The Surface Region of the Bifunctional Vaccinia RNA Modifying Protein VP39 That Interfaces with Poly(A) Polymerase Is Remote from the RNA Binding Cleft Used for Its mRNA 5’ Cap Methylation Function*

(Received for publication, May 21, 1997)

Xuenong Shi, Thomas Geoffrey Bernhardt, Shi-Mei Wang, and Paul David Gershon‡

From the Institute of Biosciences and Technology/Department of Biochemistry and Biophysics, Texas A&M University, Houston, Texas 77093-3303

VP39 is a single-domain, bifunctional viral protein, which acts at both ends of nascent mRNA. At the 5’ end, it acts as a cap-specific 2’-O-methyltransferase. At the 3’ end, it acts as a poly(A) polymerase processivity factor, requiring its direct association with poly(A) polymerase. Although crystallographic and biochemical data show the catalytic center and associated binding sites for VP39’s methyltransferase function to be juxtaposed around a superficial cleft on the protein surface, surface regions required for VP39’s mRNA 3’ end modifying functions are not known. Here, we identify a surface region that interfaces directly with poly(A) polymerase, taking three independent approaches: (i) development of a direct in vitro dimerization assay, which is applied to numerous VP39 point mutants; (ii) identification of sites within VP39 that become protected from protease cleavage upon dimerization and further mutagenesis based upon these data; (iii) site-specific photo-cross-linking of VP39 to VP55. We find that the dimerization interface lies on a surface region remote from the methyltransferase cleft and contains a 3–5-residue “hot-spot,” which is very sensitive to amino acid substitutions. Various other sites within VP39 consistently became hypersensitive to protease cleavage upon interaction with VP55, indicating the occurrence of extensive conformational changes.

Vaccinia virus protein VP39 modifies both ends of nascent mRNA. At the 5’ end, it acts as a cap-specific S-adenosyl-l-methionine:mRNA (nucleoside-O-2’)-methyltransferase, an enzyme that specifically methylates the 2’-hydroxyl group of the penultimate nucleotide of the mRNA cap structure (1). At the 3’ end, VP39 acts as a processivity factor for the vaccinia poly(A) polymerase (PAP), V.5 (2). In vitro, VP55 extends RNA 3’ ends with ~30–35-nucleotide oligo(A) tails in a rapid, processive manner, but can only extend the resulting oligoadenylylated RNA very slowly and nonprocessively (3). Upon addition of VP39 to the reaction, this slow nonprocessive tail elongation is converted to a rapid semi-processive reaction (2). Apparently, VP39 permits VP55 to remain associated with the elongating poly(A) tails. VP55 and VP39 form a heterodimer (4, 5). Since molar excess amounts of VP39 over VP55 present no apparent advantage over a 1:1 stoichiometry in vitro (2), the heterodimer appears to be the active species in poly(A) tail elongation.

Solution of the 1.85-Å x-ray co-crystal structure of VP39 with its S-adenosyl-l-methionine (AdoMet) cofactor (6) showed VP39 to be a single domain protein based upon the Rossmann fold structure found in the catalytic domains of other methyltransferases (7). It could be speculated that either five or six surface regions might be required to fulfill VP39’s two RNA modifying activities. Thus, the cap-specific 2’-O-methyltransferase activities would presumably require a methyltransferase catalytic center, along with binding sites for the AdoMet cofactor, mRNA cap structure, and RNA polynucleotide adjacent to the cap (the latter two being pre-requisites for RNA 2’-O-methylation by VP39; Ref. 8). VP39’s tail elongatory activity would require a dimerization interface for VP55, and possibly also a second site for RNA polynucleotide binding. VP39’s water-accessible surface resembles a highly oblate sphere (6). It thus possesses two major faces, one of which is scarred by a superficial cleft of sufficient length to bind 4–5 nucleotides of unstacked RNA. Surface regions required for VP39’s a 2’-O-methyltransferase function are reasonably well defined, and appear to be confined to the cleft face. Thus, the central region of the cleft contains the methyltransferase catalytic center as defined by the position of the donor methyl group of the bound AdoMet molecule (6), and one end of the cleft is punctuated by a binding pocket for the terminal (m7G) nucleotide of the mRNA cap structure (9). The cleft therefore appears to be a site for the binding for cap-proximal RNA.

Regions of VP39 required for its poly(A) tail elongatory activity are not known, but presumably include a dimerization interface. Attempts to define polyadenylylation-specific residues of VP39 by point mutagenesis in combination with functional assays for VP39’s two activities have been surprisingly unsuccessful. Despite the generation of 35 VP39 point mutants, 10–14 of which were specifically defective in 2’-O-methyltransferase activity, no mutants specifically defective in poly(A) tail elongatory activity were isolated (10, 11). Here, we identify the dimerization interface of VP39 using three biochemical approaches: (i) a novel direct dimerization assay in combination with existing mutants; (ii) a “protein footprinting” approach, which assays for the protection of VP39’s protease cleavage...
DNA Cloning and Mutagenesis—The majority of VP39 point mutants were made as described previously (11). For a few of the mutants, the "unique site-elimination" point mutagenesis procedure (12) was employed, using the Chamaeleon double-stranded site-directed mutagenesis kit (Stratagene). Templates for the unique site-elimination procedure comprised double-stranded pGEX-KG-based plasmids (13), facilitating direct protein expression from the mutated plasmids. For most mutants, plasmid pPG177 (containing the wild-type VP39 gene cloned in pGEX-KG; Ref. 11) was used as the mutagenesis template. However, the template for mutant CF6 comprised a pGEX-KG-based plasmid encoding the protein VP39-NΔC26 (described below), and the template for mutants CF1*, CF2*, CF3*, PC1*, PC3*, PC4*, and PC5* comprised a pGEX-KG-based plasmid containing a version of the VP39 gene in which both cysteine codons had been changed to serine codons (10). DNA sequencing was used to confirm the desired mutations in clones that appeared positive by restriction enzyme screening. Mutant PC2** was made by replacing the smaller NcoI-XhoI fragment of pPG177 with that from CF3** DNA.

Gene expression vectors for the expression of VP39 variants containing an appended recognition site for the cAMP-dependent protein kinase catalytic subunit (PKA), the VP39 gene was amplified in vitro as described (11), after which the PCR products were incubated with NcoI and HindIII, then cloned between the NcoI and HindIII sites of the pGEX-KG expression vector. For the expression of protein VP39-NΔC26 (i.e. VP39 containing an N-terminal PKA recognition site plus a C-terminal truncation of 26 amino acids), and VP39-CΔ36 (i.e. in which the C-terminal 36 amino acids of the wild-type protein were replaced with a PKA recognition site), the PCR template was vaccinia genomic DNA. For the expression of protein VP39-NΔC26/AS3, the PCR template comprised plasmid DNA encoding mutant AS3 in the pGEX-KG vector (10, 11). Primers for the generation of VP39-NΔC26 and VP39-NΔC26/AS3 PCR products had the following sequences: 5'-ggggggaagcttctattcatgactaactttttcagttgat-3' and 5'-ggggggaagcttccatggatgttgtgtcgttagataaaccg-3'. The first of these primers encodes the amino acid sequence RRAS.

