Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 507–511, January 1995
Biochemistry

Reaction in alphavirus mRNA capping: Formation of a covalent complex of nonstructural protein nsP1 with 7-methyl-GMP

(mRNA guanylyltransferase/covalent catalysis/Semliki Forest virus/Sindbis virus)

TERO AHOLA* AND LEEVI KÄÄRIÄINEN

Institute of Biotechnology, P.O. Box 45 (Valimotie 7), FIN-00014 University of Helsinki, Finland

Communicated by Daniel Nathans, Johns Hopkins University, Baltimore, MD, October 14, 1994 (received for review August 26, 1994)

ABSTRACT **After the start of transcription, the 5′ ends of eukaryotic mRNA molecules are modified by the addition of a guanylyl residue to form a cap structure, G(5′)ppp(5′)N. The guanylyltransferase (GTP:mRNA guanylyltransferase, EC 2.7.7.50) reaction responsible for cap formation usually proceeds via a covalent enzyme–GMP intermediate. We have studied the alphavirus-specific guanylyltransferase by incubating lysates from Semliki Forest and Sindbis virus-infected cells with [α-^{32}P]GTP, using vaccinia virus and mock-infected cells as controls. One additional ^{32}P-labeled protein was detected in alphavirus-infected cells but only in the presence of *S*-adenosylmethionine. This protein was identified as the nonstructural protein nsP1. The properties of the covalent enzyme–guanylate complex were studied with Semliki Forest virus nsP1 expressed in recombinant baculovirus-infected cells. *S*-Adenosylmethionine and divalent cations were required for the complex formation. The reaction was specific for guanylate nucleotides (GTP, dGTP) and was inhibited by pyrophosphate. nsP1 could be labeled with *S*-adenosyl[*methyl*-^{3}H]methionine but only under conditions in which the nsP1–guanylate complex was formed. 7-Methyl-GMP was released from the nsP1–guanylate complex by treatment with acid or acidic hydroxylamine. Similar treatment of vaccinia virus capping enzyme released GMP. These findings suggest that in the capping of alphavirus mRNAs the guanine is methylated before linkage to the mRNA molecule.**

The genome of alphaviruses consists of a single-stranded RNA molecule of positive polarity. Its translation in infected cells yields a polyprotein, which is processed into four nonstructural proteins (nsP1–nsP4) that form a membrane-associated replication complex. In addition to the genomic 42S RNA (11.5 kb), a subgenomic 26S RNA, which codes for the virus structural proteins, is produced during infection (1, 2). The 5′ end of both mRNAs is blocked by a cap 0 structure (3, 4). Since cap formation of cellular mRNAs is a nuclear function and alphaviruses replicate exclusively in the cytoplasm of the infected cells (5), these viruses are expected to devote a substantial part of their coding capacity for producing their own capping enzymes.

The capping of mRNA has been studied in mammalian and yeast cells and in several viral systems (reviewed in ref. 6). The capping enzymes of vaccinia virus have been characterized in great detail (7–9). Cap formation in these systems proceeds by a common mechanism:

Step 1: $pppN_1pN_2pN_3 \ldots \rightarrow ppN_1pN_2pN_3 \ldots + P_i$

Step 2: GTP + enzyme $\rightarrow$ enzyme–GMP + PP_i

Step 3: enzyme–GMP + $ppN_1pN_2pN_3 \ldots \rightarrow GpppN_1pN_2pN_3 \ldots$ + enzyme

Step 4: $GpppN_1pN_2pN_3 \ldots$ + AdoMet $\rightarrow m^7GpppN_1pN_2pN_3 \ldots$ + AdoHcy

RNA triphosphatase removes the 5′ γ-phosphate of the nascent RNA molecule (step 1), whereafter mRNA guanylyltransferase (GTP:mRNA guanylyltransferase, EC 2.7.7.50) donates a GMP moiety, derived from GTP, to form a 5′-5′ triphosphate linkage, typical for cap structures. This reaction proceeds via a covalently bound enzyme–GMP intermediate (steps 2 and 3). Thereafter, the cap is methylated by *S*-adenosylmethionine (AdoMet) at position 7 of the terminal guanosine, yielding a cap 0 and *S*-adenosylhomocysteine (AdoHcy) (step 4). Specific mRNA (nucleoside-2′-*O*)-methyltransferases may then act on the riboses of the first two nucleotides (N_1 and N_2) giving rise to cap 1 and cap 2, respectively (6, 10). There are two types of exceptions to this general strategy, both found among negative-stranded RNA viruses. First, in the synthesis of the 5′ cap of vesicular stomatitis virus and related rhabdovirus messengers, two phosphates of the 5′-5′ triphosphate linkage are derived from the capping GTP. The details and possible intermediates of this process are not known (11). Second, influenza virus mRNAs obtain their 5′ caps together with a short oligonucleotide by excision from capped nuclear mRNAs and their precursors (12). Bunyavirus-specific mRNAs also have 5′ ends, which are derived from cytoplasmic host messengers (13).

