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Capping of Viral RNA in Cultured *Spodoptera frugiperda* Cells Infected with *Autographa californica* Nuclear Polyhedrosis Virus

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Viral RNA from fall armyworm (*Spodoptera frugiperda*) cells infected with *Autographa californica* nuclear polyhedrosis virus contains cap structures. Most of the cap labeled in vivo with [^{3}H]methionine or $^{32}P_i$ cochromatographed on DEAE-cellulose with the −5 charge marker; a minor component appeared at −4 net charge. The former is probably a cap 1 structure ($m^7GpppX^m_pYp$), and the latter is probably a cap 0 ($m^7GpppXp$). On the basis of relative labeling of the two caps with [^{3}H]adenosine and [^{3}H]guanosine, we concluded that each cap contained at least one adenosine residue in addition to guanosine and, therefore, that cap 0 contained $m^7GpppAp$. Cleavage of [^{3}H]methionine-labeled viral RNA with tobacco acid pyrophosphatase released a labeled component that cochromatographed on polyethyleneimine-cellulose with m^7Gp, confirming the m^7GpppX linkage in the cap. These results were also seen with host polyadenylated RNA. The caps appeared in nearly equal abundance in viral polyadenylated and non-polyadenylated RNAs. The ratio of $^{32}P_i$ incorporated into the cap to that incorporated into mononucleotides suggested average lengths for the polyadenylated and non-polyadenylated RNAs of 1,800 and 1,200 nucleotides, respectively.

Since the cap structure was first identified by Furuichi (7) at the 5′ terminus of mRNA from cytoplasmic polyhedrosis virus, caps have been reported in most eucaryotic mRNAs. RNAs from certain animal (2, 6) and plant (4) viruses and lower eucaryotes (5, 20) contain a cap 0 structure ($m^7GpppXp$) (19). Other viral mRNAs (8, 22) and mRNAs from higher eucaryotes contain a cap 1 with one additional methyl group ($m^7GpppX^m_pYp$) (1). Mammalian mRNAs have a minor amount of a further methylated cap species, cap 2 ($m^7GpppX^m_pY^m_pZp$) (3). These cap species all resist hydrolysis by alkali or RNase T_2 because of the unusual 5′-5′ triphosphate linkage and the 2′-O-methylation on the X and Y residues that prevents formation of the 2′,3′ cyclic hydrolysis intermediate. These early studies are reviewed in reference 19.

Nuclear polyhedrosis virus (NPV) is a baculovirus whose members have been isolated from hundreds of insect species. Its virions are contained in polyhedral inclusion bodies that are composed mainly of a viral structural protein called polyhedrin. The alfalfa looper (*Autographa californica*) NPV is easily grown in cultured fall armyworm (*Spodoptera frugiperda*) cells (9) and is therefore a convenient model for studying all of the NPVs. From the host cells, we obtained three forms of RNA polymerase which, after purification, had the characteristics of the classic polymerases I, II, and III (11). Using isolated nuclei from infected cells, we have shown that most of the late viral RNA synthesis is not inhibited by α-amanitin and is therefore not effected by host RNA polymerase II (10).

This unique finding raised the question of whether the bulk of the viral RNA is capped, as capping occurs primarily on mRNA and its precursors, which are normally made by polymerase II. The present study shows for the first time that NPV RNA is indeed fully capped and elucidates some characteristics of the caps.

MATERIALS AND METHODS

Biochemicals. Tobacco acid pyrophosphatase (TAP) was purchased from Bethesda Research Laboratories, Inc. RNase T_2 (*Aspergillus oryzae*) grade VI was from Sigma Chemical Corp. $^{32}P_i$ (25 mCi/ml, carrier free) and L-[*methyl*-^{3}H]methionine (12 Ci/mmol) were from New England Nuclear Corp. [8-^{3}H]guanosine (14 Ci/mmol) was from ICN, Chemical & Radioisotope Div. [2,8-^{3}H]adenosine (33 Ci/mmol) was from Schwarz/Mann. DEAE-cellulose (fine) was from Sigma. Polyethyleneimine (PEI)-cellulose F (precoated thin-layer chromatography plastic sheets; layer thickness, 0.1 mm) was from Brinkmann Instruments, Inc. Oligodeoxythymidylate [oligo(dT)]-cellulose (type 3)

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was from Collaborative Research, Inc. *m*-Aminobenzyl-oxymethyl-cellulose was from Miles-Yeda Ltd. Complete cocktail 3a70 for liquid scintillation counting was from Research Products International Corp. All other chemicals were reagent grade.

