

Mutational Analysis of a Multifunctional Protein, with mRNA 5' Cap-specific (Nucleoside-2'-O-)methyltransferase and 3'-Adenylyltransferase Stimulatory Activities, Encoded by Vaccinia Virus*

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The vaccinia virus-encoded protein VP39 is a poly(A) polymerase subunit that stimulates the formation of long poly(A) tails as well as a cap-specific mRNA (nucleoside-2'-O-)methyltransferase. We have carried out mutagenesis studies aimed at locating regions of VP39 which are important for these activities. The open reading frame encoding VP39 was expressed in *Escherichia coli* as a glutathione S-transferase fusion protein. The affinity-purified protein had both mRNA modification activities, before and after removal of the glutathione S-transferase domain. Truncation, charge cluster → Ala scanning, and Cys → Ser substitution mutations of VP39 were made, and the proteins were synthesized, purified, and analyzed. Deletion of the RNA binding domain, experimentally localized within the carboxyl-terminal 112 amino acids, resulted in the loss of both mRNA modification activities. Eleven of the 21 charge cluster → Ala mutated proteins had low to nondetectable methyltransferase activity. Four of those 11 also lacked adenylyltransferase stimulatory function, whereas the remainder had amino acid substitutions that selectively affected methyltransferase activity. However, no mutated proteins lacking adenylyltransferase stimulatory function but possessing methyltransferase activity were isolated by the procedures used. Neither of the 2 cysteine residues in VP39 was necessary for either mRNA modification activity.

The encoding of enzymes for synthesis and modification of mRNA makes vaccinia virus and other poxviruses extraordinarily useful for biochemical and genetic studies (for review see Ref. 1). This unique feature of poxviruses is related to their cytoplasmic site of DNA replication and gene expression, which necessitates viral counterparts for host enzymes and factors that reside in the nucleus. Vaccinia viral mRNA has typical eukaryotic characteristics, possessing a 5' methylated cap 1 m⁷G(5')pppNm- structure as well as a 3' poly(A) tail. There is considerable evidence that the terminal m⁷G residue of the cap has a role in mRNA translation and stability, whereas the func-

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tion of 2'-O-methylation of the penultimate nucleotide is entirely unknown (for review see Ref. 2). The poly(A) tail of eukaryotic mRNAs has been implicated in mRNA processing, stability, and translation.

The steps in cap 1 formation are as follows.

Step i—pppN(pN)_n → ppN(pN)_n + P_i.

Step ii—pppG + ppN(pN)_n → G(5')pppN(pN)_n + PP_i.

Step iii—AdoMet¹ + G(5')pppN(pN)_n → m⁷G(5')pppN(pN)_n + AdoHcy.

Step iv—AdoMet + m⁷G(5')pppN(pN)_n → m⁷G(5')pppNm(pN)_n + AdoHcy.

The steps were worked out originally for vaccinia virus (3–7) but apply to eukaryotes as well. The first three steps are catalyzed by a single multifunctional heterodimeric viral protein containing (i) mRNA triphosphatase, (ii) GTP:mRNA guanylyltransferase (EC 2.7.7.50), and (iii) AdoMet:mRNA (guanine-7-)methyltransferase (EC 2.1.1.56) activities. In this report, we are concerned with the last step in cap 1 formation (iv), which is carried out by a mRNA (nucleoside-2'-O-)methyltransferase (EC 2.1.1.57). The viral enzyme was originally described as a monomeric protein of 38 kDa which methylated the penultimate nucleotide of RNA ending in m⁷G(5')pppN (5, 8). The acceptor specificity of the enzyme indicated that 2'-O-methylation was the last step in cap 1 formation. A cellular cap-specific mRNA (nucleoside-2'-O-)methyltransferase was also isolated, although in this case both G(5')pppN(pN)_n and m⁷G(5')pppN(pN)_n were good acceptors (9). Open reading frame (ORF) J3R was recently shown to encode the vaccinia virus mRNA (nucleoside 2'-O-)methyltransferase (10), whereas the identification of the corresponding eukaryotic gene is yet to be reported. Surprisingly, ORF J3R had previously been assigned to VP39, the 39-kDa stimulatory subunit of vaccinia virus poly(A) polymerase (polynucleotide adenylyltransferase; EC 2.7.7.19). VP39 exists both in monomeric form and in association with VP55, the catalytic subunit of the poly(A) polymerase (11). Both the monomeric and heterodimeric forms of VP39 have methyltransferase activity (10).

As indicated above, the genes encoding both subunits of the vaccinia virus poly(A) polymerase have been identified (11); they appear structurally unrelated to the gene encoding the corresponding yeast (12) or mammalian (13, 14) enzyme. Although VP55, the viral poly(A) polymerase large subunit, possesses catalytic activity necessary for the addition of adenylates to uridylate-containing RNA, processive additions cease after 30–35 adenylates (15–17). VP39, the small subunit, con-

¹ The abbreviations used are: AdoMet, S-adenosyl-L-methionine; AdoHcy, S-adenosyl-L-homocysteine; BMV, bromo mosaic virus; GS, glutathione-Sepharose; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; ORF, open reading frame.

verts the VP55-catalyzed slow, nonprocessive extension of poly(A) tails greater than 35 nucleotides in length to a more rapid, semiprocessive reaction (16). VP39 binds poly(A), a property that probably facilitates adenylate addition by the VP55 subunit (11). The formation of eukaryotic poly(A) tails appears complex and is associated with the processing of mRNA precursors (18). A poly(A)-binding protein that stimulates the formation of long poly(A) tails has been isolated from mammalian cells (19), although the gene encoding it has not yet been reported for comparison with VP39.

