

Methyltransferase-specific domains within VP39, a bifunctional protein that participates in the modification of both mRNA ends

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ABSTRACT

VP39 is a bifunctional vaccinia virus protein that acts as both a cap-dependent 2'-O-methyltransferase and a poly(A) polymerase processivity factor. An analysis of C-terminal truncation mutants of a GST-VP39 fusion protein indicated the presence of a protease-sensitive C-terminal “tail” 36–43 amino acids in length that is non-essential for VP39 function. Fourteen new VP39 point mutants, containing either single or multiple-clustered amino acid substitutions, were expressed in *Escherichia coli*. Of the eight that retained either one or both of the activities of VP39, seven were specifically methyltransferase-defective. None was specifically defective in adenylyltransferase stimulation. The nature of the methyltransferase defects in 10 of the methyltransferase-specific defectives, identified both herein and in a previous study (Schnierle BS, Gershon PD, Moss B, 1994, *J Biol Chem* 269:20700–20706), was investigated using two novel substrate-binding assays. Three of the mutants (and possibly a fourth), whose lesions were juxtaposed and centrally located within VP39, exhibited anomalous S-adenosyl-L-methionine (AdoMet) binding behavior, identifying residues important for AdoMet binding and possibly also for catalysis. A surface plasmon resonance-based assay measured the interaction of VP39 with uncapped and 5'-cap 0-terminated oligo(A). A cap 0-dependent association-rate enhancement was observed for wild-type VP39 and 4 of the 10 mutant proteins. Two others were identified as defective in cap binding, and a third as partially defective. The lesions within the latter three mutants were closely apposed, and located toward the N-terminus of VP39. We have thus identified regions of VP39 important for interaction with its two substrates for cap-dependent methyltransferase activity: AdoMet and cap 0.

Keywords: adenosyl; BIACore; cap; methionine; methyltransfer; mRNA; poly(A); vaccinia

INTRODUCTION

Vaccinia virus protein VP39 is very unusual in that it interacts with both ends of nascent mRNA. At the mRNA 5' end, the protein functions as the enzyme S-adenosyl-L-methionine:mRNA (nucleoside-O^{2'}-)-methyltransferase, specifically methylating the 2' OH of the penultimate nucleotide of the mRNA 5' cap structure to convert the cap 0 structure ($m^7G(5')pppG$) to cap I ($m^7G(5')pppG^m$) (Schnierle et al., 1992). At the 3' end, VP39 functions as the stimulatory (smaller) subunit of the vaccinia virus poly(A) polymerase (PAP). Vaccinia PAP is a heterodimer, in which the larger (VP55) subunit performs catalytic functions in

poly(A) tail formation (Gershon et al., 1991). In the absence of the VP39 subunit, VP55 polyadenylates a primer representing an mRNA 3' end bimodally: 30–35-nt tails are added in a rapid, highly processive initiating burst, followed by an abrupt transition to a slow, nonprocessive mode of adenylate addition. Consistent with this, short pre-formed tails are extended to a net length of 30–35 nt in the initiating polyadenylation burst (Gershon & Moss, 1992). Specific RNA sequences are not required for the polyadenylation burst, although the presence of uridylates within the 3'31–40 nt of the RNA primer is a prerequisite (Gershon & Moss, 1993a). Cessation of processive polyadenylation by VP55 apparently results from its translocation from the uridylate-containing RNA sequences to the nascent oligo(A) tail (Gershon & Moss, 1993a). VP39 possesses no independent PAP activity, but does convert the VP55-catalyzed slow, non-processive polyadenylation that occurs after the initial

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burst, to a rapid, semi-processive reaction (Gershon & Moss, 1993b), apparently by anchoring the VP55 subunit to the growing poly(A) tail. Consistent with this action, VP39 has properties of a poly(A)-binding protein (Gershon et al., 1991).

The above studies contribute toward a growing body of literature characterizing functional mRNA 5' and 3' interactions. Specific studies include reports of: effects of poly(A) or the poly(A) tail upon cap-dependent translational initiation (Jackson & Standart, 1990; Monroe & Jacobson, 1990a, 1990b; Gallie & Tanguay, 1994); a poly(A) nuclease that is also required for translational initiation (Sachs & Deardorff, 1992); a deadenylation-dependent decapping step in the degradation of yeast mRNA (Muhlrad et al., 1994); and a cap methylation event that is coordinated with developmental poly(A) tail elongation (Kuge & Richter, 1995). VP39 offers a unique opportunity to characterize, in detail, a single, relatively well-characterized protein that modifies both mRNA ends in a well-defined manner. An initial mutagenesis study of VP39 (Schnierle et al., 1994) indicated the presence of a unique RNA-binding domain required for both activities, located within a C-terminal 85-amino acid region of the protein. Despite the identification of several additional mutants of VP39 defective specifically for methyltransferase activity, the roles of the mutated residues in the methyl transfer reaction were not defined. Furthermore, VP39 mutants specifically defective in adenylyltransferase activity were not identified (Schnierle et al., 1994). Here, as part of an ongoing attempt to fully define the protein domains required for the two VP39 functions, we have constructed additional VP39 mutants and identified methyltransferase-specific domains by the introduction of novel assays for the interaction of VP39 with its two methyltransferase substrates: cap 0 and AdoMet.

RESULTS

C-terminal "tail" region of VP39 is not essential for protein activity

Previous studies indicated that the truncation of 26 residues from the C-terminus of VP39 did not affect the methyltransferase, adenylyltransferase stimulatory, or RNA-binding activities of VP39, whereas the truncation of 74 or 111 residues eliminated methyltransferase and adenylyltransferase activities, and either reduced or completely eliminated RNA-binding activity (Schnierle et al., 1994). We further investigated the C-terminal region of VP39 by cloning, expression, and purification of additional truncation mutants, followed by assays for adenylyltransferase-stimulatory and methyltransferase activities (Fig. 1). The newly cloned truncation mutants ($\Delta C32, 34, 36, 38, 44$, and 50) were initially analyzed for purity and abundance by Coomassie-stained

SDS-PAGE, after their recovery from crude *Escherichia coli* extracts using GSH-agarose beads (Fig. 1A). Prior to SDS-PAGE, VP39 moieties were released from the GST-VP39 fusion proteins by thrombin treatment, to ensure that recombinant proteins could be distinguished from a GSH-agarose-adsorbed *E. coli* protein, whose electrophoretic mobility was similar to that of uncleaved GST-VP39. Three previously characterized VP39 truncation mutants ($\Delta C26, 74$, and 111 [Schnierle et al., 1994]), along with wild-type VP39, were analyzed as controls. The data of Figure 1A clearly indicated that the C-terminal 36–37 amino acids of VP39 were not required for the recovery of high levels of purified *E. coli*-expressed recombinant protein, whereas further truncation severely affected protein recovery. The differences in protein recovery levels apparently resulted from differences in protein solubility in *E. coli* and/or extraction buffer, because levels of recombinant protein were much less variable upon direct solubilization of *E. coli* using SDS (as determined by SDS-PAGE and immunoblotting, data not shown). The data of Figure 1A were thus consistent with the possession by VP39 of a C-terminal "tail" 36–37 amino acids in length, coupled with an impairment of correct protein folding upon deletion of sequences beyond the putative tail.

