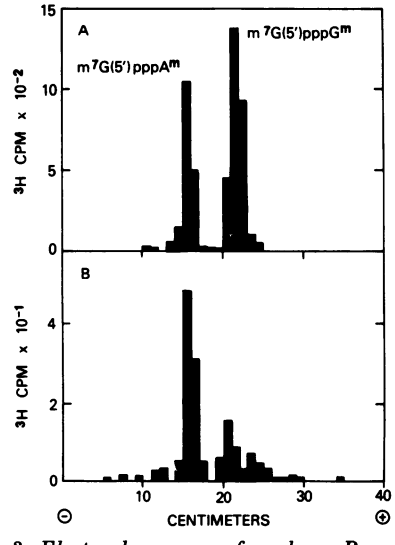

FIG. 3. *Electropherogram of nuclease P_1 and alkaline phosphatase-resistant oligonucleotides. (A) Vaccinia virus mRNA synthesized in vitro with S-adenosyl-[methyl-^3H]methionine was digested with nuclease P_1 followed by bacterial alkaline phosphatase. (B) Combined −5 and −6 peaks derived by RNase digestion as in Fig. 2A were desalted and digested with nuclease P_1 followed by bacterial alkaline phosphatase. Electrophoresis was performed as described in the text, and 1-cm strips were counted.*

matographed with m^7G (42%), G^m (19%), and m^6A^m (29%), and 10% migrated in the region in which A^m and C^m were incompletely resolved (Fig. 4A). These values were then converted to molar percentages by taking into account the presence of two methyl groups in m^6A^m and by assuming that all methyl groups have similar specific activities. The results, so obtained, were m^7G (50%), G^m (22%), m^6A^m (17%), and $A^m + C^m$ (11%). The value of 50% m^7G agrees with the suggested $m^7G(5')pppN^m$ structure, and the analysis further indicated that $m^7G(5')pppG^m$ and $m^7G(5')ppp^6A^m$ were the major sequences. This result was also supported by the electrophoretic analysis of the nuclease P_1 digest (Fig. 3) since $m^7G(5')pppA^m$ and $m^7G(5')ppp^6A^m$ comigrate under these conditions.

A similar nucleoside analysis for the putative $m^7G(5')pppN^mpN^mpNp$ (−6) peak after correction for the two methyls in m^6A^m was m^7G (31%), G^m (12%), m^6A^m (27%), and U^m (24 to 29%) (Fig. 4B). The lower value for U^m was calculated by subtracting the portion overlapping with C^m marker. Of particular significance was the nearly theoretical (33%) amount of m^7G in a structure of the type $m^7G(5')pppN^mpN^mpNp$ and the relatively large amount of U^m, which was undetected in the −5 peak. Although it is tempting to suggest that U^m is the principal methylated nucleoside in the third position, the small quantities of labeled $m^7G(5')pppN^mpN^mpNp$ precluded further analysis. In some experiments, lower percentages of m^7G were obtained, possibly due to the conversion of unlabeled $m^7G(5')pppN^mpNp$ terminals to labeled $m^7G(5')pppN^mpN^mpNp$ terminals after the addition of [*methyl*-^{3}H]-methionine.

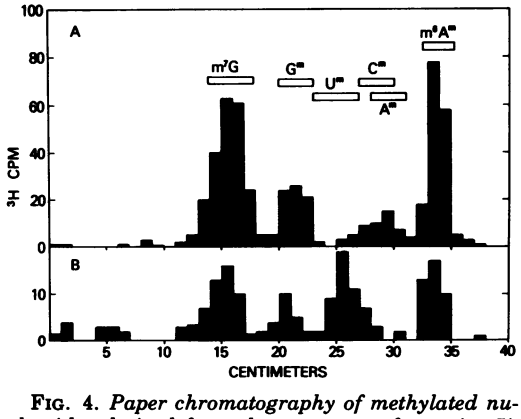

FIG. 4. *Paper chromatography of methylated nucleosides derived from the two types of putative 5'-terminal oligonucleotides. The indicated fractions of Fig. 2A comprising the separated −5 and −6 peaks were desalted, digested with snake venom phosphodiesterase and alkaline phosphatase, and chromatographed on Whatman no. 1 paper in solvent A. (A) Digest of −5 peak; (B) digest of −6 peak.*

Methyl-labeled poly(A−) HSV-specific polyribosomal RNA was analyzed as described above to be certain that no methylated 5'-terminal structures were missed by poly(U) filter purification of HSV RNA. Briefly, again we obtained −5 and −6 peaks upon DEAE-cellulose chromatography after RNase digestion, and the nucleoside analyses of the separated peaks were similar to those shown in Fig. 4A and B.

