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Evidence That the RNA Methylation and Poly(A) Polymerase Stimulatory Activities of Vaccinia Virus Protein VP39 Do Not Impinge upon One Another

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Vaccinia protein VP39 has two RNA modifying activities. In monomeric form, it acts as an mRNA cap-specific 2′-*O*-methyltransferase, specifically modifying the ribose moiety of the first transcribed nucleotide of m^7G-capped mRNA. In association with VP55, the catalytic subunit of the vaccinia poly(A) polymerase, VP39 facilitates the rapid elongation of poly(A) tails that are already greater than ~35 nt in length. Introducing new assays, we provide evidence that substrates for each of VP39's two activities do not detectably modulate the converse reaction and that VP39's 2′-*O*-methyltransferase activity is not significantly affected by its association with VP55. In an electrophoretic mobility shift assay, VP39 interacted with a short (5 nucleotide) RNA only when the latter was m^7G-capped. Complexes with longer (22 nucleotide) RNAs were more stable (i.e., cap-independent) but were further stabilized by the presence of an m^7G cap. An additional complex was observed at elevated RNA:protein molar ratios, indicating the presence of two RNA binding sites per VP39 molecule. Interaction at one of these sites was stabilized by the cap structure. Additional experiments indicated that RNA molecules undergoing poly(A) tail elongation by the VP55-VP39 heterodimer are not favored as cap-methylation substrates.

INTRODUCTION

Vaccinia virus provides a model system for studies of poxvirus replication. The virus encodes, and carries within its virion, functional counterparts of transcription and RNA modification machineries normally confined to the host cell nucleus. These counterparts include enzymes for the attachment of eukaryotic-like m^7G cap and poly(A) tail structures to the 5′ and 3′ ends, respectively, of nascent vaccinia transcripts (Gershon, 1997). The mRNA cap 0 structure (m^7G(5′)pppN) and poly(A) tail are added through the actions of the heterodimeric vaccinia capping enzyme (Shuman, 1995) and VP55-VP39 (poly(A) polymerase) heterodimer (Gershon *et al.,* 1991; Moss *et al.,* 1975), respectively. Within the latter enzyme, adenylate addition is catalyzed solely by the VP55 subunit (Gershon *et al.,* 1991). However, VP55 can catalyze the rapid and processive synthesis of tails no more than 30–35 nt in length, with longer tails being synthesized in only a very slow and nonprocessive manner (Gershon and Moss, 1992). The rapid and processive synthesis of longer (physiological-length) tails requires the presence of the VP39 subunit. The latter acts as a "processivity factor" for VP55, enabling the VP55-VP39 heterodimer to remain stably associated with poly(A) tails greater than 35 nt in length during their rapid elongation (Gershon and Moss, 1993a). VP39 has a second, entirely unrelated function, namely conversion of the mRNA 5′ cap 0 structure to its cap I counterpart (m^7G(5′)pppN^m) by methylation of the ribose moiety of the first transcribed nucleotide of the mRNA (Schnierle *et al.,* 1992).

It is unclear whether VP39's unusual bifunctionality has arisen to facilitate some form of communication between its two mRNA modifying functions. Here, we have explored potential interactions between VP39's two functions. First, we have screened for the effects of cap-specific 2′-*O*-methyltransferase substrates upon poly(A) tail elongation and vice versa. Second, although association with VP55 is implicit in VP39's action as a polyadenylation processivity factor (Gershon and Moss, 1993a), the possible effects of VP55 upon VP39's 2′-*O*-methyltransferase function have not been fully explored. We have therefore screened for such effects and also for the concurrent modification of both RNA ends by individual VP55-VP39 heterodimer molecules. Finally, in an effort to detect multiple RNA binding sites per VP39 molecule, we have adapted the electrophoretic mobility shift assay (EMSA) for analysis of VP39–RNA interactions.

RESULTS

VP39 2′-*O*-methyltransferase substrates/products do not affect poly(A) tail elongation by the VP55-VP39 heterodimer

VP39 is a bifunctional protein which acts as both a cap-specific 2′-*O*-methyltransferase and a poly(A) polymerase (PAP) processivity factor (Gershon *et al.,* 1991;

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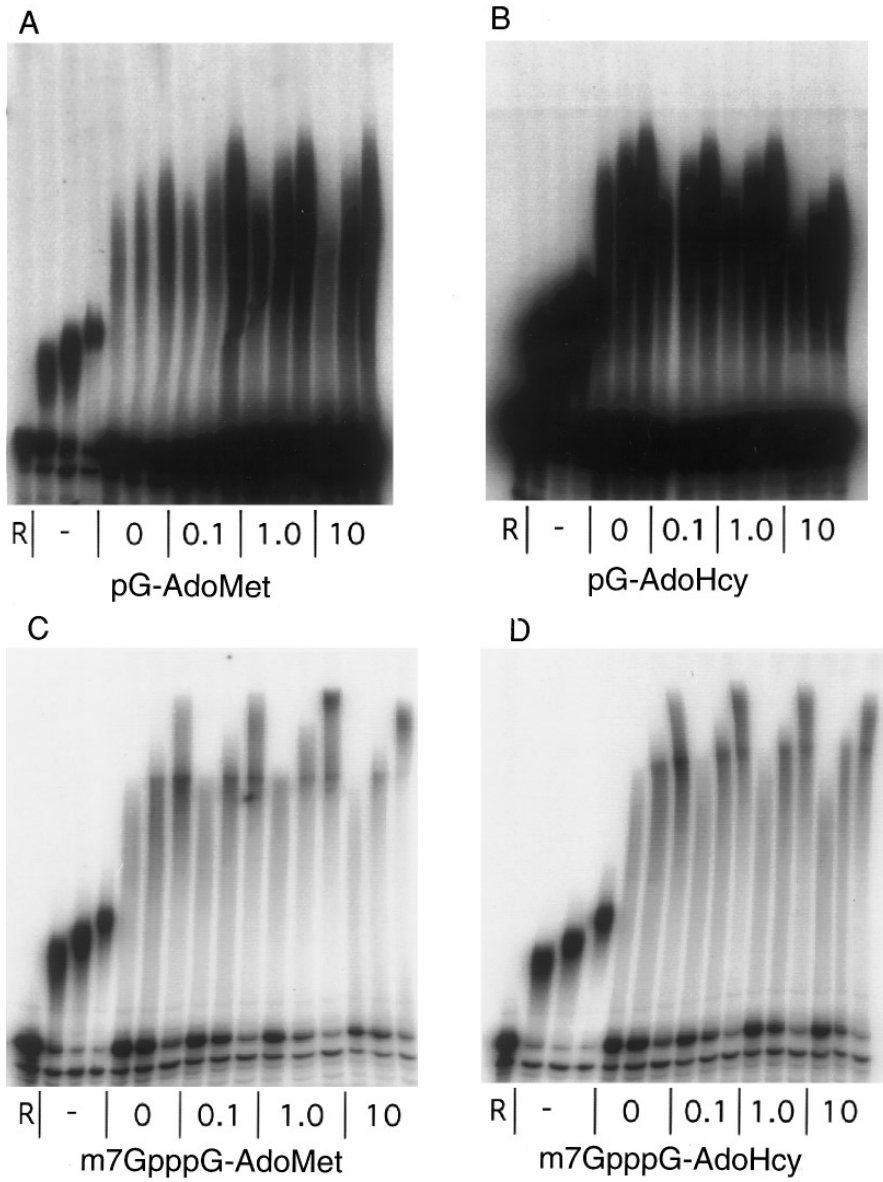

FIG. 1 2′-*O*-methyltransferase substrate and cofactor/coproduct do not affect VP39's poly(A) tail elongatory activity *in vitro.* (A and B) ^{32}P-kinase-labeled, uncapped VGF 60mer RNA substrate. (C and D) ^{32}P-cap 0-labeled VGF 60mer RNA substrate. (A and C) Assays conducted in the presence of various concentrations of AdoMet; (B and D) assays conducted in the presence of various concentrations of AdoHcy. The gel shown in each panel has an equivalent layout, in which the first lane (R, showing labeled RNA alone), is followed by four polyadenylation timecourse assays. Each assay contains VP55, the PAP catalytic subunit, and each except the first (−) also contains VP39. Numbers below each VP39-containing assay (0, 0.1, 1.0, 10) represent the concentration of AdoMet or AdoHcy (mM) present. For each assay, three lanes are shown, containing samples taken 30, 90, and 300 s after assay initiation. Standard assay conditions were employed (Materials and Methods), with reaction mixtures also containing NaCl (60 mM). Reaction products were electrophoresed in standard 8% polyacrylamide, urea–TBE gels. The faint horizontal "line" running ~one-quarter the way down the gels shown in parts (C) and (D) is an artifact of electrophoresis.

