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RNA Synthesis in Cells Infected with Herpes Simplex Virus

XIII. Differences in the Methylation Patterns of Viral RNA During the Reproductive Cycle

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Herpes simplex virus 1 (HSV-1) RNA labeled with [*methyl*-^{3}H]methionine at various times during the infectious cycle and purified by hybridization to viral DNA was analyzed for the presence of methylated nucleotides. The data indicate the following. (i) RNA labeled from 0 to 14 h postinfection and accumulating in the cytoplasm contained internal base-methylated nucleotides and terminal oligonucleotides consistent with the structure 7mG(5')ppp-(5')X^{m}pY^{m}pNp. Similar methylated nucleotides and oligonucleotides were also found in viral RNA accumulating in the cytoplasm of cells treated with cycloheximide from the time of infection. Previous studies (M. Kozak and B. Roizman, 1974) have shown that, whereas the RNA accumulating in the 14-h infected cells contains all of the sequences functioning as mRNA throughout infection, the RNA accumulating in the cytoplasm of cycloheximide-treated cells is associated with polyribosomes synthesizing the earliest (α) group of polypeptides specified by the virus. (ii) Cytoplasmic viral RNA from cells labeled 11 to 14 h postinfection as well as the total adenylated RNA in the cytoplasm and polyribosomes labeled in the same fashion contained the terminal oligonucleotide but not the internal base-methylated nucleotide.

Previous reports from this laboratory have shown that in human cells infected with human herpesvirus 1 (herpes simplex virus 1 [HSV-1]) viral polypeptides form at least three groups whose synthesis is coordinately regulated and sequentially ordered (13, 14, 21). Thus, α polypeptides are made first and reach maximum rates of synthesis between 2 and 4 h postinfection. They induce the synthesis of the second or β group made at maximum rates between 5 and 7 h postinfection. These shut off the synthesis of α polypeptides and induce the synthesis of the third or γ group. This group, consisting largely of viral structural polypeptides (12), in turn shuts off the synthesis of the β group.

Several lines of evidence indicate that the α and β mRNA's, are relatively stable and that the shutoff of synthesis of these polypeptides is a specific function of one or more β and γ polypeptides, respectively. Thus, α polypeptide synthesis continues for many hours if the synthesis of β polypeptides is blocked either by rendering α polypeptides nonfunctional (14) or by interfering with the transcription of β mRNA after α mRNA had accumulated in the infected cell (13, 21). However, α polypeptide synthesis decays at a relatively rapid rate once functional β polypeptides are made (13, 21). One expectation of a system in which the polypeptide synthesis is coordinately regulated in a cascade fashion is that the mRNA's of each group share structural features that would permit differential regulation at the level of translation (21). In the search for such differential features, we began studies on the presence and distribution of methylated nucleotides in viral RNA extracted from the cytoplasm of infected cells.

Methylated nucleotides have been demonstrated in mRNA of eukaryotic cells (1, 4, 20) and virus (17, 24, 30), but not all viruses infecting eukaryotic cells specify methylated informational RNA molecules (7). The methylated nucleotides occur internally, usually in the form of 6-methyl A, and at the 5' terminus as an oligonucleotide of the structure m7G(5')ppp(5')X^{m}pY^{m}pNp or m7G(5')ppp(5')X^{m}pNp, where X^m and Y^m represent the 2'-O-methylated derivatives of all four ribonucleosides (1). Although it has been recently demonstrated that the presence of m7G at the 5' terminus is needed for the effective translation of globin, vesicular stomatitis virus, and reovirus mRNA's (2, 19), the exact function of these methylated sequences is still unknown.

In designing the experiments described in this paper we took advantage of two observations. First, only α polypeptides are made in cells immediately after withdrawal of cycloheximide present in the medium from the time of infection (13) or in the presence of amino acid analogues added at the time of infection (14). In both instances transcripts arising from only 10 to 14% of the DNA accumulate in the cytoplasm (16, 21; N. Frenkel, H. Locker, and B. Roizman, manuscript in preparation). We have defined these transcripts as α mRNA. Second, after the onset of assembly and maturation of viruses, the transcripts accumulating in the infected cell cytoplasm are homologous to 43% of the viral DNA, and molecular hybridization studies show that this RNA includes all transcripts translocated into the cytoplasm, including the α mRNA sequences, even though the α polypeptides are no longer made (16, 21, 25).

