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mRNA Capping Enzymes Are Masked in Reovirus Progeny Subviral Particles

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We examined the enzyme activities associated with progeny subviral particles isolated from L-cells infected with reovirus at 12 h postinfection. Activities normally present in reovirus cores were also found to be present in the progeny subviral particles, with the exception of the capping enzymes. The methylase and guanyl transferase activities, which constitute the capping system, were present in a masked form that could be activated by chymotrypsin digestion. The appearance of these progeny subviral particles in infected cells coincided with the time when mRNA synthesis was maximal, suggesting that viral mRNA synthesized at later times is uncapped.

Enzymes associated with reovirions are known to catalyze at least five distinct enzymatic reactions of which only one, the oligoadenylic acid synthetase (22), is active in intact virions. The reovirus-specific enzyme activities include: an RNA-dependent RNA polymerase or transcriptase (2, 15, 17); a nucleotide phosphohydrolase (3, 11); a pyrophosphate exchange (23); and the capping activities, which consist of an RNA methylase (6, 14) and guanyl transferase (8, 14). The latter enzyme activities are masked or latent in intact virions, but are activated in vitro when intact virions are reduced to viral cores by digestion with chymotrypsin (14). (Carter [4] has reported virion-associated guanyl transferase and methylating activities in virions under conditions distinct from those resulting in mRNA methylation.) These core-associated enzyme activities are all well characterized (for review, see reference 16).

During reovirus infection, two distinct populations of subviral particles (SVPs) are responsible for transcription of the 10 double-stranded RNA genome segments. The first of these consists of parental SVPs, which result from intracellular uncoating of the infectious virions. These particles initiate the infection, producing capped mRNA's (5, 9) which are translated into viral polypeptides by the host translational apparatus. Several lines of evidence indicate that only the mRNA molecules transcribed from these SVPs serve as template for progeny minus-strand synthesis (7, 13, 16, 21). The second population of SVPs active in mRNA transcription is comprised of particles containing progeny double-stranded RNA, which are referred to as progeny SVPs. Indications are that the bulk of the mRNA that is translated into viral polypeptides is transcribed from the genome of these progeny SVPs.

In the present publication we report on the enzymatic activities associated with progeny SVPs isolated from L-cells at late times postinfection (p.i.), when viral mRNA synthesis is maximal. We find that the activities present in reovirus cores are also found in progeny SVPs, with two notable exceptions. The mRNA capping enzymes are not expressed unless the progeny SVPs are first treated with chymotrypsin. In an accompanying report (24) we show by direct analysis of the 5'-terminal structure that mRNA transcribed from progeny SVPs is indeed uncapped. This is of great interest in view of work we reported recently (19) indicating that changes in the capping pattern of viral mRNA may be involved in takeover of protein synthesis in L-cells infected with reovirus.

MATERIALS AND METHODS

Cells, reovirus, and reovirus mRNA. Reovirus, type 3 (Dearing strain), was propagated in suspension cultures of mouse L-cells as described by Smith et al. (20) and purified by freon extraction and isopycnic banding in CsCl density gradients. Reovirus mRNA was synthesized in vitro as described previously (6). When radioactive precursors were used, the concentration of the corresponding nucleoside triphosphate (NTP) was lowered to 0.4 mM. mRNA was purified as described previously (18). Radioactive *S*-adenosyl methionine (SAM) was used at a final concentration of 3 μM and a specific activity of 70 to 80 Ci/mmol. Progeny SVPs were present to a final concentration of 2 to 3 absorbancy units at 260 nm per ml.

Preparation of LP fraction from infected cells. Cells were infected at a multiplicity of infection of 5 PFU/cell, and infection was allowed to proceed for 12 h at 37°C. At this time, the infected cells were harvested by centrifugation at 800 rpm for 10 min in an IEC refrigerated centrifuge and washed twice cold phosphate-buffered saline. The cells were suspended in 3 volumes of 50 mM Tris-hydrochloride (pH 8.0), 1 mM $MgCl_2$, 0.25 M sucrose, and 1% Nonidet P-40 and

blended in a Vortex mixer to lyse the cells. Nuclei were removed by centrifugation at 800 × *g* for 10 min. This extraction was repeated, and postnuclear supernatants were combined.