Gershon and Moss, 1993a; Schnierle *et al.,* 1992). Whereas monomeric VP39 is active in cap methylation, VP39 acts in poly(A) tail formation only in association with VP55, the PAP catalytic subunit (Gershon *et al.,* 1991). We set out to investigate possible effects of 2′-*O*-methyltransferase substrates and products upon the polyadenylation reaction. To this end, *in vitro* polyadenylation timecourse assays were conducted using either 5′-monophosphate-terminated (Figs. 1A and 1B) or 5′-cap 0-terminated (Figs. 1C and 1D) versions of the VGF 60mer RNA as the polyadenylation substrate, only the latter version is a substrate for cap-specific 2′-*O*-methylation by VP39. For each polyadenylation substrate, polyadenylation reactions were conducted in the presence of various concentrations of either *S*-adenosyl-L-methionine (AdoMet) (Figs. 1A and 1C) or *S*-adenosyl-L-homocysteine (AdoHcy) (Figs. 1B and 1D), the 2′-*O*-methyltransferase cofactor and corresponding coproduct, respectively. For each of the four combinations of substrate + cofactor/coproduct, increasing amounts of co-

factor/coproduct did not significantly affect rates of RNA substrate utilization or product elongation or the lengths of tail obtained. The slightly longer tails noticeable for the capped than the uncapped RNAs (comparing Figs. 1C and 1D with 1A and 1B, respectively) in the presence of VP39 can be attributed to a greater protein:RNA ratio, due to the use of lower concentrations of capped than uncapped RNA in the assays. In Fig. 1B, the slightly reduced tail lengths observed in the presence of 10 mM AdoHcy correspond with the reduction of pH in this assay from 9.0 to ~7.5 due to the acidity of the AdoHcy stock solution. In Fig. 1C, conditions are appropriate for simultaneous polyadenylation and 2′-*O*-methylation of the substrate.

To generate the ^{32}P-cap 0-labeled RNA substrate used for the experiments of Figs. 1C and 1D, the vaccinia capping enzyme must catalyze a guanylation (^{32}P-labeled GMP transfer) reaction followed by guanine N7-methylation. Only the guanine-7-methylated (i.e., cap 0-terminated) form of the RNA is a bona fide substrate for 2′-*O*-methylation by VP39 (Barbosa and Moss, 1978). However, complete N7-methylation of the substrate's newly added guanine was not ensured, since the presence of the N7-methyl group would not have been detectable by polyacrylamide gel electrophoresis of the ^{32}P-labeled 60mer RNA product. A more rigorously defined substrate was therefore tested. This comprised cap 0-terminated VGF 60mer RNA that was [^{3}H]methyl labeled at the N7 position of the terminal guanine. The new substrate was compared with an uncapped (5′ ^{32}P-monophosphate-terminated) version of the VGF 60mer, as a polyadenylation substrate, in *in vitro* polyadenylation competition assays (Fig. 2). In these assays, either ^{32}P- or ^{3}H-labeled VGF 60mer RNA was mixed with 1-, 3-, or 10-fold excess amounts of an identical, unlabeled competitor RNA possessing a 5′-terminal OH group, and the mixtures were polyadenylated in the presence of VP55, VP55 + VP39, or VP55 + VP39 + unlabeled AdoMet (Figs. 2A and 2B). The 5′ OH-terminated RNA competed effectively for utilization of the ^{32}P-labeled RNA substrate in the presence or absence of VP39 (Fig. 2A), indicating, as would be expected, that the 5′ phosphate does not provide an advantage to the polyadenylation substrate. An equivalent result was obtained in the equivalent experiment with ^{3}H-labeled RNA substrate (Fig. 2B), indicating that a 5′ cap 0 structure confers neither an advantage nor a disadvantage over a 5′-monophosphate or a 5′-OH in the polyadenylation reaction. Conditions favoring cap I formation by VP39 (i.e., cap 0-terminated substrate in the presence of VP55 + VP39 + AdoMet, Fig. 2B) did not appear to affect the polyadenylation reaction, consistent with the lack of effect observed in Fig. 1C.

Separate studies (Lockless *et al.,* 1998) indicate that the minimum RNA chain length for cap methylation is two or three transcribed nucleotides. Chromatographically purified uncapped and m^{7}G-capped RNAs close to the minimum length (i.e., pppG(A)$_4$ and m^{7}GpppG(A)$_4$) were tested as unlabeled competitors for the polyadenylation of a ^{32}P-labeled version of a polyadenylation-elongation substrate, A$_{50}$, by the VP55-VP39 heterodimer. In molar excess amounts of up to 100×, neither pppG(A)$_4$ nor m^{7}GpppG(A)$_4$ competed detectably for the polyadenylation of ^{32}P-A$_{50}$ (Fig. 2C), despite m^{7}GpppG(A)$_4$ being a 2′-*O*-methyltransferase substrate. This indicated that VP39 might possess distinct RNA-binding sites for its two functions.

Substrates for poly(A) tail elongation by the VP55-VP39 heterodimer do not affect the cap-specific 2′-*O*-methyltransferase activity of VP39

The above experiments indicated that the polyadenylation activity of the vaccinia PAP heterodimer is not influenced by substrates for or products of the VP39 cap-specific 2′-*O*-methyltransferase reaction. In order to assess whether VP39's 2′-*O*-methyltransferase activity is influenced by an RNA substrate for poly(A) tail elongation by the heterodimer, namely an oligoadenylated RNA, oligoadenylated and nonoligoadenylated versions of an unlabeled, 5′ cap 0-terminated RNA (the VGF 60mer RNA) were allowed to compete with one another for 2′-*O*-methylation. To generate the necessary substrates, a sample of unlabeled, cap 0-terminated VGF 60mer RNA was divided into two equal portions, and one of these was appended with a ~35-nt oligo(A) tail. The oligoadenylated and nonoligoadenylated species were then mixed in equimolar amounts and included in an electrophoretic timecourse assay for 2′-*O*-methyltransferase activity in > fivefold molar excess over VP39, along with [^{3}H]AdoMet. RNA products were sampled at various times after the start of the reaction, and the appearance of [^{3}H]RNA was monitored by gel electrophoresis and fluorography. The oligoadenylated and nonoligoadenylated RNA species became 2′-*O*-methylated at comparable rates (Fig. 3A). We therefore conclude that a substrate for VP39's tail elongatory activity (i.e. an oligo(A) tailed RNA) does not significantly influence VP39's activity in methylating the RNA 5′ cap 0 structure. An additional experiment (Fig. 3B) showed that VP39's 2′-*O*-methyltransferase activity was not significantly affected by the presence of Mg:ATP, the small-molecule substrate for the polyadenylation reaction.

Association with VP55 does not significantly influence the methyltransferase activity of VP39

The experiments of Figs. 1–3 established that VP39's cap-specific 2′-*O*-methyltransferase activity is not affected by substrates for poly(A) tail elongation, and poly(A) tail elongatory activity is not affected by substrates for 5′ cap methylation. Possible explanations include: (i) monomeric VP39 targets the mRNA 5′ cap structure exclusively (presumably via its cap-enhanced RNA binding activity (Shi *et al.,* 1996)), whereas the VP55-