MATERIALS AND METHODS

Cells and virus. The procedures for the propagation of HEp-2 (human epidermoid carcinoma no. 2) cells and the assay, production, and pertinent properties of the F strain of HSV-1 were described elsewhere (6, 8, 15, 22). In these studies, confluent monolayers of HEp-2 cells were infected with HSV-1 (F) at a multiplicity of 10 PFU/cell.

Labeling of RNA. Infected cells were labeled at the times specified in the text with 20 to 25 mCi of [*methyl*-^{3}H]methionine (Amersham/Searle Co.) per ml of maintenance medium consisting of mixture 199 containing one-fifth the normal methionine concentrations, but supplemented with 1% dialyzed calf serum, 20 μM each adenosine and guanosine, and 20 mM sodium formate to inhibit the incorporation of [*methyl*-^{3}H]methionine groups into the purine rings (18).

Cell fractionation. Cells were scraped and harvested in phosphate-buffered saline (5), washed once, and resuspended in lysing buffer (50 mM Tris-hydrochloride [pH 7.4]-50 mM KCl-1 mM $MgCl_2$-0.5% Nonidet P-40 [BDH Chemicals Ltd.]). After 10 to 20 min on ice, the nuclei were separated from the cytoplasmic extract by centrifugation. Sodium dodecyl sulfate (SDS) was added to a final concentration of 0.5% to the cytoplasmic fraction.

Extraction of RNA. RNA was extracted from the cytoplasmic fraction with phenol followed by reextraction of the nonaqueous residue with Tris buffer, pH 9.0 (3). The aqueous phases were combined and reextracted with phenol-chloroform (1:1) followed by chloroform-4% isoamyl alcohol. In the final step of purification the RNA in a solvent containing 0.025 M ammonium acetate was precipitated with ethanol.

Fractionation of polyadenylated RNA. The binding and elution of adenylated RNA from poly(U) immobilized on glass-fiber columns were done as previously described (26).

Enzymatic digestion of RNA. RNA was digested with T2 RNase (30 μg/ml; Sigma Chemical Co.) in 10 mM sodium acetate, pH 4.6. P1 nuclease digestion (200 μg/ml; Yamasa, Ltd., Japan) was done in 10 mM acetate buffer, pH 6.0. For alkaline phosphatase digestion (10 μg/ml; Sigma Chemical Co.) after P1 digestion, the pH was raised to 8.2 with Tris.

DEAE-Sephadex chromatography. The nuclease digestion products were analyzed by chromatography on DEAE-Sephadex (A 25) columns equilibrated with 20 mM Tris-hydrochloride (pH 7.4), 0.05 M NaCl, and 7 M urea (29). The digest was eluted from the columns with linear gradients of 0.05 to 0.4 M NaCl in the Tris-urea buffer and cleared completely with 1 M NaCl.

The eluent fractions were pooled and desalted by the barium precipitation procedure described by Roy and Bishop (23). The elution of nucleotides and oligonucleotides with charges ranging from -2 to -7 was standardized with the aid of pancreatic RNase digest of *Escherichia coli* tRNA.

DNA-RNA hybridization. The DNA-RNA hybridizations were carried out by one of two procedures. In the first, DNA was immobilized on Sepharose as described by Gilboa et al. (11). The hybridization was done in 5 × SSC (1X SSC contains 0.15 M NaCl plus 0.015 M sodium citrate) and 50% formamide at 50°C. After hybridization, the DNA-Sepharose column was washed extensively with hybridization buffer. The hybridized RNA was then eluted with 98% formamide-10 mM Tris (pH 7.4) followed by a 1 M NaCl wash. The second procedure involved hybridization of RNA to viral DNA fixed to nitrocellulose filters as described by Kieff et al. (15). The hybridizations were done at 50°C for 48 h in 0.8 ml of hybridization buffer containing 0.75 M NaCl, 0.1 M Tris (pH 7.4), 0.5 mM EDTA, 0.1% SDS, and 50% formamide. After hybridization the filters were soaked in 1 × SSC containing 0.5% SDS, reincubated for 1 h, and then rinsed in 2 × SSC. The RNA was then released by incubating the filters in 95% formamide-30 mM Tris (pH 8.2)-0.1% SDS for 1 h at 50°C. Released RNA was precipitated with 2 volumes of ethanol and stored at −20°C.