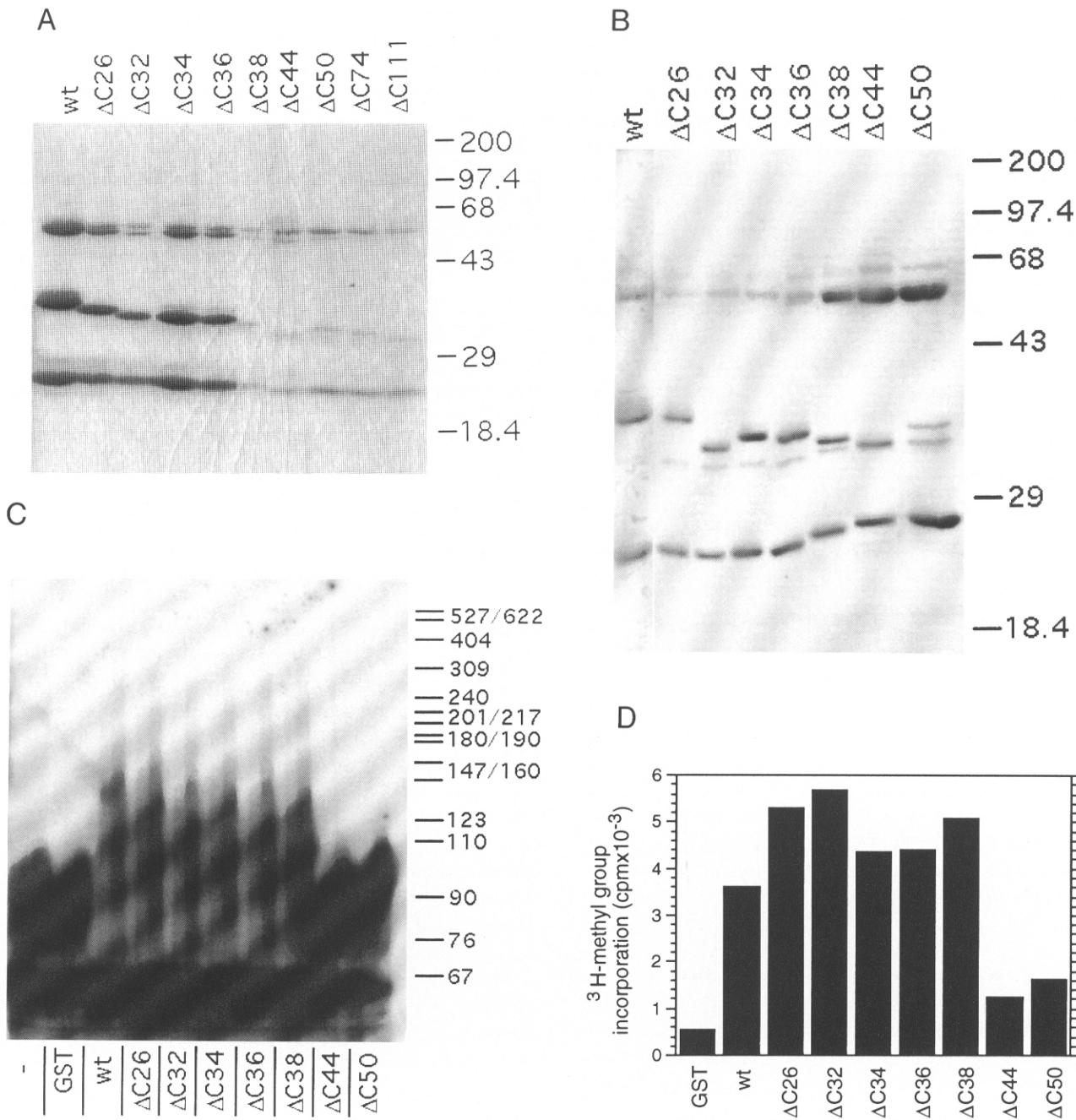
Because the GST moiety does not affect VP39 activity in either the methyltransferase or the adenylyltransferase-stimulation assay (Schnierle et al., 1994), GST-VP39 fusion proteins were not cleaved with thrombin prior to being assayed. Instead, after their purification from *E. coli* extracts using GSH-agarose, the intact proteins were eluted using GSH. Prior to activity determinations, the widely disparate levels of the recombinant proteins (Fig. 1A) were equalized by thrombin cleavage of small aliquots of the protein samples, followed by SDS-PAGE, Coomassie-staining, and digitization by scanning densitometry. The resulting data were used to equalize concentrations of the uncleaved proteins by sample dilution, followed by a second round of small-scale thrombin cleavage and SDS-PAGE, to determine the accuracy of equalization (Fig. 1B). Although some disparities remained at this stage in the levels of recombinant proteins, a second round of sample dilution was performed, based upon a digitized image of the gel shown in Figure 1B, prior to assaying the proteins. Results of adenylyltransferase-stimulatory and methyltransferase assays of the intact GST-fusion proteins are shown in Figure 1C and D, respectively. Whereas the truncation of 38 residues from the C-terminus of VP39 did not affect specific activities in either assay, truncation of 44 or 50 residues completely abrogated adenylyltransferase-stimulatory activity and reduced methyltransferase activity by ~75% (unpublished replicate assays supported the results shown). These data indicate that the C-terminal 38–43 residues of VP39 (i.e., the 36–37-residue C-terminal "tail" region plus some

additional residues) is nonessential for either of the two activities of VP39.

A closely spaced pair of bands is characteristic of VP39 obtained from vaccinia virions, virus-infected cells, or overexpression systems even when extracted directly into SDS-PAGE loading buffer (cf. Fig. 1A of Gershon & Moss, 1993b). Experiments with a C-terminally 6His-tagged version of VP39 have indicated that the slightly faster migrating (minor) form is a C-terminally truncated version of the full-length protein (data not shown). This sensitivity of the extreme C-terminus of VP39 to proteolysis is consistent with the presence of a "tail" structure, as characterized above.

Residues of VP39 required specifically for methyltransferase activity are more prevalent than those required specifically for adenylyltransferase stimulatory activity

Previous analysis of a comprehensive set of charge cluster → alanine point mutants of VP39 identified several that were specifically inactive in methyltransferase activity, but none that was specifically inactive in adenylyltransferase-stimulatory activity (Schnierle et al., 1994). To further analyze structure-function relationships within VP39, 14 new point mutants were made, targeting previously unmutated residues in all parts of



the protein with the exception of the C-terminal "tail" region. Thus, several unclustered, charged residues were targeted for charge neutralization or charge reversal. In addition, several hydrophobic clusters were targeted for conversion to charge clusters. Chemically conservative changes were also made, at positions determined to be highly conserved in the VP39 sequences from different poxviruses. Figure 2 shows the amino acid changes in the new VP39 point mutants, these being denoted with the prefix "SS" to distinguish them from the previous set of 21 alanine-scanning mutants (Schnierle et al., 1994) that are also shown (prefixed "AS").

In common with the truncation mutants (above), the new point mutants showed considerable variation in levels of recovered protein (data not shown). Low recovery correlated with the replacement of clustered hydrophobic residues with those possessing multiple-charged, "bulky" side chains (e.g., clusters of three arginines), consistent with the occurrence of inefficient protein folding. Prior to assaying for methyltransferase and adenylyltransferase stimulatory activities, levels of the point mutant proteins were equalized to that of wild-type VP39, as described for the truncation mutants, above. Figure 3A shows relative protein levels after an initial round of equalization. The results of adenylyltransferase stimulatory and methyltransferase assays are shown in Figure 3B and C, respectively. Only mutant SS14 retained full activity in both assays. Six others (SS3, 6, 7, 9, 10, and 11) were completely in-

active in both assays. This double-negative phenotype coincided with low protein expression levels (data not shown), indicating that even the small amounts of recovered, soluble protein might be misfolded. The seven remaining mutants (SS1, 2, 4, 5, 8, 12, 13) were specifically defective in methyltransferase activity. Three of them (SS1, 2, and 5) appeared to be completely defective, one (SS4) appeared to be almost completely defective (retaining ~10% of wild-type activity levels), whereas the remaining three (SS8, 12, and 13) appeared to be partially defective. Failure to specifically abolish adenylyltransferase-stimulatory activity in any of the mutants extends an earlier, comparable finding using a panel of VP39 alanine-scanning mutants (Schnierle et al., 1994). The distribution of residues whose mutation led specifically to methyltransferase defects (Fig. 2) indicated the possession by VP39 of at least two linear "domains" with roles specifically in methyltransfer. These are: residues ~14–46 (located toward the N-terminus) and residues ~138–159 (located toward the center of the protein). In the remainder of this study, we attempt to correlate methyltransferase-specific VP39 defects with the binding of methyltransferase substrates and/or catalysis.

Interaction of the specifically methyltransferase-deficient VP39 point mutants with AdoMet

Using a novel solid-phase assay, AdoMet binding activity was determined for 10 of the 14 VP39 point mutants