The implications of our finding, that VP39 participates in the modification of both the 5' and 3' ends of mRNA, are not yet fully understood. The present mutational analysis was undertaken to identify regions of VP39 which mediate the disparate activities of this multifunctional protein.

EXPERIMENTAL PROCEDURES

Cloning and DNA Mutagenesis—Routine cloning procedures were performed as described (20). Oligonucleotide primers were synthesized with an Applied Biosystems model 394 DNA synthesizer. Vaccinia virus DNA was used as a template for polymerase chain reaction (PCR) amplifications.

Plasmid pPG177 was constructed for the synthesis, in *Escherichia coli*, of glutathione S-transferase fused to unmutated VP39. ORF J3R, encoding VP39, was amplified by the PCR using 42-mer oligonucleotide primers of which 30 nucleotides overlapped ORF J3R; the 5' primer contained an additional *Nco*I site and the 3' primer an *Sall* site. The PCR product was digested with *Nco*I and *Sall* and ligated to the larger *Nco*I-*Xho*I fragment of pGEX-KG (21).

Plasmids for the expression of truncated VP39 proteins were constructed as follows. For mutant ΔC112, an *Xho*I fragment of approximately 350 base pairs was deleted by digestion and religation of pPG177. For mutant ΔN221, this 350-base pair fragment was cloned into the *Sall* site of the vector pGEX-KG. Mutants ΔC75 and ΔC26 contained PCR-amplified portions of ORF J3R cloned in pGEX-KG. Cloning protocols differed from that of wild-type VP39 (above) in that oligonucleotide primers contained 25 nucleotides that overlapped ORF J3R, and the PCR 3' primer contained a *Hind*III site instead of a *Sall* site.

Plasmids for the synthesis of VP39 with Cys → Ser mutations were generated by overlap extension PCR (22) as described (23). Briefly, each point mutation was incorporated into two complementary 25-mer PCR primers. Two PCR reactions were performed in each of which one of the two primers with mutations was paired with a primer complementary to an end of J3R containing an *Nco*I or *Hind*III site (above). The PCR products were isolated, combined, and mixed with the J3R end primers for a further PCR reaction. Resulting PCR products were isolated, cloned in pGEX-KG, and the resulting plasmids sequenced over the entire VP39 coding region using an Applied Biosystems 370A DNA Sequencer.

Plasmids for the expression of VP39, in which clusters of potentially charged amino acids (Asp, Glu, His, Lys, Arg) were changed to Ala (charge cluster → Ala mutations), were constructed by oligonucleotide-directed mutagenesis (Sculptor™ kit, Amersham Corp.) as described by the manufacturer. The mutagenesis template comprised the smaller *Bam*HI-*Hind*III fragment of pPG177, containing ORF J3R plus some vector sequences, cloned in double-stranded M13mp18 DNA. Mutagenic oligonucleotides were between 21 and 30 nucleotides in length and carried silent mutations creating a restriction site as well as the charged amino acid → Ala substitution mutations. Recombinant plasmids were analyzed by restriction analysis, and inserts from apparent positives were recloned in the vector pGEX-KG. Finally, each plasmid was sequenced over the entire VP39 coding region, as described above.

Protein Synthesis—Glutathione S-transferase fusions of VP39 and mutated variants were synthesized as described (21) with modifications. Briefly, *E. coli* strain HB101 was transformed with each plasmid, and the resulting colonies were grown overnight at 37 °C in superbroth containing ampicillin (0.1 mg/ml). After the addition of 0.4 mM isopropyl 1-thio-β-D-galactopyranoside and a further 3-h incubation at 37 °C, cells were sedimented. The pellets were resuspended in 50 mM Tris-HCl, pH 8.0, 50 mM dithiothreitol, 10 µg/ml aprotinin, 10 µg/ml leupeptin and placed on ice. Extracts were made by sonication (10 × 5-s bursts at medium power) using a Vibra cell (Sonics & Materials Inc., Danbury, CT) and then centrifuged for 5 min at 6,000 × g. Glutathione-Sepharose (GS) beads (Pharmacia Biotech. Inc.) were washed twice with 5 volumes

of phosphate-buffered saline containing 0.5% (v/v) Triton X-100 (Mallinckrodt) and resuspended 1:1 with this buffer. A 0.2-ml sample of the 50% (v/v) GS bead suspension was combined with 1 ml of supernatant from the centrifuged extract. After a 30-min agitation at 4 °C, the beads were washed five times with 1.5-ml volumes of phosphate-buffered saline containing 1% Triton X-100. The beads were resuspended twice in 0.15 ml of 50 mM Tris-HCl, pH 8, containing 10 mM glutathione, and the eluates were made 10% (v/v) in glycerol and stored at -70 °C.