RESULTS

The studies described in this paper consisted of three series of experiments. In the first we examined the methylated nucleotides in cytoplasmic virus-specific RNA labeled with [*methyl*-^{3}H]methionine from the time of exposure of cells to virus until 14 h postinfection. Previous studies from this laboratory have shown that the cytoplasm of 14-h infected cells contains RNA sequences homologous to 43% of DNA. This RNA represents all informational sequences present in cytoplasm throughout infection even though only α polypeptides are made at that time (16, 21). In this series HEp-2 cells were infected with HSV-1 (F) at a multiplicity of 10 PFU/cell and labeled with [*methyl*-^{3}H]methionine from 0 to 14 h postinfection as described above. At that time the infected cells were harvested, and nuclear and cytoplasmic fractions were separated as described. Viral

RNA was purified by hybridization to viral DNA immobilized on Sepharose (11), eluted, and then analyzed for the presence of methylated nucleotides. In these analyses the T2 RNase digest of purified RNA was chromatographed on DEAE-Sephadex in the presence of 7 M urea (29). This procedure separates nucleotides and oligonucleotides on the basis of the number of negatively charged phosphates. Thus, each internal phosphate contributes a −1 charge, whereas each free terminal phosphate contributes a −2 charge. The *methyl*-^{3}H label in the HSV-1 (F) RNA digest chromatographed as two peaks, which eluted at positions corresponding to −2 and −5 to −6 charge (Fig. 1A). Peak 1, containing approximately 37% of the total eluted activity (Table 1), consisted of base-methylated nucleotides. This conclusion is based on its charge (−2) and on the observation that subsequent treatment with alkaline phosphatase converted it to a nucleoside that did not absorb to the DEAE-Sephadex column (Fig. 1B). The material eluting in peak 1 corresponds in its properties to internal base-methylated nucleotides (1, 24, 30). The −5 to −6 charged material in peak 2 (Fig. 1A) was desalted, digested with P1 nuclease, which degrades RNA to 5′-nucleotides (9), and split into two portions. One portion, designated as the P1 digest, was chromatographed on DEAE-Sephadex in 7 M urea. The other portion, designated as P1 alkaline phosphatase digest, was redigested with alkaline phosphatase and then rechromatographed as above. The P1 digest eluted in two peaks (Fig. 1C). The first peak, containing approximately one-third of the label, eluted at a position corresponding to a mononucleotide with a −2 charge. The second peak, containing two-thirds of the label, eluted at a position (−2 to −3) as expected for a structure of the type m7G(5′)ppp(5′)X^m (10). The P1 alkaline phosphatase digestion (Fig. 1D) did not, however, affect the elution of the peak 2 of Fig. 1C. We interpret these results to indicate that P1 nuclease digestion released the two terminal nucleotides (Y^{m}p, Np) of which the methylated nucleotide eluted in peak 1 (Fig. 1C and D) and that the remaining dinucleotides of the type m7G(5′)ppp(5′)X^m eluted in peak 2 (Fig. 1C and D). The results of these analyses are consistent