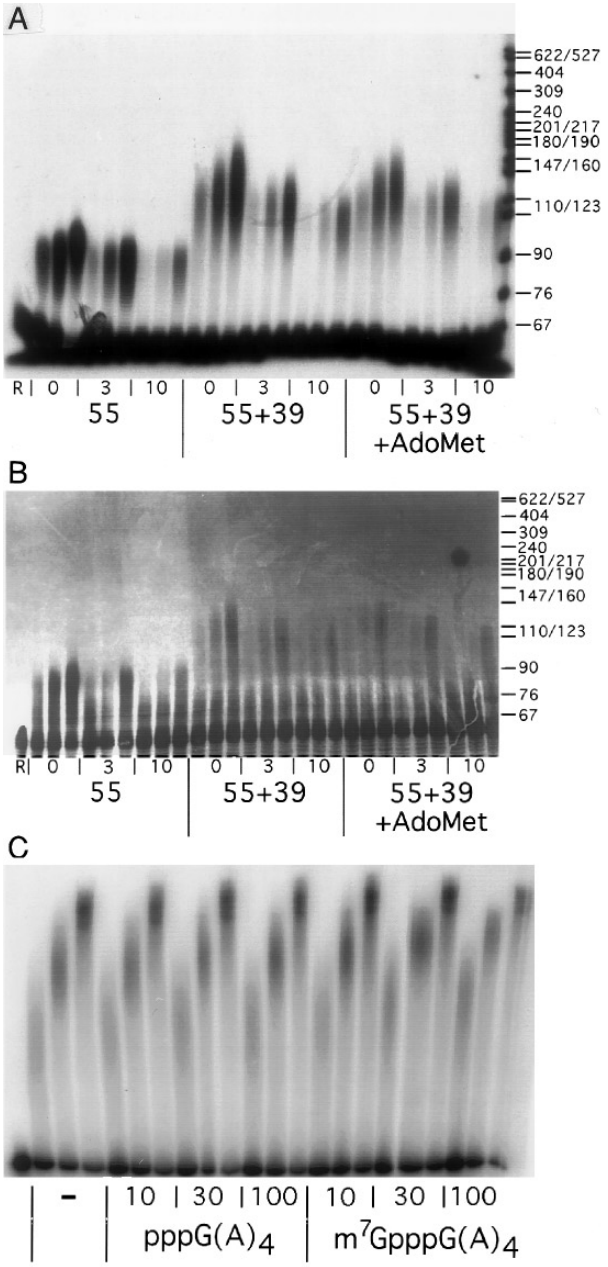

FIG. 2. The 5′ cap 0 structure does not affect VP39's poly(A) tail elongatory activity *in vitro.* (A) Control assays, showing polyadenylation of 5′ ^{32}P-labeled VGF 60mer RNA in the presence of excess unlabeled 5′ OH-terminated VGF 60mer RNA competitor. Polyadenylation reactions were conducted in the presence of vaccinia-expressed VP55 alone (55), VP55 + vaccinia-expressed VP39 (55 + 39), or VP55 + VP39 + unlabeled AdoMet (50 μM) (55 + 39 + AdoMet). For each of these combinations, 0 refers to an assay conducted in the absence of competitor; 3 and 10 refer to assays conducted in the presence of 3- and 10-fold excess amounts, respectively, of unlabeled competitor. For each assay, timepoints were taken 30, 90, and 300 s after assay initiation. R refers to RNA alone. (B) Polyadenylation of 5′ cap 0-labeled VGF 60mer RNA in the presence of excess unlabeled 5′ OH-terminated RNA competitor. The ^{3}H label was incorporated at the 7-methyl position of the terminal guanine of the cap, to ensure that all of the labeled substrate molecules possessed an authentic cap 0 structure (see text). Other details are as for (A). (C) Testing $pppG(A)_4$ and $m^7GpppG(A)_4$ as competitors in the poly(A) tail elongation reaction, using 5′ kinase-labeled A_{50} as the polyadenylation substrate. − denotes an assay conducted in the absence of competitor. 10, 30, and 100 indicate the fold excess of competitor. For each assay, timepoints were taken 30, 90, and 300 s after assay initiation. Other details are as for (A).

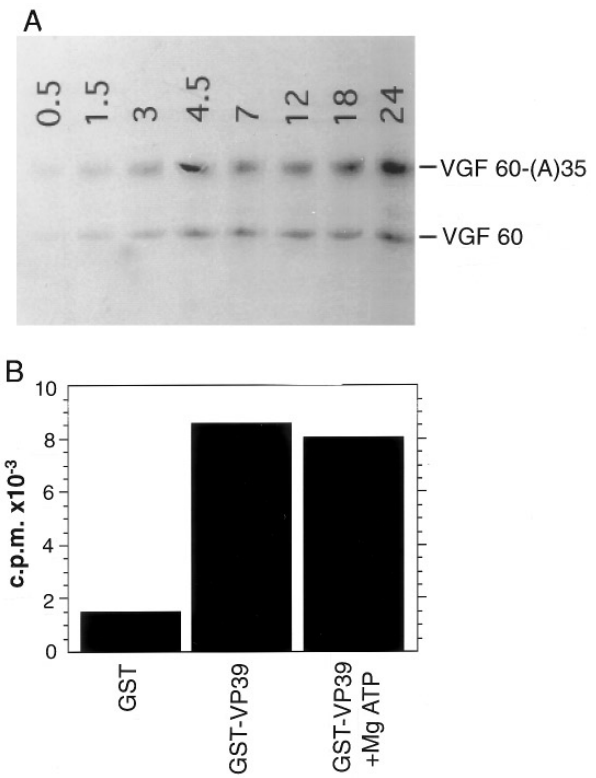

FIG. 3. Polyadenylation substrates do not affect VP39's 2′-*O*-methyltransferase activity. (A) Methylation timecourse assay, showing that polyadenylated and nonpolyadenylated versions of the cap 0-terminated VGF 60mer RNA are 2′-*O*-methylated at equivalent rates. The fluorographed gel shows the appearance of ^{3}H-labeled RNA (i.e., 2′-*O*-methyltransferase product) in a timecourse reaction containing two versions of the cap 0-terminated VGF 60mer RNA substrate: nonoligoadenylated (VGF 60) and oligoadenylated with a ~35-nt oligo(A) tail (VGF 60-$(A)_{35}$). Prior to the reaction, the two RNAs were mixed in equimolar amounts. Samples were taken at various times (given above each lane of the gel in min) after the addition of VP39 and [^{3}H]AdoMet. The dark blemish over the 4.5-min/VGF60-$(A)_{35}$ band is from the X-ray film. (B) Mg.ATP does not significantly affect the 2′-*O*-methyltransferase reaction. The standard methyltransferase assay (Barbosa and Moss, 1978) was performed in the absence and presence of 1 mM Mg.ATP. In this experiment, *E. coli*-expressed GST-VP39 fusion protein was assayed while anchored to glutathione beads. Anchored GST fragment was used as a negative control.

VP39 heterodimer (the active species in polyadenylation) targets only the mRNA 3′ end, rendering it incapable of 5′ cap methylation; and (ii) separate, noninterfering RNA binding sites of VP39 are utilized for the protein's polyadenylation and 2′-*O*-methyltransferase activities. The first possibility was addressed by determining whether VP55 switches the specificity of VP39, converting it from a 5′ end to a 3′ end modifying protein. This was done by conducting 2′-*O*-methyltransferase timecourse assays similar to that shown in Fig. 3A, only in the absence and presence of excess amounts of VP55. To control for potential inhibitors that might have been introduced into the assay along with VP55, a dimerization-defective VP39 mutant, $CF3^{c-}$ (Shi *et al.,* 1997), was also tested in the absence and presence of VP55. $CF3^{c-}$ has previously

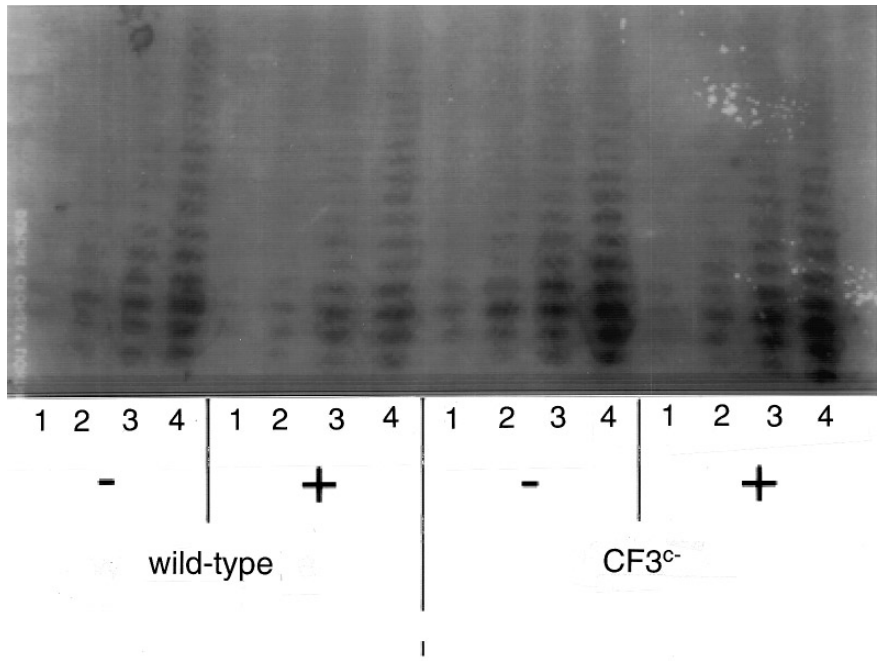

FIG. 4. VP55 does not significantly influence VP39's 2′-*O*-methyltransferase activity. A 25% polyacrylamide gel shows the appearance of ^{3}H-labeled RNA (i.e., 2′-*O*-methyltransferase product) with time. Timepoints (1, 2, 3, 4) were taken 15, 40, 120, and 300 s, respectively, after assay initiation. Wild-type VP39 (wild-type) and a mutant whose only known lesion is an inability to associate with VP55 (CF3^{c-}), were overexpressed in *E. coli* and purified as described under Materials and Methods. Assays were conducted in the absence (−) and presence (+) of 2.8-fold molar excess amounts of purified, baculovirus-expressed VP55. Amounts of VP39 and VP55 added to the assays had been accurately determined by quantitation of Coomassie-stained gels as described (Shi *et al.,* 1996).

