Vaccinia Virus mRNA (Guanine-7-)Methyltransferase: Mutational Effects on Cap Methylation and AdoHcy-Dependent Photo-Cross-Linking of the Cap to the Methyl Acceptor Site†

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ABSTRACT: The (guanine-7-) methyltransferase domain of the vaccinia virus mRNA capping enzyme is composed of the C-terminal portion of the D1 subunit, D1(498−844), heterodimerized with the D12 protein. In order to identify protein structural elements involved in cap methylation, we introduced eight alanine substitution mutations within two sequence motifs of D1(498−844)—(594)VLAIDFGNG(602) and (681)IHYSF(685)—that are conserved in the cap methyltransferase from yeast. The D1(498−844)-Ala proteins were coexpressed in bacteria with the D12 subunit, and the recombinant D1(498−844)/D12 heterodimers were purified. Alanine substitutions at five positions—Asp-598, Gly-602, Ile-681, Ser-684, and Phe-685—had little or no effect on methyltransferase activity. Mutations at three conserved residues were deleterious. Alanine substitution at Gly-600 reduced the specific activity to 4% of that of the wild-type protein. Substitutions at His-682 and Tyr-683 reduced activity to 4% and 0.05%, respectively. By further mutating Tyr-683 to Phe and Ser, we established that the aromatic group was essential for cap methylation, whereas the hydroxyl moiety was dispensable. Specific binding of the methyltransferase to the RNA cap was demonstrated by UV cross-linking to [^{32}P]GMP-labeled capped poly(A). Label transfer occurred exclusively to the D1(498-844) subunit and was competed by the cap analogs GpppA and m7GpppA. Cap-specific cross-linking to m7GpppA(pA)n was stimulated by AdoHcy, whereas cross-linking to GpppA(pA)n was unaffected by AdoHcy, but stimulated by AdoMet. We suggest that occupancy of the methyl donor site either enhances the affinity for the cap guanosine or alters the protein interface so that a photoreactive moiety is brought closer to the cap structure. The catalytically defective H682A, Y683A, and Y683S mutant methyltransferases were unable to cross-link to the cap in the presence of AdoHcy. The catalytically defective G600A mutant did cross-link to the cap in the presence of AdoHcy, suggesting that this mutation affects the chemical step of transmethylation.

Eukaryotic mRNAs contain a 5′-terminal cap structure, m7GpppN, which is synthesized by the sequential action of three enzymes—RNA triphosphatase, RNA guanylyltransferase, and RNA (guanine-7)-methyltransferase (Shuman, 1995). In the vaccinia virus capping enzyme, the triphosphatase, guanylyltransferase, and methyltransferase activities are linked physically within a multifunctional protein (Venkatesan et al., 1980). The 95 and 33 kDa subunits of the heterodimeric vaccinia capping enzyme are encoded by the viral D1 and D12 genes, respectively (Niles et al., 1986, 1989). The catalytic domains are organized in a modular fashion. The amino-terminal 60 kDa of the D1 subunit is an autonomous unit containing the guanylyltransferase and triphosphatase activities (Shuman, 1989; Shuman & Morham, 1990; Higman et al., 1992). The methyltransferase domain is a distinct, nonoverlapping, autonomous unit consisting of the carboxyl portion of the D1 subunit heterodimerized with the D12 subunit (Cong & Shuman, 1992; Higman et al., 1992).

The contributions of the individual subunits to the cap methyltransferase domain have been examined by expressing the D1 and D12 polypeptides in bacteria. The carboxyl-terminal segment of the D1 polypeptide has a weak intrinsic methyltransferase activity that can be stimulated 50−100-fold by addition of purified D12 protein, which is itself catalytically inert (Higman et al., 1994; Mao & Shuman, 1994). Therefore, the catalytic center must reside within the D1 protein. Consistent with this, the C-terminal segment of the D1 polypeptide contains binding sites for RNA, for *S*-adenosylmethionine (AdoMet; the methyl donor), and for GTP (a methyl acceptor) (Luo & Shuman, 1993; Higman et al., 1994; Higman & Niles, 1994; Niles et al., 1994). The mechanism by which the D12 subunit stimulates the methyltransferase of the D1 subunit is not understood.

We have adopted a mutational approach to the identification of protein structural elements in the D1 subunit that are important for cap methylation. Initially, we constructed a series of alanine-cluster mutations in D1(498−844), the C-terminal protein segment that contains the methyltransferase. In this strategy, two or three closely spaced residues were simultaneously replaced by alanine. The mutated versions of D1(498−844) were cotranslated in vitro with the D12 protein, and the translation products were tested for subunit association (by coimmunoprecipitation) and for cap methyltransferase activity. Most of the mutant D1 proteins we analyzed were defective both for subunit association and for enzymatic activity (Mao & Shuman, 1994). This class of mutations was not especially informative, beyond confirming that subunit heterodimerization was essential for optimal catalysis. Only one mutant D1 protein (a double

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mutant at vicinal residues His-682 and Tyr-683) displayed the properties we sought, i.e., defective methyltransferase activity but no effect on subunit interaction (Mao et al., 1994; Luo et al., 1995).

In the present study, we characterize a collection of single alanine substitution mutants of D1(498−844), the rationale being that single mutations might be less disruptive of subunit interactions than clustered changes. The D1(498−844)-Ala proteins were expressed in bacteria together with the D12 subunit and then purified to homogeneity. This approach was much better suited than in vitro translation to quantitative assessment of methyltransferase function. Whereas none of the single alanine mutations affected subunit association, three residues were specifically shown to be essential for catalysis. We show by UV cross-linking that binding of the vaccinia methyltransferase to the cap structure is stimulated by concomitant occupancy of the AdoMet/AdoHcy binding site. Using this assay, together with a nitrocellulose filter binding assay, we were able to discern distinctive mutational effects on nonspecific RNA binding and on cofactor-dependent interaction with the RNA cap.