It has been previously shown that both Sindbis virus (SIN) and Semliki Forest virus (SFV) nsP1 have guanine-7-methyltransferase activity (14, 15), and genetic evidence has also implicated nsP1 in mRNA 5′ end modification (16). Here we show that nsP1 is able to form a covalently bound enzyme–guanylate complex typical of guanylyltransferases. Furthermore, we provide evidence that the complex in this case consists of 7-methyl-GMP (m^7GMP) linked to nsP1, suggesting that alphaviruses have a unique mechanism for mRNA cap formation, where the methylation of the guanine precedes bonding with the 5′ end of the RNA.

MATERIALS AND METHODS

Cells and Viruses. The origin and cultivation of BHK21 cells, SFV prototype strain, and SIN HR strain have been described (17). Vaccinia virus vTF7-3 was kindly provided by B. Moss (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD) and grown as described (18). BHK cells were infected with 50 plaque-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: AdoMet, *S*-adenosyl-L-methionine; AdoHcy, *S*-adenosyl-L-homocysteine; SFV, Semliki Forest virus; SIN, Sindbis virus.

*To whom reprint requests should be addressed.

forming units per cell and harvested as follows: SFV at 4 h and SIN and vaccinia virus at 6 h postinfection. The construction of recombinant baculovirus AcNPV-nsP1 and infection of *Spodoptera frugiperda* (Sf9) cells have been recently described (15).

Cell Fractionation. Cells were scraped into ice-cold phosphate-buffered saline and collected by centrifugation at 250 × *g* for 5 min. They were resuspended in RS buffer (10 mM NaCl/10 mM Tris·HCl, pH 7.8), kept on ice for 15 min, and disrupted in a Dounce homogenizer. Nuclei were removed by centrifugation at 500 × *g* for 5 min and washed with RS. The cytoplasm preparation was centrifuged at 15,000 × *g* for 20 min to yield pellet (P15) and supernatant (S15) fractions. P15 membranes were washed with RS containing 50 mM KCl. All fractions were resuspended in RS buffer containing 10% glycerol and stored at −70°C.

Enzyme–Guanylate Complex. nsP1-containing cell lysates or subcellular fractions were incubated with 5 μCi of [α-^{32}P]GTP (400 Ci/mmol; 1 Ci = 37 GBq) in 50 mM Tris·HCl/10 mM KCl/2 mM $MgCl_2$/5 mM dithiothreitol/100 μM AdoMet, pH 7.5, in a total vol of 30 μl and for 20 min at 30°C unless otherwise specified. For vaccinia virus-infected cell cytoplasm, the pH was 8.0 and 4 mM $MgCl_2$ was used. The reactions were stopped by boiling in the presence of SDS (1–2%) or by adding EDTA to a final concentration of 5 mM. The proteins were separated by SDS/PAGE in 10% gels, and the radioactive bands were visualized by autoradiography as described (15). For further analysis of ^{32}P-labeled proteins, the bands were excised from the gels, and the proteins were eluted as described (19) and concentrated with Centricon 30 devices (Amicon). Quantitation of the ^{32}P-labeled nsP1–guanylate complexes was carried out by determining the relative intensities of the bands after autoradiography of the gels using an Ultrascan XL enhanced laser densitometer (LKB).

Immunological Methods. Immunoprecipitations of SDS-denatured proteins and Western blotting by enhanced chemiluminescence (ECL; Amersham) were as described (15, 20).

Materials. All radiolabeled compounds and [^{14}C]methylated molecular weight markers were from Amersham. Nucleotides, AdoMet, and AdoHcy were from Sigma.

RESULTS

Infection-Specific Radiolabeling with [α-^{32}P]GTP. To discover the alphavirus guanylyltransferase-like enzyme, we incubated lysates from mock-infected and SFV- and SIN-infected BHK cells with [α-^{32}P]GTP. The reaction mixtures were analyzed for labeled proteins by SDS/PAGE and autoradiography to visualize the enzyme–guanylate complexes (Fig. 1*A*). In all cases, a band of ≈68 kDa was seen. An additional ^{32}P-labeled protein band, specific for alphavirus-infected cells, was observed only when AdoMet was included in the reaction mixture (Fig. 1*A*, lanes 4 and 6). The size of this protein was slightly different in SFV- and SIN-infected cell lysates (64 and 61 kDa, respectively), suggesting that it was a virus-specific component. The addition of AdoMet did not affect the labeling of the 68-kDa protein. Smaller labeled proteins were sometimes observed in these experiments, especially when reactions were stopped with EDTA (21). Since they were not specific for virus-infected cells, they were not investigated further.