been shown to possess wild-type levels of 2′-*O*-methyltransferase activity (Shi *et al.,* 1997), and no defects have been detected other than an inability to associate with VP55. The 2′-*O*-methylation substrate comprised a nested ladder of capped RNAs generated by transcription of a template for the RNA G(A)$_5$ in the presence of m^{7}GpppG cap dinucleotide (Lockless *et al.,* 1998). Assay results (Fig. 4) indicated that products accumulate with time in either the absence or the presence of a ~2.8-fold molar excess of VP55, for both wild-type VP39 and mutant CF3^{c-}. Although the wild-type protein appears to have lost a very small amount of activity in the presence of VP55, no loss of activity was observed in other (replicate) experiments, including those in which the VP55: VP39 molar ratio was increased further (data not shown). Furthermore, separate experiments have shown that wild-type VP39's K_M for RNA substrate remains unchanged in the presence or absence of excess molar amounts of VP55 (H-T. Cheng and P. D. Gershon, unpublished data).

Cap-dependent interaction of short capped RNAs with VP39

Since VP39's cap-specific 2′-*O*-methyltransferase activity is apparently not affected by its association with VP55 (Fig. 4), the VP55-VP39 heterodimer can apparently act at both RNA ends. One possibility is that it interacts with the two RNA ends sequentially, i.e., via a single RNA binding site. This seems unlikely, since 2′-*O*-methyltransferase (capped) substrates do not interfere with polyadenylation and vice versa (Figs. 1–3). Furthermore, the heterodimer would need to interact with mRNA 3′ ends efficiently despite VP39's demonstrable targeting activity for capped 5′ ends (Shi *et al.,* 1996). A second possibility is that the heterodimer can interact with both ends simultaneously, i.e., via separate RNA binding sites. This possibility is explored below.

Initially, an EMSA was developed to detect VP39–RNA interaction. Highly purified VP39-ΔC26 (a 26-aa C-terminal VP39 truncation mutant whose properties are indistinguishable from those of wild-type VP39 (Schnierle *et al.,* 1994)) was used, along with capped and uncapped versions of a highly purified short RNA, G(A)$_4$. Since the capped version is almost a minimum-length 2′-*O*-methyltransferase substrate (Lockless *et al.,* 1998), EMSA complexes should possess only the minimum number of protein–RNA contacts necessary for 2′-*O*-methylation to occur. Whereas uncapped (5′ kinase-labeled) G(A)$_4$ probe did not form a detectable complex with VP39-ΔC26 in the EMSA (Fig. 5A), a diffuse complex was observed with the cap-labeled version, whose mobility was slightly lower than that of the free RNA (Fig. 5B). Moreover, whereas the complex was completely stable in the presence of at least 70-fold molar excess amounts of uncapped, unlabeled pppG(A)$_4$ RNA (Fig. 5B, left), equivalent amounts of the capped version (m^{7}GpppG(A)$_4$) completely inhibited complex formation

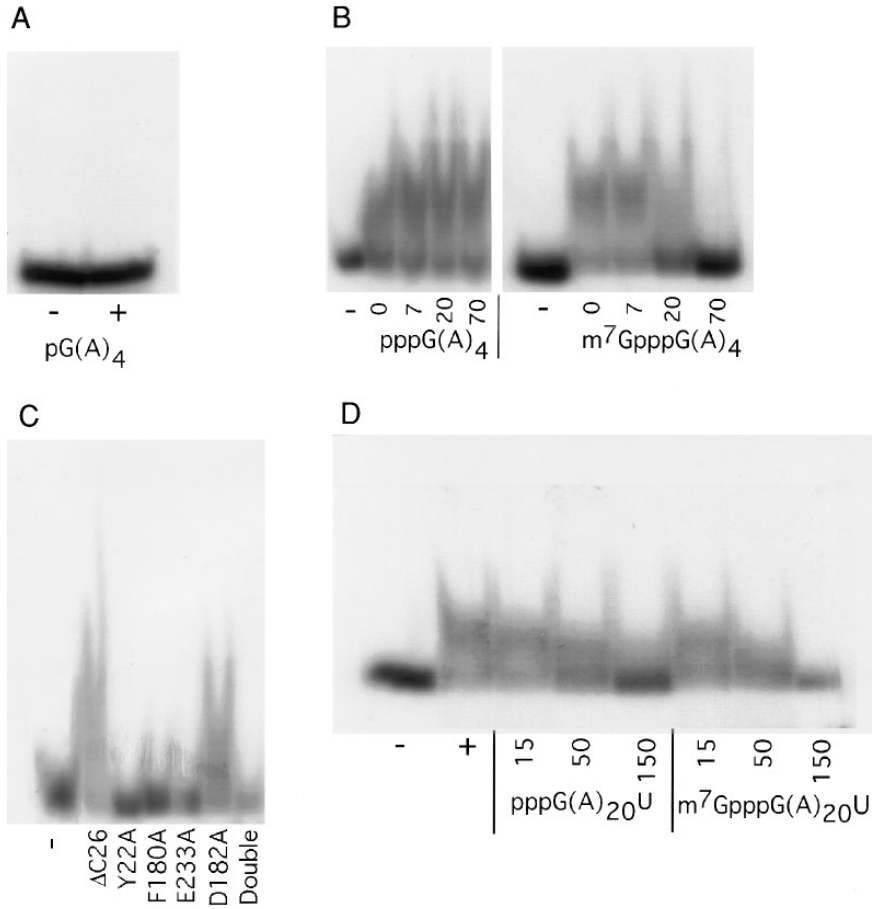

FIG. 5. Cap-dependent VP39–RNA interaction assayed by EMSA. (A) EMSA gel showing the absence of complexes with an uncapped 5-nt RNA $^{32}PG(A)_4$. −, + represent RNA incubations in the absence and presence, respectively, of VP39-ΔC26. (B) EMSA gel showing that VP39-ΔC26 forms a diffuse complex with a ^{32}P cap-labeled version of the 5-nt RNA, $m^7GpppG(A)_4$. For both left and right panels, − represents an RNA sample without added protein, the remaining assays contained VP39-ΔC26. 0 represents no competitor, 7, 20, and 70 represent fold molar excess of unlabeled competitor over cap-labeled $m^7GpppG(A)_4$ probe (2 pmol of the latter being present in each assay). (Left) Uncapped 5mer RNA competitor, $pppG(A)_4$. (Right) Capped 5mer RNA competitor ($m^7GpppG(A)_4$). (C) EMSA of VP39-ΔC26 mutants lacking m^7G-contact sidechains in VP39's m^7G-binding pocket (Hodel *et al.,* 1997). Amounts of protein in the assay were as follows: 4.8 μg (wild-type VP39-ΔC26, labeled ΔC26), 3.5 μg (Y22A), 6 μg (F180A), 5 μg (D182A), 7 μg (E233A), 2 μg (D182A + E233A, labeled Double). Other conditions and symbols were as for (B). (D) EMSA gel showing the behavior of the complex between VP39-ΔC26 and a ^{32}P $m^7GpppG(A)_4$ probe in the presence of large excess amounts of unlabeled, uncapped, and capped $G(A)_{20}U$. − and + are as described in (A). Other conditions and symbols are as for (B).