FIGURE 1. Analysis of the six new VP39 truncation mutants. **A:** Coomassie-stained SDS-PAGE, showing thrombin-cleaved, wild-type GST-VP39 ("wt"), and C-terminal truncation mutants thereof. Recombinant *E. coli*-expressed proteins were electrophoresed after making soluble *E. coli* extract, incubation of equal volumes of extract with GSH-agarose beads, washing the beads with extraction buffer, then incubating the beads with thrombin protease. The mobilities of SDS-PAGE size markers are indicated. The ~26-kDa and ~60-kDa bands, present in all lanes, represent the GST moiety and an endogenous *E. coli* protein, respectively. Residual uncleaved fusion protein migrates slightly faster than the endogenous *E. coli* protein, and the thrombin-released VP39 moieties migrate with mobilities intermediate between those of the 29- and 43-kDa markers. The apparently anomalous migration of some of the truncated proteins was not due to DNA sequence errors around the 3' ends of inserts (data not shown), and was reproducible with different plasmid isolates of specific clones. Although protease inhibitors were included during extraction procedures, we cannot rule out differential sensitivities of the mutants to nonspecific cleavage during isolation and/or at the thrombin step, or some kind of artifact of SDS-PAGE. **B:** Equalization of protein concentrations for the C-terminal truncation mutants. For each mutant, protein amounts were quantitated from a gel equivalent to that shown in A. According to the results obtained, GSH-agarose eluates were diluted to equalize protein concentrations. Equal-volume aliquots were then treated with thrombin and re-electrophoresed, as shown. The fastest migrating band corresponds to the GST moiety, the second fastest band corresponds to the mobility of thrombin protease. Other bands, with mobilities intermediate between those of the 29- and 43-kDa markers, are the VP39 moieties. Slower migrating bands correspond to endogenous *E. coli* proteins. The anomalous migration of some VP39 moieties presumably results from the factors outlined for A. **C:** Adenylyltransferase-stimulatory activities of GST-VP39 C-terminal truncation mutants. Assays for elongation of the VGF 60mer RNA primer contained either VP55 alone ("−"), or VP55 plus either the GST moiety ("GST"), wild-type GST-VP39 ("wt"), or the GST-VP39 C-terminal truncation mutants denoted in the standard manner. For each of the 10 assays shown, the left-most, center, and right-most lanes represent samples taken from in vitro polyadenylation reactions 30, 90, and 300 s after the initiation of reactions. Sizes, in nucleotides, of single-stranded DNA markers are shown. **D:** Cap-dependent 2'-O-methyltransferase activities of the GST-VP39 C-terminal truncation mutants. Initially, wild-type GST-VP39 was used to establish that the assay was linear with time at the protein concentration employed, i.e., that cpm values represent reaction rate as opposed to yield (data not shown). Consistent with this, the maximum observed cpm value of ~6,000 cpm was calculated to represent the formation of 0.33 pmol of ^3H -methylated cap, a 28% conversion of cap 0 to cap I in the assay. A representative experiment is shown; replicates yielded comparable results. To ensure that contaminating *E. coli* proteins were not influencing assay results for the more poorly expressing mutants, a mixing experiment was done, in which wild-type VP39 was mixed with either truncation mutant $\Delta\text{C}50$ or an equivalent volume of buffer. Very similar methyltransferase activities obtained with the two mixtures (data not shown), indicating that maximal inputs of *E. coli* protein did not affect assay results.

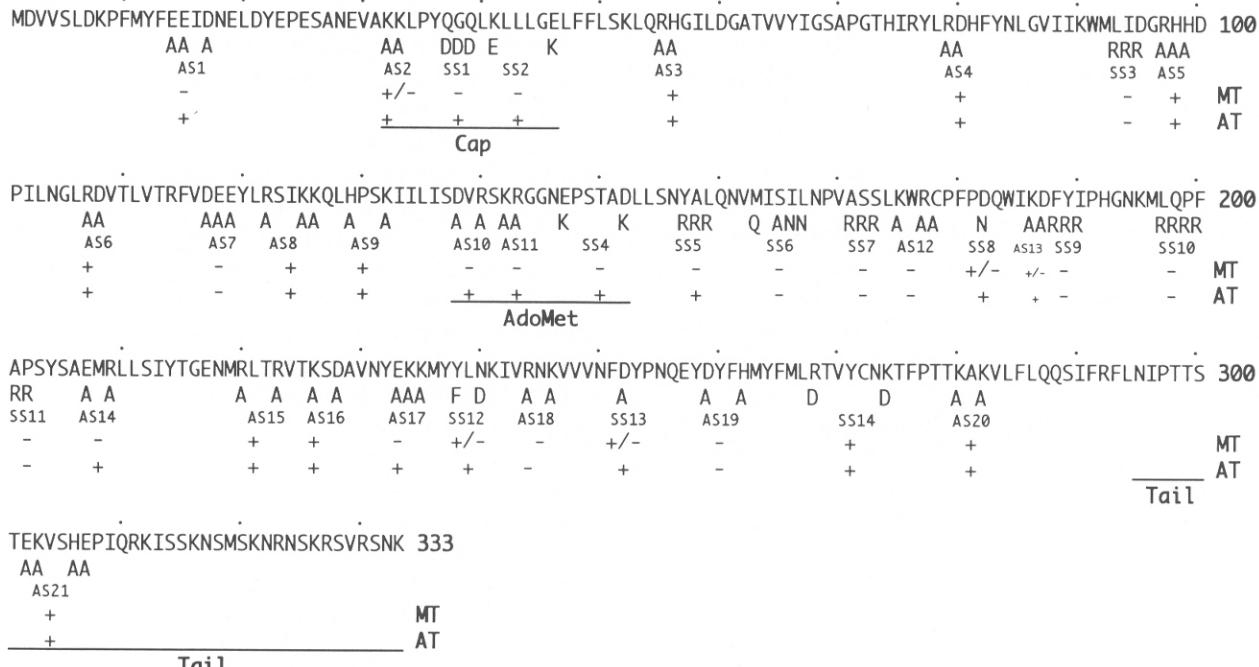


FIGURE 2. Map showing VP39 point mutations generated in both this and a previous study (Schnierle et al., 1994). Those mutants denoted AS1–AS21 comprise a set of alanine-scanning mutants previously referred to as 1–21 (Schnierle et al., 1994). Newly generated point mutants are referred to as SS1–SS14 (denoting “second series”). Denoted beneath the sequence, on consecutive lines are: the substituted amino acids; the mutant name; the methyltransferase activity (“MT”) and adenylyltransferase-stimulatory (“AT”) activities of the mutant proteins (as shown in Fig. 3B,C); and horizontal lines representing functional domains identified in the current study. The R140 → A substitution was common to mutants AS10 and AS11 (Schnierle et al., 1994).

that were shown, either here or in the previous study (Schnierle et al., 1994), to be specifically methyltransferase-defective (Fig. 2). Wild-type GST-VP39 and the GST moiety alone were included as controls. Despite its methyltransferase-defective phenotype, mutant AS1 was not analyzed further because of the impractically low expression levels obtained. Mutants SS8, 12, and 13 were also not included because of their partially methyltransferase-active phenotype. The AdoMet-binding assay comprised the immobilization of recombinant proteins from *E. coli* extracts to GSH-agarose beads and washing of the beads, followed by their incubation with ³H-AdoMet-containing buffer, rapid removal of the buffer, and quantitation of bead-associated ³H by scintillation counting. Beads were not washed after incubation with ³H-AdoMet because this was found to result in the rapid loss of VP39-bound AdoMet (data not shown). The influences of relatively high background ³H-AdoMet binding (presumably due to the entrapment of ³H-AdoMet within the bead matrix), along with variations in protein expression levels and potential for cross-contamination of mutants, were minimized by: (1) equating the AdoMet binding quotient (“AdoMet binding coefficient”) to the change in bead-associated ³H with amount of immobilized recombinant protein, after immobilizing four different amounts of protein (each in duplicate) to fixed volumes

of beads; (2) almost completely saturating the beads with GST-VP39 at the highest levels of immobilized protein employed; (3) rapidly and thoroughly draining the beads after the completion of binding reactions using an ultra-fine pipet tip; (4) for each recombinant protein, running the complete assay 2–5 times, from multiple *E. coli* transformations and multiple rounds of *E. coli* growth, induction, and harvest. The concentration range for bead-bound GST-VP39 employed in a typical assay was ~1.5–50 μM.

The mean AdoMet binding coefficients obtained for the 10 mutants are plotted in Figure 4. Those mutants (SS2, AS13, AS14, AS17) for which the range of binding coefficients enclosed by the ± average deviations from the mean overlapped the equivalent band of values for wild-type GST-VP39 were considered wild-type for AdoMet binding. Mutants AS2 and SS1, which appeared by these criteria to be slightly compromised in AdoMet binding (Fig. 4), are nonetheless also considered to be “wild-type” in AdoMet binding. Mutant SS5, although apparently compromised in AdoMet binding, is conservatively considered to be “unknown” due to the relatively large average deviations of replicate assays from the mean. This variability indicates that SS5, which exhibited significant preparation-to-preparation variability in overall levels of recovered protein, may also exhibit variability in the specific activity of the sol-