The postnuclear supernatants were made 1% in sodium deoxycholate (using 10% stock solution), and a large-particle (LP) fraction was prepared by centrifugation of the mixture in an SW40 rotor for 3 h at 186,000 × *g*. The resulting pellet was suspended by sonication in a small volume of 50 mM Tris-hydrochloride (pH 8)–1 mM $MgCl_2$ and immediately fractionated by velocity sedimentation in glycerol density gradients.

Glycerol density gradient fractionation of LP fraction. Resuspended LP fraction was layered onto a 20 to 40% glycerol gradient containing 10 mM Tris-hydrochloride (pH 8.0) and centrifuged at 25,000 rpm for 90 min in an SW27 rotor. The top 12 ml of the gradient was discarded, and the remaining portion was divided in half, collecting from the bottom. The resulting fractions are called "fast" (whole virions) and "slow" (subviral particles) sedimenting fractions. These fractions were then pelleted in an SW40 rotor at 183,000 × *g* for 4 h and suspended by mild sonication in 0.1 M Tris-hydrochloride (pH 8). Samples were then fast frozen in an acetone-Dry Ice bath and stored at −70°C until used.

Electron microscopy and polyacrylamide gel electrophoresis of the fast- and slow-sedimenting fractions. Fast- and slow-sedimenting fractions were prepared as described above. The viral particles in these two fractions were purified free from cellular debris by freon extraction (20) and centrifugation through a cushion of 15% sucrose. Fractions were negatively stained with 1% ammonium molybdate and examined in a Philips 300 electron microscope at ×50,800 magnification and an operating voltage of 60 kV. Polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (12).

Analysis of pyrophosphate exchange activity of SVPs. Analysis was essentially as described by Wachsman et al. (23). SVPs (0.5 absorbancy units at 260 nm), in a final volume of 100 μl, were incubated at 37°C for 60 min with 2 mM [^{32}P]pyrophosphate (2.5 × 10^4 cpm/nmol) and 2 mM each of the NTPs. Reactions were stopped by cooling on ice, and 0.5 ml of 0.1 M sodium pyrophosphate was added with 10 μl of 1% bovine serum albumin and 1 ml of 10% trichloroacetic acid. Samples were centrifuged in the cold for 10 min at 12,000 × *g*. To each sample was added 0.2 ml of 15% (wt/vol) activated charcoal and 1.0 of 0.5 M sodium acetate (pH 5.2). Samples were left for 30 min on ice, filtered through glass fiber paper (Whatman GF/A), washed with 0.1 M sodium acetate (pH 5.2) containing 0.01 M sodium pyrophosphate, dried, and counted in 5 ml of toluene-based scintillation fluid in an Intertechnique liquid scintillation spectrometer.

NTP phosphohydrolase assay. Assay was according to Kapuler et al. (11). Reaction mixtures contained [α-^{32}P]ATP to a final concentration of 0.4 mM (final specific activity about 1 Ci/mmol). Reaction mixtures contained everything required for mRNA synthesis (6) except the NTP regenerating system. Final reaction mixtures, in 50 μl, contained 0.1 absorbancy units at 260 nm of SVPs or virions treated with chymotrypsin. Reactions were carried out at 37°C. At the times specified, 5-μl samples were removed and spotted onto PEI-cellulose thin-layer plates (Polygram CEL 300, PEI, Machery-Nagel) along with 0.3 μmol each of AMP, ADP, and ATP. The chromatogram was developed in 1.4 M LiCl and dried. Unlabeled markers were visualized under UV light, and then the spots were cut out and the radioactivity was determined in toluene-based scintillation cocktail.