EXPERIMENTAL PROCEDURES

Mutagenesis. Mutations in the D1(498−844) protein were programmed by synthetic oligonucleotides using the two-stage PCR-based overlap extension strategy. Plasmid pET-His-D1(498−844) [described by Mao and Shuman (1994)] was used as the template for the first stage of amplification by *Pfu* DNA polymerase. The reaction products (two D1 gene fragments with overlapping ends containing the mutated sites) were gel-purified and used for a second stage of amplification primed by oligonucleotides flanking the His-D1(498−844) expression cassette. Restriction fragments of the PCR-amplified gene containing the mutated D1(498−844) gene were inserted into the T7 RNA polymerase-based coexpression vector pET-D12/His-D1(498−844) (Mao & Shuman, 1994) so as to replace a wild-type gene fragment with mutated DNA. The presence of the desired mutation was confirmed by dideoxy sequencing of plasmid DNAs isolated from individual ampicillin-resistant *Escherichia coli* transformants. We then sequenced across the entire restriction fragment that had been exchanged into the wild type background; the occurrence of unwanted PCR-generated mutations was thereby excluded.

Enzyme Purification. Wild-type and mutated pET-D1/His-D1(498−844) expression plasmids were introduced by electroporation into *E. coli* BL21(DE3). Ampicillin-resistant transformants were inoculated into 200 mL of LB medium containing 0.1 mg/mL ampicillin and grown at 37 °C until the A_{600} reached approximately 0.6. The cultures were placed on ice for 30 min and subsequently incubated for 48 h at 20 °C with continuous shaking. Cells were harvested by centrifugation, and the pellets were stored at −80 °C. All subsequent procedures were performed at 4 °C. Cell lysis was achieved by treatment of thawed, resuspended cells with lysozyme (0.2 mg/mL) and 0.1% Triton X-100 in lysis buffer (50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, and 10% sucrose). Insoluble material was removed by centrifugation at 18 000 rpm for 30 min in a Sorvall SS34 rotor. The supernatants were mixed with 1 mL of Ni−NTA−agarose resin (Qiagen) for 1 h. The slurries were poured into a column and then washed with lysis buffer. The columns were eluted stepwise with IMAC buffer (20 mM Tris-HCl, pH 7.9, 0.5 M NaCl, 1 mM PMSF, and 10% glycerol) containing 5, 25, 65, and 200 mM imidazole. The polypeptide composition of the column fractions was monitored by SDS−PAGE. In every case, the D1(498−844) and D12 capping enzyme subunits were coeluted at 65 mM imidazole. This fraction was dialyzed against buffer B (25 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 1 mM dithiothreitol, 0.05% Triton X-100, and 5% glycerol). Each dialysate was applied to a 1 mL column of phosphocellulose that had been equilibrated with buffer B. The columns were eluted stepwise with buffer A (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 2 mM dithiothreitol, 0.1% Triton X-100, and 10% glycerol) containing 0.05, 0.1, 0.5, and 1 M NaCl. The capping enzyme subunits were recovered in the 0.5 M NaCl step. The protein concentrations of the capping enzyme preparations were determined using the BioRad dye reagent with bovine serum albumin as a standard. Enzyme molarity was calculated from the protein concentration using molecular weight values predicted from the amino acid sequences of the subunits.

Methyltransferase Assay. RNA (guanine-7)-methyltransferase was assayed by conversion of ^{32}P cap-labeled poly(A) to methylated capped poly(A) in the presence of unlabeled AdoMet (Shuman, 1989). Assay mixtures (10 μL) contained 50 mM Tris-HCl, pH 7.5, 5 mM dithiothreitol, 50 mM AdoMet, ^{32}P cap-labeled acceptor RNA as specified, and enzyme. After incubation at 37 °C for 5 min, the reaction mixtures were made 50 mM in sodium acetate, pH 5.5, and then digested with 5 μg of nuclease P1 for 30−60 min at 37 °C. The digests were then spotted on polyethyleneimine−cellulose TLC plates that were developed with 0.35 M $(NH_4)_2SO_4$. Labeled products were detected by autoradiography. The extent of methylation of the cap [expressed as m7GpppA/(m7GpppA + GpppA)] was determined either by cutting out the labeled dinucleotides and counting in liquid scintillation fluid or by scanning the chromatogram with a Fujix BAS1000 Bio-imaging Analyzer.

UV Cross-Linking of the Methyltransferase to Cap-Labeled RNA. Cap-labeled poly(A) and methylated cap-labeled poly(A) were prepared by transfer of [^{32}P]GMP from [α-^{32}P]-GTP (3000 Ci/mmol) to triphosphate-terminated poly(A) using purified vaccinia virus capping enzyme in the absence or presence of unlabeled AdoMet. The cap-labeled RNA products—G**p**ppA(pA)n in reactions lacking AdoMet and m7G**p**ppA(pA)n in reactions containing AdoMet—were isolated free of protein and unincorporated GTP by multiple rounds of trichloroacetic acid precipitation, followed by phenol extraction and ethanol precipitation. The efficiency of in vitro capping was calculated on the basis of the extent of [^{32}P]GMP incorporation relative to the input level of 5′-triphosphate poly(A) ends. In the preparation of GpppA-(pA)n, 5% of the available 5′ ends were cap-labeled; in the m7GpppA(pA)n preparation, 19% of the 5′ ends were capped and methylated. RNA cross-linking reaction mixtures (30 μL) contained 50 mM Tris-HCl, pH 7.5, 5 mM dithiothreitol, cap-labeled RNA as specified, AdoMet or AdoHcy as specified, and purified methyltransferase. After incubation on ice for 15 min, the mixtures were transferred to individual wells of a 96-well microtiter dish and irradiated for 15 min at 22 °C using a 254 nm transilluminator (Fotodyne 3-3000) situated 5 cm above the sample. After UV irradiation, the mixtures were made 50 mM in sodium acetate, pH 5.5, and digested with 15 μg of nuclease P1 for 30−60 min at 37 °C. The samples were denatured by addition of SDS−PAGE sample buffer, heated for 5 min at 95 °C, and then