Protein Expression, Extraction, Purification, and Quantitation—Wild-type and mutant GST-VP39 were expressed in Escherichia coli as described (14). E. coli strain DH2S was used for the expression of VP39-CΔ36, and either DH2S or HB101 for the expression of other VP39-based proteins. Briefly, 3-ml cultures of LB broth containing 100 μg/ml ampicillin were inoculated with single plasmid-containing colonies and shaken at 37 °C until turbid. The cells were used to inoculate ~40-ml or 1,000-ml superbroth cultures containing carbenicillin or ampicillin (100 μg/ml), respectively, which were then shaken overnight, at 25 °C for all EB and C mutants and 37 °C for other VP39-based proteins. Isopropyl-1-thio-galactopyranoside was then added to a final concentration of 0.4 mM. After an additional 2.5-h incubation at the temperature used for overnight growth, cells were harvested by centrifugation. Pellets were resuspended in 5 ml of cells of buffer E (10 mM HEPES-NaOH, pH 8.0, 10% glycerol, 5 mM 2-mercaptoethanol, 0.1% Nonidet P-40) supplemented with NaCl (0.25 M), phenylmethylsulfonyl fluoride (0.1 mg/ml), and pepstatin (2 μg/ml), then lysed by sonication (five 15-s bursts). Lysates were supplemented with MgCl2 to 1 mM and DNase I (5 units, Boehringer Mannheim), incubated at room temperature for 15 min, then centrifuged (20 min at 20,000 rpm in an SW40 rotor). Supernatants were collected and stored at -70 °C for use in the in vitro dimerization assay (IVDDA, below) or purification of the recombinant protein.

For purification of the recombinant protein, protein extracts, made as described above, were combined with GSH-agarose beads (Sigma, 0.5 ml of packed bead volume/50 ml of E. coli extract) and the mixture rotated at room temperature for 30 min. The beads were then pelleted and washed four times with 5 ml of buffer E containing 0.25 mM NaCl. Thrombin protease (10 units, Novagen) was then added, and incubation continued at room temperature for 3–4 h. The beads were then pelleted and the supernatant collected. After washing the beads three more times with additional 2-ml aliquots of buffer E containing 0.25 mM NaCl, all supernatants were pooled, diluted 4-fold with buffer A (20 mM Tris-HCl, pH 7.8, 5 mM 2-mercaptoethanol, 10% glycerol, 0.01% Nonidet P-40), and passed through a 1-ml heparin-agarose column. The column was washed with buffer A, and the beads were eluted with a linear gradient of 0–0.5 M NaCl in buffer A. Fractions (1 ml) were collected and stored at -70 °C. Those containing wild-type/mutant VP39 were identified by SDS-PAGE. Purified VP39 and mutants thereof were quantitated by Coomassie-stained SDS-PAGE, as described previously (11). Wild-type and mutant VP39 proteins for photo-cross-linking studies were desalted as described above, except that 2-mercaptoethanol was omitted from buffers A and E. After purification and prior to photo-cross-linking, some of the protein samples were incubated briefly with 1 mM dithiothreitol followed by spin gel filtration.

Protein Footprinting Assay—Sequencing grade proteases were obtained from Boehringer Mannheim. Stocks of trypsin and chymotrypsin were prepared by dissolving lyophilized protease in 1 ml HCl, Arg-C, and Lys-C stocks by dissolving in water. Proteinase K (U.S. Biochemical Corp.) was initially dissolved at 20 mg/ml in 2% SDS, and this stock diluted 1280-fold in water prior to use. Terminally modified VP39 ( appended with a PKA recognition site) was terminally labeled with 32P using PKA (New England Biolabs). A typical 100-μl reaction contained 20 μCi of (γ-32P)ATP and 5 μg PKA, the resulting reaction mixture containing the recommended concentration of buffer and protein substrate. After a 60-min incubation at 30 °C, four 10-μl aliquots of the labeling reaction were mixed with equimolar amounts of VP55, and four additional aliquots with buffer A (above, after normalizing the latter for NaCl concentration by blending the gradient buffers used for the heparin-agarose column step of VP55 purification). Paired aliquots of CP-32P-labeled VP39 + buffer and CP-32P-labeled VP39 + VP55 were supplemented with 0, 75, 225, or 625-ng aliquots of chymotrypsin, Arg-C, or
Lys-C (Arg-C digestions also containing manufacturer’s “activator”), or with 0-, 12-, 36-, or 108-ng aliquots of proteinase K. After 1-min incubations at room temperature, digestions were terminated by the addition of either 5 μl of 0.1 M 4-(2-aminoethyl)benzenesulfonyl fluoride (Pefabloc SC, Boehringer Mannheim, for trypsin, chymotrypsin, Arg-C, or Lys-C) or 1 μl of 50 mM EGTA (for proteinase K, although EGTA affected proteolytic activity only minimally), followed by snap freezing in a dry ice ethanol bath. The digests were resolved in 16 × 20-cm 16.5% T, 3% C discontinuous SDS-PAGE gels (containing glycerol) capped with a 2-cm spacer and ~1.5-cm stack, following published procedures (15). After electrophoresis, gels were soaked in two 500-ml changes of 50% methanol, 10% glacial acetic acid for a total of 3-4 h, then dried and imaged.

Methionine- and cysteine-terminated peptide size markers were generated by partial degradation of 35S-end-labeled with either cyanogen bromide (16) or 2-nitro-5-thiocyanobenzoic acid (TNB-CN, Ref. 17). For CNBr cleavage, labeled protein (30 μl) was supplemented with 4.5 μl of 1 M HCl and 4.5 μl of 0.1 M CNBr, then incubated overnight at room temperature. Reactions were terminated by the addition of 1 ml of 3 M Tris-HCl, pH 8.5 (4.5 μl), and peptide gel loading buffer (15), then stored at ~70 °C. For TNB-CN cleavage, labeled protein (20 μl) was mixed with 10 mM urea (20 μl) and incubated for 20 min at room temperature, before supplementing reactions with 0.1% TNB-CN (4 μl) and 1 M NaOH (10 μl), and incubating at room temperature overnight. Reactions were terminated by the addition of 1 ml of 0.1 M NaOH (10 μl), 3 mM Tris-HCl, pH 8.5 (3 μl), and peptide gel loading buffer, then stored at ~70 °C. 14C-Labeled standards were obtained from Amersham Life Science.