When SFV-infected cell lysates were fractionated to obtain nuclei and 15,000 × *g* pellet (P15) and supernatant (S15), the 68-kDa protein was found in the nuclear and S15 fractions (Fig. 1*B*, lanes 1 and 3). It probably represents the cellular mRNA guanylyltransferase, for which a similar size and intracellular distribution have been reported in HeLa and rat liver cells (6, 21, 22). The SFV-specific 64-kDa protein was mainly found in the P15 fraction (lanes 2 and 5). A smaller amount was associated with the crude nuclear fraction (lanes 1 and 4). The SIN-specific 61-kDa protein had a similar distribution (data not shown). If deoxycholate, which has been used to activate the alphavirus-specific methyltransferase (23), was included in the reaction mixtures, the cellular 68-kDa protein could not be labeled, while the SFV-specific 64-kDa protein remained active (lanes 4–6). Thus, treatment with deoxycholate allowed specific labeling of the virus-specific protein.

Identification of the Virus-Specific Protein. Lysates from alphavirus-infected cells were incubated with [α-^{32}P]GTP in the presence of AdoMet and subjected to immunoprecipitation with antisera against the individual SFV nonstructural proteins. Only anti-nsP1 antiserum precipitated a ^{32}P-labeled protein from SFV-infected cell lysates (Fig. 2, lane 3), whereas no labeled products were precipitated from mock-infected cell lysates (lane 2). When [^{35}S]methionine-labeled infected cell lysates were subjected to immunoprecipitation, all SFV-specific nonstructural proteins were visualized (lanes 7–10). Our antisera recognized also the SIN-specific nonstructural proteins (data not shown), allowing the precipitation of the ^{32}P-labeled 61-kDa protein with anti-nsP1 antiserum (lane 1).

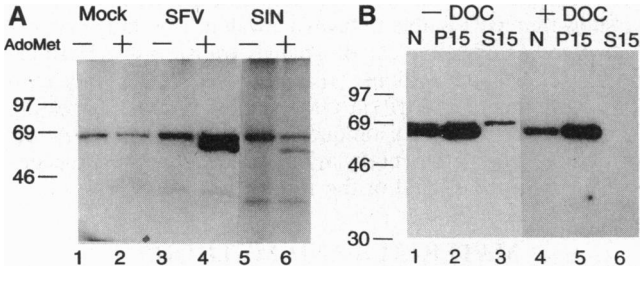

FIG. 1. Formation of enzyme–guanylate complexes in mock-infected and alphavirus-infected cell lysates and subcellular fractions. (*A*) Lysates from mock-infected (lanes 1 and 2), SFV-infected (lanes 3 and 4), and SIN-infected (lanes 5 and 6) cells were incubated with [α-^{32}P]GTP under standard conditions (see *Materials and Methods*) in the absence or presence of 100 μM AdoMet as indicated. Autoradiogram after separation of proteins by SDS/PAGE is shown. Positions of molecular mass markers (kDa) are indicated on the left. (*B*) Subcellular fractions N (nuclear fraction), P15, and S15 from SFV-infected cells were incubated with [α-^{32}P]GTP in the presence of AdoMet and in the absence (lanes 1–3) or presence (lanes 4–6) of 1% deoxycholate (DOC) and analyzed as described above.

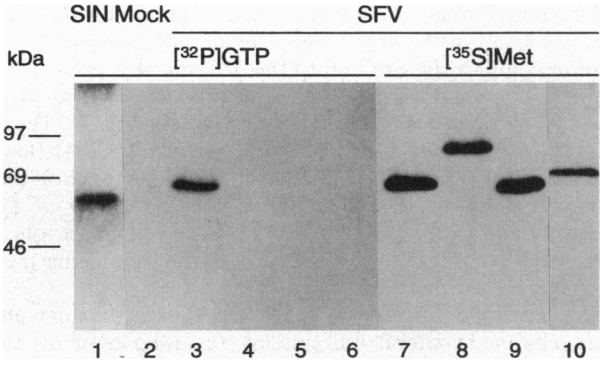

FIG. 2. Identification of the ^{32}P-labeled alphavirus-specific proteins. Lysates from SIN-infected (lane 1), mock-infected (lane 2), and SFV-infected (lanes 3–6) cells were incubated in the presence of [α-^{32}P]GTP under standard conditions. The reaction was stopped by boiling in 1% SDS for 2 min. SFV-infected BHK cells were labeled with 300 μCi of [^{35}S]methionine (1000 Ci/mmol) per 10^7 cells 2–3 h postinfection followed by a chase with 2 mM methionine for 60 min (lanes 7–10). Cell lysate was boiled in SDS as described above. Portions of these denatured samples were subjected to immunoprecipitation with anti-nsP1 (lanes 1–3 and 7), anti-nsP2 (lanes 4 and 8), anti-nsP3 (lanes 5 and 9), and anti-nsP4 (lanes 6 and 10) antisera and analyzed by SDS/PAGE followed by fluorography.

These results identified the ^{32}P-labeled protein as nsP1 in both SFV- and SIN-infected cell lysates.