Radiochemicals. [*methyl*-^{3}H]SAM (about 70 Ci/mmol), [^{3}H]CTP (24 Ci/mmol), [α-^{32}P]ATP (340 Ci/mmol), and [^{32}P]sodium pyrophosphate (about 2,500 Ci/mmol) were purchased from New England Nuclear Corp, Boston, Mass.

RESULTS

Preparation and characterization of SVPs. The LP fraction from infected cells (12 h p.i. at 37°C) was prepared as described in Materials and Methods. This material was fractionated in a glycerol gradient and divided into fast-sedimenting and slow-sedimenting fractions to be assayed for enzyme activities. The morphology of particles in both fractions was examined by electron microscopy. Figure 1A shows a typical field of particles found in the slow-sedimenting fraction of the glycerol gradient. The particles displayed the characteristic morphology of SVPs as distinguished from whole virions. A count of 503 particles revealed that only 12 (2.4%) had the morphology of whole virions, indicating that the level of contamination of the slow-sedimenting fraction with whole virions is very low. Electron microscopy of the fast-sedimenting fraction showed it to contain exclusively particles with the morphology of whole virions (Fig. 1B).

The polypeptides of the morphologically distinct particles in these two fractions were analyzed by polyacrylamide gel electrophoresis (Fig. 2). Viral particles in the fast-sedimenting fraction gave a polypeptide pattern (Fig. 2, lane C) indistinguishable from that of purified virions (Fig. 2, lane A). On the other hand, the polypeptide pattern of the SVPs in the slow-sedimenting fraction (Fig. 2, lane B) was quite distinct from that of purified virions. Although the relative intensity of staining in the λ and σ_1 regions was similar for the two fractions, polypeptides μ-1, μ-2, and σ-3 were clearly absent in the SVPs found in the slow-sedimenting fraction. Although a very faint band was observed in the region of μ-2, we tentatively ascribe this to a low level of contamination (2 to 3%, as judged from electron micrographs) of the slow-sedimenting fraction with whole virions.

Assay of RNA polymerase and capping activities in the fast- and slow-sedimenting fractions extracted from L-cells infected with reovirus. Standard RNA polymerase re-