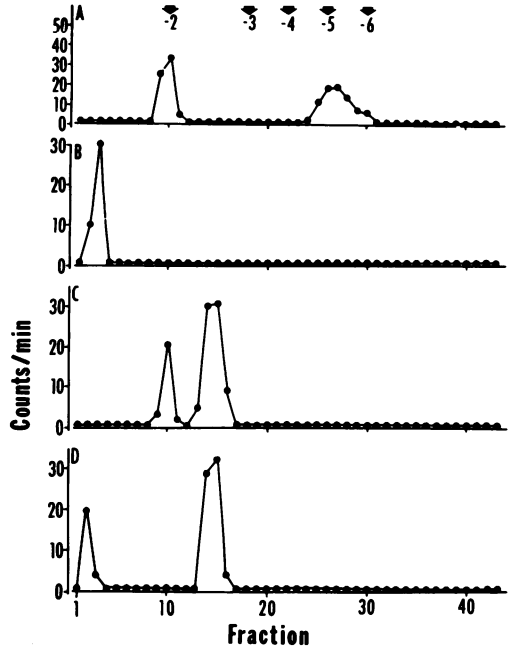

FIG. 1. *Elution profile of HSV-1-specific RNA digest on DEAE-Sephadex columns. The RNA was labeled with [methyl-^{3}H]methionine from 0 to 14 h postinfection. (A) T2 RNase digest of HSV-1 RNA. (B) Rechromatography of peak 1 of (A) after desalting and alkaline phosphatase digestion. (C) Rechromatography of peak 2 of (A) after desalting and digestion with P1 nuclease. (D) P1 digest of (C) further digested with alkaline phosphatase. The position of charges −2 to −7 was standardized, using a pancreatic RNase digest of E. coli tRNA.*

TABLE 1. *Distribution of methyl-^{3}H-labeled nucleotides in selected RNAs from HSV-1-infected cells*

Infected cell RNA digested with T2 RNase	Labeling interval after infection (h)	[*methyl*-^{3}H]methionine-labeled mononucleotides in digest (−2 charge)		[*methyl*-^{3}H]methionine-labeled oligonucleotides in digest (−5 to −6 charge)	
		cpm	% of total	cpm	% of total
Cytoplasmic HSV-1 specific RNA[a]	0–14	60	37	99	63
Cytoplasmic HSV-1 specific RNA[b]	0–8	25	55	20	45
Adenylated polyribosomal HSV-1 specific	11–14	2	5	48	95
Adenylated total cytoplasmic	11–14	80	1	6,880	99
Adenylated polyribosomal	11–14	50	1	3,550	99

[a] HSV-1-specific RNA refers to RNA selected by hybridization to HSV-1 DNA as described in text.
[b] From cells infected and maintained in the presence of cycloheximide.

with the hypothesis that HSV-1 RNA accumulating in the cytoplasm has a modified 5′ terminus predominantly of the type m7G(5′)-ppp(5′)X^{m}pY^{m}pNp, designated as cap 2, although we cannot exclude the possibility that modified 5′ termini of the type m7G(5′)ppp(5′)-X^{m}pNp, designated as cap 1, are also present. This conclusion is based on a comparison of label in peaks 1 and 2 of Fig. 1C. Assuming that each nucleotide has a single methylation, the predicted ratio of label in peak 1 to peak 2 of Fig. 1C is 1:2 for cap 2. P1 nuclease digestion of a cap 1 structure should yield no counts in peak 1. Since we are observing the maximum ratio we could expect for pure cap 2, the extent of contamination with cap 1 structures is probably not very high.

In the second series of experiments we examined RNA accumulating in the cytoplasm of cells treated with cycloheximide from the time of infection. As indicated in the introduction, the RNA extracted from polyribosomes or from total cytoplasm of cells treated with cycloheximide is homologous to 10 to 12% of viral DNA (16, 21). On withdrawal of the drug, the cells make only α polypeptides (13, 21). In these experiments HEp-2 cells were infected with HSV-1 (F) at a multiplicity of 10 PFU/cells, in the presence of cycloheximide (50 μg/ml), and then maintained in the presence of the drug for 8 h. The cells were then fractionated, and RNA was extracted from the cytoplasm as described above. Cytoplasmic viral RNA purified by hybridization to viral DNA was digested with T2 RNase and chromatographed as above. The *methyl*-^{3}H-labeled material from HSV-1-specific RNA eluted from the column in two peaks (Fig. 2A and Table 1) in a pattern similar to that of the digest of HSV-1 RNA labeled for 0 to 14 h postinfection (Fig. 1A). The two peaks with -2 and -5 to -6 charges, respectively, were collected and desalted for further analysis. Peak 1 (Fig. 2A) was shown to consist of a mononucleotide because subsequent digestion with alkaline phosphatase converted it to a nucleoside that did not absorb to the column (Fig. 2B). Peak 2 (Fig. 2A) was digested with P1 nuclease and alkaline phosphatase. The products were then chromatographed as above (Fig. 2C). Approximately one-third of the *methyl*-^{3}H label activity was converted to nucleosides that did not absorb to the column (peak 2, Fig. 2C). However, the remaining *methyl*-^{3}H-labeled material retained a -2 to a -3 charge (peak 2, Fig. 2C), corresponding in its elution properties to a structure of the type m7G(5′)ppp(5′)X^m (10). It appears from this analysis that HSV-1 RNA made in the presence of cycloheximide contains