RNA (Nucleoside-2'-O-)methyltransferase Assay—The methyltransferase assay was performed as described previously (10). Briefly, 100-µl reaction mixtures contained 25 mM HEPES/NaOH, pH 7.5, 1 mM dithiothreitol, 1 µM Ado[methyl-³H]Met (6.9 Ci/mmol, DuPont NEN), 5 µg of bromo mosaic virus (BMV) RNA, and GS eluate in the amounts indicated in figure legends. After 30-min incubations at 37 °C, samples were heated at 100 °C for 2 min. Radioactively labeled RNA was quantitated by DEAE-81 filter (Schleicher & Schuell) binding and scintillation counting (Beckman LS 3801).

Immunoblot Analysis and Protein Quantitation—Proteins in GS eluates were resolved by sodium dodecyl sulfate 10% polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes as described (11). Known amounts of a previously described preparation of vaccinia virus-expressed VP39 (16) were analyzed in parallel. Blots were incubated with either of two antisera, VP39-N-pep or VP39-colI (11), developed with ¹²⁵I-protein A and washed. Membrane-bound ¹²⁵I was quantitated using a PhosphorImager (Molecular Dynamics). The VP39 in GS bead eluates was quantitated by comparison with the VP39 standards on the same blot.

RNA Binding Assay—Plasmid pSP64-poly(A) (Pharmacia) was digested with EcoRI and transcribed with SP6 RNA polymerase using an *in vitro* transcription kit (Boehringer Mannheim). Transcription reactions contained [α -³²P]UTP. After digestion of the template with DNase I, the 80-nucleotide RNA product, which contained a 3' tract of 30 adenylates, was separated from unincorporated nucleotides by Sephadex G-50 chromatography, phenol/chloroform extracted, ethanol precipitated, and redissolved in diethyl pyrocarbonate-treated water.

GS bead eluates were incubated with 50 ng of RNA for 10 min at room temperature in binding buffer (50 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 10% glycerol, 20 mM NaCl) and applied to a nitrocellulose filter (BA85, Schleicher & Schuell), which was then washed with 5 ml of binding buffer. After drying, the amount of radioactive material on the filters was determined by scintillation counting.

Adenyllyltransferase Stimulatory Assay—GS bead eluates were assayed for stimulatory activity basically as described by Gershon and Moss (16). Eluates were mixed with VP55, such that VP39 or mutated VP39 was present in 2–6-fold molar excess over VP55. Assay components (50 mM Tris-HCl, pH 9.0, 5 mM dithiothreitol, 60 mM NaCl, 1 mM MgATP, 5' end-labeled vaccinia virus growth factor 60-mer primer) were then added, such that primer was present in 2-fold molar excess over VP55, and reactions were incubated at 30 °C. Products were sampled at 30, 90, and 300 s, mixed with formamide, and resolved by electrophoresis in 8% polyacrylamide, 7 M urea gels. Autoradiograms were digitized with an Arcus Plus (AGFA) scanner, labeled using the Adobe Photoshop™ version 2.5 software on an Apple Macintosh Quadra 800 computer, and reproduced with a Rasterops CorrectPrint 3000 dye-sublimation printer.

RESULTS

Effects of Carboxyl- and Amino-terminal Truncations on the Activities of Affinity-purified VP39 Fusion Proteins—The 333-codon ORF of VP39 was expressed in *E. coli* as a glutathione S-transferase fusion protein regulated by an inducible Tac promoter. A thrombin cleavage site was present at a position appropriate for the enzymatic removal of the glutathione S-transferase segment. The fusion protein was purified by affinity chromatography on GS beads (21) and assayed, directly or after thrombin cleavage, for activities previously associated with authentic viral VP39 (10, 11). Cap-specific methyltransferase, adenyllyltransferase stimulatory, and RNA binding activities were detected (data not shown). Since thrombin cleavage did not significantly affect the assayable properties of VP39, the uncleaved proteins were used unless otherwise stated.

To identify regions of VP39 responsible for methyltransferase and adenyllyltransferase stimulatory activities, truncations of the VP39 ORF were expressed as indicated above. Three truncations resulting in deletions of 26, 75, and 112 carboxyl-ter-

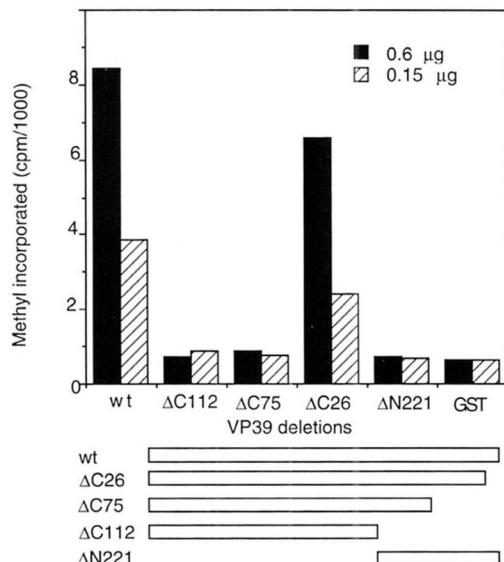


FIG. 1. Methyltransferase activities of truncated VP39 fusion proteins. Incorporation of the methyl group from Ado[methyl-³H]Met into BMV RNA was measured in the presence of 0.15 or 0.6 μg of affinity-purified glutathione *S*-transferase as a negative control or full-length (*wt*) or truncated VP39 sequences fused to glutathione *S*-transferase. The names and VP39 segments of the full-length and truncated fusion proteins are shown.

minal amino acids (ΔC26, ΔC75, and ΔC112, respectively) and one truncation of 221 amino-terminal amino acids (ΔN221) were engineered (Fig. 1, bottom). The sizes and amounts of GST-VP39 and of the mutated proteins were determined by PAGE and immunoblotting (data not shown), using an antibody raised to an amino-terminal peptide of VP39 (VP39-N-pep antibody; Ref. 11). Since the truncated protein ΔN221 did not bind this antibody, an alternative one directed against the whole VP39 protein (VP39-colic antibody; Ref. 11) was used.