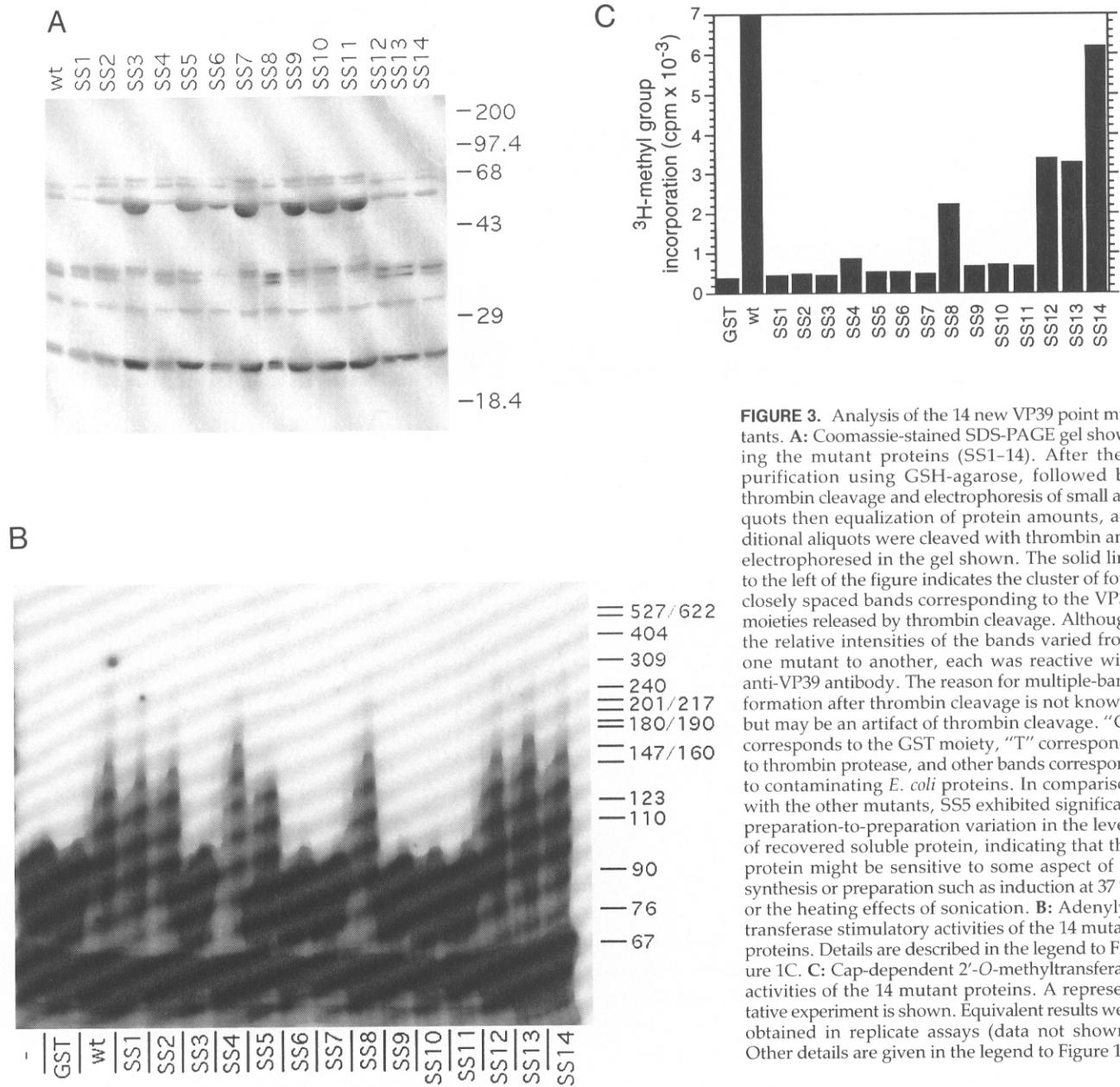


FIGURE 3. Analysis of the 14 new VP39 point mutants. **A:** Coomassie-stained SDS-PAGE gel showing the mutant proteins (SS1-14). After their purification using GSH-agarose, followed by thrombin cleavage and electrophoresis of small aliquots then equalization of protein amounts, additional aliquots were cleaved with thrombin and electrophoresed in the gel shown. The solid line to the left of the figure indicates the cluster of four closely spaced bands corresponding to the VP39 moieties released by thrombin cleavage. Although the relative intensities of the bands varied from one mutant to another, each was reactive with anti-VP39 antibody. The reason for multiple-band formation after thrombin cleavage is not known, but may be an artifact of thrombin cleavage. "G" corresponds to the GST moiety, "T" corresponds to thrombin protease, and other bands correspond to contaminating *E. coli* proteins. In comparison with the other mutants, SS5 exhibited significant preparation-to-preparation variation in the levels of recovered soluble protein, indicating that this protein might be sensitive to some aspect of its synthesis or preparation such as induction at 37 °C or the heating effects of sonication. **B:** Adenylyl-transferase stimulatory activities of the 14 mutant proteins. Details are described in the legend to Figure 1C. **C:** Cap-dependent 2'-O-methyltransferase activities of the 14 mutant proteins. A representative experiment is shown. Equivalent results were obtained in replicate assays (data not shown). Other details are given in the legend to Figure 1D.

uble fraction. One mutant, AS10, was clearly totally defective in AdoMet binding. The two remaining mutants, AS11 and SS4, possessed significantly greater AdoMet binding activity than wild-type VP39, despite their inactivity in the methyltransferase assay. The amino acid changes in AS10, AS11, and SS4 were closely apposed, and centrally located within VP39 (Fig. 2).

Interaction of the specifically methyltransferase-deficient VP39 point mutants with the mRNA 5'-Cap

A surface plasmon resonance-based assay was developed to investigate the interaction of VP39 with the

mRNA 5' cap 0 structure. For this assay, equal amounts of uncapped and 5'-cap 0-terminated versions of an otherwise identical oligoadenylate RNA were immobilized, via their 3'-ends, to the sensing surfaces of adjacent flow cells of a BIACore biosensor chip. Initially, wild-type GST-VP39 was injected to each of the two flow cells in the presence of either 20, 60, or 100 mM NaCl, and each injection was followed by a washout of the flow cell with running buffer containing 60 mM NaCl. The resulting sensorgrams are shown in Figure 5A. Whereas no cap-dependent characteristics could be detected in the dissociation phases of the sensorgrams, VP39-RNA association rates were significantly greater for the capped RNA (Fig. 5A). The association-rate enhancement due to the cap was most striking at

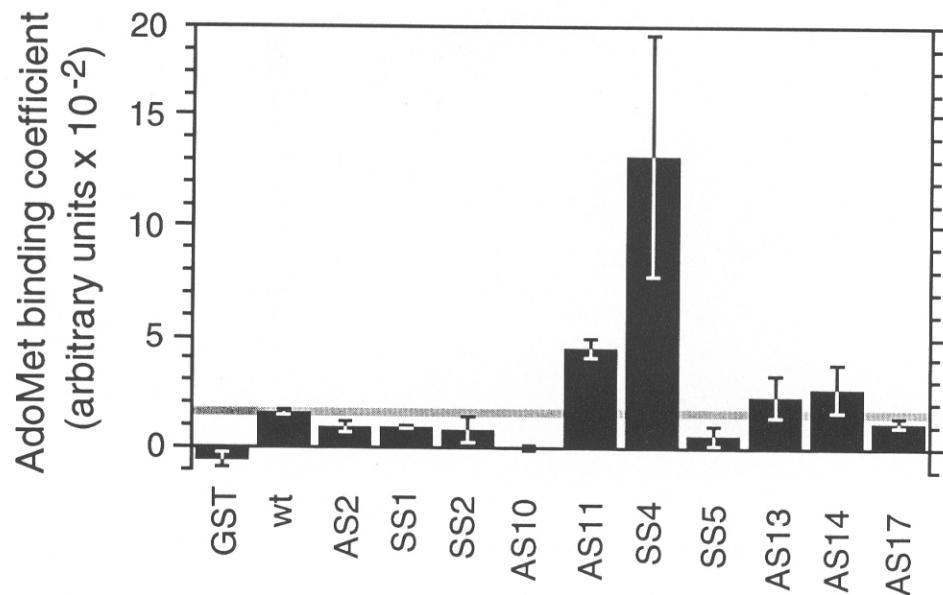
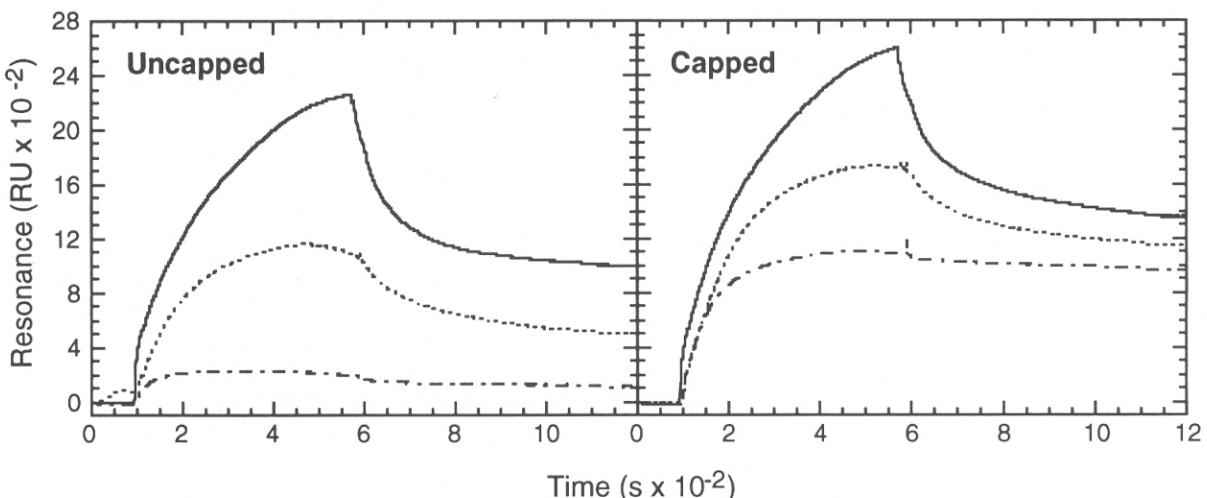