(Fig. 5B, right). The inactivity of smaller excess amounts of the capped competitor (e.g., sevenfold) was likely due to the molar excess of protein over RNA in the assay. These data strongly indicated that VP39 can interact with the short RNA $G(A)_4$ in a cap 0-dependent manner. Confirmation that the EMSA complex was cap 0-dependent came from an EMSA experiment employing mutants with alanine substitutions in m^7G-contact residues (Hodel *et al.,* 1997), namely mutants Y22A, D182A, F180A, E233A, and a double mutant in which both D182 and E233 had been converted to alanine. Mutants Y22A, F180A, E233A, and the double mutant, each of which had previously been shown to exhibit a loss of cap 0-dependent RNA binding enhancement in a BIAcore-based assay for capped RNA–VP39 interaction (Hodel *et al.,* 1997; Shi *et al.,* 1996), were also defective in the EMSA (Fig. 5C). Mutant D182A, which showed wild-type levels of cap-enhanced RNA binding in the BIAcore assay (Hodel *et al.,* 1997), also showed wild-type levels of EMSA complex formation (Fig. 5C). Next, capped and uncapped versions of a longer RNA substrate, $G(A)_{20}U$, were synthesized and tested as competitors for VP39-capped 5mer RNA interaction (Fig. 5D). At 50- to 150-fold molar excess over the labeled probe, both the capped and the uncapped versions of the longer RNA were effective competitors. This experiment indicated that the stability conferred to the VP39–5mer RNA interaction by the cap (Figs. 5A and 5B) may be insufficient to resist competition by larger amounts of a longer uncapped RNA (Fig. 5D). Thus, both the cap and an associated RNA chain of ~5 nt appear to contribute to VP39 binding stability.

Evidence that VP39 possesses two independent RNA binding sites

To further investigate the effects of cap and chain length on VP39 binding, kinase-labeled and cap-labeled

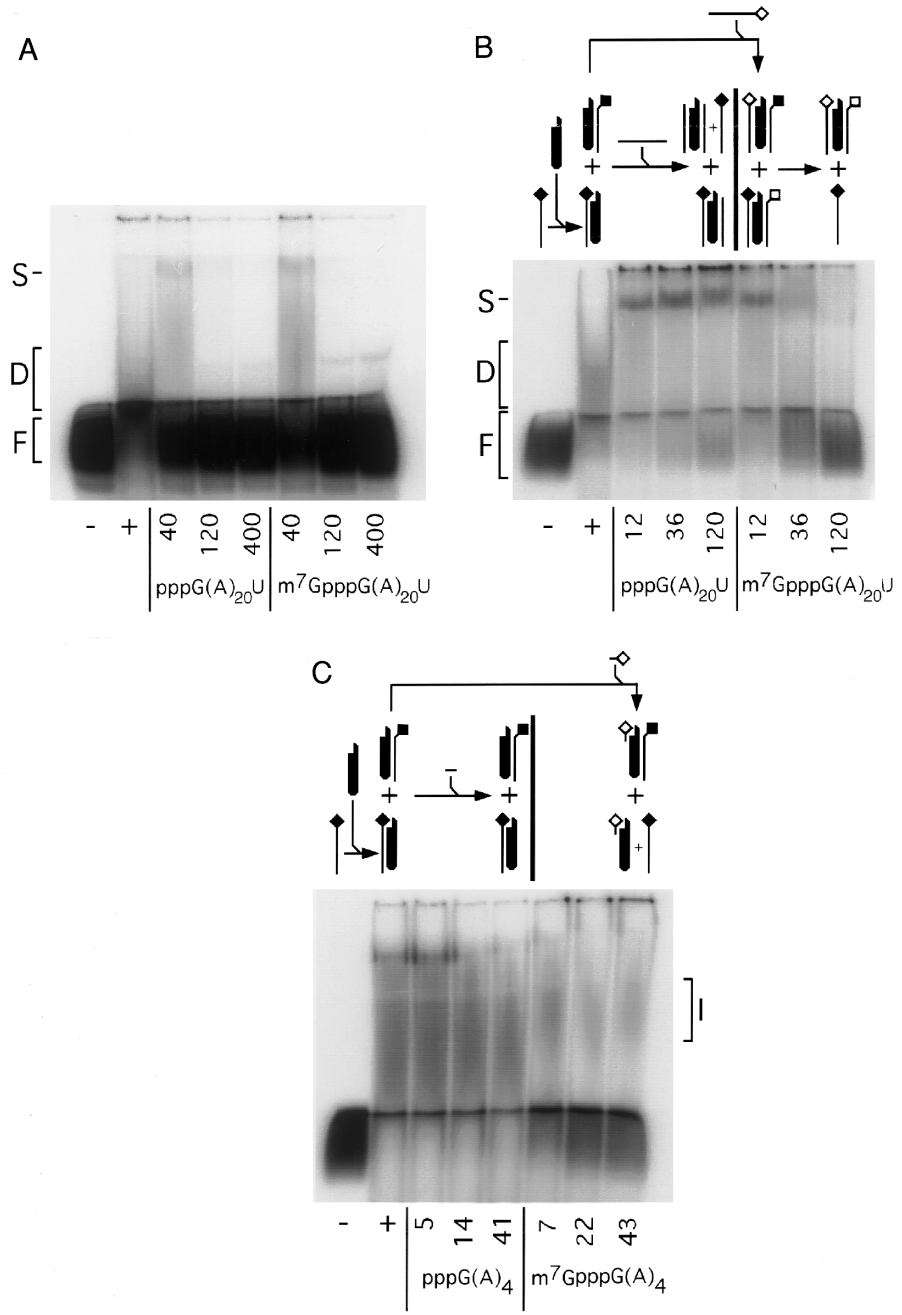

FIG. 6. EMSA analysis of the interaction of VP39-ΔC26 with labeled $G(A)_{20}U$. The faint horizontal line running across all gels, just above the free (uncomplexed) labeled $G(A)_{20}U$ RNA (F) is an artifact of electrophoresis. (A) EMSA gel showing the interaction of ^{32}P-labeled uncapped $G(A)_{20}U$ probe with VP39-ΔC26. F indicates free (uncomplexed) RNA. D indicates the diffuse complex observed at low RNA:protein stoichiometry. S represents the slow (second) complex observed upon elevation of RNA:protein stoichiometry by the addition of unlabeled $G(A)_{20}U$ in either uncapped ($pppG(A)_{20}U$) or capped ($m^7GpppG(A)_{20}U$) form. − and + are as in Fig. 5; 40, 120, and 400 represent fold mass excess of unlabeled over labeled RNA. Each assay contained 130 pmol VP39-ΔC26 and 2 pmol of ^{32}P-labeled RNA. (B) EMSA gel showing the interaction of ^{32}P cap-labeled $m^7GpppG(A)_{20}U$ probe with VP39-ΔC26. −, +, S, D and F are as in (A). 12, 36, and 120 represent fold mass excess of unlabeled competitor RNA. A schematic interpretation of the data is shown above the figure, in which ▮ represents VP39-ΔC26, with the two vertical faces representing the two proposed RNA binding sites. The site on the lefthand face is cap-specific, with a nick at the upper lefthand corner representing the cap-binding site. | represents unlabeled, uncapped $G(A)_{20}U$, ♀ represents unlabeled $m^7GpppG(A)_{20}U$ (with the open diamond representing the unlabeled m^7G cap structure), ♀ represents unlabeled $m^7GpppG(A)_4$, and ♀ represents ^{32}P cap-labeled $m^7GpppG(A)_{20}U$ (with the filled diamond representing the labeled cap structure). $m^7GpppG(A)_{20}U$ depicted with a bend in the stem represents binding of the capped RNA in a cap-independent manner. (C) EMSA gel showing the interaction of VP39-ΔC26 with ^{32}P-cap-labeled $m^7GpppG(A)_{20}U$ in the presence of unlabeled $pppG(A)_4$ or $m^7GpppG(A)_4$. I represents a slow complex with intermediate mobility. 5, 14, 41, 7, 22, and 43 represent fold mass excess of unlabeled over labeled RNA, other symbols are as described in (A) and (B).

versions of the longer $G(A)_{20}U$ RNA were tested as EMSA probes and competitors (Fig. 6). An initial EMSA experiment was performed using kinase-labeled $G(A)_{20}U$ probe (Fig. 6A). In the absence of unlabeled "competitor" RNA (+ lane), a smear was observed migrating more slowly than the free probe. The low abundance and

diffuse nature of the smear indicated dissociation during electrophoresis, presumably due to an unstable VP39–probe interaction. Addition of an unlabeled form of the probe RNA, either uncapped or capped, in 40-fold excess over the labeled probe (40 lane of Fig. 6A), led to the formation of a faint second complex with low electrophoretic mobility (labeled S). We suggest that the S complex represents the binding of two RNA molecules to distinct RNA binding sites of VP39. The requirement for an increased total amount of RNA in the assay for the S complex to be observed would result from the large amounts of VP39 present in the assay with respect to probe alone. Addition of still greater amounts of the unlabeled competitor RNA (120 and 400 lanes of Fig. 6A) led to loss of all complexes, apparently due to true competition.