Cap-specific methyltransferase activity was measured using Ado[methyl-³H]Met as the methyl donor and BMV RNA, which has an m7G(5')pppG-cap structure, as the methyl acceptor. Previous studies demonstrated that the label was incorporated solely into the penultimate G residue of the cap (10). Methyltransferase activities were determined at two protein concentrations. The activity of ΔC26 was similar to that of the wild-type enzyme, whereas no activity was detected with the other truncated proteins (Fig. 1, top).

Adenylyltransferase stimulatory activity was measured using a 60-nucleotide RNA primer corresponding to the 3' end of an early mRNA encoding the vaccinia virus growth factor. Previous studies had demonstrated that VP55 catalyzed the rapid and processive addition of about 35 adenylylates to the 3' ends of RNA primers (11). This initial burst was followed by two concurrent reactions: the processive adenylylation of additional primer molecules and the slow nonprocessive addition of adenylylates to poly(A) tails longer than 35 nucleotides. Added VP39 stimulated the formation of long poly(A) tails. Of the four truncated proteins, only ΔC26 possessed stimulatory activity and also appeared to prevent recycling of VP55 on new primer molecules (Fig. 2). Thus, the carboxyl-terminal 26 amino acids are not required for either methyltransferase or adenylyltransferase stimulatory activity.

The association of truncated VP39 fusion proteins with RNA was tested using a nitrocellulose filter binding assay. The RNA consisted of an internally labeled 80-mer polyribonucleotide possessing 30 consecutive adenylylates at its 3' end. The amount of RNA retained by the ΔC26 protein was similar to that of VP39; however, RNA binding decreased progressively with the more extensive carboxyl-terminal truncations (Fig. 3). Very

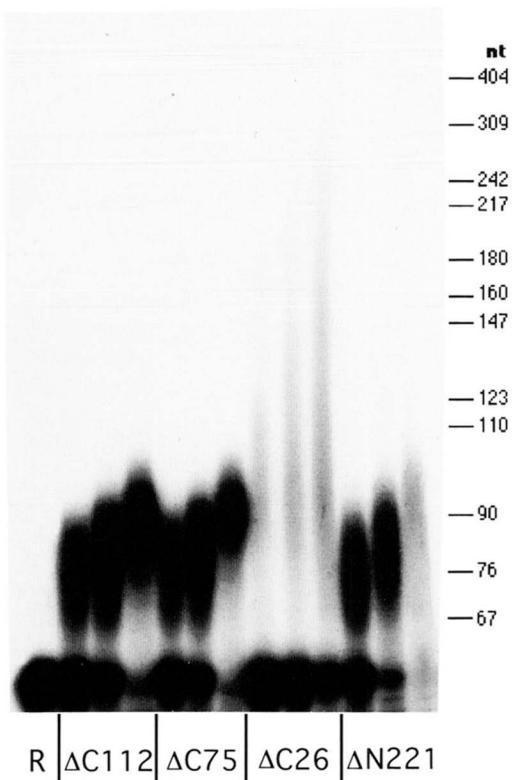


FIG. 2. Adenylyltransferase stimulatory activities of truncated GST-VP39 fusion proteins. The stimulatory activities of the full-length and truncated proteins indicated in Fig. 1 were assayed in the presence of VP55 and the vaccinia virus growth factor 60-mer RNA primer. Samples were removed after 30, 90, and 300 s and analyzed by PAGE and autoradiography. *R*, RNA primer alone; identities of GST-VP39 fusion proteins containing mutations are indicated in Fig. 1. Controls containing RNA primer with VP55 alone or with VP55 and unmutated GST-VP39 are shown in Fig. 7. The sizes in nucleotides (*nt*) of single-stranded DNA markers are shown.

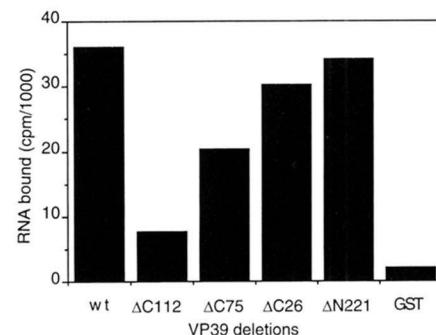


FIG. 3. RNA binding activities of truncated GST-VP39 fusion proteins. Glutathione *S*-transferase, wild-type (*wt*), and truncated fusion proteins (described in Fig. 1) were incubated with ³²P-labeled polyadenylated RNA and applied to a nitrocellulose filter. The labeled RNA retained on the filter was determined by scintillation counting.

little of the RNA incubated with the ΔC112 protein, containing the amino-terminal 221 amino acids of VP39, was retained on the filter. The reciprocal truncated protein ΔN221, containing only the carboxyl-terminal 112 amino acids of VP39, bound RNA as well as the fusion protein containing all 333 amino acids of VP39 (Fig. 3). Taken together, these data suggest that the RNA binding domain of VP39 lies between amino acids 221 and 307. The difference in RNA binding properties of ΔC75 and ΔC112 highlights the importance of the region between amino acids 221 and 257.