FIGURE 4. AdoMet binding activities of mutants that were specifically defective in methyltransferase activity. Each recombinant protein was assayed between two and five times. For each assay of each protein, four different volumes of *E. coli* extract containing GST-VP39 proteins were each incubated with 25- μ L aliquots of GSH beads. After washing, beads were divided into three aliquots. Two of these were incubated with 3 H AdoMet in methyltransferase assay buffer, after which supernatants were removed and radioactivity associated with the beads quantitated by scintillation counting. The third aliquot of beads was used for protein quantitation after SDS-PAGE. The resulting protein amount values were plotted against the corresponding values for bound 3 H AdoMet (data not shown). The slopes of the lines obtained by linear regression of the plots (denoted “AdoMet binding coefficient”) were taken as an overall measure of binding. Good fits of data to the regression lines were obtained in most cases. Finally, the AdoMet binding coefficients for multiple assays of individual mutants were averaged, and the mean values plotted. Values are plotted in the same linear order as that of the mutated residues in the protein. Error bars denote average deviations from mean values. The gray bar represents the band of values enclosed by the \pm average deviation from the mean for wild-type GST-VP39. Levels of contaminating *E. coli* proteins were undetectable in the AdoMet binding assays, presumably because of the relatively high extract:bead ratios employed.

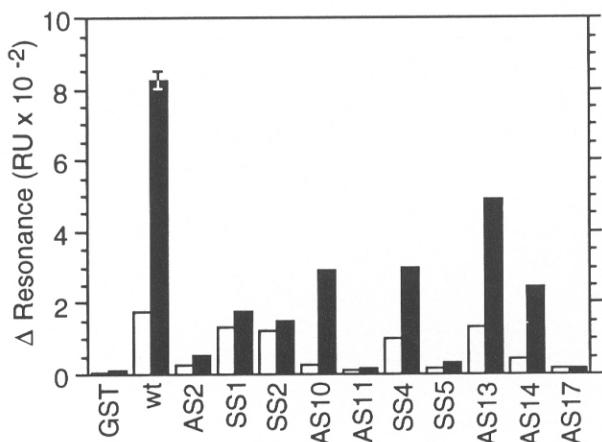
elevated NaCl concentration, due largely to the NaCl-induced abrogation of VP39-uncapped RNA interaction. We next assayed each of the 10 specifically methyltransferase-defective GST-fused VP39 point mutants that had been assayed previously for AdoMet binding (above), after equalization of protein amounts. In the presence of 100 mM NaCl, none of the mutants interacted as strongly with either capped or uncapped RNA as wild-type VP39 and only mutants SS4, AS13, and AS14 showed detectable RNA binding (data not shown). The experiment was then repeated in the presence of 60 mM NaCl. For simplicity, association rate was equated to the difference between resonance values immediately before and after protein injection (“ Δ resonance”). Δ resonance values were determined from each sensorgram, then plotted (Fig. 5B). In the experiment shown, along with additional similar experiments (data not shown), mutants AS10, SS4, AS13, and AS14 exhibited the cap-dependent association-rate enhancement characteristic of wild-type VP39, whereas mutants SS1 and SS2 did not. Levels of RNA binding by mutants AS2, AS11, SS5, and AS17 were insufficient for a determination to be made. The assay was

therefore repeated for these four mutants, in the presence of a lower concentration of NaCl (20 mM). Results are shown in Figure 5C. Although levels of RNA binding by mutants AS11, SS5, and AS17 were still insufficient for a determination to be made, a cap-dependent association-rate enhancement was apparent for mutant AS2. We conclude that cap binding by mutant AS2 exhibits a heightened sensitivity to NaCl, and is therefore partially defective. The fully defective mutants (SS1 and SS2) showed no cap-dependent RNA-binding enhancement even at 20 mM NaCl (data not shown). The salt-sensitivity of mutant AS2 in cap-binding would be consistent with its partially defective methyltransferase phenotype, observed previously (Schnierle et al., 1994). In addition, when assayed in the presence of various concentrations of NaCl, the methyltransferase activity of mutant AS2 dropped almost to zero in the range 20–100 mM NaCl, whereas, in parallel assays, the activity of wild-type GST-VP39 was undiminished (data not shown). The lesions within the three mutants exhibiting defects in cap-binding (AS2, SS1, and SS2) are juxtaposed, and located toward the N-terminus of VP39 (Fig. 2).

A



B



C

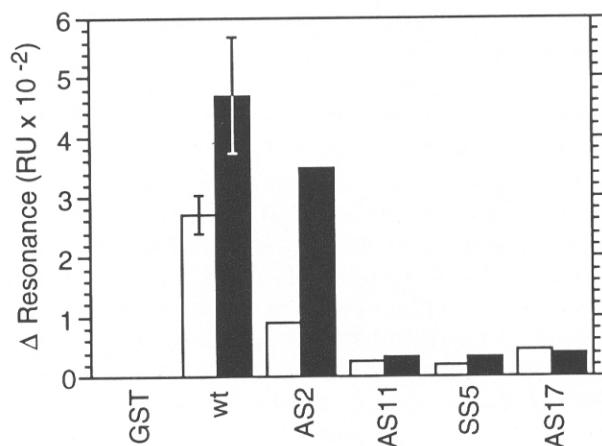


FIGURE 5. Cap binding by GST-VP39 and point mutants thereof. A: Differential salt sensitivity of VP39 binding to uncapped and capped RNAs. Left panel, uncapped; right panel, capped. Equal amounts of 3'-biotinylated uncapped and capped RNA (753 and 756 RU, respectively) were immobilized to adjacent streptavidin-derivatized flow cells of a sensor chip. GST-VP39 (42 nM in running buffer containing either 20, 60, or 100 mM NaCl) was then injected to each of the two flow cells (8-min injections at flow rates of 5 μ L/min). Protein injections were followed immediately by a 10-min washout of the flow cells with running buffer (9 min at 100 μ L/min followed by 1 min at 5 μ L/min), then RNA-surface regeneration as described above (see the Materials and methods). Constant values were subtracted from the association phases of the 60- and 100-mM NaCl sensorgrams to eliminate jumps in resonance due to glycerol and/or NaCl in the GST-VP39 preparation, after which the ordinate of each sensorgram was adjusted to set the resonance value immediately before protein injection to zero. Resulting sensorgrams were grouped according to the flow cell used (uncapped or capped RNA). The upper (—), middle (···), and lower (—·—) traces represent VP39 injection in the presence of 20, 60, and 100 mM NaCl, respectively. B: Cap binding activities of the methyltransferase-defective GST-VP39 mutant proteins. Values are plotted in the same linear order as that of the mutated residues in the protein. Exactly 750 RU of each RNA was immobilized in each of two adjacent flow cells. Each protein was diluted to 42 nM in sufficient running buffer containing ribonuclease inhibitor for two injections (except wild-type GST-VP39, for which sufficient buffer was diluted for four injections). Each protein was then subjected to two sequential cycles of injection/surface regeneration, one to each of the two flow cells. Cycles were similar to the cycles employed in A, except for the omission of the dissociation phase. For each cycle, the difference between resonance values recorded immediately before and after protein injection (Δ Resonance), representing the net increase in resonance during the protein association phase, was plotted. Unfilled bars, uncapped RNA; filled bars, capped RNA. Wild-type protein (wt) was injected to the two flow cells at both the start and the end of the experiment and mean values were plotted, along with error bars representing range for the replicate injections. Error bars for the wt uncapped plot are not visible because replicate values were so similar. C: Those mutants that showed minimal RNA-binding activity in the presence of 60 mM NaCl (panel B) were re-assayed in the presence of 20 mM NaCl. Experimental details were similar to those described for B, except that the running buffer contained 20 mM NaCl; 753 RU of each of the two RNAs were immobilized in adjacent flow cells.

DISCUSSION

It could be envisaged that at least five domains are required for the two activities of the bifunctional VP39

protein, viz: (1) an RNA-binding domain; (2) a domain that interacts with the RNA 5' cap structure; (3) an AdoMet-binding domain; (4) a methyltransferase catalytic center; and (5) a dimerization domain for inter-

action of VP39 with the VP55 subunit of vaccinia PAP. The data presented here indicate that all of the functional domains of VP39 are likely enclosed within a "core" region of the protein, to which is attached a nonessential C-terminal "tail" approximately 37–43 amino acids in length. Despite its being encoded in the VP39 genes of vaccinia and other mammalian poxviruses (Plucienniczak et al., 1985; Goebel et al., 1990; Jackson & Bults, 1990; Shchelkunov et al., 1993; Massung et al., 1994), the tail is not encoded in the more distantly related Fowlpox virus VP39 gene (Drillien et al., 1987; Binns et al., 1988), in which a stop codon truncates the protein just at the point where the vaccinia tail begins. Furthermore, in the vaccinia genome, a downstream, overlapping, open reading frame initiates within this tail region (Plucienniczak et al., 1985). This genomic data may indicate that the tail, in addition to being nonessential, became either added to or lost from the C-terminus of VP39 later during poxvirus evolution, after the divergence of the mammalian from the avian poxviruses.