New Mutants Targeting Superficial Aromatics—We define the “reverse” face of VP39 as the one that does not contain the methyltransferase catalytic center. The reverse face is depicted in Fig. 3 (C and F) of Ref. 6. At the outset of the current study, a set of seven point mutants was made in which superficial aromatic residues on VP39’s reverse face were mutated to alanine. These mutants were constructed for four major reasons. 1) When assayed for VP39’s two RNA modifying functions (namely cap-specific methyl group transfer and stimulation of poly(A) tail elongation), none of the 35 pre-existing VP39 point mutants (the “AS” and “SS” series mutants (10, 11) shown in Fig. 2 of Ref. 11) was specifically defective in tail elongation activity. 2) The absence of protein surface assignable to VP39’s adenylyltransferase function complemented the absence of known functions for VP39’s reverse face. 3) Although superficial aromatic side chains were considered likely to have roles in polyadenylation-specific functions such as RNA binding or dimerization, most of the pre-existing VP39 mutants possessed changes only in charged/polar side chains. 4) The AS and SS series mutants had been made prior to crystal structure determination, and therefore did not necessarily target superficial residues.

Of the seven new mutants, denoted EB1–EB7 (Fig. 1), EB4, -6, and -7 each contain a single aromatic to alanine change, whereas EB1, -2, -3, and -5 each contain multiple such changes, with a cluster of surface-exposed aromatic side chains (deduced from Fig. 3C of Ref. 6) being ablated in each mutant. Cap-specific 2′-O-methyltransferase and poly(A) tail elongation activities were determined for these EB mutants (Table I). All appeared wild-type in methylation transferase activity, providing further evidence that the reverse face plays no role in VP39’s 2′-O-methyltransferase activity. This would be consistent with VP39’s methyltransferase functions being confined to the face containing the methyltransferase catalytic center. The EB mutants also appeared wild-type in poly(A) tail elongation activity (Table I). Since a very large portion of VP39’s total surface had now been mutated without polyadenylation-specific defects was then placed inside a borosilicate glass tube. Samples were then irradiated with 350 nm light for 10 s at room temperature using a photochemical mini-reactor (RMR-600, Southern New England Ultraviolet), and subjected to SDS-PAGE in the absence of reducing agent.

RESULTS

Site-specific protein-protein cross-linking Assay—Site-specific protein-protein cross-linking assays followed published protocols (18, 19), with slight modifications. All procedures were performed in the dark or under safelight illumination. To 103-label N-(2-pyridyldithio)ethyl-4-azidosalicylame (PEAS, Molecular Probes), 8 nmol of PEAS, dissolved in 100 μl of 0.1 M sodium borate (pH 8.4), 0.5% (v/v) Me2SO, was supplemented with 2 nmol of 6 M NaCl (NEN Life Science Products) and 6 nmol E1. The mixture was exposed to an IODOGEN-coated reaction vial. After 30 s at room temperature, the mixture was quenched by transferring to a vial containing 15 μl of 8 M methionine, 1 mM tyrosine in 0.1 M sodium borate (pH 8.4), and incubating at room temperature for an additional 10 min. 12 μl of the resulting mixture, containing labeled PEAS, was mixed with 0.1 nmol purified VP39 or mutants thereof in 100 μl of buffer X (20 mM Tris-HCl, pH 8.0, 10% glycerol, 0.01% Nonidet P-40) containing ~0.25 M NaCl. After 120 min at room temperature, protein was separated from free 125I and 125I-labeled PEAS by gel filtration using columns (Bio-Spin 6, Bio-Rad) that had been pre-equilibrated with buffer X. The resulting ~100 μl of labeled protein was stored at ~70 °C until use. For cross-linking, 10 μl of desalted 125I-labeled PEAS-derivatized VP39, or a mutant thereof, was mixed with an equimolar amount of VP55 in a 1.5-ml microtube, which was
being observed, we concluded that either (i) surface residues (or groups of residues) contributing specifically to VP39's polyadenylation function were very elusive/nonexistent/redundant, or (ii) the tail elongation assay was inadequate for defining polyadenylation-specific residues.

Identification of Dimerization-defective Mutants Using the IVDDA—Since the tail elongation assay may have been inadequate for characterization of VP39's dimerization interface, a novel assay was developed in which the stability of VP55-VP39 interaction was assessed more directly. In this assay, the IVDDA, GST-VP39 fusion proteins were anchored to glutathione-agarose beads, which were then incubated with soluble VP55. The amount of bead-associated VP55 remaining after successive washes was determined by immunoblotting. The IVDDA was applied to 31 mutants, i.e., the seven "EB" series mutants (above), along with 24 of the 25 AS and SS series mutants (11), which retained the tail elongatory activity of wild-type VP39 (but only a subset of which retained VP39's cap-specific methyltransferase activity (11)). Fig. 2A shows results of an initial round of IVDDAs, which were conducted in the presence of 0.25 mM NaCl to confer a high stringency to the protein-protein interaction. With the exception of EB1, AS3, AS4, SS6, EB3, and EB6 (indicated in Fig. 2A with arrowheads), all mutants were wild-type in dimerization. Of the six defective mutants, EB1, AS3, and EB6 were completely defective, whereas AS4, SS6, and EB3 appeared to interact with VP55, but with a lower stability than wild-type VP39 (i.e., after initial association, steady dissociation of VP55 was detected during bead washing).

To determine the severity of the dimerization defect in mutants EB1, AS3, AS4, SS6, EB3, and EB6, these were re-assoayed in the presence of 60 mM NaCl, the ionic condition employed in the in vitro poly(A) tail elongation assay (Fig. 2B). No dimerization defect was detected for any of the six mutants at the lower NaCl concentration, consistent with their wild-type tail elongatory activities.

Protein Footprinting Analysis—Since the dimerization defects in the above VP39 mutants could have resulted either directly or indirectly from lesions in the mutant protein at 0.25 M NaCl, a more direct approach was taken to identify VP39's dimerization domain, namely protein footprinting. In this approach, VP39 was terminally labeled, the labeled protein partially proteolyzed in the presence and absence of unlabeled VP55, and the resulting peptides electrophoresed. Sequestration of cleavage sites within the dimerization interface with VP55 was expected to lead to reduced intensities of specific labeled peptide bands. The study utilized terminally modified versions of VP39 in which either the N or C terminus was appended with the sequence RRASVEF. This sequence contains the recognition site for PKA, permitting the serine residue to be 32P-labeled. For clarity of data, the N- and C-terminally tagged version of VP39 (denoted VP39-NAC26 and VP39-CAC36, respectively) possessed C-terminal truncations of 26 and 36 amino acids, respectively, to remove most or all of VP39's tail (above).