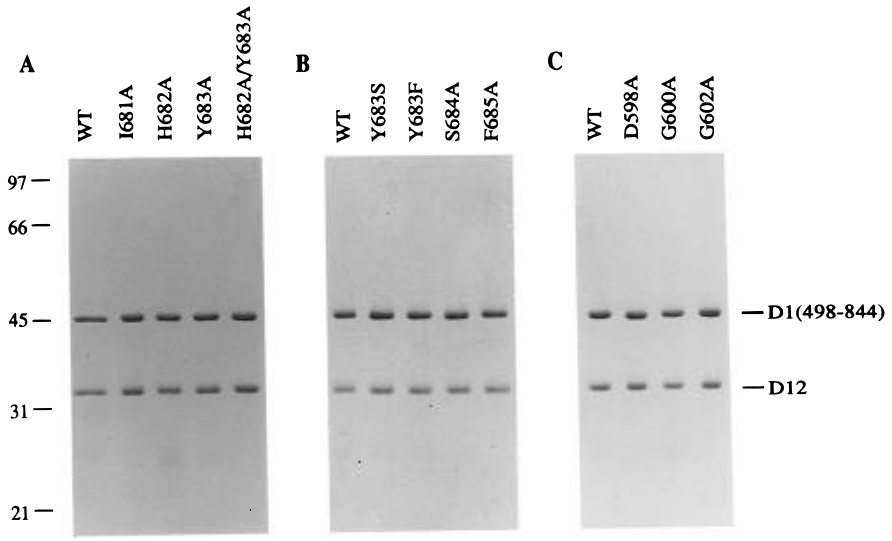

FIGURE 1: Purification of wild-type and mutant methyltransferase domains expressed in bacteria. The polypeptide composition of the peak phosphocellulose fractions of the wild-type and mutant D1(498−844)/D12 proteins was assessed by SDS−PAGE. Aliquots of the indicated preparations (2 μg of protein) were electrophoresed through a 10% polyacrylamide gel. Polypeptides were visualized by staining with Coomassie blue. The positions and sizes (kDa) of coelectrophoresed marker polypeptides are indicated at the left. The positions of the D1(498−844) and D12 methyltransferase subunits are indicated at the right.

electrophoresed through a 10% polyacrylamide gel containing 0.1% SDS. The gels were dried and autoradiographed. Photoadduct formation was quantitated by scanning the gel with a Fujix BAS1000 Bio-imaging Analyzer.

RESULTS

Mutational Analysis of a Motif Conserved among Cap Methyltransferases. Mutations were targeted to a five amino acid segment of D1(498−844) from residues 681 to 685. The sequence of this region—IHYSF—is conserved in the viral capping enzymes encoded by Shope fibroma virus and African swine fever virus and in the RNA (guanine-7-)-methyltransferase of the budding yeast *Saccharomyces cerevisiae* [for complete protein sequences, see Niles et al. (1986), Upton et al. (1991), Pena et al. (1993), and Mao et al. (1995)]. We had shown previously that a double alanine substitution at the neighboring residues His-682 and Tyr-683 abrogated cap methyltransferase activity, but did not affect the interaction of the mutant D1 protein with the D12 subunit (Mao & Shuman, 1994; Luo et al., 1995). In the present study, we introduced single alanine substitutions at each of the five positions: Ile-681, His-682, Tyr-683, Ser-684, and Phe-685.

His-tagged versions of the wild-type and Ala-substituted D1(498−844) proteins were expressed in bacteria together with the D12 capping enzyme subunit. The polypeptides normally form a stable heterodimer when coexpressed (Higman et al., 1994; Mao et al., 1994). Purification of the wild-type and Ala-substituted proteins was achieved by Ni-affinity chromatography, which selects for the His-tagged D1(498−844) subunit, followed by phosphocellulose chromatography. SDS−PAGE analysis revealed that the phosphocellulose preparations were essentially homogeneous with respect to the D1(498−844) and D12 polypeptides and that the two capping enzyme subunits were present in near-equimolar amounts in every case (Figure 1A,B). Thus, none of the mutations in the IHYSF motif affected subunit heterodimerization.

Methyltransferase activity was assayed by the conversion of ^{32}P cap-labeled poly(A) to methylated cap-labeled poly-

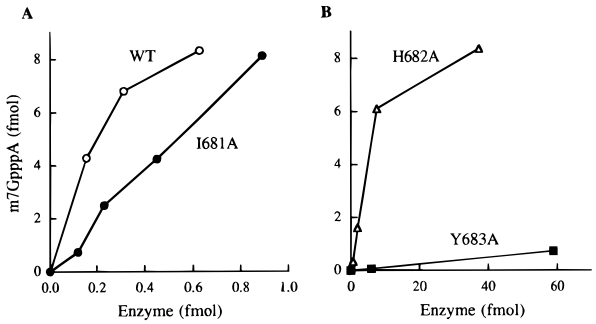

FIGURE 2: Effects of the I681A, H682A, and Y683A mutations on cap methyltransferase activity. Methyltransferase reaction mixtures contained 8.5 fmol of cap-labeled poly(A) and the indicated amounts (fmol) of the phosphocellulose preparation of the wild-type, I681A, H682A, or Y683A proteins. The titrations were performed simultaneously; the results are plotted in two graphs for clarity. Note that the *x*-axis scale is different in panels A and B.