Labeling of Recombinant nsP1 with [α-^{32}P]GTP. We have previously studied the methyltransferase activity of SFV nsP1, which was expressed in Sf9 cells, infected with the recombinant baculovirus AcNPV-nsP1 (15). We used this expression system to characterize the nsP1–guanylate complex. In AcNPV-nsP1-infected cells, nsP1 was a major component of the P15 cytoplasmic membrane fraction (Fig. 3*A*, lane 2), as evidenced by Western blotting with anti-nsP1 antiserum (Fig. 3*A*, lane 4). When the P15 fraction of AcNPV-nsP1-infected cells was incubated with [α-^{32}P]GTP in the presence of AdoMet, a 64-kDa protein was labeled (Fig. 3*B*, lane 2). This 64-kDa protein could be immunoprecipitated by anti-nsP1 antiserum (Fig. 3*B*, lane 4) but not with preimmune serum (Fig. 3*B*, lane 3). No ^{32}P-labeled proteins could be detected in the P15 fraction of the wild-type virus (AcNPV)-infected cells (Fig. 3*B*, lane 1). Thus, nsP1 expressed in the absence of other alphavirus-specific proteins was able to form the enzyme–guanylate complex. The P15 fraction from AcNPV-nsP1-infected cells was used for further experiments.

Requirements for Complex Formation. The labeling of recombinant nsP1 with [α-^{32}P]GTP was absolutely dependent on AdoMet even in the presence of higher concentrations (up to 100 μM) of unlabeled GTP. AdoMet was effective also in small concentrations: 1 μM AdoMet caused 15% of the complex formation as compared with 100 μM AdoMet (total GTP concentration in this experiment, 100 μM). No labeling of nsP1 was observed if AdoMet was not included, under any conditions tested, either in alphavirus-infected BHK cell fractions or in P15 from AcNPV-nsP1-infected cells. AdoHcy could not substitute for AdoMet in this reaction.

Divalent cations were also required for the reaction, since the addition of 1 mM EDTA, in the absence of added divalent cations, quantitatively inhibited the reaction. The optimal concentration of Mg^{2+} or Mn^{2+} was $\approx$2 mM, but Mn^{2+} was less effective in promoting the complex formation. The nsP1 from AcNPV-nsP1-infected cells also remained active in the presence of deoxycholate. Under standard conditions, the synthesis of the complex was complete within 20 min. Pyrophosphate at 50 μM inhibited the reaction 90%, whereas phosphate had no effect even at 1 mM.

Nucleotide Specificity. Attempts to form a complex of nsP1 with other labeled nucleotides showed that it was specific for guanine-containing nucleotides. [α-^{32}P]dGTP had 38% activity as compared with GTP. No complex was formed with [α-^{32}P]ATP, [α-^{32}P]CTP, or [α-^{32}P]UTP (detection limit, <0.1%). As expected, nsP1 could not be labeled with [γ-^{32}P]GTP, because if it forms a covalent complex with GMP only the α-phosphate would be retained. No labeling with any nucleotide was seen in the absence of AdoMet, and solubilizing of nsP1 with deoxycholate had no effect on the nucleotide specificity of the complex formation.

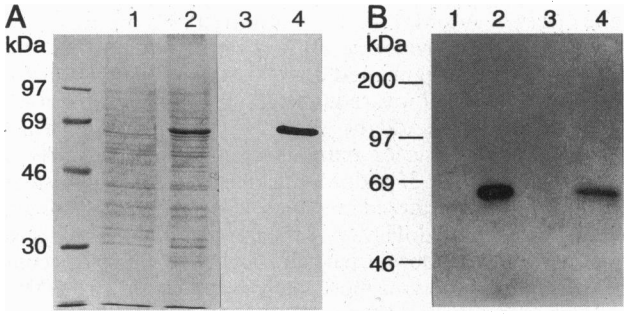

FIG. 3. Expression and ^{32}P-labeling of nsP1 in the recombinant baculovirus (AcNPV-nsP1)-infected cells. (*A*) P15 fractions from the wild-type virus AcNPV-infected (lane 1) and AcNPV-nsP1-infected (lane 2) cells were analyzed by SDS/PAGE and stained with Coomassie blue. Samples of AcNPV-infected (lane 3) and AcNPV-nsP1-infected (lane 4) cell P15 diluted 1:50 were analyzed by Western blotting with anti-nsP1 antiserum. (*B*) P15 fractions from AcNPV-infected (lane 1) and AcNPV-nsP1-infected (lane 2) cells were incubated with [α-^{32}P]GTP followed by SDS/PAGE and autoradiography. Equal samples from reaction mixtures, respective to lane 2, were subjected to precipitation with preimmune serum (lane 3) or anti-nsP1 antiserum (lane 4) followed by SDS/PAGE and autoradiography.