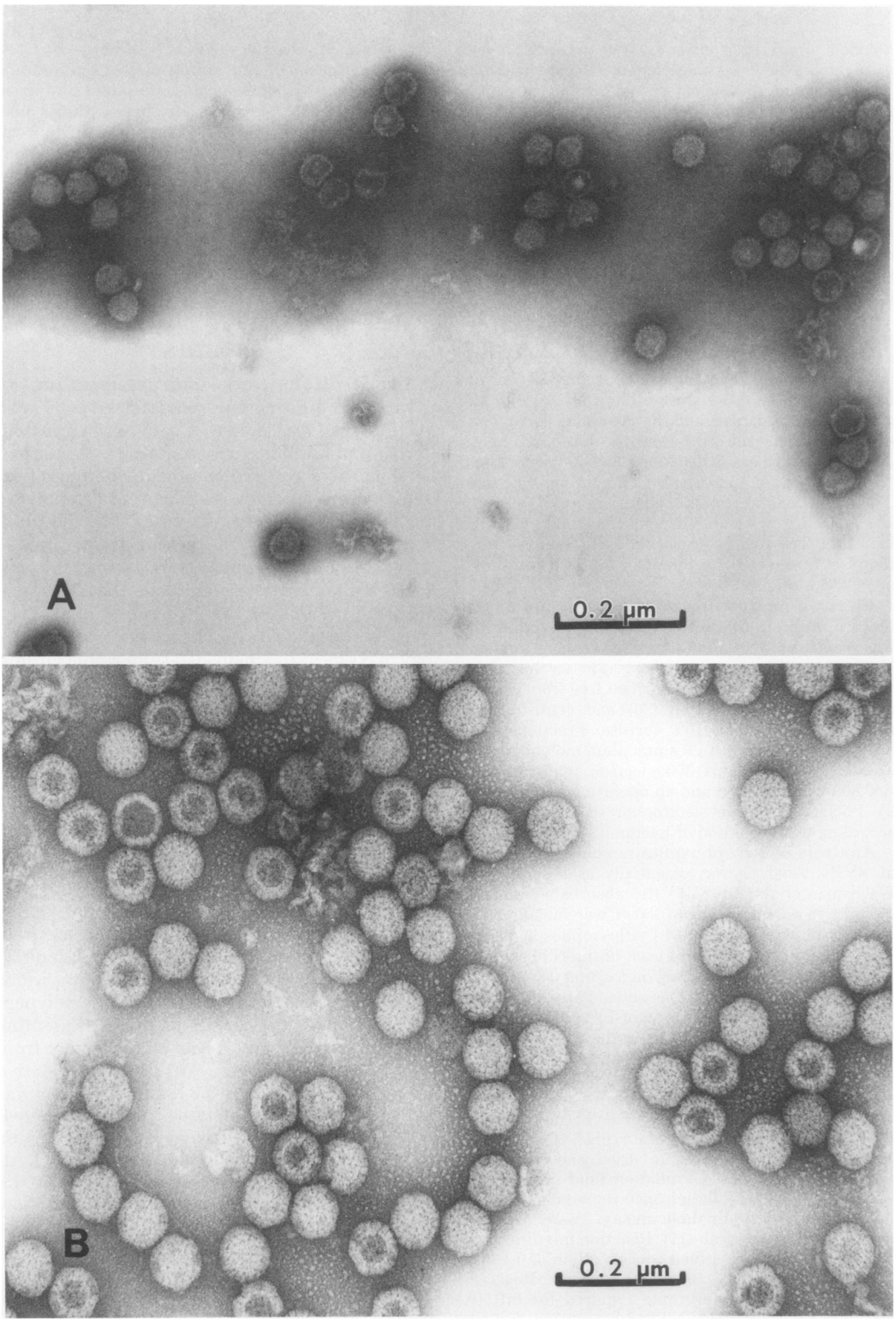

FIG. 1. *Electron micrographs of slow- and fast-sedimenting fractions. Viral particles prepared as described in the text were negatively stained with 1% ammonium molybdate. Final magnification is ×100,000. (A) Slow-sedimenting fraction. (B) Fast-sedimenting fraction.*

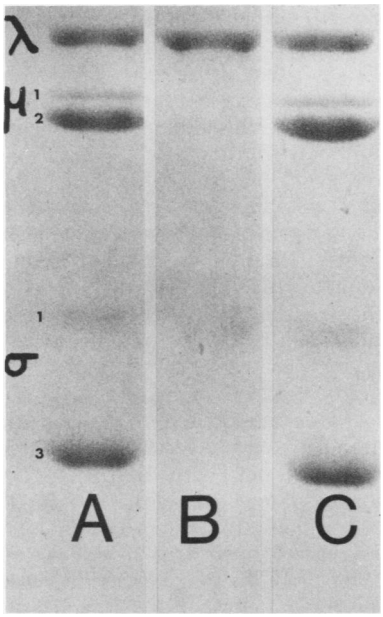

FIG. 2. *Polypeptide analysis of viral particles from the fast- and slow-sedimenting fractions. Viral particles were electrophoresed in a 10% polyacrylamide gel according to Laemmli (12), and gels were stained with Coomassie blue. (A) Purified reovirions. (B) Slow-sedimenting fraction. (C) Fast-sedimenting fraction. Electrophoresis is from top to bottom. The symbols λ, μ, and σ represent the three size classes characteristic of reovirus structural polypeptides (20).*