Since the methylated 5' terminals of poly(A+) and poly(A−) HSV-specific RNA are similar, the −5 and −6 peaks from another RNase digest of HSV-specific polyribosomal RNA that had not been fractionated on the basis of poly(A) content were pooled to obtain sufficient amounts of radioactively labeled materials to confirm the identities of the methylated nucleosides. The methylated nucleosides obtained from this material were subjected to high-voltage paper electrophoresis with authentic markers. Under these conditions m^7G migrated furthest toward the cathode, followed closely by C^m, which was well separated from the comigrating A^m and m^6A^m. The remaining two methylated nucleosides, G^m and U^m, moved only slightly off the origin and were incompletely resolved. The regions of the electropherogram corresponding to the markers were eluted with water, concentrated, and analyzed in a second dimension, using two different paper chromatographic systems. As shown in Fig. 5, radioactively labeled materials from the appropriate regions of the electropherogram cochromatographed with G^m, U^m, A^m, m^6A^m, C^m, and m^7G.

Identification of internal methylated nucleosides. Base methylated nucleotides derived by RNase T_2, T_1, and A digestion of internal regions of an RNA would be expected to elute with a net charge of −2. Approximately 11 to 29% of the total methyl-labeled material from different preparations of poly(A+) HSV-specific RNA eluted in this position. After digestion with bacterial alkaline phosphatase, the major component comigrated with m^6A (Fig. 6). The materials preceding m^6A were present in submolar amounts and have not been identified, although the possibility of m^5C (4) and small amounts of purine ring-labeled adenosine that migrate in this region of the chromatogram were considered.

The −3 peak of Fig. 2A never represented more than 8 to 10% of the recovered methyl-labeled material from poly(A+) HSV-selected RNA. However, as much as 28% of the methyl-labeled material from some preparations of

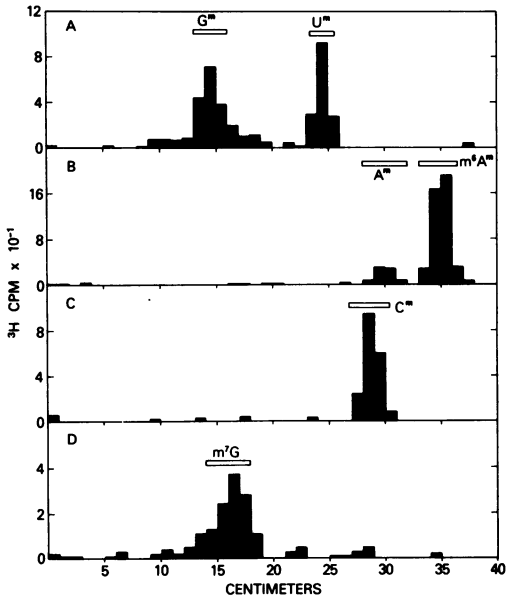

FIG. 5. *Further identification by electrophoresis and chromatography of the methylated nucleoside components of the 5'-terminal oligonucleotides. Methylated nucleosides derived from the combined −5 and −6 peaks of HSV-specific polyribosomal RNA were separated by paper electrophoresis at pH 3.5. The areas of the electropherogram corresponding to G^m + U^m (A), A^m (B), C^m (C), and m^7G (D) were eluted with water and chromatographed. Solvent B was used for panel (A), and solvent A was used for the remaining panels.*

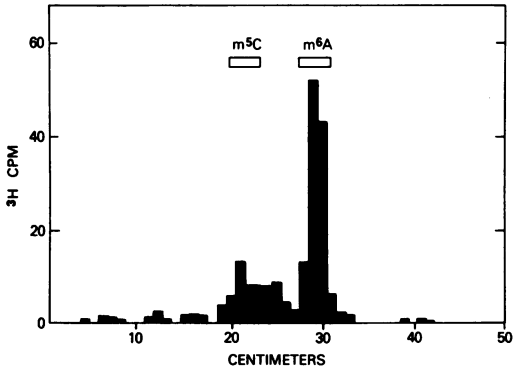

FIG. 6. *Paper chromatography of internal methylated nucleosides. The peak with a −2 charge from an experiment similar to that shown in Fig. 2A was desalted, digested with bacterial alkaline phosphatase, and chromatographed in solvent A.*

poly(A−) HSV-selected RNA eluted in that position. It appears to represent the RNase-resistant structure N^mpNp since the charge decreased to −1 after digestion with alkaline phosphatase. The recovery of larger amounts of this material in the poly(A−) fraction suggested that it is probably derived from trace amounts of very highly methylated rRNA. A 1% contamination of poly(A+) HSV RNA with rRNA and a 3% contamination of poly(A−) HSV RNA with rRNA would give the obtained results. Since rRNA contains neither cap structures nor m^6A, this level of contamination had no effect on our other results.