(A) in the presence of unlabeled AdoMet. Reaction products were digested to cap dinucleotides with nuclease P1 and then analyzed by PEI−cellulose thin-layer chromatography, which resolves the GpppA cap from the methylated cap, m7GpppA. Cap methylation by the wild-type methyltransferase domain was proportional to the amount of input protein, and quantitative methylation of the input substrate [8.5 fmol of cap-labeled poly(A)] was readily achieved (Figure 2A). The yield of methylated cap was in excess of the molar amount of protein added. Assuming all enzyme molecules were active, we estimated that the protein catalyzed ~4 mol of cap methylation (mol of enzyme)$^{-1}$ min^{-1}. The purified H682A/Y683A double mutant was inert in cap methylation, even when added in vast enzyme excess (data not shown). Significant catalytic defects were encountered with the H682A and Y683A single mutants (Figure 2B). The specific methyltransferase activities of the H682A and Y683A proteins were 4% and 0.05% of the wild type, respectively. In contrast, alanine substitutions at the flanking residues were well tolerated. The specific activity of the I681A mutant was ~50% of that of wild-type methyltransferase (Figure 2A). The specific activities of the S684A and F685A proteins were 60−80% of the wild-type enzyme (Figure 3A).

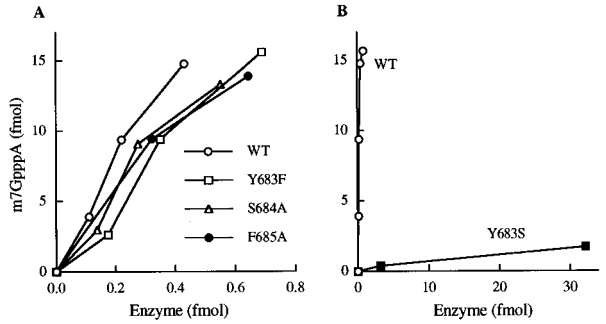

FIGURE 3: Effects of the Y683S, Y683F, S684A, and F685A mutations on cap methyltransferase activity. Methyltransferase reaction mixtures contained 16 fmol of cap-labeled poly(A) and the indicated amounts (fmol) of the phosphocellulose preparation of the wild-type, Y683F, S684A, F685A, or Y683S proteins. The titration results are plotted in two graphs for clarity; note that the *x*-axis scale is different in panels A and B.

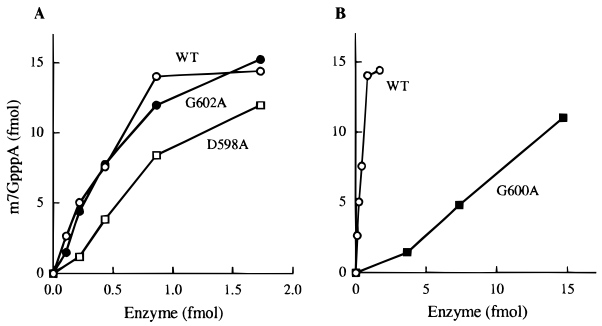

FIGURE 4: Effects of the D598A, G600A, and G602A mutations on cap methyltransferase activity. Methyltransferase reaction mixtures contained 16 fmol of cap-labeled poly(A) and the indicated amounts (fmol) of the phosphocellulose preparations of the wild-type, D598A, G602A, or G600A proteins. The titration results are plotted in two graphs for clarity; note that the *x*-axis scale is different in Panels A and B.

Alanine substitution at Tyr-683 elicited the most severe effect on enzyme activity. To better understand the essential role of this residue, Tyr-683 was substituted with serine and phenylalanine. The Y683S and Y683F mutant D1(498−844) polypeptides were coexpressed with the D12 subunit and purified to homogeneity as heterodimers (Figure 1B). Whereas replacement of Tyr-683 by Ser reduced the specific methyltransferase activity to 0.1% of wild type (Figure 3B), the specific activity of the Y683F mutant was ~60% of that of the wild-type enzyme (Figure 3A). We conclude that the aromatic ring of Tyr-683 is critical for methyltransferase activity and that the hydroxyl moiety is dispensable.

Mutational Analysis of a Motif Conserved among AdoMet-Dependent Methyltransferases. The D1 subunit of the cap methyltransferase domain contains a motif, (594)-VLAID-FGNG-(602), that is conserved in the Shope fibroma virus and African swine fever virus capping enzyme subunits and in the yeast RNA (guanine-7)-methyltransferase encoded by the *ABD1* gene (Mao et al., 1995, 1996). This motif, which is referred to as motif I, is found in a multitude of other AdoMet-requiring enzymes that catalyze methyl group transfer to diverse acceptor molecules and is likely to be a component of the AdoMet binding site (Ingrosso et al., 1989; Cheng et al., 1993; Kagan & Clarke, 1994). We reported previously that a triple alanine cluster mutation of residues Asp-598, Gly-600, and Gly-602 of D1(498−844) resulted in defective subunit association and loss of methyltransferase activity (Mao & Shuman, 1994). To better assess the function of this motif, we have now introduced single alanine mutations at these three positions. The wild-type and mutant D1(498−844) proteins were coexpressed with D12 and purified from bacterial lysates. SDS−PAGE analysis showed that none of the single alanine mutations affected subunit heterodimerization (Figure 1C). The specific activity of the G602A mutant was identical to the wild-type enzyme, and the D598A mutant was half as active as wild type (Figure 4A). In contrast, the specific activity of the G600A protein was 4% that of the wild-type methyltransferase (Figure 4B). Thus, of the three lesions in the original alanine cluster mutant, only Gly-600 was important for catalysis.