actions were carried out in the presence of 2 μg of actinomycin D per ml to avoid possible DNA-dependent RNA transcription. Parallel reactions contained either tritiated SAM or tritiated CTP to analyze, respectively, the methylase and RNA polymerase activities present in these fractions. In Fig. 3 we see the plot of RNase-sensitive, trichloroacetic acid-precipitable radioactivity versus time of incubation of the polymerase reaction. It is clear that the fast-sedimenting fraction behaved as we would expect if it consisted of whole virus. Almost no polymerase or methylase activity was present prior to chymotrypsin treatment (Fig. 3c). When particles were pretreated with chymotrypsin (Fig. 3d), both polymerase and methylase activities displayed the same linear kinetics over 90 min of reaction time. The behavior of the subviral particles present in the slow-sedimenting fraction, however, was clearly different. RNA polymerase activity was present before chymotrypsin digestion (Fig. 3a), but no significant incorporation of methyl groups was detected. After chymotrypsin treatment (Fig. 3b), there was no change in polymerase activity, but the methylase activity was expressed at that point and showed the same kinetics as those of the polymerase. We conclude from these observations that the subviral particles that make up this slow-sedimenting fraction have an active polymerase and a masked methylase activity, which can be activated by chymotrypsin digestion.

Pyrophosphate exchange activity of SVPs. Wachsman et al. (23) have reported that reovirus cores have an enzyme activity that can transfer phosphate groups from inorganic pyrophosphate into an organic form as assayed by adsorption to activated charcoal. This activity was found to occur in the presence of all four NTPs. The only single triphosphate that could support this activity was found to be GTP (23). In view of present knowledge of the capping reaction, the activity with GTP alone can be assumed to be due to the guanyl transferase activity of these particles (8).

Table 1 shows that the transfer of [^{32}P]phosphate from inorganic pyrophosphate to an organic form by the progeny SVPs was supported only in the presence of all four NTPs. We note that before treatment with chymotrypsin, these particles did not exhibit GTP-supported pyro-

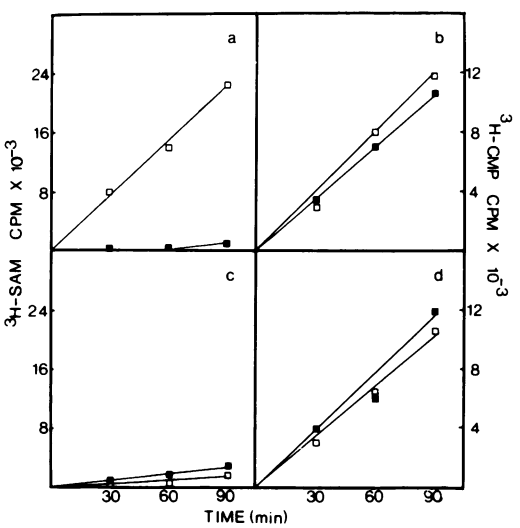

FIG. 3. *Assay of methylase and polymerase activities in progeny SVPs. Standard reaction mixtures, containing about 2 absorbancy units at 260 nm of SVPs and either tritiated SAM or tritiated CTP as described in the text, were incubated at 44°C for the times indicated. Samples were divided in half and precipitated with trichloroacetic acid before and after RNase A digestion. Plotted is trichloroacetic acid-precipitable, RNase-sensitive radioactivity. (a) and (b), Slow-sedimenting fraction; (c) and (d), fast-sedimenting fraction. (a) and (c), Without chymotrypsin digestion; (b) and (d), after chymotrypsin. (■) Incorporation of [methyl-^{3}H]SAM; (□) incorporation of [^{3}H]CMP.*

phosphate exchange activity, although the activity with all four triphosphates was considerable. Chymotrypsin digestion, however, although having no effect on the activity of the enzyme using all four triphosphates, activated exchange dependent on GTP alone. As reported by Wachsman et al. (23), the three NTPs lacking GTP cannot support the reaction. This pattern is consistent with an inactive guanyl transferase in the native SVP, which can be activated by chymotrypsin treatment.