A panel of 21 "alanine-scanning" point mutants of VP39 (in which clusters of charged residues were converted to alanines) was constructed and assayed previously for methyltransferase, adenylyltransferase-stimulatory, and RNA-binding activities (Schnierle et al., 1994). In the current study, a new panel of VP39 point mutants was constructed and also assayed for methyltransferase and adenylyltransferase-stimulatory activities. Mutants that were specifically defective in methyltransferase activity, collected from both studies, were assayed for AdoMet and cap-binding activity using novel assays. The combined data of the two studies enabled each of the five predicted functional domains of VP39 outlined above, with the exception of the dimerization domain, to be addressed. Defects within the dimerization domain would have been expected to cause loss of adenylyltransferase stimulatory activity without affecting methyltransferase activity (Schnierle et al., 1994), yet no specifically adenylyltransferase-defective mutants were found.

With respect to the RNA-binding domain (RBD) of VP39, the previous mutagenesis study showed that the truncation mutants $\Delta N222$ (previously referred to as $\Delta N221$) and $\Delta C26$ are both active in RNA binding (Schnierle et al., 1994), indicating that an RBD is present within an 85-amino acid region (the 111 amino acids of $\Delta N222$, minus 26 amino acids) located toward the C-terminus of the protein. In the current study, truncation of a total of 38–43 residues from the VP39 C-terminus did not affect either methyltransferase or adenylyltransferase-stimulatory activity, indicating that this RBD is enclosed within an even smaller, 68–73-amino acid region, i.e., the region between residues 223 and 290–295. The presence within this region of the altered residues of mutants AS18 and 19, which were previously shown to be defective in RNA binding

(Schnierle et al., 1994), lends further support to this region being involved in RNA binding. No significant similarity has been found between the RBD of VP39 and the RBDs of other known RNA-binding proteins (data not shown). However, phenylalanine and tyrosine residues occur prominently within one of the most common classes of RBD (Bandziulis et al., 1989; Query et al., 1989), in which they have been shown to contact RNA—often in the context of a β -stranded structure (Merrill et al., 1988; Hoffman et al., 1991; Gorlach et al., 1992). Interestingly, despite the RBD region of VP39 (i.e., residues 223–295) comprising only 22% of the protein, it contains nearly 50% of the total number of phenylalanine and tyrosines. In the current study, mutants AS11 and 17 were found to be defective in binding to 3' end-anchored oligo(A) in the BIACore (Fig. 5B,C), despite their wild-type levels of binding to solution-phase mixed-sequence RNA in the filter-binding assay (Schnierle et al., 1994). Although AS17 lies within the RBD region, AS11 does not. These data point toward additional subtleties in the RNA-binding characteristics of VP39 that might have been highlighted by the use of different substrates in the two assay methods. Although the RNA-binding properties of VP39 might be complex, due to the disparate RNA-binding requirements of the two functions of the protein, a thorough treatment of this area is beyond the scope of the current study.

The cap binding activities of VP39 mutants were examined using a qualitative "difference" assay, in which the binding propensities of proteins for capped and uncapped RNAs anchored at their 3' ends were compared in the BIACore biosensor. In addition to the relative ease with which it could be quantitatively capped, poly(A) was chosen for binding studies because of its efficacy as a 2'-O-methyltransferase substrate (Barbosa & Moss, 1978). Significant steps were taken to avoid ribonuclease contamination in the BIACore assay because scission at any position within the 3' end-anchored capped RNA chain would have resulted in cap loss, abrogating the entire basis of the assay. In each experiment, wild-type GST-VP39 was assayed both prior to, and after, all of the other proteins. Only experiments in which the cap-dependent association-rate enhancement for GST-VP39 was similar at both the outset and the end of the experiment were considered meaningful. Our finding that VP39 has a significantly greater affinity for capped than uncapped RNA was not unexpected, because vaccinia-specific mRNA contains only one 5' cap per ~1,000–1,250 nt of polynucleotide chain (Boone & Moss, 1977). Therefore, within the infected cell, the enzyme must locate the cap against a potentially very high background of polynucleotide chain binding. Furthermore, the effect of the type 0 cap on VP39-RNA association rate, as opposed to dissociation rate, was consistent with the presumed catalytic cycle for VP39, namely the association of cap 0-ended

RNA with VP39 followed by dissociation only after its conversion to cap I. Three mutants were clearly defective in cap 0 binding. The amino acid changes in these mutants were closely apposed, and located within a 15-residue stretch toward the N-terminus of the protein. Because the uncapped RNA employed in the assay would have possessed a 5'-pppG structure, the capped and uncapped RNAs would have differed only in the terminal (m^7G) nucleoside of the cap structure, indicating that it is this nucleotide that interacts with the 15-residue stretch of VP39. Although this degree of specificity is informative, defects in the interaction of VP39 with the triphosphate-bridge of the cap might not be detected in the assay, due to the presence of the triphosphate moiety at the 5' end of the uncapped RNA.

Residues of VP39 required for AdoMet binding were investigated experimentally, using a novel assay for AdoMet-protein interaction. To compensate for the use of relatively low AdoMet concentrations (2 nM), necessary for the achievement of low backgrounds in the assay, high protein concentrations were employed (typical assays employing immobilized GST-VP39 concentrations in the range ~1.5–50 μ M). We could not formally exclude the possibility that the assay was detecting, in addition to AdoMet binding, an expedited expulsion of the tritiated CH_3 group from the AdoHcy moiety. However, such a reaction is considered unlikely due to the absence from the AdoMet-binding reaction of an appropriate nucleophile, such as the oxygen of the target RNA ribose 2'-OH. Upon assaying the AdoMet binding activities of 10 specifically methyltransferase-defective mutants, most of them exhibited minor variations in specific binding activity that could be attributable to small variations in protein-folding efficiency. However, variable folding efficiency would be unlikely to account for the significantly abnormal AdoMet-binding properties of three of the mutants (AS10, AS11, SS4) because the adenylyltransferase-stimulatory activities of these mutants were all comparable. Mutant AS10 exhibited a total inability to bind AdoMet, whereas AS11 and SS4 exhibited significantly greater AdoMet binding than wild-type VP39 and were thus termed AdoMet "super-binders." The altered residues in the three mutants were juxtaposed and located toward the center of VP39. Two residues are substituted with alanine in the AdoMet nonbinding mutant AS10, namely D138 and R140. Because mutant AS11 also possesses the R140 → alanine substitution (Schnierle et al., 1994), yet has the opposite (i.e., an AdoMet super-binding) phenotype, the loss of AdoMet binding by AS10 can apparently be attributed solely to the D138 → alanine substitution. This would suggest that the loss of a single COOH group from VP39, namely that of D138, can lead to a loss of AdoMet binding. In the methyltransferase assay, this AdoMet-binding lesion could not be overcome by a 10-fold elevation in 3 H-AdoMet concentration (data not shown). The al-

tered AdoMet-binding phenotypes of mutants AS10 and AS11 are perhaps consistent with the presence of the mutated residues within the 138-DVRSKRGG-145 region of VP39. This region shows very weak similarity with a region (named "motif I") that is found within several families of actual and putative AdoMet-dependent methyltransferases (Schnierle et al., 1994). Because the co-crystal structure of *Hha* I DNA methyltransferase with AdoMet (Cheng et al., 1993) shows only a single residue within Motif I interacting with AdoMet, and this residue is not conserved in VP39, the significance of the weak similarity is unclear.

Two alternative explanations might reconcile the AdoMet "super-binding" phenotype of mutants AS11 and SS4 with their lack of methyltransferase activity. One explanation might be an inability to turn over substrate. Thus, tight AdoMet binding might correlate with slow AdoHcy release after RNA methylation. However, because the molar ratio of enzyme to limiting (i.e., RNA) substrate was close to unity in the assay, detectable product formation could have been expected in a single round of catalysis. A more intriguing explanation would be an increase in the activation energy of the enzyme. Thus, the AdoMet substrate appears to be bound only very weakly by wild-type VP39 (see the Results). If methyl-group transfer by wild-type VP39 involves the stabilization of a transition state, the enzyme might bind the latter more tightly than substrate. The "super-binding" mutants may have lost their catalytic potential by instead stabilizing substrate binding. Because substrate and transition state would differ only around the atoms where covalent chemistry occurs, the mutated residues in the AdoMet "super-binders" could be at, or very close to, the catalytic center of VP39. Two additional mutants (AS13 and AS14) were specifically defective in methyltransferase activity, yet wild-type in the binding of both cap and AdoMet substrates (Figs. 3, 4, 5). The substituted residues in these mutants might therefore also function at the catalytic center of VP39. Figure 6 summarizes the functional domains of VP39 characterized in both this and the previous mutagenesis study.