UV Cross-Linking of Cap-Labeled RNA to the Methyltransferase Domain. We employed a UV cross-linking assay to assess the interaction of the methyltransferase domain with the 5′ terminus of the RNA substrate. Our aim was to first determine the substrate binding properties of the wild-type enzyme and then to apply the method to analyze the mutant

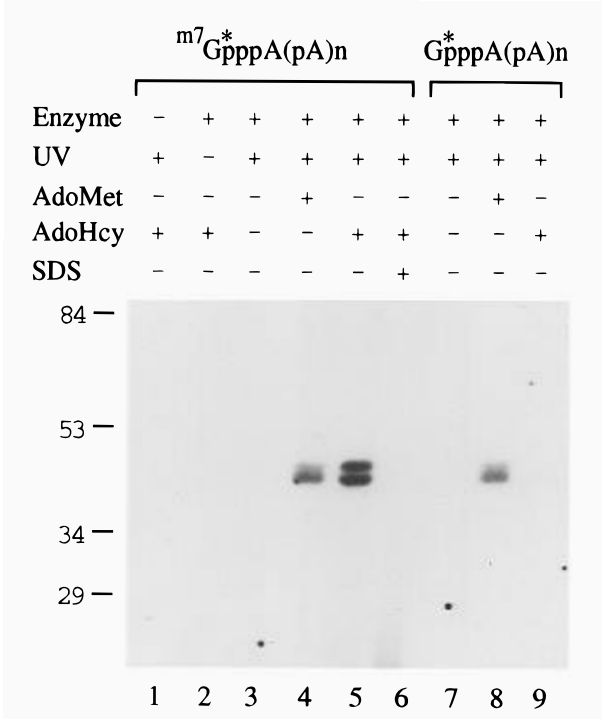

FIGURE 5: UV-induced cross-linking of cap-labeled RNA to the methyltransferase domain. Reaction mixtures (30 μL) contained 35 fmol of ^{32}P-labeled m7G**p**pppA(pA)n (lanes 1−6) or 35 fmol of ^{32}P-labeled G**p**pppA(pA)n (lanes 7−9), 700 fmol of purified D1(498−844)/D12 (phosphocellulose fraction), and either 50 μM AdoMet or 50 μM AdoHcy as indicated. After 15 min incubation on ice, the samples were UV-irradiated, then digested with nuclease P1, and denatured with SDS. The reaction mixture in lane 6 was SDS-denatured prior to UV irradiation. The ^{32}P-labeled photoadducts were analyzed by SDS−PAGE. An autoradiogram of the gel is shown. The positions and sizes (kDa) of coelectrophoresed marker polypeptides are indicated on the left.

enzymes described above. The purified wild-type protein was incubated with cap-labeled poly(A) [G**p**pppA(pA)n] on ice for 15 min, and the mixtures were irradiated using a short-wave UV transilluminator. The samples were digested with nuclease P1 [to degrade the unlabeled poly(A) portion of the RNA ligand] and then analyzed by SDS−PAGE. We expected that photo-cross-linking of the methyltransferase to the 5′ cap structure would be evinced by ^{32}P label transfer to one or both of the capping enzyme subunits.

We readily detected label transfer from G**p**pppA(pA)n to the cap methyltransferase domain (Figure 5). Two properties of the UV cross-linking reaction are noteworthy: (i) only the D1(498−844) subunit was labeled, and (ii) photoadduct

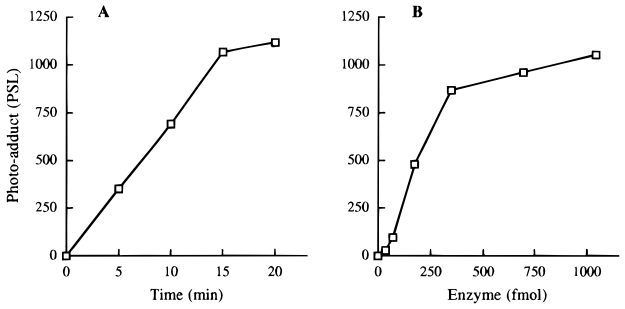

FIGURE 6: Time course and enzyme dependence of UV cross-linking of methylated capped RNA to the methyltransferase domain. (Panel A) A reaction mixture (30 μL) containing 70 fmol of ^{32}P-labeled m7G**p**pppA(pA)n, 50 μM AdoHcy, and 1.4 pmol of purified D1(498−844)/D12 was preincubated for 15 min and then irradiated with UV light. Aliquots were removed after 5, 10, 15, and 20 min of UV exposure and then digested with nuclease P1. The digests were analyzed by SDS−PAGE. (Panel B) Reaction mixtures containing 35 fmol of ^{32}P-labeled m7G**p**pppA(pA)n, 50 μM AdoHcy, and purified D1(498−844)/D12 as indicated were preincubated for 15 min and then UV-irrradiated for 15 min. The samples were digested with nuclease P1 and analyzed by SDS−PAGE. The extent of label-transfer to D1(498−844) was quantitated by scanning the gels with a Bio-imaging Analyzer. The phosphorimage signal (photostimulatable luminescence; PSL) is plotted as a function of the time of UV exposure in panel A or as a function of input methyltransferase in panel B.

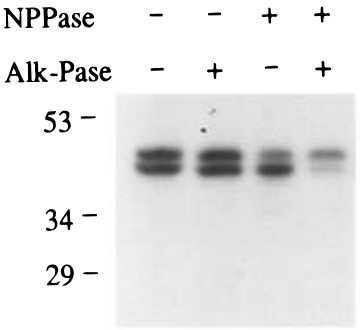

FIGURE 7: Characterization of the photoproduct. A reaction mixture (120 μL) containing 140 fmol of ^{32}P-labeled m7G**p**pppA(pA)n, 50 μM AdoHcy, and 5.5 pmol of purified D1(498−844)/D12 was preincubated for 15 min and then UV-irradiated for 15 min. The mixture was digested for 60 min at 37 °C with 60 μg of nuclease P1 and then adjusted to 5 mM $MgCl_2$, 33 mM Tris-HCl, pH 8.0. Aliquots (60 μL) were distributed to new tubes and then incubated for 5 h at 37 °C with (+) or without (−) 44 μg of nucleotide pyrophosphatase (NPPase, from *Croatalus adamanteus*; type II, Sigma). The mixtures were then supplemented with 0.1 volume of 10× dephosphorylation buffer (Boehringer). Aliquots (30 μL) were distributed into new tubes and incubated for 30 min at 37 °C with (+) or without (−) addition of 2 units of alkaline phosphatase (Alk-Pase, from calf intestine; Boehringer). The reaction products were resolved by SDS−PAGE; an autoradiogram of the gel is shown. The positions and sizes (kDa) of marker polypeptides are indicated on the left.