Chemical Nature of the Enzyme–Guanylate Linkage. The stability of the enzyme–guanylate complex was studied after the isolation of ^{32}P-labeled nsP1 from polyacrylamide gels (Table 1). The labeled guanylate moiety was detached from the protein by treatment with acid and was partially released by treatment with 0.1 M NaOH. The complex was dissociated with hydroxylamine at pH 4.75 but was otherwise stable at this pH, while neutral hydroxylamine did not affect the complex. Although similar results have been reported for other capping enzymes, with respect to hydroxylamine treatments, those enzyme–guanylate complexes were stable under basic conditions (21, 24–26). We therefore controlled our results with the vaccinia virus capping enzyme, isolated after SDS/PAGE (see legend to Fig. 5). The ^{32}P-labeled GMP–95-kDa protein complex was stable when subjected to base treatment but was hydrolyzed by treatment with acid (data not shown) as described (24). Results similar to those in Table 1 were obtained when the nsP1–guanylate complex was isolated by gel filtration under nondenaturing conditions.

Sensitivity to acidic but not neutral hydroxylamine is indicative of an amide-type bond between nsP1 and the guanylate moiety and tends to exclude an ester or anhydride-type bond (24, 27). Amide bonds are usually stable in base but hydrolyzed by acid treatment as has been observed for other capping enzymes. However, suitably reactive groups in nearby amino acids or in the nucleotide base moiety can render an amide bond sensitive to base (27). Therefore, the guanylate moiety seems to be connected to an amino group in nsP1 by a phosphoamide-type bond, possibly to a lysine residue in the enzyme, as is the case in other guanylyltransferase-like proteins (6, 28).

Characterization of the Guanylate Moiety. We then wanted to examine the reason for the absolute AdoMet requirement for nsP1–guanylate complex formation. As a control, we used a cytoplasmic extract from vaccinia virus-infected cells. Both nsP1 from P15 of AcNPV-nsP1-infected cells and the vaccinia virus-specific 95-kDa protein from infected BHK cells were labeled with [α-^{32}P]GTP in the presence of AdoMet (Fig. 4, lanes 6 and 7). AdoMet was not required by the vaccinia virus guanylyltransferase (data not shown) (9). When Ado[*methyl*-^{3}H]Met was used in the reaction mixture, the label was found

Table 1. Stability of nsP1–guanylate complex

Treatment	cpm
Exp. 1	
0.1 M Tris·HCl (pH 7.5, 5 min, 65°C)	2310
0.1 M HCl (5 min, 65°C)	280
0.1 M NaOH (5 min, 65°C)	900
Exp. 2	
H_2O (10 min, 37°C)	3360
3.86 M hydroxylamine (pH 4.75, 10 min, 37°C)	50
0.2 M hydroxylamine (pH 7.5, 10 min, 37°C)	3210
4 M NaOAc (pH 4.75, 10 min, 37°C)	3250

Exps. 1 and 2 were performed separately with unequal amounts of label. The labeled nsP1–guanylate complex was isolated after SDS/PAGE. Portions (10 μl) of the concentrated sample were treated with 100 μl of the solutions indicated. After incubations the tubes were transferred to ice, and 100 μl of 0.1% bovine serum albumin/0.1% SDS was added, followed by 2 ml of 7.5% trichloroacetic acid. After 30 min, the precipitates were collected on glass fiber filters and assayed by liquid scintillation spectrometry.

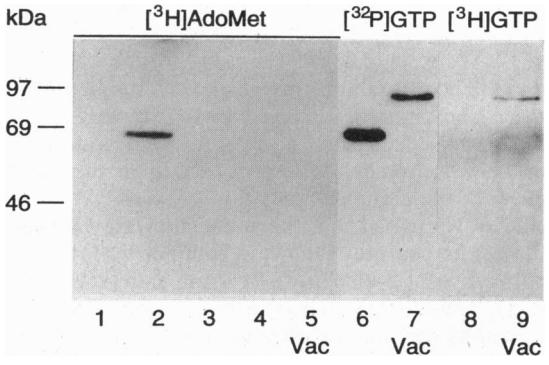

FIG. 4. Comparison of nsP1 with vaccinia virus capping enzyme. P15 from AcNPV-nsP1-infected Sf9 cells (lanes 1–4) or cytoplasm from vaccinia virus-infected BHK cells (lane 5) were incubated with 5 μCi of Ado[*methyl*-^{3}H]Met (total concentration, 5 μM) in the presence (lanes 2–5) or absence (lane 1) of 10 μM GTP. Incubation buffer was as described in *Materials and Methods* (lanes 1, 2, and 5), or was modified by adding 200 μM pyrophosphate (lane 3) or 1 mM EDTA (lane 4) instead of $MgCl_2$. In other experiments, P15 from AcNPV-nsP1-infected Sf9 cells (lanes 6 and 8) or cytoplasm from vaccinia virus-infected BHK cells (lanes 7 and 9) was incubated either with [α-^{32}P]GTP (lanes 6 and 7) or with [8-^{3}H]GTP (5 μCi; total GTP concentration, 10 μM; lanes 8 and 9) under standard conditions in the presence of 100 μM AdoMet. Reaction mixtures were analyzed by SDS/PAGE and fluorography.