Five proteases were used for the footprinting analysis: Lys-C (which cleaves to the C-terminal side of lysine residues), Arg-C (which cleaves to the C-terminal side of arginine residues), trypsin (which cleaves to the C-terminal side of either lysine or arginine residues), and chymotrypsin and proteinase K. The latter two are broad spectrum proteases. Chymotrypsin cleaves predominantly to the C-terminal side of tyrosine, phenylalanine, and tryptophan residues, but can also cleave to the C-terminal side of leucine, methionine, alanine, aspartate, and glutamate residues. Proteinase K cleaves predominantly to the C-terminal side of aliphatic and aromatic residues. A combination of methods was used to assign 32P-labeled electrophoretic bands to specific cleavage sites, in the absence of the VP55 subunit. First, labeled peptides generated by partial cleavage with CNBr or TNB-CN were assigned to specific methionine and cysteine residues, respectively, by comparison with each other and with 14C-labeled protein standards. Next, specific lysine and arginine residues were identified by comparison of trypsin, Lys-C, and Arg-C digestion patterns with each other, and with cyanogen bromide and TNB-CN cleavage patterns. In this manner, 17 of the 18 basic residues occurring between Met-11 and Met-163 of VP39's amino acid sequence were assigned to specific cleavage products using N-terminally labeled VP39-NAC26 (Fig. 3A). Some assignments were confirmed by mutation of specific residues, followed by direct comparison of the digestion patterns for the wild-type and mutant proteins. This method was used to confirm the assignment of Arg-55 (Fig. 3B).

We next examined the effect of VP55 upon the VP39-NAC26 cleavage ladders with trypsin and chymotrypsin (Fig. 3C). The left-hand portion of Fig. 3C shows typical ladders for trypsin cleavage of N-terminally labeled VP39-NAC26 in the absence and presence of VP55. Arg-55 always became fully protected. In addition, Arg-122 and Lys-125/126 always became protected to a large extent, and Lys-32/33 and Lys-41 consistently became partially protected, although to a different extent in different experiments. The right-hand portion of Fig. 3C shows the pattern of cleavage with chymotrypsin. Arrows 4 and 6 show two bands that always became fully protected. Arrow 4 is believed, by comparison with the trypsin cleavage pattern, to correspond to either Leu-59, Asp-60, or Ala-62. Arrow 6 was tentatively assigned to Phe-115, by making a mutant in which Phe-115 was converted to alanine (mutant CF6, Fig. 1), then comparing the chymotrypsin cleavage pattern for this mutant with that for the wild-type protein (Fig. 3D). With the mutant protein, a single band was missing in the relevant portion of the gel. Arrow 5 shows a site that became partially protected here, but whose extent of protection was variable in different experiments. This site, which by comparison with the trypsin cleavage pattern was located between Arg-55 and Arg-76/79 in the linear sequence, was not assigned due to the paucity of nearby basic residues to serve as markers.

Fig. 3C highlights an additional effect, namely the apparent hypersensitivity to cleavage of various sites in VP39 upon dimerization with VP55. Although detectable with trypsin, the
Dimerization Interface of Poly(A) Polymerase Processivity Factor

**A**

| GST        | AS6 | AS15 |
|------------|-----|------|
| WT         | AS8 | AS16 |
| **EB1**    | AS10| AS17 |
| AS1        | AS11| EB4  |
| AS2        | SS4 | EB5  |
| SS1        | SS5 | SS13 |
| SS2        | SS8 | SS14 |
| **AS3**    | AS13| EB6  |
| **AS4**    | EB3 | EB7  |
| AS5        | AS14| AS20 |

**B**

| EB1        | AS4  | EB3  |
| AS3        | AS6  | EB6  |

**C**

| CF4        | CF6  | CF7  |
| CF5        |

**D**

| CF1C       | PC1C | CF4  |
| CF2C       | CF3C | CF5  |

**Fig. 2. IVDDAs with VP39 mutants.**

A, assays in the presence of 0.25 M NaCl, of the seven new EB series mutants (Fig. 1), along with GST and wild-type VP39 (WT) control proteins, and 24 of the 25 AS and SS series mutants available from previous studies that retained VP39’s tail elongatory activity (10, 11). The 25th mutant (AS21) was not assayed because the substitutions fell within VP39’s 36-amino acid “tail” region, which plays no detectable role in VP39’s activities (11). Briefly, N-terminally GST-tagged VP39, or mutants thereof, were immobilized to glutathione-agarose beads, after which soluble VP55 was added. The beads were then washed, divided into seven equal aliquots, and subjected to one to seven additional washes. Proteins associated with the samples of washed beads were subjected to SDS-PAGE followed by immunoblotting using anti-VP55 antibody (see “Experimental Procedures”). An immunoblot is shown for each mutant, to the right of the mutant-identifying name. On each immunoblot, a bead sample taken prior to VP55 addition is followed, successively (left to right), by bead samples taken after washes 2–8. For half of the assays, an antibody-control lane containing soluble VP55 alone (400 ng) is shown to the left of the lanes described above. Since samples from pairs of assays were combined onto single immunoblots along with a single antibody-control lane, the control lane is shown for only approximately half of the assays. For simplicity, only the region around the immunoreactive VP55 band is shown for each assay. However, in the immunoblots for mutants SS2, AS5, AS8, AS9, AS11, SS4, AS14, AS15, AS17, and EB6, this region contains a cross-reacting band migrating slightly more slowly than VP55, corresponding with the mutant GST-VP39 protein itself. These protein bands are detected as the sum of all VP55 associated with the bead sample taken prior to VP55 addition. Arrowheads denote those six mutants that showed either weak or undetectable affinity for VP55. Although mutant SS14 may appear slightly defective, gold staining of the membrane indicated that the loss of signal was equivalent for both subunits, indicating loss of beads during washing. Immunoblots are arranged in three columns according to the position, in the linear VP39 amino acid sequence, of the N-terminal-most amino acid substitution of the mutant (top of left column to bottom of right column = N terminus to C terminus, respectively). B, re-assay of those mutants that were defective in A, in the presence of 60 mM NaCl. Other details as for A. Although EB6 was positive, the mobilities of the VP55 and cross-reacting bands were very similar on the blot. C, IVDDAs, in the presence of 250 mM NaCl, of CF mutants (i.e., mutants made to confirm the protein footprinting data shown in Figs. 3 and 4). D, IVDDAs, in the presence of 60 mM NaCl, of those CF mutants that were dimerization-defective in the presence of 250 mM NaCl, and mutant PC1C (which was also defective at 250 mM NaCl). The cysteine-to-serine changes comprising the background for some of the mutants did not affect dimerization (data not shown), consistent with the unpaired 2′-O-methyltransferase and tail elongatory activities of the cysteine to serine mutants (10). The Cys-minus background was introduced into mutants CF1C, CF2C, and CF3C inadvertently, during their preparation.