formation required that AdoMet, the methyl donor, be included in the binding reaction mixture (Figure 5, lane 8). Furthermore, *S*-adenosylhomocysteine (AdoHcy), a product of the cap methylation reaction, was unable to stimulate cross-linking of the methyltransferase to G**p**pppA(pA)n (Figure 5, lane 9). Because inclusion of AdoMet will result in quantitative methylation of the cap-labeled RNA, we considered the possibility that m7G**p**pppA(pA)n might be the photoreactive moiety in this experiment. If the role of AdoMet were simply to allow conversion of the RNA to the methylated form, then we would expect that m7G**p**pppA-(pA)n, the product of the methylation reaction, should cross-link to the enzyme in the absence of AdoMet. Yet, when the cross-linking reactions were performed using methylated capped RNA as the ligand, there was no apparent label transfer to the D1(498−844) subunit (Figure 5, lane 3). Photoadduct formation was stimulated by the addition of AdoMet, just as was seen for the unmethylated capped RNA ligand (Figure 5, lane 4), but the extent of cross-linking was even higher when AdoHcy was substituted for AdoMet (Figure 5, lane 5). The basis for the stimulation of cross-linking by nucleotide cofactor is considered in detail below. Note that the photoadduct formed in reactions containing m7GpppA(pA)n and AdoHcy migrated as a ^{32}P-labeled doublet (Figure 5, lane 5). Label transfer to these polypeptides in the presence of AdoHcy was completely dependent on UV exposure (Figure 5, lane 2).

Additional experiments were performed in order to optimize the conditions for cross-linking of D1(498−844)/D12 to m7G**p**pppA(pA)n in the presence of 50 μM AdoHcy. The RNA−protein complexes were exposed to UV light; aliquots were withdrawn at various times, and then digested with nuclease P1 and analyzed by SDS−PAGE. The extent of label transfer to the D1(498−844) polypeptide was quantitated by scanning the gel with a Bio-imaging Analyzer. The signal intensity of the labeled photoadduct was plotted as a function of time of UV exposure (Figure 6A). Cross-linking was linear with time of irradiation for 15 min and plateaued thereafter. About 0.1% of the input ^{32}P label was transferred from the methylated RNA cap to the D1(498−844) polypeptide; guanosine nucleotides are known to be very poorly photoreactive in protein−nucleic acid cross-linking assays (Hockensmith et al., 1986). The yield of photoproduct increased linearly with the amount of input enzyme up to 350 fmol; a more shallow increase was observed at higher protein levels (Figure 6B). The ratio of the two ^{32}P-labeled species in the photo-cross-linked doublet did not change as a function of the time of UV irradiation or as a function of the amount of methytransferase included in the reactions (data not shown).

Characterization of the Photoproduct. Photo-cross-linking of methylated capped RNA to the heterodimeric methyltransferase domain in the presence of AdoHcy yielded a ^{32}P-labeled polypeptide doublet after digestion with nuclease P1 (Figure 7). Cross-linking occurred exclusively to the D1-(498−844) polypeptide; no cross-linking could be detected to the 33 kDa D12 subunit (Figure 7). When cross-linking reactions were performed using purified recombinant full-sized capping enzyme (a heterodimer of the 97 kDa full-length His-D1 subunit and the D12 subunit), we observed that label transfer from m7GpppA(pA)n occurred exclusively to the 97 kDa D1 subunit (not shown).

The more rapidly migrating component of the ^{32}P-labeled doublet generated by cross-linking to the D1(498−844)/D12 domain was precisely superimposable on the D1(498−844) polypeptide. (This was determined by fixing the SDS gel, staining the polypeptides with Coomassie brilliant blue, and then visualizing the photoadducts by autoradiography of the dried stained gel.) This indicated that the heterogeneity of the photoproduct was not caused by UV-induced fragmentation of the polypeptide. That the upper species of the photo-cross-linked doublet migrated more slowly than the intact His-D1(498−844) polypeptide suggested that size heterogeneity was caused by a difference in the nature of the RNA component of the photoproduct. This would occur if nuclease P1 were not able to digest all photoproducts to the same extent. Under the conditions used in our assay, the

nuclease P1 digested the *free* m7GpppA(pA)n to completion to yield ^{32}P-labeled cap dinucleotide m7GpppA and unlabeled 5′AMP (not shown).