Viruses and cells. The continuous *S. frugiperda* cell line IPLB-SF-21 was cultured and infected by nonoccluded virions of *A. californica* NPV on a supplemented TC-100 medium as described previously (10).

Labeling of RNA in vivo. For labeling host polysomal RNA, *S. frugiperda* cells were grown to near confluency (ca. 2 days after cells were transferred). The medium was removed, and each plate of cells was labeled for 4 h with 100 μCi of [*methyl*-^{3}H]methionine in 5 ml of methionine-free medium, 2.5 mCi of $^{32}P_i$ in 5 ml of phosphate-free medium, or 100 μCi of [8-^{3}H]guanosine or [2,8-^{3}H]adenosine in 5 ml of normal medium. After being labeled, the cells were harvested and the RNA was isolated. For labeling the viral RNA, the cells were infected at a multiplicity of infection of 10 to 20 50% tissue culture infective doses per cell 24 to 48 h after being seeded. From 1.0 to 1.5 h was allowed for adsorption. Infected cells were labeled as described above for 4 h (16 to 20 h postinfection). Polyhedral inclusion bodies were clearly visible in the infected cells under a light microscope. At 24 h postinfection, about 90% of the cells contained polyhedral inclusion bodies.

Isolation of host poly(A)$^+$ RNA. The polysomes were isolated from the cells by the procedure of Palmiter (16), modified as follows. Labeled cells from 10 plates were harvested by scraping and then centrifuged at 4,000 rpm in a Beckman JS13 rotor for 5 min. The cell pellet was suspended in 8 ml of buffer A (25 mM NaCl, 5 mM $MgCl_2$, 25 mM Tris-hydrochloride [pH 7.5], 2% Triton X-100, 1 mg of heparin per ml) and homogenized with 10 passes of a type A rod in a Dounce homogenizer. The extract was then centrifuged at 8,000 rpm (JS13 rotor) for 20 min. The supernatant was collected, mixed with an equal volume of buffer B (25 mM NaCl, 200 mM $MgCl_2$, 25 mM Tris-hydrochloride [pH 7.5], 2% Triton X-100, 1 mg of heparin per ml), and then put on ice for 1 h. The polysome suspension was layered slowly over 8 ml of buffer C (25 mM NaCl, 5 mM $MgCl_2$, 25 mM Tris-hydrochloride [pH 7.5], 1 M sucrose) and spun down at 8,000 rpm (JS13 rotor) for 50 min. All of the processes described above were performed at 0 to 4°C. The supernatant was decanted, and the pellet of polysomes was dissolved in 4 ml of NETS (10 mM Tris-hydrochloride [pH 7.5], 0.1 M NaCl, 0.5% sodium dodecyl sulfate [SDS], 5 mM EDTA). This polysome preparation was used immediately or stored at −70°C until needed.

Before extraction, 3 M sodium acetate (pH 5.2) was added to the polysome solution to a final concentration of 0.05 M. The extract was mixed with 4 ml of phenol saturated with NETS in 0.05 M sodium acetate and blended with a Vortex mixer for 3 min. Next, 4 ml of Sevag solution (chloroform-isoamyl alcohol, 24:1) was added, and the mixture was blended with a Vortex mixer for 3 min and then centrifuged at 8,000 rpm in the JS13 rotor for 10 min. The aqueous phase was collected and extracted once more as described above. It was then extracted once with Sevag solution only and twice with ether. The residual ether was removed with a stream of nitrogen. One-tenth volume of 3 M sodium acetate (pH 6.0) was added to the aqueous phase, and the RNA was precipitated with 2 volumes of 95% ethyl alcohol (EtOH) at −20°C overnight. The pellet was collected by centrifugation at 8,000 rpm (JS13 rotor) for 20 min. It was washed with cold 95% EtOH and dried under vacuum. The polysomal RNA was dissolved in 1 ml of TE buffer (10 mM Tris-hydrochloride [pH 7.5], 1 mM EDTA).