Effects of Charge Cluster → Ala Substitutions on the Activities of Affinity-purified VP39 Fusion Proteins—A second set of

FIG. 4. Predicted amino acid sequence of the J3R ORF encoding VP39. Asterisks have been placed under those charged amino acids that were changed to alanine. Each mutation, indicated by numbers from 1 to 21, contains a cluster of two to four substitutions. Arg-140 was changed to alanine in mutant proteins 10 and 11. Mutated proteins that exhibit methyltransferase (*MT*) or polyadenylation stimulatory (*PS*) activities comparable to wild-type are indicated by a *plus sign* and those that exhibit little or no activity by a *minus sign*. *+/-* denotes low activity.

MDVVSLSDKPFMYYFEEIDNELDYEPESANEVAKKLPYQQQLKLLLGELFFSKLQRHGILDGATVYIGSAPGTHIRYLRODHFYNLGVIIK	90
***	**
1	2
-	+/-
+	+
***	**
5	6
+	+
+	+
***	***
7	8
-	+
-	+
***	***
9	10 11
+	-
+	+
+	+
***	***
12	#
-	-
-	-
***	***
13	14
+/-	-
+	+
***	***
15	16
-	+
+	+
***	***
17	18
-	-
+	+
***	***
19	#
-	-
-	-
***	***
20	21
+	+
+	+
***	***
YCNKTFPTTKAKVLFQQSIFRFLNIPPTSTEKVSHPEIQRKISSKNSMSKNRNSKRSVRNSK	333
333	
#	
MT	
PS	

mutated proteins was prepared by systematically replacing clusters of potentially charged amino acids (Asp, Glu, His, Lys, Arg) with Ala (24). Twenty-one mutated proteins were constructed to change all charge clusters (defined as a local charge density of >50%) in VP39. The amino acid substitution mutations are indicated in Fig. 4. The amount of each protein was determined by immunoblotting using VP39-N-pep antibody (11) for each of the mutated proteins except for protein 1, for which the VP39-coli antibody (11) was used (data not shown). The E14A, E15A, and D17A substitutions in mutant protein 1 prevented binding of the antibody raised to the amino-terminal peptide. The purities of the fusion proteins used for enzyme assays are shown in Fig. 5. Methyltransferase activity was determined with 0.3, 1, and 1.6 μ g of each of the 21 charge cluster \rightarrow Ala mutated proteins. Ten (proteins 3–6, 8, 9, 15, 16, 20, and 21) had methyltransferase activities that were comparable to wild-type; two (proteins 2 and 13) had reduced activities; three (proteins 1, 7, and 17) had very low activities; and six (proteins 10–12, 14, 18, and 19) had background activities (Fig. 6). Similar results were obtained using thrombin-cleaved fusion proteins except for mutated protein 2, which had increased methyltransferase activity after cleavage (data not shown). The above assays were carried out at concentrations of BMV RNA and Ado[methyl- 3 H]Met which were $>100 \times$ and 0.5 \times the respective K_m values (5). When mutated protein 2 was also assayed at a 10-fold higher Ado[methyl- 3 H]Met concentration, activity was stimulated only 2.5-fold (data not shown).

Although methyltransferase was routinely assayed at 37 °C, the effect of each charge cluster \rightarrow Ala substitution was also determined at 30 and 42 °C. The methyltransferase activities were uniformly reduced at 42 °C compared with 30 or 37 °C such that the wild-type/mutant activity ratios were similar at all temperatures (data not shown).

Adenylyltransferase assays were performed with each of the mutated proteins in the presence of VP55. The majority stimulated the formation of long poly(A) tails and reduced the amount of primer with short 30–35 adenylylate tails (Fig. 7). Mutated proteins 12, 18, and 19 were severely defective in adenylyltransferase stimulatory activity, and the assay results were similar to that obtained with VP55 alone (Fig. 7). Mutated protein 7 also appeared to inhibit adenylylation by VP55, since the abundance of short poly(A)-tailed molecules was reduced. These mutated proteins (proteins 7, 12, 18, and 19) had little or no methyltransferase activity (Fig. 6). Other methyltransferase-deficient proteins (proteins 1, 10, 11, 14, and 17) retained adenylyltransferase activity (Fig. 7).

RNA filter binding assays were also performed with the mutated proteins. Each fusion protein caused a greater retention

of labeled RNA than glutathione S-transferase (Fig. 8). The most severely affected in RNA binding (Fig. 8; proteins 18 and 19) had substitutions within the RNA binding region (amino acids 221–307), defined by deletion mutagenesis (Fig. 3), and had lost methyltransferase (Fig. 6) and adenylyltransferase stimulatory (Fig. 7) activities. However, other mutated proteins such as 5 and 6 also exhibited lower than wild-type RNA binding yet were not notably affected in methyltransferase or adenylyltransferase stimulatory activities. In view of this apparent anomaly, the 21 mutated proteins were again expressed in *E. coli*, purified, and assayed for RNA binding. However, the relative activities of the reexpressed proteins (data not shown) were similar to the values in Fig. 8.