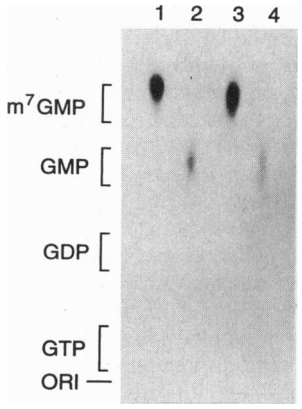

FIG. 5. TLC analysis of guanylate moieties. P15 from AcNPV-nsP1-infected Sf9 cells (lanes 1 and 3) or cytoplasm from vaccinia virus-infected BHK cells (lanes 2 and 4) was incubated with [α-^{32}P]GTP. Proteins were separated by SDS/PAGE, and ^{32}P-labeled proteins were eluted from the gel and concentrated. Portions of the samples were treated with HCl (lanes 1 and 2) or acidic hydroxylamine (lanes 3 and 4; see Table 1). The treated samples were chromatographed on polyethylenimine-cellulose plates with 4 M sodium formate (pH 3.4) as eluent followed by autoradiography. Positions of origin (ORI) and authentic markers, detected under UV light, are shown.

in nsP1 in the presence (lane 2) but not in the absence (lane 1) of unlabeled GTP. The label seemed to be bound covalently, since it was not released during analysis by SDS/PAGE. Labeling was inhibited by the addition of pyrophosphate (lane 3) or EDTA (lane 4) to the reaction mixture. These agents also inhibited guanylate–enzyme complex formation (see above). No proteins were labeled with Ado[*methyl*-^{3}H]Met in the cytoplasm of vaccinia virus-infected cells (lane 5). Thus, the SFV-specific nsP1 differs from the vaccinia capping enzyme in its ability to incorporate the labeled methyl group from AdoMet.

Finally, we incubated vaccinia virus-infected cell lysate and P15 from AcNPV-nsP1-infected cells in the presence of [8-^{3}H]GTP and unlabeled AdoMet. Only the vaccinia virus enzyme could be visualized after SDS/PAGE and fluorography (Fig. 4, lane 9). Under the same conditions, no label was associated with nsP1 (lane 8). Assuming that nsP1–guanylate complex forms between m^{7}GMP and nsP1, this result is understandable, since alkylation of N-7 is known to cause a rapid exchange of H-8 with water (9, 29). This process would release the ^{3}H label at position 8 in the nsP1–guanylate complex. Taken together, these findings suggest that nsP1 covalently binds m^{7}GMP, while vaccinia virus enzyme binds GMP. To directly confirm this, we labeled these proteins with [α-^{32}P]GTP, isolated the enzyme–guanylate complexes after SDS/PAGE, and subjected them to hydrolysis with acid (Fig. 5, lanes 1 and 2) or acidic hydroxylamine (lanes 3 and 4). The products of these reactions were analyzed by TLC. The results indicate that the ^{32}P-labeled guanylate moieties released from these enzymes are different. Comparison with authentic markers confirmed that m^{7}GMP was exclusively released from nsP1 (lanes 1 and 3), while only GMP was released from the vaccinia virus capping enzyme (lanes 2 and 4). Therefore, complexing with m^{7}GMP seems to be an obligatory step in the reaction catalyzed by SFV-specific nsP1.

DISCUSSION

Exposure of cell lysates from SFV- and SIN-infected cells to [α-^{32}P]GTP in the presence of AdoMet, under conditions used for the detection of covalent capping enzyme–GMP complexes, resulted in the labeling of 64- and 61-kDa proteins, respectively. The labeled proteins were identified by immunoprecipitation as alphavirus-specific nonstructural protein nsP1. The enzymatic activity, responsible for the nsP1–guanylate complex, was mainly found in a cytoplasmic membranous fraction, P15, which is also the source of alphavirus-specific RNA polymerase activity (30, 31). The virus-specific enzyme–guanylate complex was studied in more detail with recombinant baculovirus-infected cells, which expressed large amounts of SFV-specific nsP1.

The synthesis of the nsP1–guanylate complex required divalent cations and was inhibited by pyrophosphate. The reaction was specific for GTP and dGTP. The guanylate was presumably bound to nsP1 via a phosphoamide bond. These features are typical for mRNA guanylyltransferases of viral and cellular origin, which act through an enzyme–GMP intermediate (7, 8, 24–26, 32, 33). However, the synthesis of the alphavirus-specific complex differs from the previously characterized guanylyltransferase–GMP complexes in its absolute requirement in the reaction mixture for AdoMet, which could not be replaced by its structural homolog AdoHcy. An allosteric effect of AdoMet has been reported (34) on the virion-associated RNA polymerase of the cytoplasmic polyhedrosis virus, which has a segmented double-stranded RNA genome. In this case, AdoMet was required for chain initiation but not for elongation. It could be substituted by AdoHcy, which allowed the synthesis of unmethylated capped mRNAs in normal ratios (34). If AdoMet is needed as an allosteric effector for nsP1-catalyzed reactions, its effects in this case are not mimicked by AdoHcy. Another, not mutually exclusive, possibility is that AdoMet participates in the guanylate complex formation reaction, most likely resulting in a m^{7}GMP–nsP1 complex. Three different approaches were used to test this hypothesis. In these experiments, we used cytoplasm from vaccinia virus-infected cells as a control for the synthesis of a well-characterized GMP–enzyme complex.