NTP phosphohydrolase activity of SVPs. Another enzymatic activity associated with reovirus cores is the NTP phosphohydrolase (3, 11). This activity makes it necessary to add an NTP-regenerating system to polymerase reactions to maintain linear kinetics over extended periods. To further investigate the similarity of SVPs to chymotrypsin cores, we carried out an assay to determine whether or not this activity is present in our slow-sedimenting fraction (Fig. 4). A 60-min incubation was sufficient to convert 90% of ATP present to ADP. This activity was absent when no viral particles were present. This activity in progeny SVPs was comparable to the activity shown by an approximately equivalent amount of chymotrypsin cores.

Time course of methylase and polymerase activities in slow-sedimenting fraction. We have shown earlier in this report that progeny SVPs purified at the time of maximal RNA synthesis have a masked methylase (Fig. 3). We then looked for the appearance of such particles as a function of time p.i. LP fractions were prepared every 2 h from time 0 to 18 h (37°C) p.i. and divided into fast- and slow-sedimenting fractions as described in Materials and Methods. SVPs were contained in the slow-sedimenting fraction, and whole virions were in the fast-sedimenting fraction (Fig. 1). Each fraction was assayed for methylase and polymerase activities by carrying out transcriptase reactions in the presence of [*methyl*-^{3}H]SAM and [α-^{32}P]CTP. Samples were taken, and trichloroacetic acid-precipitable radioactivity was determined before and after digestion with RNase A. Nuclease digestion was done because this fraction also has double-stranded RNA-synthesizing activity. Figure 5 shows the RNase-sensitive, trichloroacetic acid-precipitable radioactivity generated with the SVPs before (Fig. 5a) and after (Fig. 5b) chymotrypsin digestion. Polymerase activity followed the same time course as single-stranded RNA synthesis in vivo (10) with a two-step increase showing maximal RNA synthesis around 12 h p.i. The pattern of the methylase, however, was totally different: a rapid onset of activity, presumed to belong to parental SVPs that were present in this slow-sedimenting fraction. The methylase activity reached a plateau around 4.5 h and began to drop after 8 h, with little activity remaining by 12 h p.i. When the SVPs were pretreated with chymotrypsin before the assay (Fig. 5b), the methylase activity paralleled the polymerase throughout the whole infectious cycle. This is a direct indication that the methylase in the particles had been activated, as seen already for particles isolated at 12 h p.i. (Fig. 3).

TABLE 1. *Pyrophosphate exchange activity of SVPs*[a]

NTP added	Charcoal-adsorbable pyrophosphate (nmol)	
	No chymotrypsin	Plus chymotrypsin
None	0.01	0.01
ATP + UTP + CTP + GTP	1.12	1.15
ATP + UTP + CTP	0.02	0.02
GTP	0.01	0.29

[a] Assays were performed as described in the text.

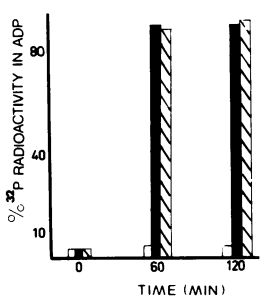

FIG. 4. *ATP-phosphohydrolase activity of progeny SVPs. Assay was as described in the text. Data are plotted as percent radioactivity present as ADP. Open bars, No viral particles; solid bars, reovirus cores; hatched bars, progeny SVPs.*

DISCUSSION

In a recent report (19) we showed that as a result of reovirus infection a transition occurs in the cap-dependent translational machinery of L-cells. This transition occurs gradually throughout the course of infection, and, as an end result, uncapped mRNA is preferentially translated in extracts from cells harvested late in the infectious cycle. The present publication supports the view that changes in the capping pattern of viral mRNA occur during the reovirus replicative cycle. Progeny SVPs isolated at late times p.i. were assayed for the enzyme activities known to be associated with reovirus cores. The SVPs were found to have all the enzyme activities found in cores, with the exception of the capping enzymes. Both the guanyl transferase and the