Effects of Cys to Ser Substitutions on the Activities of Affinity-purified VP39 Fusion Proteins—Three glutathione S-transferase fusion proteins, in which the 2 cysteine residues of VP39 were changed to serine either individually or together, were constructed. These mutations had no significant effect on either methyltransferase (Fig. 6) or adenylyltransferase stimulatory (data not shown) activities.

DISCUSSION

Although VP39 is a relatively small protein, composed of 333 amino acids, it has two distinct and apparently unrelated mRNA modification activities. The nucleoside-2'-O-methyltransferase activity is specific for the capped 5' end of the mRNA, whereas the adenylyltransferase stimulatory activity is manifested at the 3' end. Whether the multifunctional nature of the protein enhances its role or is a by-product of an evolutionary process is unknown. It will be interesting to compare VP39 with its eukaryotic counterparts after the structures of genes encoding them have been reported. In the absence of sequence homologies or defined structural motifs, we carried out broad mutagenesis studies aimed at locating regions of VP39 which are important for its various activities. Most significantly, we found amino acid substitutions that eliminated methyltransferase without detectably affecting adenylyltransferase stimulatory activity.

To facilitate the preparation of mutated forms of VP39, the ORF was expressed as an inducible glutathione S-transferase fusion protein in *E. coli*. Soluble extracts were then purified using GS affinity beads. Methyltransferase, adenylyltransferase stimulatory, and RNA binding activities could be measured without removing the glutathione S-transferase moiety. We started our analysis by making deletions in VP39. Several amino-terminal truncations proved to be insoluble (data not shown), and we therefore made carboxyl-terminal truncations. Although removal of the last 26 amino acids had no detectable

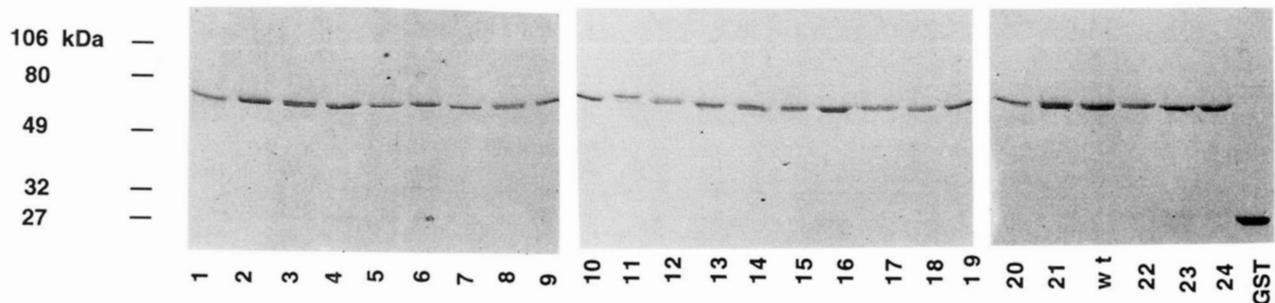


FIG. 5. SDS-PAGE analysis of mutated GST-VP39 fusion proteins. Recombinant fusion proteins and glutathione *S*-transferase were expressed by *E. coli*, affinity purified using GS beads, and quantitated by SDS-PAGE and immunoblotting. Similar amounts of each protein were analyzed by SDS-PAGE and stained with Coomassie Brilliant Blue. Charge cluster → Ala mutated proteins, defined in Fig. 4, are numbered from 1 to 21. The proteins 22, 23, and 24 contain Cys-178 → Ser; Cys-272 → Ser; and both Cys-178 → Ser and Cys-272 → Ser substitutions, respectively. Wild-type VP39 fused to glutathione *S*-transferase is labeled *wt*. Glutathione *S*-transferase alone is also shown. The electrophoretic mobilities and masses of standard proteins are indicated.

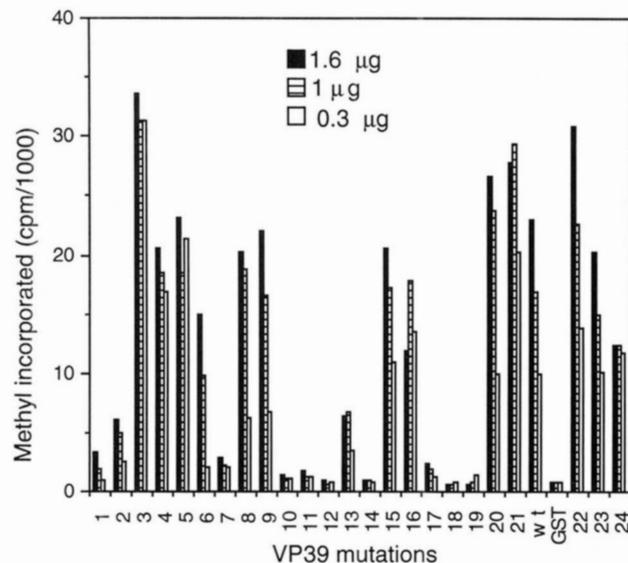


FIG. 6. Methyltransferase activities of charge cluster → Ala and Cys → Ser mutated VP39-GST fusion proteins. Incorporation of the methyl group from Ado[methyl-³H]Met into BMV RNA was measured in the presence of 0.3, 1.0, or 1.6 μg of affinity-purified glutathione *S*-transferase or wild-type (*wt*) or mutated VP39 sequences fused to glutathione *S*-transferase. The mutated proteins numbered 1–21 contain charge cluster → Ala substitutions as indicated in Figs. 4 and 5. The proteins 22–24 contain Cys-178 → Ser; Cys-272 → Ser; and both Cys-178 → Ser and Cys-272 → Ser substitutions, respectively.