Formally, there are two ways in which the P1-resistant cap moiety may be photo-cross-linked to the D1(498−844) protein: either via the cap guanosine base or via one of the 5′ proximal adenosine bases. Because guanosine and adenosine bases in nucleic acids have similarly poor efficiencies of UV cross-linking to protein (Hockensmith et al., 1986), no assumptions can be made about the nature of the RNA moiety that is cross-linked to the protein. This issue was addressed by subjecting the nuclease P1-treated photoadducts to further digestion with nucleotide pyrophosphatase and/or alkaline phosphatase (Figure 7). Treatment with alkaline phosphatase alone had no effect on the amount or distribution of the two photoproducts that comprise the doublet (Figure 7). This was expected, because the phosphates of the cap dinucleotide are protected from hydrolysis by alkaline phosphatase. Nucleotide pyrophosphatase (NPPase) liberates ^{32}P-labeled m7GMP from the cap dinucleotide of m7G**p**pppA-(pA)n (Mao et al., 1995). If the 5′ cap end of the poly(A) is cross-linked to the protein via the cap guanosine, then the label should remain protein-associated after NPPase treatment. The lower band of the doublet was unaffected by NPPase treatment, suggesting that this species was indeed cross-linked to the cap guanosine. After treatment with NPPase, most of the ^{32}P label in the lower band became susceptible to hydrolysis by alkaline phosphatase (Figure 7). This confirmed that NPPase digestion of the protein-bound cap dinucleotide had in fact occurred. In contrast, the upper band of the doublet was diminished in intensity by NPPase treatment alone, which indicated that a fraction of the cap guanylate was not attached to protein via the guanosine base. The fraction of the upper band that remained after NPPase treatment was also resistant to further digestion with alkaline phosphatase (Figure 7), implying that this material had not been digested by NPPase in the first place, perhaps because the 5′ RNA end cross-linked to the enzyme was partially protected from the pyrophosphatase. (Heating the photoadduct at 95 °C for 5 min prior to NPPase treatment did not render the ^{32}P-labeled upper band more susceptible to NPPase, nor did increasing the NPPase digestion time to 20 h; data not shown.) These results indicate that cross-linking of cap-labeled poly(A) to the methyltransferase domain occurred via protein contacts with the cap guanosine and with the 5′ proximal adenosine base (or bases). We cannot say with certainty whether the protein is cross-linked in the upper complex to the 5′ adenosine of the cap dinucleotide or to one of the flanking adenosines. A plausible hypothesis is that cross-linking to the adenosine of the cap dinucleotide might impede complete digestion of the bound RNA by nuclease P1, and that this could explain why the adduct cross-linked to adenosine migrated more slowly during SDS−PAGE.

Cross-Linking Is Cap-Specific. The site of cross-linking to the 5′ cap resides on the D1(498−844) subunit that contains the active site of the methyltransferase. To substantiate that cross-linking is actually occurring at the RNA acceptor site on the methyltransferase, we tested the effects of cap dinucleotides on the cross-linking of m7GMP-labeled poly(A) to the D1(498−844) polypeptide in the presence of AdoHcy (Figure 8A). GpppA, which is an effective substrate for the cap methyltransferase (Martin & Moss, 1976),

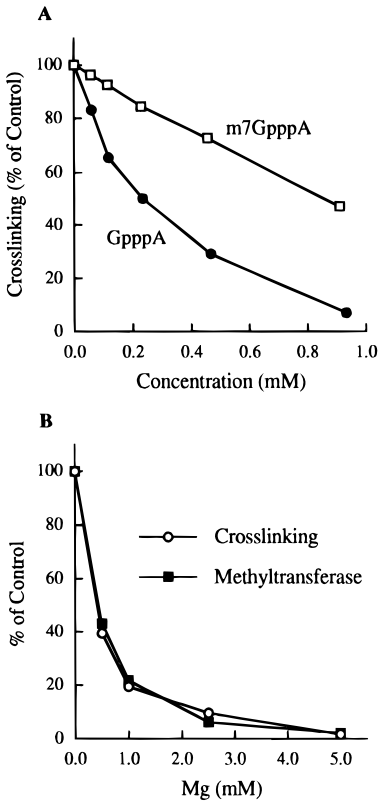

FIGURE 8: Cap-specificity of UV cross-linking. (Panel A) Competition by cap analogs. Reaction mixtures contained 35 fmol of ^{32}P-labeled m7G**p**pppA(pA)n, 50 μM AdoHcy, 700 fmol of D1(498−844)/D12, and cap dinucleotides GpppA or m7GpppA as indicated. The samples were UV-irradiated for 15 min, digested with nuclease P1, and analyzed by SDS−PAGE. The extent of label-transfer to D1(498−844) was quantitated by scanning the gels with a Bioimaging Analyzer. The signal intensities of the doublet photoproduct were normalized to that of a control reaction lacking cap dinucleotide and then plotted as a function of cap analog concentration. There was no selective effect of cap dinucleotides on one or the other components of the doublet (not shown). (Panel B) Effect of magnesium on cap methylation and cap cross-linking. UV cross-linking reaction mixtures contained 35 fmol of ^{32}P-labeled m7G**p**pppA-(pA)n, 50 μM AdoHcy, 700 fmol of D1(498−844)/D12, and $MgCl_2$ as indicated. Cap methyltransferase reaction mixtures contained 46 fmol of ^{32}P-labeled G**p**pppA(pA)n, 2.3 fmol of D1(498−844)/D12, and $MgCl_2$ as indicated. The extents of photoadduct formation and cap methylation were normalized to those of control reactions lacking $MgCl_2$ and then plotted as a function of magnesium concentration (18 fmol of capped ends was methylated in the control methyltransferase assay).

inhibited the cross-linking reaction. Inhibition was proportional to dinucleotide concentration; 50% inhibition was observed at 200 μM GpppA, a value that agrees with the affinity of the methyltransferase for GpppG (K_m = 120 μM) as determined by Martin and Moss (1976). m7GpppA, which is an analog of the methylation reaction product, was a weaker inhibitor of cross-linking than was GpppA; 50% inhibition was observed at about 1 mM m7GpppA (Figure 8A). These competition results suggest that the 5′ end of the cap-labeled RNA ligand is being cross-linked at the methyl acceptor site on the enzyme.