The experiment of Fig. 6A was repeated using cap-labeled $G(A)_{20}U$ as the EMSA probe (Fig. 6B). As with the uncapped $G(A)_{20}U$ probe (Fig. 6A), a diffuse smear was observed for VP39 in the presence of probe alone (Fig. 6B + lane, labeled D), and inclusion of excess unlabeled, uncapped $G(A)_{20}U$ competitor along with the capped probe led to the appearance of an S complex (Fig. 6B, 3rd lane from left). However, the smear and the S complex with the capped 22mer probe (Fig. 6B) were consistently far more abundant, with respect to the uncomplexed RNA, than were the equivalent complexes observed using the equivalent uncapped probe (Fig. 6A). Furthermore, in contrast to the situation with uncapped probe (Fig. 6A), the S complex observed with capped probe could not be competed by large excess amounts of the unlabeled, uncapped RNA (Fig. 6B, third–fifth lanes from left). The increased abundance of complexes with the capped probe, and their resistance to competition by the uncapped competitor, suggested a cap-dependent stabilization of VP39–22mer probe interaction equivalent to that observed using the shorter (5mer) probe (Figs. 5A and 5B). Taken together, the experiments of Figs. 5, 6A, and 6B suggested that VP39 possesses distinct cap-stabilized and cap-independent RNA binding sites, interactions at the former also being stabilized by an increased RNA chain length from 5 to 22 nt. In Fig. 6B (final three lanes) capped instead of uncapped $G(A)_{20}U$ competitor was used. As with the uncapped competitor, the lowest concentration of capped competitor titrated the diffuse complex into the S complex. Since only capped RNA was present in the assay, both of the RNA molecules bound to VP39 in the S complex would be capped, providing evidence that the cap-independent binding site does not exclude segments of RNA chain associated with capped RNA. Greater excess amounts of capped competitor led to a loss of the slow complex (Fig. 6B, final two lanes). This is not surprising, since the capped $G(A)_{20}U$ competitor, unlike the uncapped version, can displace its cap-labeled counterpart from the cap-stabilized RNA binding site. The slight increase in amount of free probe that occurs upon addition of uncapped competitor to the cap-labeled probe (Fig. 6B, third–fifth lanes) can be explained by the ability of the cap-independent binding site to bind capped RNA chain (above). Thus, the capped $G(A)_{20}U$ would bind most stably to the cap-stabilized site, but would also form a relatively unstable complex with the cap-independent site. The D band of Fig. 6B (+ lane) would therefore contain a mixture of these two complexes (see schematic above the figure). The uncapped competitor would selectively displace the capped probe from the cap-independent site, leading to the reappearance of some free probe (fifth lane from left).

The above interpretation of Fig. 6B was tested by combining the capped 22mer probe with shorter unlabeled RNAs (uncapped and capped GA_4) to create complexes of distinct mobility (Fig. 6C). Uncapped GA_4 did not significantly affect the smear resulting from VP39-capped $G(A)_{20}U$ interaction (Fig. 6C, lanes 2–5), consistent with its inability to interact with VP39 (Figs. 5A and 5B). However, when unlabeled, capped GA_4 was used, a distinct, diffuse complex was observed (Fig. 6C, lanes 6–8, labeled I) whose mobility was intermediate between the mobilities of the diffuse complex and the slow (S) complex observed in Fig. 6B. Its electrophoretic mobility suggested that it was equivalent to the S complex of Fig. 6B, but contained unlabeled $m^7GpppG(A)_4$ in place of unlabeled $m^7GpppG(A)_{20}U$. Since stable EMSA complex formation with the short (GA_4-based) RNA is cap-dependent (Fig. 5A), the binding of $m^7GpppG(A)_4$ in the I complex of Fig. 6C presumably occurs exclusively at VP39's cap-stabilized RNA binding site. Therefore, the cap-labeled $m^7GpppG(A)_{20}U$ probe in the I complex presumably binds at VP39's cap-independent RNA binding site. If this is so, then the I complex should also be observed using an uncapped (5′ P-labeled) $G(A)_{20}U$ probe in combination with unlabeled $m^7GpppG(A)_4$. Under these conditions a very diffuse I complex was indeed observed (data not shown), supporting the above interpretation of the I complex and thereby our interpretation of the S complex based upon Fig. 6B.

Substrate molecules whose 3′ ends interact with the VP55-VP39 heterodimer are not strongly favored for 5′ cap-specific 2′-*O*-methylation

Since VP39 appears to have two independent RNA binding sites (Fig. 6), and can also 2′-*O*-methylate the cap structure while associated with VP55 (Fig. 4), we wondered whether VP39 might preferentially 2′-*O*-methylate the cap while engaged in poly(A) tail elongation. Since heterodimer molecules acting at the RNA 3′ end might be expected to preferentially 2′-*O*-methylate the capped 5′ end of the same RNA molecule due to the higher local concentration of the latter over the 5′ ends of other RNA molecules, we addressed the issue by assaying for the preferential 2′-*O*-methylation of RNAs undergoing 3′ end modification by the heterodimer. The assay employed cap 0-terminated A_{50} as a substrate for both

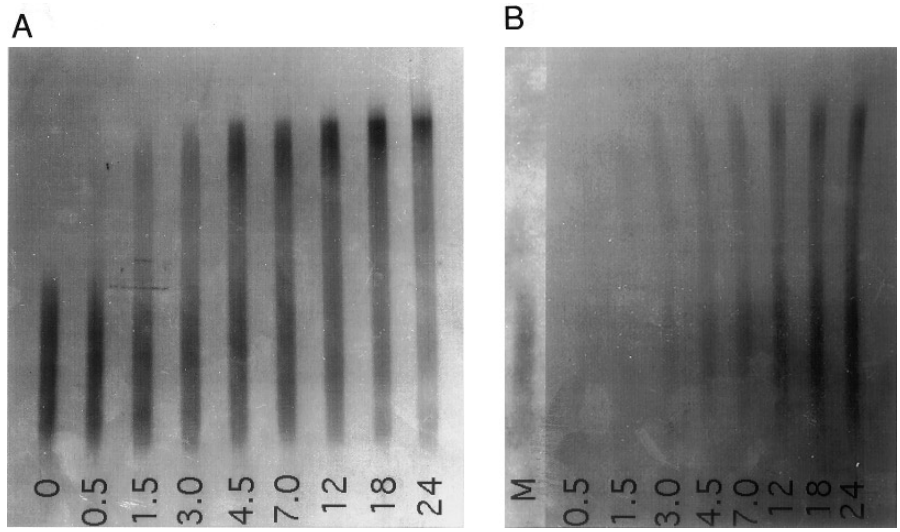

FIG. 7. Substrate molecules whose 3′ ends interact with the VP55-VP39 heterodimer are not strongly favored for 5′ cap-specific 2′-*O*-methylation. (A) Polyadenylation timecourse assay, in which the polyadenylation substrate comprised ~20 pmol of cap I-terminated A_{50} RNA possessing a ^{3}H-labeled 2′-*O*-methyl group. The polyadenylation reaction contained ~1 pmol each of VP55 and VP39. Numbers are time (min) at which samples were taken. (B) Simultaneous 2′-*O*-methylation and poly(A) tail elongation of ~20 pmol of cap 0-terminated A_{50}, in which the transferred methyl group is ^{3}H labeled. M denotes a separate 2′-*O*-methylation reaction in the absence of polyadenylation (i.e., ATP omitted from the reaction). Experimental details are given under Materials and Methods; other details are given in the legend to (A).

tail elongation and cap 2′-*O*-methylation. 2′-*O*-methylation of this substrate in the presence of [^{3}H]AdoMet would lead to the appearance of labeled product, and polyadenylation would change the RNA's electrophoretic mobility. In a control experiment to determine the activity of the capped A_{50} substrate at pH 8.0 (a compromise between the optimal polyadenylation and methylation pH values of 9.0 and 7.5, respectively), the substrate was first [^{3}H]methyl labeled by VP39 (not shown), then subjected to poly(A) tail elongation by the VP55-VP39 heterodimer in a timecourse assay (Fig. 7A). In a second experiment, the substrate was simultaneously 2′-*O*-methylated and polyadenylated by the heterodimer, in a reaction containing a ~20-fold molar excess of RNA over the heterodimer (Fig. 7B). During the course of the reaction, tritium appeared to be incorporated into bands corresponding to the polyadenylation substrate and products at equivalent rates, indicating that RNA molecules whose 3′ ends have interacted with the heterodimer (polyadenylation products) are not strongly favored for cap-specific 2′-O-methylation.