The previous footprinting experiments, with N-terminally labeled VP39, addressed only the N-terminal half of the 333-residue protein because of insufficient resolving power of the peptide gel for peptides greater than ~150 amino acids in length. To examine the C-terminal half of VP39, a C-terminally labeled, C-terminally tagged VP39 derivative, VP39-CAC36, was used. As with the N-terminally labeled protein, size markers were generated, and Arg and Lys cleavage sites assigned to specific labeled bands (Fig. 4A). Fig. 4B shows the effect upon the trypsin and chymotrypsin cleavage patterns of dimerization with VP55. Although several trypsin cleavage sites appeared to be desensitized in the presence of VP55, this is probably due to either an incomplete normalization of buffer conditions or the increased amount of protein substrate present. However, dimerization apparently sensitized specific basic residues to the C-terminal side of R244 to trypsination. For chymotrypsin cleavage, the protection of a single site was apparent (arrow 5). Migrating between the products of CNBr cleavage at Met-196 and Met-208, the band corresponding to arrow 5 may have resulted from cleavage at either Phe-200 or Tyr-204. These are both surface residues, located on the top of VP39 with respect to the view of VP39 in Fig. 6A. Seven of the most clearly observable chymotrypsin-hypersensitive sites are labeled with arrows 1–4 and 6–8 (Fig. 4B). Arrows 1–3 correspond with the C-terminal region of VP39, which also became hypersensitive to trypsin digestion. The site labeled with arrow 4 apparently resides between Tyr-232 and Tyr-238. Hypersensitivity at the sites corresponding to arrows 6 and 7 was also apparent after chymotrypsin treatment of N-terminally labeled VP39 (arrows 8 and 7, respectively, in Fig. 3C), and occurs at residues ~150 and ~130, respectively. Arrow 8 indicates a hypersensitive N-terminal region that corresponds with arrows 1–3 in Fig. 3C. To confirm the VP55-induced proteinase K not affect dimerization (data not shown), consistent with the unpaired 2′-O-methyltransferase and tail elongatory activities of the cysteine to serine mutants (10). The Cys-minus background was introduced into mutants CF1C, CF2C, and CF3C inadvertently, during their preparation.
hypersensitivities observed with N-terminally labeled VP39, C-terminally labeled VP39-CΔC36 was also treated with proteinase K (Fig. 4C). As with the N-terminally labeled protein (Fig. 3E), no protections were detected, but many hypersensitive sites were observed. These filled a broad region extending from residue −270 toward the N terminus of VP39. Two of the
sites, corresponding to arrows 1 and 2, could be mapped quite accurately to the regions of residues −270 and −230, respectively. Their positions were comparable to arrows 3 and 4, respectively, marking sites that become hypersensitive to chymotrypsin cleavage (Fig. 4B). The bracket (numbered 3, Fig. 4C) showed a region that was hypersensitive to protease K upon dimerization. The combined data from Figs. 3E and 4C indicated that the proteinase K-hypersensitive region extended between residues −70 and −270. The experiments of Figs. 3 and 4 (summarized in Table III) strongly suggest that dimerization correlates with extensive conformational changes throughout a large portion of VP39.

Confirmation of Protein Footprinting Data Using the IVDDA—Some of the protected residues in the footprinting assays were further investigated by analysis of corresponding point mutants in the IVDDA. Two clusters of basic residues, namely Lys-32/Lys-33/Lys-41 and Arg-122/Lys-125/Lys-126, consistently became partially protected in the presence of VP35 (Fig. 3C). Fortunately, Lys-32 and Lys-33 had already been coordinately changed to alanine in mutant AS2, Lys-41 had been changed to glutamate in mutant SS2, and Arg-122, Lys-125, and Lys-126 had been changed to alanine in mutant AS8 (11). No dimerization defects were apparent with AS2, SS2, or AS8 in the IVDDA (Fig. 2A, above). To evaluate the role of Ser-123, an additional mutant was created containing the change S123A in the context of the AS8 mutant (CF7, Fig. 1). However, consistent with the AS8 result, CF7 was wild-type in the IVDDA (Fig. 2C), and also in the 2'-O-methyltransferase and tail elongatory assays (Table I). Residue Arg-55 was consistently fully protected upon dimerization with VP55. Arg-55 had previously been mutated to alanine in mutant AS3, which also contained an additional change, H56A (Fig. 2 of Ref. 11). Mutant AS3 was completely defective in dimerization at 0.25 m NaCl (Fig. 2A, above). Since AS3 also contained the H56A substitution, an additional mutant was made to confirm that changes at Arg-55 alone were sufficient to disrupt dimerization. Changing Arg-55 to either glutamate (radical charge-swap; mutant CF1C, Fig. 1), glutamine (loss of charge but retention of some potential hydrogen bonding; mutant CF2C, Fig. 1) or cysteine (removal of side chain; mutant PC1C, Fig. 1), completely eliminated dimerization in the presence of 0.25 m NaCl (data not shown), although, as with other mutants, dimerization activity of each was retained in the presence of 60 m NaCl (Fig. 2D). The three mutants were wild-type in both 2'-O-methyltransferase and tail elongatory activities (Table I). We next explored the adjacent His-56/Ile-58 region of the VP39 surface more fully. An attempt was made to create a PKA site in this region with the substitutions H56R and I58S (mutant CF3C', Fig. 1). Interestingly, this mutant turned out to be unusually defective in the IVDDA, being incapable of dimerization at either 250 or 60 m NaCl (data not shown and Fig. 2D). In addition, the mutant was completely defective in tail elongatory assay (Table I), consistent with its lack of dimerization activity at the equivalent NaCl concentration (60 m, Fig. 2D). The defects in CF3C' activity could not be attributed to a global folding defect, since the mutant was completely wild-type in 2'-O-methyltransferase activity (Table I). While many specifically methyltransferase-defective mutants had been made in previous studies (10, 11), the isolation of specifically polyadenylylation-defective mutants had previously proven intractable. CF5C' (along with its derivative, PC2C'), Table I) was the only mutant made to date that was specifically defective in polyadenylation activity. Mutant CF3C' strongly implicated residues His-56 and/or Ile-58 in dimerization. To characterize a substitution at Ile-58 alone, this residue was changed to either glutamate or aspartate (mutants CF4 and CF5, respectively, Fig. 1). These changes added a negative charge to an aliphatic chain comparable to that of isoleucine. Both mutants were partially defective in the 0.25 m NaCl IVDDA (Fig. 2C), but were not defective in either the 60 m NaCl IVDDA (Fig. 2D), or the 2'-O-methyl-
Site-specific Photo-cross-linking—A site-specific photo-cross-linking assay (19) was employed to confirm footprinting and mutagenesis data. In this approach, specific residues of VP39 only through the unique cysteine, samples of 125I-PEAS-derivatized mutants were subjected to SDS-PAGE in the presence of reducing agent, to confirm loss of label (data not shown). As further confirmation of thiol-dependent PEAS attachment, the cysteine-minus VP39 mutant used as a basis for the introduction of unique thios was shown to be refractory to 125I-PEAS labeling (C178S/C272S, Fig. 5). Third, to confirm that photo-cross-linking was dimerization-dependent, a dimerization-defective VP39 mutant, PC2C, was tested and found not to produce VP55-dependent, UV-dependent photo-cross-linking products (Fig. 5). PC2C was based upon the dimerization-defective mutant CF3 (Fig. 1) after re-introduction of one of the two wild-type cysteines (Cys-272). Cys-272 was known to be able to photo-cross-link to VP55 in the context of wild-type VP39, since 125I-PEAS-derivatized versions of both wild-type VP39 and a VP39 mutant in which the other cysteine (Cys-178) was changed to serine, could be photo-cross-linked to VP55 (WT and C178S, respectively, in Fig. 5). Mutant PC5C, in which the unique cysteine replaced F115, did not photo-cross-link to VP55 (Fig. 5).