effect on methyltransferase or adenyllyltransferase stimulatory activities, both activities were lost after removal of an additional 49 amino acids. The latter truncation diminished RNA binding, which was abolished when 112 amino acids were deleted from the carboxyl terminus of VP39. The finding that a glutathione *S*-transferase fusion containing the carboxyl-terminal 112 amino acids of VP39 retained RNA in the filter binding assay indicated that this segment is necessary and sufficient for RNA binding. Nevertheless, the carboxyl-terminal fragment lacks both methyltransferase and adenyllyltransferase stimulatory activities. This region contains basic amino acids but lacks known RNA-binding protein signatures, like RGG boxes or RNP motifs (25, 26).

Structure-function studies were continued using a charge cluster → Ala scanning mutagenesis strategy (24). This approach was chosen to minimize gross changes in protein folding, with the rationale that clusters of charged residues are solvent-exposed and may not contribute greatly to tertiary structure. The VP39 ORF predicts 21 clusters of amino acids with greater than 50% charged residues. Twenty-one GST-VP39 fusion proteins, each of which contained one of the charge

cluster → Ala mutations in VP39, were synthesized and purified. The characteristics of these mutated proteins are summarized in Fig. 4. Nine had severely or completely defective methyltransferase activity, and two more had moderately reduced activity. Although the 11 mutations were distributed throughout the protein, six that completely inhibited methyltransferase activity occurred in two sets of three consecutive charge clusters (Fig. 4, proteins 10–12 and 17–19). Charge cluster → Ala mutants frequently show temperature sensitivity (27); however, this was not detected when the methyltransferase activities of the mutated VP39 proteins were measured at 30 and 42 °C. Only four mutant proteins had severely inhibited adenyllyltransferase stimulatory function (measured only at 30 °C; Fig. 4, proteins 7, 12, 18, and 19), and these also had impaired methyltransferase activity. Thus, we had several mutations that selectively and severely affected methyltransferase (Fig. 4, proteins 1, 10, 11, 14, and 17) but none that only affected adenyllyltransferase stimulatory function. The former mutations could affect either AdoMet binding or catalysis. The changes in mutated proteins 10 and 11 lie within the segment D VR SK R G G, which shows some similarity to a conserved sequence in the amino-terminal region of the NS5 protein of flaviviruses (28). The conserved NS5 sequence, which may play a role in AdoMet-dependent cap methylation by flaviviruses, can in turn be aligned with sequences in enzymes that methylate small molecules (28, 29) and may form part of a site that interacts with AdoMet. The above segment of VP39 can also be aligned with motif I of a DNA (cytosine-5-)methyltransferase (30). One amino acid in this region has been shown to contact AdoMet in the crystal structure of the *Hha*I DNA methyltransferase (31). However, the significance of the relationship between these methyltransferases is uncertain since a cysteine residue is the active site nucleophile in DNA methyltransferases, and cysteine is not required for the activity of the vaccinia virus mRNA (nucleoside-2'-O-)methyltransferase.

Cysteine residues have been implicated in the transfer of methyl groups by thymidylate synthase (32), tRNA (uracil-5-)methyltransferase (33), and DNA (cytosine-5-)methyltransferases (34), and their replacement abolishes activity. We found that when either or both of the 2 cysteine residues in VP39 were replaced by serine, there was no effect on methyltransferase (or adenyllyltransferase stimulatory) activity, consistent with a fundamentally different mechanism of methyl transfer.

The mechanism by which VP39 stimulates adenyllyltransferase activity is not precisely known. One possibility is that VP39 enhances the association of VP55 with poly(A) tails. In that case, mutations that affect either VP39-VP55 or VP39-polyribonucleotide interactions would be deleterious. Because of the ready dissociability of the VP39-VP55 dimer (11), we have not yet determined the effects of mutations on subunit