DISCUSSION

Vaccinia protein VP39 is bifunctional. The aim of this study was to investigate possible relationships between the two functions, using biochemical approaches. We found that substrates for one reaction did not have a significant effect upon the other. For example, the presence of an oligo(A) tail (substrate for VP39's tail elongatory activity) did not significantly affect the rate at which VP39 could 2′-*O*-methylate a 5′ cap 0 structure attached to the same RNA molecule (Fig. 3). This indicates that the tail does not play a role in bringing monomeric VP39 into the proximity of the cap. Although the polyadenylation cofactor, Mg.ATP, had no effect on cap 2′-*O*-methylation (Fig. 3), equivalent concentrations of free adenine are inhibitory (S.W. Lockless and P.D. Gershon, unpublished data). This is presumably because adenine is more closely related than ATP to the adenosyl moiety of the methyltransferase cofactor AdoMet. In additional experiments, the 2′-*O*-methyltransferase substrate (cap 0-terminated RNA), cofactor (AdoMet), and coproduct (AdoHcy) did not appear to affect poly(A) tail elongation. One precedent for examining the effect of AdoHcy upon polyadenylation is the unusual effect of AdoHcy upon poly(A) tail length in vesicular stomatitis virus (VSV). In VSV, the multifunctional viral L protein mediates increased poly(A) tail lengths in response to AdoHcy (Hunt *et al.,* 1988; Hunt and Hutchinson, 1993), presumably as a consequence of the protein's roles in both cap and poly(A) tail formation. Since tail lengths due to the VP55-VP39 heterodimer did not change significantly in response to AdoHcy *in vitro* (Figs. 1B and 1D), VP39 apparently does not mediate such a coupling through an interaction between its AdoHcy-binding (Barbosa and Moss, 1978) and poly(A) tail elongatory (Gershon and Moss, 1993a) activities. This would be consistent with the complete dissimilarity of vaccinia virus and VSV in terms of replication mechanisms, genome structure and size, and gene expression mechanisms.

One explanation for the absence of detectable substrate cross-modulation could be that VP39's two functions are mutually exclusive, such that a single VP39 molecule cannot carry out both functions simultaneously.

Since VP39 is able to modify RNA 3′ ends only in the context of the VP39-VP55 heterodimer, heterodimerization would provide a simple and attractive mechanism to switch VP39 from 5′ to 3′ end modifying functions. Although an earlier experiment (Fig. 2 of Schnierle *et al.,* 1992) indicated the heterodimer to be active in cap-specific 2′-*O*-methylation *in vitro,* the previous experiment also suggested the heterodimer to be slightly less active than free VP39. Moreover, the heterodimer concentrations used previously ($<\sim 10^{-8}$ M) were comparable to the K_d for heterodimer dissociation to monomeric subunits (P. D. Gershon, unpublished data), raising questions about the heterodimer's association status in the experiment. The experiments of Fig. 4 employed higher protein concentrations, multiple datapoints per assay, and a dimerization-defective mutant as a negative control. Furthermore, replicate experiments at still higher protein concentrations (data not shown) also showed dimerization to have no detectable effect upon 2′-*O*-methyltransferase activity. We therefore conclude that association with VP55 does not switch the function of VP39. An alternative explanation for the apparent absence of cross-modulation between VP39's two functions might be that they employ completely independent, noninterfering domains of the protein. This would require two separate RNA binding sites for VP39's two activities. In an attempt to detect more than one RNA binding site, an EMSA was developed. The complexes observed in the absence of unlabeled competitor RNA were diffuse, indicating the occurrence of equilibrium dissociation during electrophoresis. This may have been encouraged by VP39's p*I* of ~8.3 (Barbosa and Moss, 1978), close to the pH of the electrophoresis buffer. High protein concentrations (10 μM) were required for any EMSA complexes to be observed at all, indicating that dissociation could be retarded through mass action. Consistent with this, additional increases in protein concentration beyond those used here led to further retardation of the average electrophoretic mobility of the diffuse complexes (data not shown). Despite the above factors, surprisingly reproducible data were obtained in the EMSA. Cap-dependent (Fig. 5) and cap-enhanced (Fig. 6) complex formation were observed, consistent with the results of previous studies using the BIAcore instrument (Shi *et al.,* 1996), in which VP39–RNA complexes exhibited a cap-dependent enhancement in binding stability. The observation of a slow second complex in EMSAs when using longer (22mer) RNA probes under conditions of elevated RNA:protein stoichiometry indicated the occurrence of two distinct RNA binding sites per VP39 molecule. This latter observation contrasts with the behavior of VP55 in the EMSA, in which slow complexes arise under the converse conditions (elevated protein:RNA stoichiometry), due to the presence of multiple protein binding sites per RNA molecule (e.g., Fig. 2B (Gershon and Moss, 1993b)). Other data (not shown), in which experiments similar to those of Figs. 6A and 6B were performed using 50-nt in place of 20-nt RNAs, also showed the binding of two RNAs per VP39 molecule. This indicated that VP39's two RNA binding sites are not parts of a single, much larger RNA binding domain that can be bridged by a single 50mer RNA.

Whereas unlabeled $G(A)_{20}U$ was able to titrate cap-labeled $m^7GpppG(A)_{20}U$ into a slow EMSA complex (Fig. 6B), it displaced cap-labeled $m^7GpppG(A)_4$ from the protein entirely (Fig. 5D). This would indicate that VP39 interacts with the longer capped RNA more strongly than with the capped 5mer. Consistent with this, VP39's K_M for 2′-*O*-methylation of $m^7GpppG(A)_{20}U$ is significantly lower than for 2′-*O*-methylation of $m^7GpppG(A)_4$ (Lockless *et al.,* 1998). VP39 possesses a cleft adjacent to its m^7G binding pocket, which passes though the 2′-*O*-methyltransferase catalytic center and accommodates the first three transcribed nucleotides of the RNA chain (Hodel *et al.,* 1996, 1997, 1998). The functional data shown here are consistent with the conclusion of a sister study (Lockless *et al.,* 1998) that an RNA binding site located beyond the cleft plays a role in VP39's 2′-*O*-methyltransferase function.

Under *in vitro* polyadenylation conditions, the act of polyadenylation by the heterodimer had little or no effect on its 2′-*O*-methyltransferase activity and did not promote selective cap-specific 2′-*O*-methylation of the RNA molecule undergoing polyadenylation (as opposed to other RNA molecules; Fig. 7). Indeed, RNA molecules undergoing tail elongation appeared to be neither preferred nor disadvantaged for cap-specific 2′-*O*-methylation. Thus, there is no evidence to suggest the occurrence of polyadenylation-dependent cap 2′-*O*-methylation, as has recently been detected for certain *Xenopus* cytoplasmic mRNAs *in ovo* (Kuge and Richter, 1995). Since heterodimer molecules participating in RNA 3′ end modification might be expected to experience an elevated local concentration of RNA 5′ ends, it is not clear why RNA molecules undergoing tail elongation did not experience some degree of preferential 2′-*O*-methylation. One explanation might be that the VP55-VP39-poly(A) ternary complex lacks 2′-*O*-methyltransferase activity.

In summary, the evidence presented herein suggests that VP39's two functions act independently of one another, via independent RNA binding sites on the protein surface.

MATERIALS AND METHODS

Materials

The vaccinia-expressed VP55 and VP39 used here have been described previously (Gershon and Moss, 1992, 1993a). For some experiments (e.g., Fig. 4) baculovirus-expressed VP55 (Shi *et al.,* 1997) was employed. *Escherichia coli*-expressed VP39 was generated as described previously (Gershon and Moss, 1996; Shi *et al.,* 1996). Very high purity VP39-ΔC26 (a 26-amino-acid C-

terminal truncation mutant of VP39 (Schnierle *et al.,* 1994)) was expressed in *E. coli* and purified as described previously (Gershon and Moss, 1996; Hodel *et al.,* 1996). The final column (heparin–agarose) yielded highly purified protein at a concentration of 4.8 mg/ml, which was used for the experiments described herein as well as for crystallographic studies (Hodel *et al.,* 1997). The generation, expression, and purification of the VP39 point mutant proteins used for the experiment shown in Fig. 5C have been described (Hodel *et al.,* 1997; Lockless *et al.,* 1998). After heparin–agarose column chromatography, and before use, each of the mutant proteins was concentrated by ultrafiltration (Centricon, Amicon) to 3.5 mg/ml (Y22A), 6 mg/ml (F180A), 5 mg/ml (D182A), 7 mg/ml (E233A), and 2 mg/ml (D182A + E233A). Vaccinia virus capping enzyme was purified from soluble virion extract by passage through two diethylaminoethyl (DEAE)–cellulose columns, followed by binding and salt-gradient elution from sequential columns of single-stranded DNA–agarose and heparin–agarose. The final preparation appeared by electrophoresis to be free of VP39, though small amounts of capped RNAs generated by the capping enzyme preparation (up to 25%) were found, by C18 column chromatography (Lockless *et al.,* 1998; data not shown) to be in the cap I form. This indicated the presence of trace amounts of contaminating VP39.