DISCUSSION
Vaccinia PAP has been isolated from both virion extracts and infected cells as a heterodimer (4, 5, 20). Furthermore, heterodimer formation between the isolated subunits, VP55 and VP39, has been specifically demonstrated in vitro upon mixing the two purified, recombinant subunits (2). Despite VP39 being expressed in vaccinia-infected cells and packaged in the virion in five fold molar excess over VP55 (4), the observation that 1:1 subunit stoichiometry is adequate for full tail elongation activity in vitro (2) indicates that the heterodimer is the active species in poly(A) tail elongation. Subunit interaction therefore appears to be central to VP39’s mechanism of action as a PAP processivity factor. Previous attempts to reveal VP39’s dimerization domain had assumed that a dimerization-defective VP39 mutant would be specifically defective in poly(A) tail elongatory activity. However, studies using 35 point mutants (mainly charge and charge-cluster → alanine mutants) in combination with assays for VP39’s two known functions, viz. caps-specific 2′-O-methylolation and poly(A) tail elongation, were unfruitful in revealing a VP39 mutant that was specifically polyadenylation-defective, despite 14 mutants being isolated that were specifically 2′-O-methyltranslase-defective (10, 11). The following possibilities were considered as possible explanations: (i) the dimerization domain might be shared with essential methyltransferase-specific domains; (ii) it could involve only very few residues (and/or contain no charged residues); (iii) it could involve sufficient numbers of residues that ablation of individual side chains had little effect on the overall dimerization energy; and (iv) the poly(A) tail elongatory assay might be insensitive to major changes in dimerization energy.

At the outset of the current study, we attempted to address each of the above possibilities. Thus, three direct yet distinct biochemical approaches were employed to investigate the dimerization domain of VP39, viz. a novel direct dimerization assay, the IVDDA; a protein footprinting assay for protease-
cleavage sites that become protected upon dimerization; and a direct site-specific photochemical cross-linking assay (previously used to define the targets of transcriptional activators (18, 21), as opposed to the current application of exploring the extent of a dimerization interface). Furthermore, knowing the crystal structure of VP39, uncharged (aromatic) surface side chains decorating an uncharacterized region of the protein surface were chosen as targets for additional mutants. Finally, in the IVDDA, we attempted to maximize stringency by assaysing dimerization at a much higher ionic strength (250 mM NaCl) than that employed previously in the polyadenylation assay (60 mM NaCl). Our suspicion that increased ionic strength would lead to increased stringency came from observations, using surface plasmon resonance methodology (Ref. 22 and data not shown), of increased rates of heterodimer dissociation at elevated ionic strength. The destabilizing effect of ionic strength accounted for the previously observed dissociability of PAP heterodimer purified from virion extracts, upon anchoring via a VP55-specific antibody (4). Unfortunately, elevated salt concentrations could not be used to increase stringency in the poly(A) tail elongatory assay due to inhibition of VP55's catalytic activity (5, 23). Although no one of the three approaches was considered conclusive, site-specific photo-cross-linking was used to resolve inconsistencies between the other two approaches. For example, dimerization consistently led to the protection of residue Phe-115 (which maps adjacent to the methyltransferase catalytic center) from chymotrypsin cleavage, yet removal of the Phe-115 side chain did not affect dimerization activity in the IVDDA. This might have been because either the side chain contributes little individual dimerization energy to an otherwise large overall dimerization interface, or its protection from cleavage resulted from conformational changes within VP39 remote from the dimerization interface itself. The inactivity of site-specific photo-cross-linker anchored at residue Phe-115 in photo-cross-linking to VP55, favored the latter possibility. The consistent protection of other VP39 residues upon dimerization (e.g. Lys-32, Lys-33, Lys-41, and Arg-122, Lys-125, Lys-126), whose combined mutation did not affect dimerization in the IVDDA, was also presumed to result from conformational change remote from the dimerization interface.

2 X. Shi and P. D. Gershon, unpublished data.

methyltransferase catalytic center; B, 90° rotation about vertical axis with respect to A (side view); C, 180° rotation about vertical axis with respect to A, showing VP39's reverse face. Surface residues are color-coded as follows. Very pale blue, residues that are protected from protease digestion by VP55, but whose mutation does not lead to a dimerization defect (Lys-32, Lys-33, Lys-41, Arg-122, Ser-123, Lys-126). Darker blue, residues protected from protease digestion by VP55, but no confirmatory data (Leu-59, Asp-60, Ala-62). Pale green, mutant incorporating substitution at this residue is partially defective in the IVDDA at 250 mM NaCl, but defect not necessarily due to substitution at this residue due to multiple substitutions in the mutant (e.g. Asp-80, Asp-108, Tyr-189, Tyr-214). Mid-green, mutant incorporating substitution at this residue is completely defective in the IVDDA at 250 mM NaCl, but defect not necessarily due to substitution at this residue due to multiple substitutions in the mutant (Tyr-12, Phe-48). Dark green, mutant whose only substitution is at this residue is completely defective in the IVDDA at 250 mM NaCl (Tyr-271). Red, this residue (i) photo-cross-links directly to VP55 AND is protected in footprinting experiments AND cannot be changed without multiple/serious defects (e.g. Arg-55), (ii) photo-cross-links directly to VP55, but cannot be changed without either multiple/serious defects (e.g. His-56) or significant defects despite only subtle changes (e.g. Ile-58), or (iii) photo-cross-links directly to VP55, but cannot be changed without multiple/serious defects (e.g. Arg-79, Arg-107), (iv) cannot be changed without either multiple/serious defects (e.g. His-56) or significant defects despite only subtle changes (e.g. Ile-58), or (v) can be changed without multiple/serious defects (e.g. Arg-55).