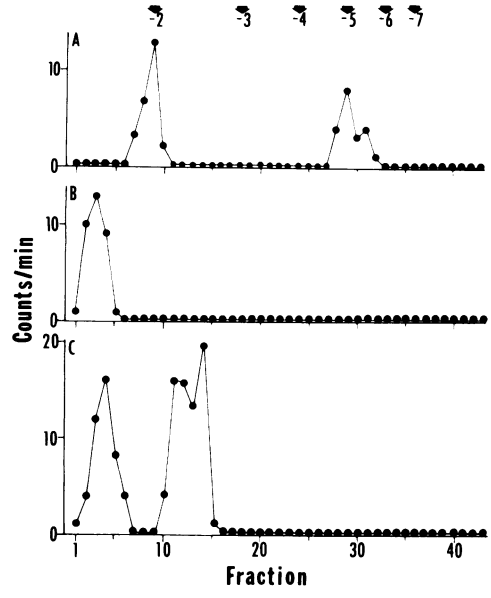

FIG. 2. *Elution profile of HSV-1-specific RNA digest on DEAE-Sephadex columns. [^{3}H-methyl]methionine from 0 to 8 h postinfection in the presence of cycloheximide (50 μg/ml) as described in the text. (A) T2 RNase digest of HSV-1-specific RNA. (B) Rechromatography of peak 1 of (A) after desalting and alkaline phosphatase digestion. (C) Peak 2 of (A) rechromatographed after desalting and digestion with P1 and alkaline phosphatase.*

both terminal and internal methylated nucleotides.

The objective of the third series of experiments was to examine the mRNA specifying the last or γ group of viral polypeptides. However, because (i) the RNA sequences associated with polyribosomes of infected cells making α polypeptides are conserved and cosediment with polyribosomes even after α polypeptide synthesis ceases (16, 21) and (ii) current evidence strongly suggests that the templates for the three groups of polypeptides are intermixed rather than clustered (P. Jones, G. S. Hayward, and B. Roizman, J. Virol., in press), it was not feasible to obtain purified γ mRNA. We have therefore examined only the RNA labeled and entering into the cytoplasm late in infection without prejudice as to whether this RNA is capable of specifying all classes or only γ polypeptides. In designing this experiment we took advantage of the observation that all or most species of viral RNA accumulating in the cytoplasm of infected cells are adenylated and bind to poly(U)bound to glass-fiber filters (26). In one experiment the (poly)A-containing RNA was hybridized to viral DNA,

eluted, and then digested with T2 RNase as described above. The elution profile showed one double peak of radioactivity at the position of tetra- and pentanucleotides (−5 to −6 charge) (Table 1 and Fig. 3A). A minor peak, usually less than 5% of the total, was detected at the position of mononucleotides (−2 charge) (Fig. 3A). The radioactivity eluting from the column with a charge of −5 to −6 was pooled, desalted, and digested with P1 nuclease. On chromatography, this peak eluted with a −2 and −2 to −3 charge (Fig. 3B). This pattern is consistent with a "capped" 5′ end structure m7G(5′)ppp(5′)X^mpY^mpNp as described above, although in this instance a cap 1 structure could also be present. The absence of internal base-methylated nucleotides in HSV-1 (F) RNA entering cytoplasm late in infection raised questions as to whether HSV-1 (F) RNA differed from all other adenylated RNA species made at that time. Accordingly, we examined the total cytoplasmic and polyribosomal RNA bound and eluted from poly(U) immobilized on glass-fiber columns. Table 1 shows that all adenylated RNA species accumulating in the cytoplasm share with viral RNA species a deficiency in internal base-methylated sequences.