First, we showed that the [^{3}H]methyl group from radiolabeled AdoMet was covalently associated with nsP1 but only when GTP was added to the reaction mixture. The vaccinia virus 95-kDa guanylyltransferase was not labeled under these conditions. When [α-^{32}P]GTP was used, both nsP1 and vaccinia 95-kDa protein were readily labeled. Second, the vaccinia virus guanylyltransferase was labeled when [8-^{3}H]GTP was used in the reaction mixture. Under these conditions, nsP1 was

not labeled. This result was to be expected, since methylation of position 7 in the guanine ring releases the ^3H label at position 8 (29). Third, acid hydrolysis of nsP1–[α-^{32}P]guanylate yielded a labeled product, which was identified as m^{7}GMP. Under the same conditions, GMP was released from the ^{32}P-labeled vaccinia virus 95-kDa protein. Since no GMP was released from the nsP1–guanylate complex, we conclude that the covalently bound guanylate was exclusively m^{7}GMP. This is in contrast to guanylyltransferases of vaccinia virus (8, 35), reovirus (36), HeLa cells (32), rat liver cells (26), and yeast *Saccharomyces cerevisiae* (33), which are not able to bond with m^{7}GMP but form a linkage only with GMP (and dGMP). An exception is presented by rhabdoviruses, where no guanylate–protein intermediate has been found in the virion-associated transcription complex or in the infected cells (11).

We have recently shown that SFV nsP1, expressed in recombinant baculovirus-infected Sf9 cells and in *Escherichia coli*, is a guanine-7-methyltransferase, which is able to transfer the methyl group from AdoMet to GTP, dGTP, and G(5′)ppp(5′)G but to neither G(5′)ppp(5′)A nor capped unmethylated SFV-specific RNAs (15). These results are compatible with our present findings. Since nsP1 forms a bond only with m^{7}GMP, methylation of the capping guanosine would take place prior to the synthesis of the 5′-5′ bond to the penultimate adenosine residue, creating the typical cap 0 of alphavirus mRNAs. Direct methylation of capped RNA and the cap analog GpppA would be incompatible with this type of capping mechanism. In contrast to nsP1, the HeLa cell methyltransferase methylates only RNAs and cap analogues but does not methylate GTP or dGTP (37). The guanine-7-methyltransferase of vaccinia virus can methylate GTP and dGTP only very inefficiently compared with unmethylated RNA substrates (8, 9).

The guanine-7-methyltransferase reaction catalyzed by nsP1 did not require divalent cations, and the best substrate for methylation was dGTP (15). These features differ from the synthesis of the guanylate–nsP1 complex, indicating that the two enzymatic reactions, carried out by the same protein, can be distinguished from each other, at least *in vitro*. The characterization of the detailed mechanism of synthesis of the enzyme–m^{7}GMP complex, as well as the acceptor specificity of the putative guanylyltransferase, will have to wait for the purification of nsP1. This has been hampered by heavy aggregation of nsP1 in all expression systems, even after detergent solubilization of the membranes (15). One possibility is that the methylation and the reaction of m^{7}GTP with nsP1 take place simultaneously, so that free m^{7}GTP would not be a necessary intermediate in the reaction. Schematically, capping reactions 2 and 3 (see Introduction) of alphavirus mRNAs would be as follows:

$$\text{Reaction 2: GTP + AdoMet} \rightarrow \text{m}^7\text{GTP + AdoHcy}$$

$$\text{Reaction 3: m}^7\text{GTP + nsP1} \rightarrow \text{m}^7\text{GMP–nsP1 + PP}_i$$

By analogy to the previously identified reactions we assume that the next reaction would be

$$\text{Reaction 4: m}^7\text{GMP–nsP1} + \text{ppN}_1\text{pN}_2\text{pN}_3 \ldots \rightarrow \text{m}^7\text{GpppN}_1\text{pN}_2\text{pN}_3 \ldots + \text{nsP1}$$

A guanylyltransferase-like enzyme has recently been described in tobacco mosaic virus-infected plant cells (38). Tobacco mosaic virus is a member of the alphavirus-like superfamily of positive-stranded RNA viruses and shares a similar strategy for RNA replication and some sequence homology with alphavirus nonstructural proteins (39). The tobacco mosaic virus replicase protein formed a covalent complex with GMP in the absence of added AdoMet (38). Thus, the alphavirus mode of capping may not be generally used even within the superfamily. Last, since the synthesis of the nsP1–m^{7}GMP complex is clearly a virus-specific reaction, it may offer possibilities to interfere with virus replication by drugs specifically inhibiting this reaction.