The mechanism of methyl group transfer by VP39 is not known. The most extensively studied methyltransferases to date are those of the DNA (cytosine-5)-methyltransferase family, which also provide the most completely deduced mechanism (Verdine, 1994). This mechanism involves a covalent enzyme-substrate base intermediate, generated by an initial nucleophilic attack upon C6 of the substrate cytosine by a cysteine thiolate of the enzyme. Methyl-group transfer by VP39 clearly does not involve a cysteine, because conversion of the two cysteine residues of VP39 to serines does not affect VP39 catalytic activity (Schnierle et al., 1994). Perhaps there is no covalent intermediate, and the DNA (cytosine-5)-methyltransferase mechanism is not an accurate model for the ribose methylation activity of

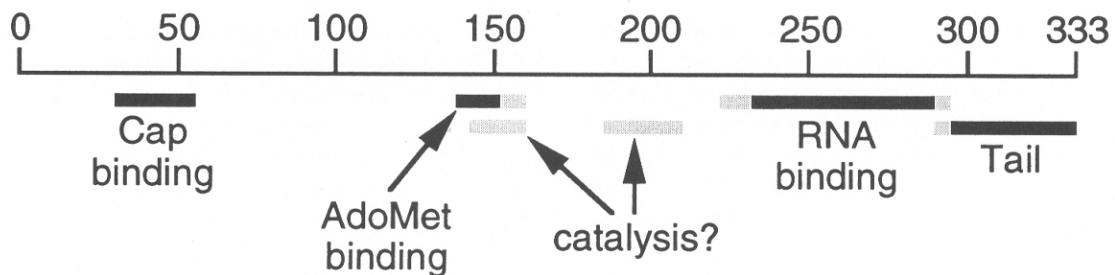


FIGURE 6. Domains within the 333-amino acid VP39 protein, as characterized in both this and the previous mutagenesis study (Schnierle et al., 1994). The cap and AdoMet-binding domains were identified by using VP39 point mutants in substrate-binding assays. The RBD was identified previously using an RNA-binding assay (Schnierle et al., 1994) and its 3' end refined here using C-terminal truncation mutants. Possible catalytic domains are those in which mutants either have an AdoMet “super-binding” phenotype, or are wild-type in both cap and AdoMet binding, yet methyltransferase-defective. The “tail” represents the nonessential, protease-sensitive C-terminus of VP39. Gray lines represent a lower degree of certainty than solid lines. The gray line at the 5' end of the RBD represents the distance between the N-terminus of mutant Δ N222 and the position of the amino acid substitutions in mutant AS17. Other gray lines represent uncertainties explained in the text.

VP39. The mechanism of methyl group transfer could involve a direct nucleophilic attack of the oxygen of the RNA target ribose 2'-OH upon the methyl group of AdoMet. To our knowledge, the only oxygen-nucleophile methyltransferase for which structural information is available is catechol O-methyltransferase (Vidgren et al., 1994). Here, a deprotonated hydroxyl is proposed as the nucleophile, which attacks the CH_3 group of AdoMet, promoting the facile loss of AdoHcy. This could be a reasonable initial paradigm for the methyltransferase activity of VP39. Obviously, further mechanistic questions would be enhanced by the availability of three-dimensional structures.

MATERIALS AND METHODS

Materials

Alanine-scanning mutants 1-21 (referred to here as AS1-AS21), along with the C-terminal truncation mutants Δ C26, 76, and 112 were kindly provided by B. Schnierle and B. Moss. DNA sequence analysis of the 3' ends of inserts revealed that mutants Δ C76 and Δ C112 encode proteins that diverge from wild-type VP39 after residues 259 and 222, respectively, and possess vector-derived C-terminal tails 5 and 6 amino acids in length, respectively. The two mutants are therefore referred to herein as Δ C74 and Δ C111, respectively. Brome Mosaic Virus (BMV) RNA was kindly provided by Drs. Tim Hall and Rohit Duggal, Department of Biology, Texas A&M University.

DNA cloning and mutagenesis

Plasmids for the expression of VP39 C-terminal truncation mutants from plasmid pGEX-KG (Guan & Dixon, 1991) were constructed after the in vitro amplification of “insert” sequences using vaccinia DNA as the template. Amplification reactions for the expression of Δ C34, 36, and 50 contained a 5' primer possessing an *Nco* I restriction site followed by wild-

type VP39 sequences. The 5' amplification primer for the expression of Δ C32, 38, and 44 encoded an additional amino acid sequence, RRASVEF, inserted between codons 2 and 3 of the wild-type VP39 sequence, for reasons unrelated to this project. 3' Primers each contained, in a 5'-3' direction, a *Hind* III restriction site and an in-frame stop codon, immediately followed by the region of homology to the VP39-gene truncation end-point. A mixture of processive and proofreading thermostable DNA polymerases (“Expand,” Boehringer) was employed for amplifications, in combination with manufacturers buffer #1. Twenty-five cycles of amplification were performed, each with an annealing temperature of 55 °C. After digestion of the amplification products with *Nco* I and *Hind* III, “insert” fragments were ligated to the larger *Nco* I-*Hind* III fragment of pGEX-KG. After cloning, DNA sequences were verified at the 3'-end regions of the inserts.

Point mutants in the VP39 gene were constructed as described previously (Schnierle et al., 1994), with the following modifications. Prior to mutagenesis, the VP39 gene was excised from plasmid pPG177 (containing the wild-type VP39 gene cloned in pGEX-KG [Schnierle et al., 1994]) and re-cloned in plasmid pBluescript KS⁺ (Stratagene) as a *Bam*H I-*Hind* III fragment. Single-stranded DNA generated by phagemid rescue was used as the mutagenesis template. Mutants were initially screened by single-track DNA sequencing, using members of a set of primers originally synthesized for verification of the complete VP39-gene sequence (Gershon & Moss, 1993b). After cloning positives back into plasmid pGEX-KG, DNA sequences through the region covered by the mutagenesis primer were confirmed in all four tracks.

Expression, extraction, and purification of GST-VP39 and mutants thereof

For the expression of GST-VP39 fusion proteins, *E. coli* strains HB101 or DH12S were transformed with cloned plasmid, and single colonies were aerobically expanded into various volumes of superbroth containing 20 $\mu\text{g}/\text{mL}$ carbenicillin, with vigorous overnight shaking at 37 °C. After reaching stationary phase, IPTG was added to 0.4 mM final concentration, and shaking was continued for an additional 2.5 h. Cells were

harvested by centrifugation, then lysed by sonication ($5 \times 15\text{-s}$ bursts) in 5 mL of buffer E (10 mM HEPES-NaOH, pH 8.0, 10% glycerol, 5 mM 2-mercaptoethanol, 0.1% NP40) per gram of cells (wet weight) containing 0.25 M NaCl, 0.1 mg/mL PMSF, and 2 $\mu\text{g}/\text{mL}$ pepstatin. The lysate was then supplemented with MgCl₂ (1 mM, final concentration) and DNase I (Boehringer) and, after 15 min incubation at RT °C, the extract was subjected to ultracentrifugation (20 min at 20,000 rpm in an SW41 rotor).

GST-tagged mutant proteins were purified from crude *E. coli* extracts using reduced glutathione (GSH)-agarose beads (Pharmacia) as described by Schnierle et al. (1994), but with the following modifications. GSH-agarose beads (from which manufacturers storage buffer had been drained) were rotated along with a 20× volume excess of extract, at RT °C for 20 min. Beads were then washed three or four times with a 20× volume excess of buffer E supplemented with 0.25 M NaCl. Bead-bound proteins were eluted using buffer E containing 60 mM NaCl and 10 mM reduced glutathione (GSH, Sigma) that had been adjusted to pH 7.5 with NaOH.

Proteolytic release of VP39 moieties from GST-VP39 fusion proteins and immunoblotting

In some instances, after GSH-agarose adsorption of GST-VP39 or mutants thereof from *E. coli* extract, followed by bead washing as described above, GSH elution was substituted with thrombin treatment to specifically release VP39 moieties. Thus, 50 μL samples of beads containing adsorbed GST-VP39 were supplemented with 50 μL samples of buffer E containing 0.25 M NaCl, 10 μL of 10× thrombin cleavage buffer (Novagen) and 0.25–0.5 units of thrombin protease (Novagen), then incubated at RT °C for 30–60 min with rotation. Reactions were terminated by the addition of SDS-PAGE loading buffer, then heated at 90 °C for 3 min followed by a brief centrifugation step and SDS-PAGE of 40- μL aliquots of the resulting supernatants.