The polysomal RNA was boiled in a water bath for 60 s and then chilled in ice water. It was then brought to 0.4 M in NaCl by the addition of a 5 M salt solution and poured onto a 0.2-ml oligo(dT)-cellulose column. The flow-through was collected, and the column was then washed with 1 ml of wash buffer (100 mM Tris-hydrochloride [pH 7.5], 0.4 M NaCl, 1 mM EDTA, 0.2% SDS). The flow-through and wash, combined, comprised the non-polyadenylated [poly(A)$^-$] RNA fraction. The column was washed three more times with 0.5 ml of wash buffer. The polyadenylated [poly(A)$^+$] RNA was then eluted with 0.3 ml of elution buffer (10 mM Tris-hydrochloride [pH 7.5], 0.1% SDS, 1 mM EDTA). The column was cleaned with 1 ml of 0.1 N NaOH and washed twice with 1 ml of sterile water and once with 1 ml of wash buffer. The poly(A)$^+$ and poly(A)$^-$ RNAs were purified again by being passed through the cleaned oligo(dT)-cellulose column and precipitated with 2 volumes of 95% EtOH as described above but with an Eppendorf centrifuge at 15,000 rpm for 10 min.

Isolation of viral mRNA. The polysomal RNA was isolated from infected cells at 20 h postinfection and separated into poly(A)$^+$ and poly(A)$^-$ fractions as described above. Virus-specific RNA was purified from these fractions by hybridization to viral DNA-cellulose as follows. Viral DNA-cellulose was prepared as described by Noyes and Stark (14). The *A. californica* NPV DNA was isolated from inclusion bodies as described by Miller and Dawes (13). The viral DNA-cellulose contained about 10 μg of DNA per mg. The pellet of polysomal RNA from infected cells was dissolved in 600 μl of hybridization buffer (0.1 M Tris-sulfate [pH 7.4], 10 mM EDTA, 0.1% SDS, 0.6 M NaCl, 50% formamide) at a concentration of 0.5 to 1 mg of RNA per ml. This solution was supplemented with 20 μl of polyadenylate (10 μg/μl of sterile water) and then mixed with 12 to 24 mg of viral DNA-cellulose and heated at 80°C for 1 min.

Hybridization was performed for 24 h at 37°C; the cellulose was kept in suspension by rocking. To stop hybridization, the cellulose was spun down in the Eppendorf centrifuge for 5 min. The unhybridized RNA was then removed by two washes with hybridization buffer and two washes with cold 2× SSC (0.15 M NaCl, 0.015 M sodium citrate [pH 7.5]). The virus-specific RNA was then eluted by suspending the DNA-cellulose twice in 150 μl of 99% formamide–0.1% SDS solution and heating at 60°C for 1 min. The eluted viral RNA was diluted with sterile water to give 66% formamide and precipitated with EtOH at −20°C. The efficiency of hybridization was 60 to 80%.

Thin-layer chromatography. To detect the blocking terminal nucleotide of the cap (m^7Gp), we labeled host poly(A)$^+$ and virus-specific RNAs with [*methyl*-^{3}H]methionine in vivo, isolated them as described above, and then hydrolyzed them by TAP in assay buffer (50 mM sodium acetate [pH 5.4], 10 mM 2-mercaptoethanol, 1 mM disodium EDTA). The RNAs were dissolved in 30 μl of assay buffer, mixed with 12

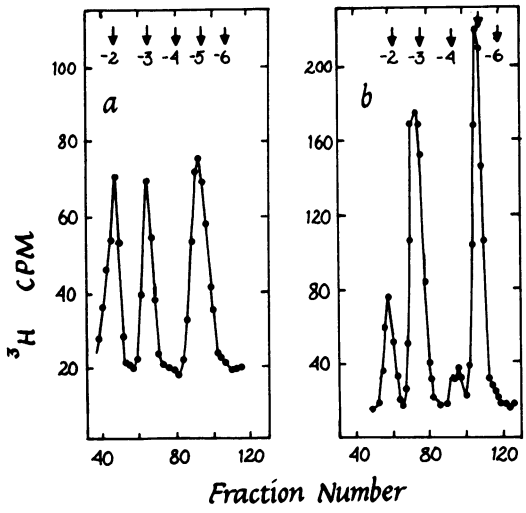

FIG. 1. Isolation of putative cap structures from host and viral RNAs. Host poly(A)$^+$ RNA (a) and total viral polysomal RNA (b) were labeled with [*methyl*-^{3}H]methionine in vivo, purified, base hydrolyzed, and chromatographed on DEAE-cellulose as described in the text. Arrows mark the positions of oligo(A) markers of the indicated charges.