DISCUSSION

HSV-1 polyribosomal RNA labeled with [*methyl*-^{3}H]methionine between 2.5 and 6 h after infection contains RNase-resistant structures that were identified as $m^7G(5')pppN^mpNp$ and $m^7G(5')pppN^mpN^mpNp$ based on the following: (i) their net negative charges, (ii) presence of only a single phosphate residue that could be removed by alkaline phosphatase, (iii) coelectrophoresis of nuclease P_1 and alkaline phosphatase-resistant fragments with authentic $m^7G(5')pppA^m$ and $m^7G(5')pppG^m$, (iv) sensitivity to snake venom phosphodiesterase, (v) molar ratio of m^7G to 2'-*O*-methylribonucleosides of 1:1 for the peak with a −5 charge, and (vi) molar ratio of m^7G to 2'-*O*-methylribonucleosides of approximately 1:2 for the −6 peak. Analysis of ^{32}P-labeled HSV-specific RNA indicated one terminal per 2,780 nucleotides. Since the average size of HSV-specific poly(A+) polyribosomal RNA, as determined by sedimentation in denaturing sucrose density gradients in this and previous work (18), is of the order of 2,200 nucleotides, at least 75% of the HSV-specific poly(A+) polyribosome-associated RNA chains contain RNase-resistant, methylated cap structures.

Under our conditions of labeling, the major 5' terminals were $m^7G(5')pppG^mpNp$, $m^7G(5')ppp^{m6}A^mpNp$, and $m^7G(5')pppA^mpNp$. The specific presence of U^m in the $m^7G(5')pppN^mpN^mpNp$ terminals suggested that this methylated nucleoside may occupy the third position of some mRNA chains. The relatively small amounts of labeled HSV RNA, however, precluded a complete catalog of all methylated 5'-terminal sequences.

Separate analyses of the methylated 5'-terminal oligonucleotides of poly(A+) and poly(A−) HSV RNA revealed no significant differences, in agreement with previous studies, indicating that both classes of HSV RNA are transcribed from the same DNA sequences (18). The labeling times, 2.5 to 6 h after infection, included the period of early and late HSV RNA synthesis (20). A comparison of the 5'-terminal sequences of early and late classes of HSV RNA has not yet been made, although there is evidence that both are methylated (2). We have

noted that poly(A+) HSV-specific RNA isolated from nuclei contained methylated RNase-resistant oligonucleotides, but insufficient amounts were obtained for further analysis.

HSV-specific polyribosomal RNA was also found to contain base methylated nucleosides derived from internal sites. The major component co-chromatographed with m^6A, a nucleoside that appears to be absent from rRNA and the majority of tRNA's (8, 28). The nucleoside m^6A is present in poly(A+) cell mRNA (3, 7, 14, 24) and the mRNA's of some other DNA viruses (9, 10, 12, 17), but is absent from globin (15) and histone mRNA's (11a, 19) and apparently from mRNA synthesized very late in HSV infection (2). Although the function of m^6A residues in mRNA is unknown, a limited sequence specificity around this methylated nucleoside in HeLa cell poly(A+) mRNA has been demonstrated (25, 27a).

In conclusion, the modifications of HSV RNA appear to be similar to those of host cell mRNA and presumably are formed and function in a similar manner.