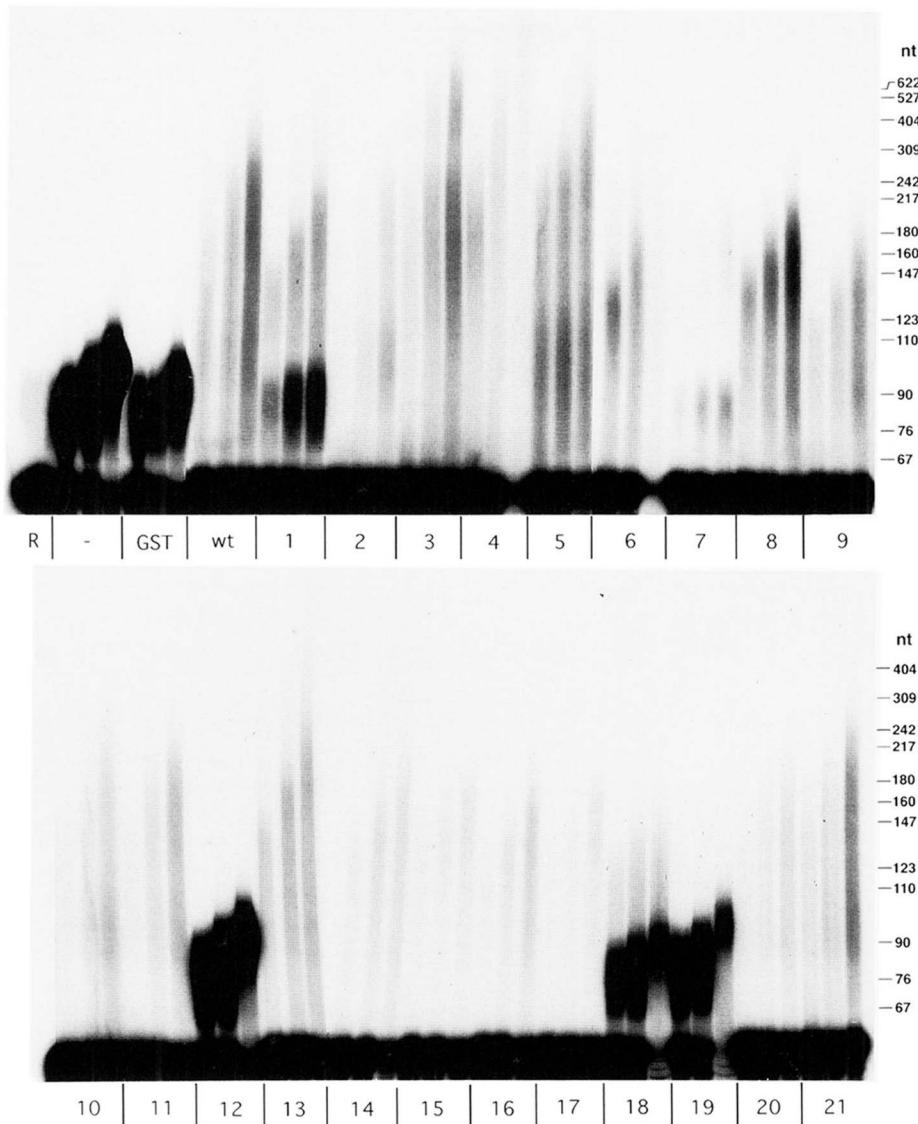


FIG. 7. Adenylyltransferase stimulatory activities of charge cluster → Ala mutated GST-VP39 fusion proteins. Glutathione S-transferase or wild-type and truncated GST-VP39 fusion proteins were assayed in the presence of VP55 and the vaccinia virus growth factor 60-mer RNA primer. Samples were removed after 30, 90, and 300 s and analyzed by PAGE and autoradiography. Reactions contained RNA primer alone (*R*), with VP55 (–), with VP55 and unmutated GST-VP39 (*wt*), or with VP55 and GST-VP39 fusion proteins containing mutations indicated in Fig. 4. The sizes in nucleotides (*nt*) of single-stranded DNA markers are shown.

interactions. Mutations that affect RNA binding could well alter both RNA methyltransferase and poly(A) stimulatory activities, *e.g.* the carboxyl-terminal deletion mutations and charge cluster → Ala substitution mutations 18 and 19. However, mutations that affect only VP55 binding might be specific for the adenylyltransferase stimulatory activity. Our failure to detect such a mutant may simply be a consequence of only choosing high amino acid charge density regions for mutagenesis.

Despite the presence of a 2'-*O*-methylated nucleotide in the cap structure of all animal and animal virus mRNAs, its role is entirely unknown. *In vitro* studies have shown little effect of the ribose methylation on translation or ribosome binding (35). Moreover, the presence of this cap modification in cytoplasmic viral mRNAs makes an exclusively nuclear role very unlikely. Efforts² to isolate a mutant vaccinia virus with a disrupted VP39 gene have failed, implying that expression of this protein is essential. However, the interpretation of this result is ambiguous since VP39 has two activities. A better approach, made possible

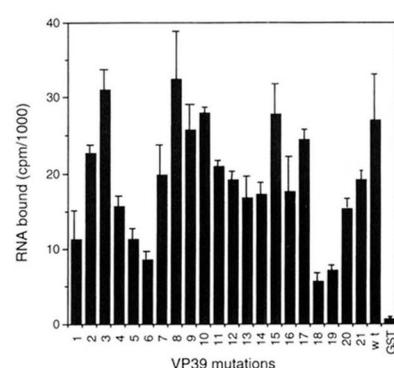


FIG. 8. RNA binding activities of charge cluster → Ala mutated GST-VP39 fusion proteins. The glutathione S-transferase or wild-type and charge cluster → Ala mutated GST-VP39 fusion proteins described in Fig. 4 and 5 were incubated with ³²P-labeled polyadenylated RNA and applied to a nitrocellulose filter. The labeled RNA retained on the filter was determined by scintillation counting. Standard error bars are shown.

² B. S. Schnierle, unpublished data.

by the present study, would be to mutagenize vaccinia virus to impair selectively the methyltransferase function of VP39 while retaining adenylyltransferase stimulatory function.

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