U of TAP, and incubated at 37°C for 3.5 h, after which the reaction was stopped by boiling the mixture for 3 min. The total digests were then applied to thin layers of PEI-cellulose that had been run earlier with distilled water. GMP and m^{7}Gp were used as markers in a parallel lane and visualized under UV light. The strip of PEI-cellulose was washed for 10 min in methanol, dried, and subjected to ascending chromatography with 1 N acetic acid up to 4 to 5 cm and then with 0.3 M LiCl up to 15 to 16 cm (18). After drying, the strip was divided into 1-cm-wide sections, and the PEI-cellulose from each section was scraped from the sheet into 2.5 ml of 3a70 scintillation fluid and counted.

DEAE-cellulose chromatography. Labeled host or viral RNAs were digested with 0.3 M KOH at 37°C for 20 h. The digest was neutralized with 70% perchloric acid, and after removal of the insoluble potassium perchlorate by centrifugation, the products were diluted 10 times with 10 mM Tris-hydrochloride (pH 7.5)–7 M urea and mixed with the unlabeled marker oligoadenylate [oligo(A)]. The sample was applied to a DEAE-cellulose column (0.6 by 20 cm) equilibrated with 10 mM Tris-hydrochloride buffer (pH 7.6) containing 7 M urea and then eluted in six-drop fractions (270 μl) with 50-ml linear gradients of 0 to 0.35 M NaCl in the same buffer (21).

Absorbance at 254 nm was monitored and recorded continuously, and the radioactivity in each fraction was counted in a complete scintillation fluid (3a70).

RESULTS

Detection of a putative cap structure in viral and host RNAs. Polysomal RNAs in infected cells were labeled with [*methyl*-^{3}H]methionine, and viral RNA was purified by hybridization to viral DNA-cellulose. Host RNA was purified from similarly labeled, uninfected cells and separated into poly(A)$^+$ and poly(A)$^-$ fractions by oligo(dT)-cellulose chromatography. All three classes of RNA were then hydrolyzed with 3 M KOH, and the digests were chromatographed on DEAE-cellulose. The viral RNA digest showed four well-defined, methylated peaks comigrating with markers with charges of −2, −3, −4, and −5 (Fig. 1b). The peak at charge −5 was most prominent and eluted in a position consistent with its assignment as a cap 1 (m^{7}GpppX$_p^m$Yp) (19).

Identification of a cap 1 structure on viral RNA. The peak eluting in the −5 position was also prominent in the host poly(A)$^+$ RNA digest (Fig. 1a). This suggested that it was a cap 1 structure, because cap 1 predominates in mRNA of higher eucaryotes (19). This was confirmed by chromatographing L-cell [^{32}P$_i$] poly(A)$^+$ RNA, which is known to contain mostly cap 1 (17); in our system it eluted close to the −5 marker, just as the putative viral and host cap structures did (Fig. 2). This in turn indicated that the peak at −4 was probably a cap 0. The peak at −2 probably represented methylated mononucleotides (mXp) in which the base is methylated, and the peak at −3 probably represented internal 2′-*O*-methylation, which gives rise to X$_p^m$Yp upon hydrolysis.

Typical cap structures contain m^{7}G linked to the penultimate nucleotide through a triphosphate group. The presence of such a structure in viral RNA was verified as follows: viral and host [*methyl*-^{3}H]methionine-labeled RNAs were digested with TAP, and the products were subjected to thin-layer chromatography on PEI-cellulose. Unlabeled markers (GMP and m^{7}Gp) were run in a parallel lane. Both viral and host RNAs showed peaks of radioactivity that coincided with the position of the m^{7}Gp marker (Fig. 3). In fact, this was the only significant radioactive peak aside from that of the residual RNA at the origin. The radioactivity in these peaks relative to that in the residual RNA peaks was about half what would be expected from the quantitative recovery of a single methyl group in m^{7}Gp.