DISCUSSION

The salient features of the data presented in this paper are as follows. (i) In conformity with the mRNA's of eukaryotic cells (1, 4, 20) and of some viruses infecting them (17, 24, 30), HSV RNA accumulating in the cytoplasm on polyribosomes of infected cells contains a modified 5′ terminus. The modified terminus was present in α mRNA, in viral RNA labeled throughout infection, and in viral RNA labeled and transported into the cytoplasm late in infection. Because current methodology does not permit the recovery of adequate amounts of viral RNA, we have not sequenced the modified 5′-ologonucleotide. The chromatographic behavior of the oligonucleotide and its digestion products indicates that the form m7G(5′)ppp(5′)X^mpY^mpNp, commonly seen in many other mRNA species, predominates on viral RNAs accumulating in the cytoplasm. We do not know whether all modified termini are identical; these studies await purification of individual mRNA's.

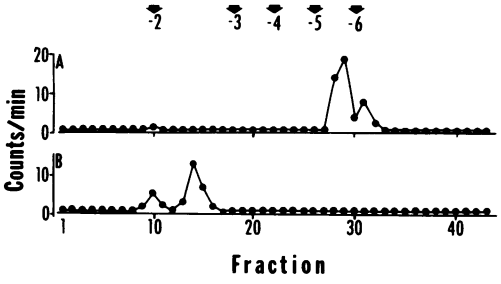

FIG. 3. *Elution profile of HSV-1-specific RNA digest on DEAE-Sephadex columns. The RNA was labeled from 11 to 14 h postinfection. (A) T2 RNase digest of HSV-specific RNA. (B) Rechromatography of material eluting with a −5 to −6 charge after desalting and digestion with P1 nuclease and alkaline phosphatase.*

(ii) In conformity with mRNA of other species, the T2 RNase digest of HSV-1 (F) RNA yielded base-methylated mononucleotides. Two observations are of special interest. The first relates to the fact that viral RNA sequences normally made and translocated into cytoplasm immediately after infection contain internal base-methylated nucleotides, whereas the RNA made and translocated into the cytoplasm at the time of peak synthesis of γ polypeptides does not contain appreciable amounts of these nucleotides. We cannot at this time determine whether the absence of internal base-methylated nucleotides in RNA made 11 to 14 h after infection is the consequence of the absence of signals for base methylation in RNA, inhibition of the enzyme by a virus-specific product, or rapid turnover coupled with a lack of new synthesis of the enzyme because of viral inhibition of host protein synthesis (27, 28). The fact that all adenylated RNA, which includes both host and virus-specific RNA, lacked the base-methylated nucleotides suggests the absence of active enzyme, but this remains to be determined. It is perhaps useful to note that among the various possibilities left open by this observation are extreme ones. The first is that the disappearance of a host enzyme due to rapid decay and overall inhibition of host protein synthesis may in fact serve a regulatory function in that it imparts on the RNA a structural alteration which could serve to differentiate this RNA from other species. At the other extreme is the possibility that internal base-methylated nucleotides have no regulatory function. Experiments are in progress to differentiate between these possibilities.

The second observation of interest relates to the fact that cytoplasmic viral RNA labeled from 0 to 14 h postinfection contained internal methylated nucleotides, whereas the RNA labeled from 11 to 14 h did not. We do not know the precise time after infection when internal base methylation ceases; the data indicate, however, that the RNA containing the internal *methyl*-^{3}H-label is conserved; the data also reinforce the conclusion reached from experi-

ments published earlier that α RNA sequences are present in the cytoplasm of infected cells many hours after α polypeptides cease to be made (16, 21).

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