**FIG. 6.** Positions, on the VP39 surface, of residues implicated in dimerization with VP55. A, major face of VP39 possessing the
Dimerization Interface of Poly(A) Polymerase Processivity Factor

TABLE II

| Residue | Substitution | IVDDA (250) | IVDDA (60) | Footprint | Photo |
|---------|--------------|-------------|------------|-----------|-------|
| Tyr-12  | Ala          | ? Comp in EB1 | OK         |           |       |
| Lys-32  | Ala          | OK in AS2    | Yes (Part) |           |       |
| Lys-33  | Ala          | OK in AS2    | Yes (Part) |           |       |
| Lys-41  | Ala          | OK in SS2    | Yes (Part) |           |       |
| Phe-48  | Ala          | ? Comp in EB1 | OK         |           |       |
| Arg-55  | Ala          | ? Comp in AS3 | OK         | Prot (Fig. 3C) | Yes |
| Glu     | Comp in CF1c- | OK         | Prot (Fig. 3C) |           |       |
| Cys     | Comp in PC1c- | OK         | Prot (Fig. 3C) |           |       |
| Gln     | Comp in CF2c- | OK         | Prot (Fig. 3C) |           |       |
| His-156 | Ala          | ? Comp in AS3 | OK         |           |       |
| Arg     | ? Comp in CF3c- | As 250   |           |           |       |
| Arg     | [PC2(5)]     | OK         |           |           |       |
| Ile-58  | Ser          | ? Comp in CF3c- | As 250   |           |       |
| Ser     | [PC2(5)]     | OK         |           |           |       |
| Glu     | Part in CF4   | OK         |           |           |       |
| Asp     | Part in CF5   | OK         |           |           |       |
| Leu-59  | Ala          | OK         | OK         | Maybe (Fig. 3C, chymo.) |       |
| Asp-60  | Ala          | OK         | OK         | Maybe (Fig. 3C, chymo.) |       |
| Ala-62  | Ala          | OK         | OK         | Maybe (Fig. 3C, chymo.) |       |
| Arg-79  | Ala          | ? Part in AS4 | OK         | In “10” region (Fig. 3C) | Yes |
| Cys     | OK in AS4    | OK         |           |           |       |
| Asp-80  | Ala          | ? Part in AS4 | OK         | In “10” region (Fig. 3C) | Yes |
| Arg-107 | Ala          | ? Part in AS8 | OK         | In “10” region (Fig. 3C) | Yes |
| Cys     | ? Part in AS8 | OK         |           |           |       |
| Asp-108 | Ala          | ? Part in AS6 | OK         |           |       |
| Phe-115 | Ala          | OK         | OK         | Prot (Fig. 3C) | No |
| Cys     | OK in CF6    | OK         |           |           |       |
| Arg-122 | Ala          | OK         | OK         | Yes (Part) |       |
| Ser-123 | Ala          | OK         | OK         | Yes (Part) |       |
| Lys-125 | Ala          | OK         | OK         | Yes (Part) |       |
| Lys-126 | Ala          | OK         | OK         | Yes (Part) |       |
| Cys-178 | Ala          | OK         | OK         | No         |       |
| Tyr-189 | Ala          | ? Part in EB3 | OK         |           |       |
| Tyr-214 | Ala          | ? Part in EB3 | OK         |           |       |
| Tyr-271 | Ala          | Comp in EB6  | OK         |           |       |
| Cys-272 | Ala          | OK         | OK         | Yes         |       |

TABLE III

Regions of VP39 that become hypersensitive to protease cleavage upon interaction with VP55

| Region | Single or multiple sites | Depiction in figure |
|--------|--------------------------|---------------------|
| Before Met-11 | Single | Fig. 3C, arrow 1 |
| Met-11 to Lys-32 | Single | Fig. 3C, arrow 2 |
| Lys-41 to Arg-55 | Single | Fig. 3C, arrow 3 |
| ~130/Arg-122 to Arg-140 | Single | Fig. 3C, arrow 7/Fig. 4B, arrow 7 |
| ~150/Arg-143 to Met-163 | Single | Fig. 3C, arrow 8/Fig. 4B, arrow 6 |
| ~230/Tyr-232 to Tyr-238 | Single | Fig. 4C, arrow 2/Fig. 4B, arrow 4 |
| ~270 | Single | Fig. 4C, arrow 1/Fig. 4B, arrow 3 |
| Arg-244 to ~280 | Multiple | Fig. 4B |
| ~70 to ~270 | Multiple | Fig. 3E, bracket 4/Fig. 4C, bracket 3 |

The combined data of this study define a dimerization interface that is remote from the cleft implicated in RNA binding for VP39’s cap-specific 2’-O-methyltransferase activity. Thus, the dimerization domain falls at the edge of the major face of VP39 that does not possess methyltransferase-specific sites (VP39’s reverse face). Although the reverse face is relatively featureless and corresponds with no previously assigned functions, it possesses an extended basic region surrounded by superficial aromatic side chains (6). The presence within the dimerization interface of at least three/four charged side chains (Arg-55, Arg-79, Arg-107, and His-56) (the latter below pH 6.0) accounts for the salt sensitivity of dimerization (4). Although the current analysis indicates two major sets of dimerization contacts (one centered upon Arg-55, the other on Arg-107), suggesting that the dimerization domain might be bipartite (Fig. 6B), there is insufficient data to show whether this is really the case. If the dimerization interface is continuous as opposed to bipartite, it would presumably include the region of VP39’s surface falling between the two major sets of contacts characterized here (Fig. 6B). This region includes residues Tyr-83 through Gly-86, Ile-88, and Asn-273. No mutants in these residues were available for characterization in the IVDDA and, although one of them (Tyr-83) could be expected to have been protected in our experiments (from chymotrypsin), no clear data were obtained. We doubt that the dimerization interface covers a much larger area than the two parts shown in Fig. 6 and possibly also the area between them, due to the relatively low numbers of residues protected in the footprinting analysis. In general, protein interfaces can vary in size from ~600 to ~3000 Å2 (24). The surface area contributed by residues Arg-55, His-56, Ile-58, Tyr-271, and Cys-272 (Fig. 6) combined, covers about 370 Å2, and the combined area for this region plus Phe-48, Tyr-214, Tyr-59, Tyr-60, Arg-79, and Arg-107 and the six residues falling between two major sets of characterized contacts (above) is...
erodimer topology in which VP55 covers VP39's cleft, face to be remote from the cleft argue strongly against a heterodimer. However, the current data showing the dimerization interaction reconfigure the cleft for VP39's poly(A) tail elongatory activity is affected at low salt (60 mM NaCl), might indicate a particularly significant contribution of this trio of side chains to dimerization. This would be consistent with previous observations with other heterodimers that small subsets of dimerization interface residues can contribute disproportionately large amounts of binding energy to dimerization (25). Due to its high degree of protrusion (Fig. 6C) and consequently large contribution to the surface area of the dimerization interface, Arg-107 might be expected to make a sizable contribution to binding energy (24).