This conclusion is derived from an inspection of the relative amounts of label in the various peaks shown in Fig. 1; these data are shown in Table 1, along with data on host poly(A)$^-$ RNA. Host cap 1 contained 45% of the methyl label in host poly(A)$^+$ RNA, whereas caps 1 and 0 from viral RNA contained 41 and 4%, respectively (Table 1). Because the m^{7}Gp portion of cap 1 would contain only half of the methyl label in the cap (the other half being in the 2′-*O*-methyl group on residue X), the expected percentage of methyl label in m^{7}Gp after TAP digestion would be about 20% of the total, assuming a quantita-

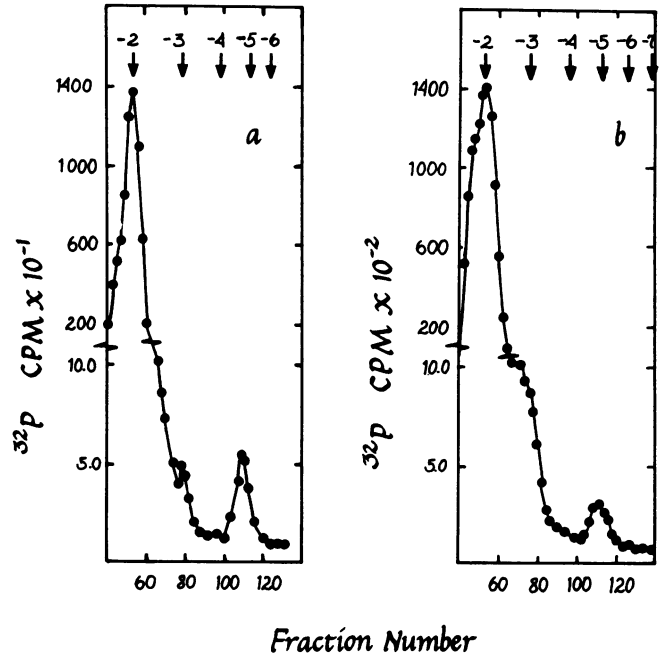

FIG. 2. Host and L-cell poly(A)$^+$ RNAs containing cap structures with identical chromatographic properties. RNAs were labeled with $^{32}P_i$ in vivo and purified, base hydrolyzed, and chromatographed on DEAE-cellulose as described in the text. (a) Host poly(A)$^+$ RNA; (b) L-cell poly(A)$^+$ RNA. Arrows show the positions of oligo(A) markers of the indicated charges.

tive yield. In fact, it constituted 10 and 8%, respectively, in host poly(A)$^+$ and viral RNAs. These numbers may reflect incomplete hydrolysis of RNA by TAP or the trapping of m^{7}Gp in the residual RNA at the origin.

Detection of adenosine and guanosine in the viral caps. Host and viral RNAs were labeled with [^{3}H]adenosine or [^{3}H]guanosine, hydrolyzed, and subjected to chromatography on DEAE-cellulose as described above. The radioactivity incorporated by the total viral RNA was 6.1 × 10^5 and 2.2 × 10^5 cpm of guanosine and adenosine, respectively. Cap 1 was labeled with adenosine and guanosine (210 and 65 cpm, respectively), demonstrating the presence of both in the cap. Cap 0 was also labeled with adenosine (65 cpm), suggesting that at least one cap structure in viral RNA is m^{7}GpppAp. The rela-

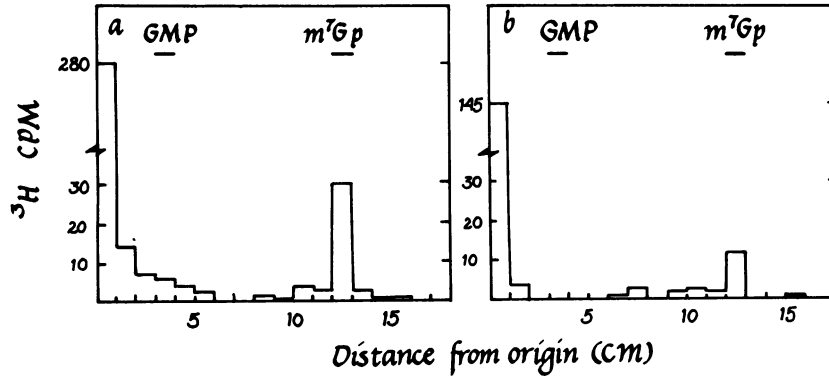

FIG. 3. Identification of the blocking nucleotide in host poly(A)$^+$ and total viral polysomal RNAs. The RNAs were labeled with [*methyl*-^{3}H]methionine in vivo, purified, hydrolyzed with TAP, and subjected to thin-layer chromatography on PEI-cellulose as described in the text. The positions of the markers (GMP and m^{7}Gp) are shown with horizontal bars. (a) Host poly(A)$^+$ RNA; (b) total viral polysomal RNA.