LITERATURE CITED

1. **Bachenheimer, S., and B. Roizman.** 1972. Ribonucleic acid synthesis in cells infected with herpes simplex virus. VI. Polyadenylic acid sequences in viral messenger ribonucleic acid. J. Virol. 10:875–879.
2. **Bartkoski, M., and B. Roizman.** 1976. RNA synthesis in cells infected with herpes simplex virus. XIII. Differences in the methylation patterns of viral RNA during the reproductive cycle. J. Virol. 20:583–588.
3. **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.
4. **Dubin, D. T., and R. H. Taylor.** 1975. The methylation state of poly(A)-containing-messenger RNA from cultured hamster cells. Nucleic Acids Res. 2:1653–1668.
5. **Eagle, H.** 1959. Amino acid metabolism in mammalian cell cultures. Science 130:432–437.
6. **Fujimoto, M., A. Kuninaka, and H. Yoshino.** 1974. Substrate specificity of nuclease P_1. Agric. Biol. Chem. 38:1555–1561.
7. **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.
8. **Harada, F., F. Kimura, and S. Nishimura.** 1969. Nucleotide sequence of oligonucleotides derived from *Escherichia coli* valine transfer RNA by ribonuclease T_1 digestion: comparison of the sequences neighboring 3'- and 5'-terminals and anticodon region of *Escherichia coli* valine transfer RNA with those of yeast valine transfer RNA. Biochim. Biophys. Acta 182:590–592.
9. **Hashimoto, S.-I., and M. Green.** 1976. Multiple methylated cap sequences in adenovirus type 2 early mRNA. J. Virol. 20:425–435.
10. **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.
11. **Lewis, J., J. Atkins, C. Anderson, P. Baum, and R. Gesteland.** 1975. Mapping of late adenovirus genes by cell free translation of RNA selected by hybridization to specific DNA fragments. Proc. Natl. Acad. Sci. U.S.A. 72:1344–1348.

11a. **Moss, B., A. Gershowitz, L. Weber, and C. Baglioni.** 1977. Histone mRNAs contain blocked and methylated 5'-terminals but lack methylated nucleotides at internal positions. Cell 10:113–120.

12. **Moss, B., and F. Koczot.** 1976. Sequence of methylated nucleotides at the 5'-terminus of adenovirus-specific RNA. J. Virol. 17:385–392.
13. **Palmiter, R.** 1974. Magnesium precipitation of ribonucleoprotein complexes: expedient techniques for the isolation of undegraded polysomes and messenger ribonucleic acid. Biochemistry 13:3606–3614.
14. **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.
15. **Perry, R. P., and K. Scherrer.** 1975. The methylated constituents of globin mRNA. FEBS Lett. 57:73–78.
16. **Silverstein, S., R. Millette, P. Jones, and B. Roizman.** 1976. RNA synthesis in cells infected with herpes simplex virus. XII. Sequence complexity and properties of RNA differing in extent of adenylation. J. Virol. 18:977–991.
17. **Sommer, S., M. Salditt-Georgieff, S. Bachenheimer, J. E. Darnell, Y. Furuichi, M. Morgan, and A. J. Shatkin.** 1976. The methylation of adenovirus-specific nuclear and cytoplasmic RNA. Nucleic Acids Res. 3: 749–765.
18. **Stringer, J. R., L. E. Holland, R. I. Swanstrom, K. Pivo, and E. K. Wagner.** 1977. Quantitation of herpes simplex virus type 1 RNA in infected HeLa cells. J. Virol. 21:889–901.
19. **Surrey, S., and M. Nemer.** 1976. Methylated blocked 5'-terminal sequences of sea urchin embryo messenger RNA classes containing and lacking poly(A). Cell 9:589–595.
20. **Swanstrom, R. I., K. Pivo, and E. K. Wagner.** 1975. Restricted transcription of the herpes simplex virus genome occurring early after infection and in the presence of metabolic inhibitors. Virology 66:140–150.
21. **Tener, G. M.** 1967. Ion-exchange chromatography in the presence of urea. Methods Enzymol. 12A:398–401.
22. **Wagner, E. K., and B. Roizman.** 1969. Ribonucleic acid synthesis in cells infected with herpes simplex virus. Patterns of ribonucleic acid synthesis in productively infected cells. J. Virol. 4:36–46.
23. **Wagner, E. K., R. I. Swanstrom, and M. G. Stafford.** 1972. Transcription of the herpes simplex virus genome in human cells. J. Virol. 10:675–682.
24. **Wei, C.-M., A. Gershowitz, and B. Moss.** 1975. Methylated nucleotides block 5'-terminus of HeLa cell messenger RNA. Cell 4:379–386.
25. **Wei, C.-M., A. Gershowitz, and B. Moss.** 1976. 5'-Terminal and internal methylated nucleotide sequences in HeLa cell mRNA. Biochemistry 15:397–401.
26. **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.
27. **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.

27a. **Wei, C.-M., and B. Moss.** 1977. Nucleotide sequences at the N^6-methyl-adenosine sites of HeLa cell messenger RNA. Biochemistry 16:1672–1676.

28. **Yaniv, M., and B. G. Barrell.** 1969. Nucleotide sequence of *E. coli* B $tRNA_1^{Val}$. Nature (London) 222: 278–279.