Triphosphate-terminated VGF 60mer RNA was synthesized enzymatically, as described previously (Gershon and Moss, 1993a). Triphosphate- and cap 0-terminated $G(A)_n$ and -$G(A)_nU$ transcripts were synthesized enzymatically, using T7 RNA polymerase, as described (Lockless *et al.,* 1998; Milligan and Uhlenbeck, 1989). For RNA synthesized using m^7GpppG cap analog (unlabeled, capped RNA), 30–50% of transcripts would be expected to possess cap dinucleotide incorporated in the forward ($m^7GpppG(N)_n$) as opposed to the reverse ($Gpppm^7G(N)_n$) direction (Pasquinelli *et al.,* 1995). Transcripts possessing oligo(A) tracts were generally somewhat heterogeneous in length, typically varying between the target size and a size corresponding to ~five additional adenylates. Where necessary, individual uncapped and forward-capped RNA species were purified from the mixtures of transcription products generated when using short templates (i.e., those with 5-nt transcribed regions), by using C18 column chromatography (Lockless *et al.,* 1998).

RNA 5′ end modification

Labeled, monophosphate-terminated RNA was generated by dephosphorylation of enzymatically synthesized, triphosphate-terminated RNA (above), followed by rephosphorylation using phage T4 polynucleotide kinase and [γ-^{32}P]ATP (Gershon and Moss, 1993a). Some of the resulting 5′ end-labeled RNAs (e.g., $G(A)_4$) were subsequently purified by denaturing PAGE. Triphosphate-terminated RNAs were modified to the cap 0 form using the vaccinia capping enzyme (Materials, above). For the addition of unlabeled cap, reactions contained 50 mM Tris–HCl, pH 7.5; 50 mM DTT; 12.5 mM $MgCl_2$; 0.5 mM *S*-adenosyl-L-methionine; 20 U RNAsin (Promega); 1 mM GTP; 5′ triphosphate-terminated RNA; and 2 μl of vaccinia capping enzyme, in a total volume of 20 μl. Reactions were incubated for 2 h at 37°C. Conditions for the generation of ^{32}P- or ^{3}H-labeled (at the m^7G position) cap 0 were identical except for the replacement of unlabeled GTP or AdoMet with either [α-^{32}P]GTP (20 μCi) or [^{3}H]AdoMet (11 μCi; 11 Ci/mMol; DuPont NEN), respectively. The proportion of RNAs which became capped was not determined. Unlabeled and ^{3}H-containing capping reactions were terminated by supplementing with 40 μl of RNase-free water, then extracting with an equal volume of phenol/chloroform, followed by chloroform alone. Following extraction, aqueous phases were exchanged into an equivalent volume (60 μl) of RNase-free water using a Bio-spin 6 column (Bio-Rad). ^{32}P cap-labeled RNA was supplemented with an equal volume (20 μl) of formamide and purified from a 20% polyacrylamide/TBE/urea gel. This would ensure the removal of unlabeled AdoMet from the cap-labeling reaction. For the experiment shown in Fig. 7A, enzymatically synthesized cap 0-terminated $G(A)_{50}U$ RNA (Materials, above) was converted to the ^{3}H cap I-form using VP39 (0.4 μg) and [^{3}H]AdoMet (13.75 μCi; 55 Ci/mMol) in 25 mM Tris–HCl, pH 8.0, 1 mM DTT, for 60 min at 30°C. It was then extracted with phenol/chloroform, followed by chloroform alone. Following extraction, the aqueous phase was exchanged into an equivalent volume of 25 mM Tris–HCl, pH 8.0, 1 mM DTT using a Bio-spin 6 column. The volume of the resulting material was reduced ~two-fold under vacuum before use in the polyadenylation timecourse assay (below).

3′-Oligoadenylation of unlabeled, 5′-cap 0-modified VGF 60mer RNA

For the experiment shown in Fig. 3A, VGF 60mer RNA whose 5′ end had been modified to unlabeled cap 0 followed by exchange into RNase-free water (previous section) was divided into two equal portions. One portion was incubated with VP55 (50 ng) in a buffer comprising 1.25 mM $MgCl_2$, 1.25 mM ATP, 62.5 mM Tris–HCl (pH 9.0), 6.25 mM DTT, in a total volume of 40 μl. Following a 10-min incubation at 37°C, the reaction was extracted once with phenol/chloroform, then once with chloroform alone. The resulting aqueous phase was mixed with the portion of cap 0-modified RNA that had not been subjected to polyadenylation, and the RNA mixture exchanged into RNase-free water by spin-gel filtration as described above.

2′-*O*-methyltransferase and polyadenylation assays

The standard cap-specific 2′-O-methyltransferase assay, using Brome mosaic virus RNA as a substrate

and DEAE filter binding for [^{3}H]RNA product isolation (Fig. 3B), was performed as described previously (Shi *et al.,* 1996). For the methyltransferase electrophoretic timecourse assay shown in Fig. 3A, the desalted RNA mixture described in the previous section (70 μl) was supplemented with 25 mM *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES)-NaOH (pH 7.5), 1 mM DTT, and [^{3}H]AdoMet (3.3 μCi; 11 Ci/mmol) in a total reaction volume of 90 μl. *E. coli*-expressed VP39 was then added, and 9-μl samples were taken after various times and mixed with 6-μl aliquots of formamide. After heating for 2 min at 95°C, 12-μl portions of the RNA samples were loaded on an 8% polyacrylamide/urea/TBE gel. Reaction conditions for the experiment shown in Fig. 4 were similar except for (i) the use of 2′-*O*-methylation substrate comprising unpurified products from an m^7GpppG-initiated, $G(A)_5$-templated T7 transcription reaction; (ii) the inclusion of baculovirus-expressed VP55 in some reactions in 2.75-fold molar excess over 2.74 pmol *E. coli*-expressed VP39 or point mutant CF3^{c-} (Shi *et al.,* 1997); and (iii) the omission of a heat pulse immediately prior to electrophoresis. After electrophoresis, methyltransferase timecourse gels were soaked in 30% methanol/5% acetic acid (30 min), then in either Entensify (NEN) or Fluoro-Hance (RPI) following the manufacturers' directions. After drying, gels were exposed to preflashed X-ray film at −70°C.

The standard polyadenylation timecourse assay (Figs. 1 and 2) was conducted as described previously (Gershon and Moss, 1993a). A similar protocol was employed for the polyadenylation timecourse experiments using ^{3}H-labeled RNA (Figs. 2B and 7A). However, the polyadenylation timecourse assay shown in Fig. 7A differed from others in that equimolar amounts of vaccinia-expressed VP55 and VP39 (~50 ng of each) were used, instead of the standard excess amount of VP39, and in the replacement of 50 mM Tris–HCl, pH 9.0, in the assay buffer with 25 mM Tris–HCl, pH 8.0. For the simultaneous 2′-*O*-methylation and polyadenylation timecourse reaction shown in Fig. 7B, ~20 pmol of RNA substrate was incubated with 25 mM Tris–HCl, pH 8.0, 1 mM DTT, [^{3}H]AdoMet (27.5 μCi; 55 Ci/mmol) and 1 mM Mg.ATP at 30°C for 5 min, before initiating the reactions by combining the above mixture with vaccinia-expressed VP55 and VP39 (~1 pmol of each). For the experiments shown in Fig. 7, timepoint samples were mixed with equal amounts of formamide and electrophoresed in 8% polyacrylamide, 7 M urea, TBE-buffered gels which were fluorographed as described above.

EMSA

RNA samples were cap-labeled or kinase-labeled with ^{32}P, as described above, then purified from 20% polyacrylamide gels. Labeled and unlabeled RNA samples were mixed in methyltransferase assay buffer (above) supplemented with 10% glycerol, then 14-μl aliquots of the buffered RNA mixture were supplemented with 4.8-μg aliquots of very high purity *E. coli*-expressed VP39-ΔC26 or mutants thereof (described above). After incubation at room temperature for ~5 min, samples were electrophoresed in 10 or 12% polyacrylamide, 0.5× TBE (pH 8.3) gels, at 160 V (max)/20 mA (max). Dried gels were imaged using a PhosphorImager (Molecular Dynamics).

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