TABLE 1. Distribution of methylated components in host and viral RNAs

RNA	*methyl*-^{3}H incorporation (cpm)[a] into species of charge:			
	−2	−3	−4	−5
Host poly(A)$^+$	325 (27)	335 (28)		545 (45)
Host poly(A)$^-$	59,800 (13)	384,000 (87)		1,840 (0.03)
Total viral	325 (11)	1,320 (44)	120 (4)	1,200 (41)

[a] The radioactivity in each peak shown in Fig. 1 [and in a similar experiment involving host poly(A)$^-$ RNA] is presented. Numbers in parentheses indicate the percentages of total radioactivity in all of the peaks.

tive inefficiency of labeling caps with [8-^{3}H]guanosine probably accounts for the lack of detectable label in cap 0 with this substrate.

Viral poly(A)$^+$ and poly(A)$^-$ RNAs capped. Viral and host RNAs were labeled with $^{32}P_i$ in vivo, extracted, separated into poly(A)$^+$ and poly(A)$^-$ fractions, hydrolyzed, and chromatographed as described above. Viral poly(A)$^+$ and poly(A)$^-$ RNAs both showed prominent cap 1 peaks and easily detectable cap 0 peaks (Fig. 4).

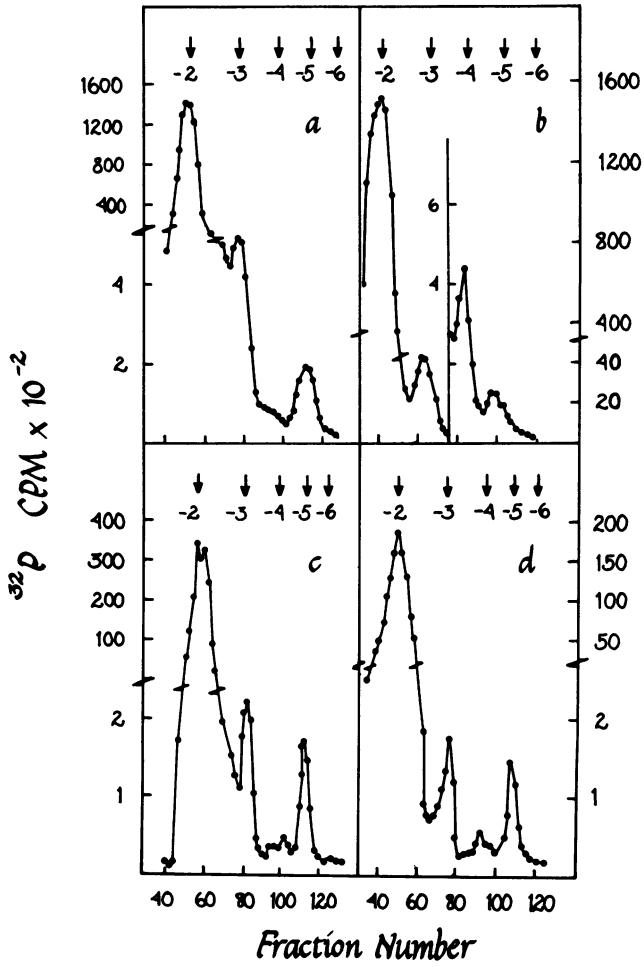

FIG. 4. Detection of cap structures in host and viral poly(A)$^+$ and poly(A)$^-$ RNAs. Host polysomal RNA in uninfected cells was labeled with $^{32}P_i$, purified, separated into poly(A)$^+$ and poly(A)$^-$ fractions, hydrolyzed, and chromatographed on DEAE-cellulose as described in the text. Viral polysomal RNA was treated the same way, except that it was purified from cells at 20 h postinfection and separated into viral and nonviral species by hybridization to viral DNA-cellulose after oligo(dT)-cellulose chromatography. (a) Host poly(A)$^+$ RNA; (b) host poly(A)$^-$ RNA; (c) viral poly(A)$^+$ RNA; (d) viral poly(A)$^-$ RNA. Arrows show the positions of oligo(A) markers of the indicated charges.