Protease cleavage sites that became hypersensitive upon dimerization with VP55, were identified in all parts of VP39 (Table III). Although different patterns of hypersensitivity were observed for each protease used, several sites were detected with more than one protease, and/or independent of which end of the protein was labeled. The trypsin- and chymotrypsin-hypersensitive sites appear to fall almost exclusively within loops and helices (which cover the protein surface; Ref. 6), and not within the β-strands (which are sequestered within the VP39 core). With proteinase K, the core also appeared to be affected. The hypersensitization of several cleavage sites upon dimerization suggest the occurrence of extensive conformational changes which alter the solvent accessibility of some residues. Indeed, the occurrence of spurious protections (such as that observed at residue Phe-115, above) would require dimerization-specific changes in VP39’s conformation. Roles for conformational change might relate to achieving dimerization specificity without an initially high affinity. Thus, a small interface area may not initially present many opportunities for high affinity between the subunits, but conformational changes during docking resulting from protein flexibility, might optimize the number of contacts and the resulting affinity, so that the equilibrium is biased toward heterodimer. Another role for conformational change could be related to the bifunctionality of the protein. If the RNA binding cleft employed for VP39’s 2′-O-methyltransferase activity is also used for tail elongation, then dimerization-induced conformational changes might serve to reconfigure the cleft for VP39’s poly(A) tail elongatory activity. However, the current data showing the dimerization interface to be remote from the cleft argue strongly against a heterodimer topology in which VP55 covers VP39’s cleft, “clamping” the elongating poly(A) tail within it, leading to increased processivity of tail elongation by the heterodimer.

Whether identical, overlapping, or separate RNA binding sites are employed for the two RNA modifying functions of VP39 remains to be seen, but will doubtless be central to the mechanism of action of VP39 in poly(A) tail elongation. If the two RNA modifying activities employ distinct RNA binding sites, then it could be speculated that a polyadenylation-specific site might run between the two parts of a bipartite dimerization domain (Fig. 6B). Further experiments would be required to address these possibilities. Identification of additional interface residues might resolve this issue, although positioning the photo-cross-linker between the two sets of currently defined interface regions might not conclusively show whether this region forms a polyadenylation-specific RNA binding channel, since the cross-linker might reach across such a channel.

Acknowledgments—We are indebted to Dr. Richard Ebright and Youngyu Kim for helpful discussions; Dr. Alec Hodel for kindly preparing Fig. 6 and calculating protein surface areas; Drs. B Moss and B Schnierle for plasmids encoding VP39 mutants C178S, C272S, and C178/272S; Dr. J. Putkey for providing the RMR-600 light source; and X. Sui for help in the construction, production, and characterization of a recombinant baculovirus expressing GST-VP55.

REFERENCES
1. Schnierle, B. S., Gershon, P. D., and Moss, B. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 2897–2901
2. Gershon, P. D., and Moss, B. (1993) J. Biol. Chem. 268, 2203–2210
3. Gershon, P. D., and Moss, B. (1992) Genes Dev. 6, 1575–1586
4. Gershon, P. D., Ahn, B.-Y., Garfield, M., and Moss, B. (1991) Cell 66, 1269–1278
5. Moss, B., Rosenblum, E. N., and Gershowitz, A. (1975) J. Biol. Chem. 250, 4722–4729
6. Hodel, A. E., Gershon, P. D., Shi, X., and Quiocio, F. A. (1996) Cell 85, 247–256
7. Schluckebier, G., O’Gara, M., Saenger, W., and Cheng, X. (1995) J. Mol. Biol. 247, 16–20
8. Barbosa, E., and Moss, B. (1978) J. Biol. Chem. 253, 7698–7702
9. Hodel, A. E., Gershon, P. D., Shi, X., Wang, S.-M., and Quiocio, F. A. (1997) Nat. Struct. Biol. 4, 550–554
10. Schnierle, B. S., Gerson, P. D., and Moss, B. (1994) J. Biol. Chem. 269, 20700–20706
11. Shi, X., Yao, P., Jose, T., and Gershon, P. D. (1996) RNA 2, 88–101
12. Deng, W. P., and Nickoloff, J. A. (1992) Anal. Biochem. 209, 81–88
13. Guan, K. L., and Dixon, J. E. (1991) Anal. Biochem. 192, 262–267
14. Gershon, P. D., and Moss, B. (1996) Methods Enzymol. 275, 208–227
15. Schagger, H., and von Jagow, G. (1987) Anal. Biochem. 166, 360–379
16. Gross, E. (1967) Methods Enzymol. 11, 238–255
17. Jacobson, G. R., Schaffer, M. H., Stark, G. R., and Vanaman, T. C. (1973) J. Biol. Chem. 248, 6583–6591
18. Chen, Y., Ebright, Y. W., and Ebright, R. H. (1994) Science 265, 90–92
19. Ebright, Y. W., Chen, Y., Kim, Y., and Ebright, R. H. (1996) Bioconjugate Chem. 7, 380–384
20. Nevins, J. R., and Joklik, W. K. (1977) J. Biol. Chem. 252, 6939–6947
21. Miller, A., Wood, D., Ebright, R. H., and Rothman-Denes, L. B. (1997) Science 275, 1655–1657
22. Gershon, P. D., and Khilko, S. N. (1995) J. Immunol. Methods 183, 65–76
23. Thomson, J. G., and Gershon, P. D. (1995) Biotechniques 19, 416–425
24. Jones, S., and Thornton, J. M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 13–20
25. Clackson, T., and Wells, J. A. (1995) Science 267, 385–386