Immunoblotting was performed as described by Gershon and Moss (1993b), using VP39-N-pep antiserum and chemiluminescent detection.

Protein quantitation, correction of gel-to-gel staining differences

For use as staining standards, four different amounts of BSA (1, 3, 10, 30 μg , always taken from the same stocks frozen in SDS-PAGE loading buffer) were loaded on each SDS-PAGE gel. Prior to loading samples onto all SDS-PAGE gels (other than those employed in the solid-phase AdoMet-binding assays, below), VP39 moieties were released by thrombin cleavage (as described above). After electrophoresis, gels were stained with Coomassie Brilliant Blue R250, then scanned using a computing densitometer with scanning laser (Molecular Dynamics). The relevant protein bands were quantitated by volume integration with background subtraction to yield “protein amount” values. For correction of gel-to-gel staining differences, protein amount values for the BSA standards loaded on a specific gel were plotted against the amounts of BSA loaded (μg), and the slope of the line obtained by linear regression employed as the “staining correction factor” for that gel. Protein amount values for sample lanes could

then be divided by the staining-correction factor for that gel, to yield staining-corrected protein amount values for gel-to-gel comparison.

RNA (nucleoside-2'-O-)methyltransferase and adenylyltransferase-stimulation assays

BMV RNA for the methyltransferase assay was kindly provided by Drs. Tim Hall and Rohit Duggal, Department of Biology, Texas A&M University. Methyltransferase activity was assayed as described by Schnierle et al. (1994) with minor modifications. Approximately 1 pmol of GST-VP39 protein was employed per assay, along with 1 μg (~0.3 pmol) BMV RNA (i.e., ~1.2 pmol BMV RNA 5' ends), in a total assay volume of 50 μL and an assay time of 45 min. The adenylyltransferase-stimulation assay was performed as described by Schnierle et al. (1994). Approximately 0.5 pmol of GST-VP39 protein was employed per assay.

Solid-phase assay for interaction of VP39, and mutants thereof, with AdoMet

For AdoMet binding assays of individual VP39 mutants, *E. coli* strains DH12S or HB101 were freshly transformed with the plasmid encoding the mutant protein. Usually, a fresh transformation was performed prior to each replicate assay of each individual mutant. Almost every time an assay was performed, fresh soluble protein extract was made by expanding a single colony of transformed *E. coli* into 1 L of superbroth, followed by induction and harvesting of the culture, and extraction and centrifugation of the extract, as described above for GST-VP39 and mutants thereof. Sometimes, protein extracts were stored at -70 °C. For each assay, an adequate volume of GSH-agarose beads was washed twice with an excess volume of buffer E containing 0.25 M NaCl, then made to a 50% slurry with this buffer. Four aliquots of protein extract (typically 0.4, 1.3, 4, and 12 mL) were mixed with four 50- μL aliquots of the 50% GSH-agarose bead slurry, by rotation for 60 min at ambient temperature. For the most abundantly expressed proteins, 12 mL of protein extract was typically sufficient to almost saturate a 25- μL aliquot of packed beads with GST-VP39 fusion protein.

Beads were sedimented by centrifugation, supernatants carefully removed, and the beads washed three times with 1-mL volumes of buffer E containing 0.25 M NaCl, followed by two washes with 0.3-mL volumes of AdoMet-binding buffer (40 mM HEPES-NaOH, pH 7.5, 1 mM DTT, referred to as buffer AB). Prior to the centrifugation step of the second wash with buffer AB, bead suspensions were divided into three 0.1-mL aliquots in 0.6-mL tubes. One aliquot was mixed with 4× SDS-PAGE loading buffer, and stored at -70 °C. The two remaining aliquots were centrifuged and supernatants replaced with 0.5-mL volumes of buffer AB containing 5 nM S-[methyl-³H]-AdoMet (10.6 Ci/mmol, DuPont NEN). S-[methyl-³H]-AdoMet was stored as recommended by the manufacturer, and only diluted with buffer AB immediately before use. Bead suspensions were rotated for 30 min at ambient temperature, then centrifuged briefly. After careful removal of supernatants, using an ultra-fine pipet tip (Eppendorf) to fully drain the beads without bead loss, 0.4-mL aliquots of scintillation fluid (Ultima gold, Packard) were

added to the drained beads, which were then suspended by vigorous agitation. The 0.6-mL assay tubes were placed inside miniature pony vials (Packard), and duplicate counts obtained for each vial in the tritium channel of a scintillation counter (Packard Tri-Carb 2500 TR). Mean tritium cpm values were calculated from the duplicate values.

For protein quantitation, the stored aliquots of beads in SDS-PAGE loading buffer (above) were heated, centrifuged briefly, and 20- μ L aliquots of the supernatants subjected to SDS-PAGE followed by Coomassie staining. Staining-corrected protein amount values, obtained from the relevant Coomassie-stained protein bands as described above, were plotted against the corresponding mean tritium cpm values (above). Because two S-[methyl- 3 H]-AdoMet binding reactions corresponded to each lane of the stained gel, two mean tritium cpm values were plotted against each staining-corrected protein amount value. Linear regression of the resulting plot yielded a slope that was employed as the final AdoMet binding coefficient. AdoMet binding coefficient values determined for the GST moiety alone were multiplied by 0.4, in order to normalize all values with respect to the molar concentration of protein. Each mutant was assayed between 2 and 5 times. Final plots of AdoMet binding coefficients (e.g., Fig. 4) showed mean values from the replicate assays of individual mutants, along with error bars indicating the average deviation of individual values from the mean.

Synthesis of 3'-biotinylated capped and uncapped A₅₀ RNA

Two oligonucleotides with the sequences: 5'-A(T)₅₀CTATA GTGAGTCGTATTAGG-3' and 5'-CCTAACGACTCACTA TAGA-3', were mixed in DEPC-treated water, then incubated for 5 min at 70 °C, followed by incubation for 15 min at 37 °C. The resulting partially double-stranded transcription template comprised a double-stranded T7 promoter, followed by a single-stranded region templating the synthesis of RNA with the sequence G(A₅₀)U. Standard T7 transcription reactions (Megashortscript, Ambion, Inc.) were performed following the manufacturers instructions, except for the nucleotide compositions employed. Thus, for the synthesis of uncapped RNA, reactions contained GTP (15 mM), ATP (30 mM), and Biotin-21-UTP (2 mM, Clontech Laboratories, Inc.). For the synthesis of quantitatively 5'-cap 0-terminated RNA, the nucleotide composition was as for uncapped RNA, except for the replacement of GTP with the cap 0 analog m⁷GpppG (0.9 mM, Boehringer). The successful incorporation of biotinylated UMP at the RNA 3' end was confirmed by electrophoresis of aliquots of the transcription reactions, followed by electroblotting to Hybond N+ membranes (Amersham Inc.), incubation of membranes with streptavidin-horseradish peroxidase conjugate, and chemiluminescent detection. Before use, transcription reactions were incubated for 15 min with DNase I, and the RNA products purified by PAGE.

Surface plasmon resonance-based assay for protein interaction with capped and uncapped RNA

For all procedures, running buffer comprised 10 mM HEPES-NaOH, pH 8.0, 0.005% Tween 20 and, unless otherwise

stated, 60 mM NaCl. After ~48 h of use, running buffer was freshly made using DEPC-treated water. Before use, the BIACore instrument (Pharmacia biosensor) was subjected to two cycles of "desorb" as described by the manufacturer (each of which includes an injection of SDS) in order to eliminate possible residual ribonuclease contamination. Streptavidin (~2,000 RU) was then immobilized to the sensor surfaces of each of two BIACore flow cells, using standard methods (O'Shannessy et al., 1992). The capped and uncapped RNAs (~750 RU of each) were then separately immobilized to the individual streptavidin surfaces, each in a single injection that was manually interrupted after the appropriate amount of RNA had become immobilized. Analytical cycles were initiated by injection to immobilized RNA surfaces of 40 μ L samples of GST-VP39, or mutants thereof, diluted to a final concentration of 42 nM in running buffer containing ribonuclease inhibitor (5' → 3' Inc.), at a flow rate of 5 μ L/min. Some protein injections were immediately followed by a 10-min washout of the flow cell with running buffer at a flow rate of 100 μ L/min. Analytical cycles were terminated with three 40- μ L injections of regeneration buffer (10 mM HEPES-NaOH, 0.5 M NaCl, 0.005% Tween 20), each at a flow rate of 40 μ L/min, to regenerate RNA surfaces. For each protein sample, the two RNA surfaces were subjected to consecutive analytical cycles before continuing to the subsequent sample. For all experiments, the GST moiety was employed as the negative control protein. Wild-type GST-VP39 was used as the positive control, and was injected to the two RNA surfaces in both the first two and the last two cycles of each experiment. RNA-derivatized flow cells were not used for more than 36 h or if association parameters for the final GST-VP39 injection of an experiment differed from those for the initial injection by more than ~25%.

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