We thank Drs. Marja Makarow, Ossi Renkonen, and Patrick Russo for critical reading of the manuscript and Ms. Tarja Välimäki for technical assistance. This work was supported by grants from the Academy of Finland and Sigrid Jusélius Foundation.

1. Strauss, E. G. & Strauss, J. H. (1986) in *Replication of Togaviridae and Flaviviridae*, eds. Schlesinger, S. & Schlesinger, M. J. (Plenum, New York), pp. 35–90.
2. Kääriäinen, L., Takkinen, K., Keränen, S. & Söderlund, H. (1987) *J. Cell Sci. Suppl.* **7**, 231–250.
3. Dubin, D. T., Stollar, V., Hsuchen, C.-C., Timko, K. & Guild, G. M. (1977) *Virology* **77**, 457–470.
4. Wengler, G., Wengler, G. & Gross, H. J. (1979) *Nature (London)* **282**, 754–756.
5. Follet, E. A. C., Pringle, C. R. & Pennington, T. H. (1975) *J. Gen. Virol.* **26**, 183–196.
6. Mizumoto, K. & Kaziro, Y. (1987) *Prog. Nucleic Acid Res. Mol. Biol.* **34**, 1–28.
7. Martin, S. A. & Moss, B. (1975) *J. Biol. Chem.* **250**, 9330–9335.
8. Martin, S. A. & Moss, B. (1976) *J. Biol. Chem.* **251**, 7313–7321.
9. Shuman, S. & Moss, B. (1990) *Methods Enzymol.* **181**, 170–180.
10. Barbosa, E. & Moss, B. (1978) *J. Biol. Chem.* **253**, 7698–7702.
11. Banerjee, A. K. (1987) *Microbiol. Rev.* **52**, 66–87.
12. Plotch, S. J., Bouloy, M., Ulmanen, I. & Krug, R. M. (1981) *Cell* **23**, 847–858.
13. Simons, J. F. & Pettersson, R. F. (1991) *J. Virol.* **65**, 4741–4748.
14. Mi, S. & Stollar, V. (1991) *Virology* **184**, 423–427.
15. Laakkonen, P., Hyvönen, M., Peränen, J. & Kääriäinen, L. (1994) *J. Virol.* **68**, 7418–7425.
16. Scheidel, L. M. & Stollar, V. (1991) *Virology* **181**, 490–499.
17. Keränen, S. & Kääriäinen, L. (1979) *J. Virol.* **32**, 19–29.
18. Fuerst, T. R., Niles, E. G., Studier, F. W. & Moss, B. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 8122–8126.
19. Hager, D. A. & Burgess, R. R. (1980) *Anal. Biochem.* **109**, 76–86.
20. Peränen, J., Takkinen, K., Kalkkinen, N. & Kääriäinen, L. (1988) *J. Gen. Virol.* **69**, 2165–2178.
21. Shuman, S. (1982) *J. Biol. Chem.* **257**, 7237–7245.
22. Venkatesan, S., Gershowitz, A. & Moss, B. (1980) *J. Biol. Chem.* **255**, 2829–2834.
23. Cross, R. K. (1983) *Virology* **130**, 452–463.
24. Shuman, S. & Hurwitz, J. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 187–191.
25. Venkatesan, S. & Moss, B. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 340–344.
26. Mizumoto, K., Kaziro, Y. & Lipmann, F. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 1693–1697.
27. Shabarova, Z. A. (1970) *Prog. Nucleic Acid Res. Mol. Biol.* **10**, 145–182.
28. Fresco, L. D. & Buratowski, S. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 6624–6628.
29. Hendler, S., Fürer, E. & Srinivasan, P. R. (1970) *Biochemistry* **9**, 4141–4153.
30. Ranki, M. & Kääriäinen, L. (1979) *Virology* **98**, 298–307.
31. Barton, D. J., Sawicki, S. G. & Sawicki, D. L. (1991) *J. Virol.* **65**, 1496–1506.
32. Venkatesan, S. & Moss, B. (1980) *J. Biol. Chem.* **255**, 2835–2842.
33. Itoh, N., Mizumoto, K. & Kaziro, Y. (1984) *J. Biol. Chem.* **259**, 13930–13936.
34. Furuichi, Y. (1981) *J. Biol. Chem.* **256**, 483–493.
35. Roth, M. J. & Hurwitz, J. (1984) *J. Biol. Chem.* **259**, 13488–13494.
36. Mao, Z. & Joklik, W. K. (1991) *Virology* **185**, 377–386.
37. Ensinger, M. J. & Moss, B. (1976) *J. Biol. Chem.* **251**, 5283–5291.
38. Dunigan, D. D. & Zaitlin, M. (1990) *J. Biol. Chem.* **265**, 7779–7786.
39. Strauss, E. G., Strauss, J. H. & Levine, A. J. (1990) in *Virology*, eds. Fields, B. N. & Knipe, D. M. (Raven, New York), 2nd Ed, pp. 167–190.