Host poly(A)$^+$ RNA also showed a cap 1 peak, but cap 0, if present at all, was barely detectable. The host poly(A)$^-$ pattern was somewhat difficult to interpret because this fraction contained massive quantities of methylated rRNA and tRNA. This resulted in a large methylated dinucleotide peak appearing at charge −3 (Table 1) and another major species at charge −4 (Fig. 4b). The latter is probably mostly pXp from the 5′ terminus of poly(A)$^-$ RNA, because it appeared when poly(A)$^-$ RNA was labeled with $^{32}P_i$ but not [*methyl*-^{3}H]methionine. The peak at charge −5 (Fig. 4b) was not as easy to identify. It was methylated; it appeared in RNA labeled with $^{32}P_i$ or methionine. It is possible that it represents a cap 1 structure in a small proportion of the poly(A)$^-$ fraction.

Length estimation of host and viral RNAs. The amount of label in each cap peak shown in Fig. 2, 4c, and 4d was determined. The ratio of the radioactivity incorporated into the cap to that incorporated into mononucleotides (Table 2) enabled us to estimate the maximum average length of the RNA species in each class assuming that all of the RNA species were capped. If some were not capped, then the average length would have been smaller. For these calculations, we assumed that the numbers of $^{32}P_i$-labeled nuclei incorporated into cap 1, cap 0, and the mononucleotide were five, four, and one, respectively. Using these calculations, we found that the maximum average lengths of viral poly(A)$^+$ and poly(A)$^-$ RNAs were 1,800 and 1,200 nucleotides, respectively, as compared with the values for L-cell and host poly(A)$^+$ RNA lengths of 2,400 and 5,400 nucleotides, respectively (Table 2).

TABLE 2. Estimated average sizes of viral and host RNAs[a]

RNA	$^{32}P_i$ (cpm) incorporated into:			Estimated size (nucleotides)
	Cap 1	Cap 0	Total RNA	
L-cell poly(A)$^+$	330		1.60×10^5	2,400
Host poly(A)$^+$	1,520		1.65×10^6	5,400
Viral poly(A)$^+$	800	120	3.37×10^5	1,800
Viral poly(A)$^-$	760	190	2.38×10^5	1,200

[a] The data shown in Fig. 2 and 4 are presented here. RNA sizes were estimated as described in the text.

DISCUSSION

These studies showed that both poly(A)$^+$ and poly(A)$^-$ NPV RNAs contain structures that have the properties of typical caps 0 and 1. We left unanswered the question of the exact chemical nature of the bases in the caps, other than noting that both adenosine and guanosine were present. Because of the partial exchange of ^{3}H in the 8 position of guanosine upon methylation to m^{7}G (19) and the possible differences in the intracellular specific activities of GTP and ATP, the ratio of labeled adenosine to labeled guanosine incorporation cannot be taken as a measure of the ratio of adenosine to guanosine in a cap. Because our RNA preparations were mixtures of different species, it did not seem productive to pursue the identification of specific bases. Identification will have to await purification of mRNAs. However, it is worth noting that the cap peaks seemed considerably broader in host and L-cell RNAs than in viral RNA. This suggests a more heterogeneous population of caps in the host poly(A)$^+$ RNA than in the viral mRNAs.

The peaks in the host poly(A)$^-$ RNA fraction were intriguing but difficult to interpret confidently, because this fraction consists mostly of rRNA and tRNA, which are not capped but are highly methylated. This means that the $^{32}P_i$ peaks at charges −3 and −4 could plausibly be interpreted as X_p^mYp and pXp, respectively. The *methyl*-^{3}H-labeled peak at charge −5 most likely represents cap 1 of poly(A)$^-$ RNA, which is well known in eucaryotes (12, 15). The only difficulty with this interpretation is the high absolute amount of this species [ca. three times higher than that of host poly(A)$^+$ RNA].

As suggested above, the calculated average lengths of viral mRNAs could be somewhat inflated bacause of the possibility that some noncapped species were present in the viral RNA populations. However, these calculations yielded reasonable values that were consistent with the sizes observed upon electrophoresis of viral RNAs (data not shown). These experiments thus indicate that most of the viral polysomal poly(A)$^+$ and poly(A)$^-$ RNAs are capped and therefore probably mRNAs.

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