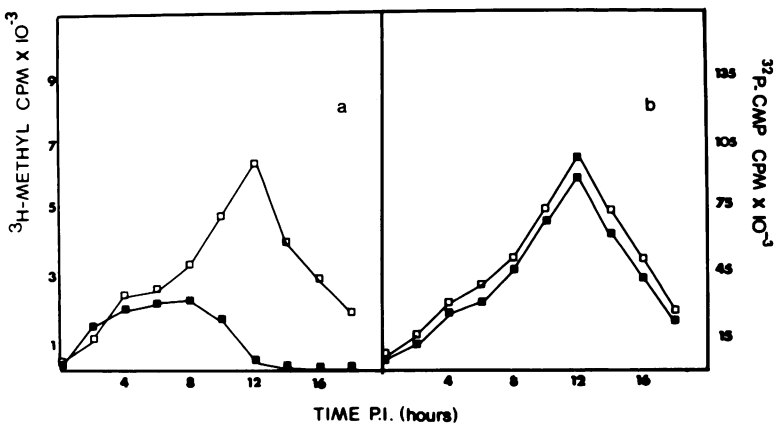

FIG. 5. *Time course of methylase and polymerase activities in SVP preparation. Cells were infected, and samples were taken at the times indicated p.i. SVPs (slow-sedimenting fraction) were prepared from each sample and used to catalyze standard transcriptase reactions containing [methyl-^{3}H]SAM and [α-^{32}P]CTP. Reactions were incubated for 60 min at 44°C. Samples were divided in half and precipitated with trichloroacetic acid before and after RNase digestion. Data are presented as RNase-sensitive, trichloroacetic acid-precipitable radioactivity. (a) Progeny SVPs; (b) progeny SVPs treated with chymotrypsin.* (■) *Tritium;* (□) ^{32}P.

mRNA methylase in progeny SVPs were masked (Table 1 and Fig. 3, respectively). These findings indicate that mRNA transcribed by these SVPs should be uncapped. This prediction is supported by evidence in the accompanying paper (24) that mRNA synthesized by progeny SVPs terminates at the 5′ end with pGpC ... The SVPs studied were isolated from infected cells at the time when intracellular mRNA synthesis was maximal (Fig. 5). One can therefore infer that the bulk of the mRNA translated in infected cells at late times p.i. is transcribed from particles having inactive capping enzymes. This contention is supported by results (manuscript in preparation) indicating that mRNA isolated from polysomes at late times p.i. is also uncapped.

In this study we also looked for the appearance of progeny SVPs having an active polymerase but a masked methylase as a function of time p.i. The results presented in Fig. 5 indicate that the production of uncapped mRNA begins between 4 and 6 h, achieving a maximum at 12 h p.i. A decline in the synthesis of uncapped mRNA occurs thereafter. The methylase activity begins to drop much earlier, being essentially absent by 12 h p.i. This is probably due to reassembly of the parental SVPs into virus, which would cause them to sediment in the fast-sedimenting fraction of the glycerol gradients. Astel et al. (1) have reported that in vitro addition of the σ_3 protein to parental SVPs inactivates their enzyme activities. They did not, however, find that this form of inactivation occurred in vivo. Our data indicate that some form of inactivation of parental SVPs does occur, but we cannot at this stage speculate on its nature.

The enzyme activities in SVPs treated with chymotrypsin also declined after 12 h p.i. (Fig. 5). It seems likely that this is due to maturation of the progeny SVPs, causing them to sediment in the fast region of the glycerol gradients.

We have shown that progeny SVPs from L-cells infected with reovirus differ from reovirus cores in that they contain mRNA capping enzymes in a latent or masked form. These SVPs are distinct from mature virions both morphologically and in their polypeptide composition (Fig. 1 and 2). In the accompanying paper (24), we discuss some of the implications of these results as they relate to the reovirus life cycle.

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