Further evidence that the UV cross-linking assay reflects a binding phenomenon relevant to the cap methylation reaction was provided by the virtually identical concentration-dependent inhibition of the RNA cap methylation and RNA cross-linking reactions by magnesium (Figure 8B). Our results agree with previous reports of magnesium inhibition of methyl group transfer to free GTP and of UV cross-linking

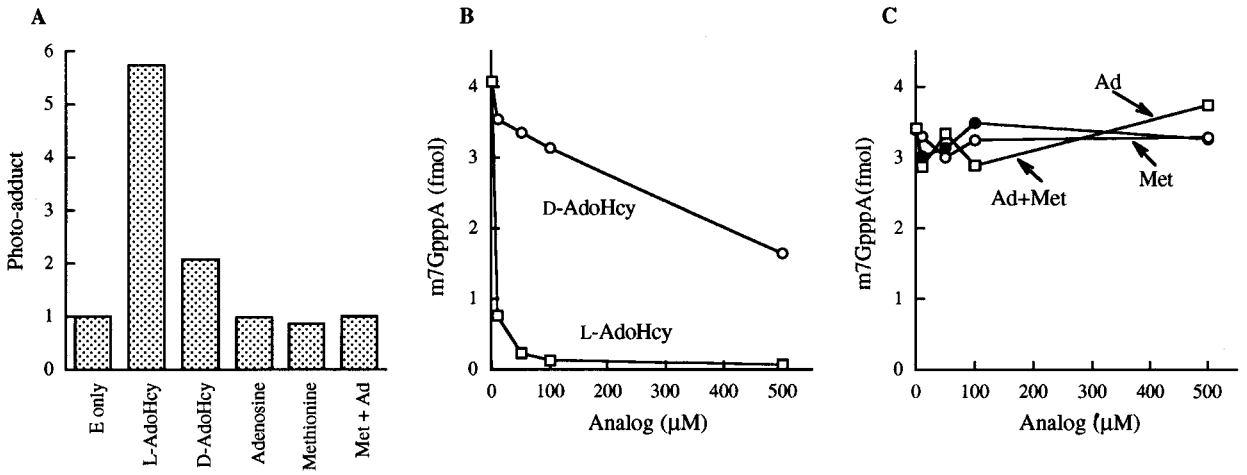

FIGURE 9: Stimulation of cap cross-linking by AdoHcy. (Panel A) Cross-linking reaction mixtures contained 35 fmol of ^{32}P-labeled m7G**p**pppA-(pA)n, 700 fmol of D1(498−844)/D12, and AdoHcy or related compounds as indicated at 1 μM concentration. The phosphorimage signal intensity of the D1(498−844) protoproduct in reactions containing methyltransferase plus added cofactor was normalized to that of the control reaction containing only enzyme (E only; signal = 1.0). (Panel B) Methyltransferase reaction mixtures contained 10 fmol of ^{32}P-labeled G**p**pppA(pA)n, 0.9 fmol of D1(498−844)/D12, 10 μM L-AdoMet, and L-AdoHcy or D-AdoHcy as indicated. (Panel C) Methyltransferase reaction mixtures contained 6.5 fmol of ^{32}P-labeled G**p**pppA(pA)n, 0.9 fmol of D1(498−844)/D12, 10 μM L-AdoMet, and the indicated concentrations of adenosine and L-methionine, added separately or together.

of the methyltransferase to free GTP (Higman et al., 1994; Niles et al., 1994).

Stimulation of UV Cross-Linking to the 5′ Cap by Occupancy of the Methyl Donor Site. It was noted above that photolinkage of unmethylated capped RNA to D1(498−844) was stimulated by AdoMet, but was unaffected by AdoHcy. In contrast, cross-linking to methylated capped RNA was stimulated by either AdoHcy or AdoMet (Figure 5). The results are suggestive of an allosteric effect of occupancy of the methyl donor site on either the affinity of the enzyme for the RNA ligand or the proximity of the cap to a moiety on the enzyme to which it can be cross-linked.

A testable prediction is that allosteric stimulation of UV cross-linking should correlate with specific binding of the activator compound to the methyl donor site. Accordingly, we examined the structural features of AdoHcy that were responsible for the stimulation of cross-linking to m7GpppA-(pA)n. We first compared the effector strength of the D- and L-stereoisomers of AdoHcy. The relative affinity of AdoHcy stereoisomers for the enzyme was assayed by their ability to competitively inhibit RNA methylation (Figure 9B). In reactions containing 10 μM L-AdoMet, methyltransferase was inhibited 80% by 10 μM L-AdoHcy and 95% by 50 mM L-AdoHcy. In contrast, D-AdoHcy was an extremely weak inhibitor of cap methylation; 50% inhibition required ~400 mM D-AdoHcy (Figure 9B). In agreement with the stereospecificity of cofactor binding, we found that UV cross-linking was stimulated 6-fold by as little as 1 μM L-AdoHcy, whereas the D-stereoisomer had only a 2-fold effect (Figure 9A). Neither adenosine nor L-methionine had any inhibitory effect on cap methylation at concentrations up to 0.5 mM (Figure 9C). The combination of adenosine and methionine was also not inhibitory (Figure 9C), implying that neither the nucleoside nor the amino acid was bound to the methyltransferase. Adenosine and methionine, added separated or together, did not enhance the cross-linking of the methyltransferase to capped RNA (Figure 9A).

UV Cross-Linking of Methylated Capped RNA to Mutant Methyltransferases. The mutated versions of the methyltransferase domain were tested in parallel with the wild-type domain for their ability to cross-link to cap-labeled m7G**p**pppA-(pA). Cross-linking of the wild-type D1(498−844) subunit to the RNA cap was dependent on AdoHcy. In reactions lacking the nucleotide cofactor, only trace amounts of photoproduct were detected, and this cross-linked species corresponded to the lower band of the doublet seen in the presence of AdoHcy (Figures 10 and 11). Addition of AdoHcy enhanced cross-linking efficiency ~10-fold in this experiment; note that the yield of the doublet photoproduct did not differ appreciably at 5 μM versus 50 μM AdoHcy (Figures 10 and 11). Three of the mutant methyltransferases that displayed normal or near-normal catalytic activity—the I681A, S684A, and F685A proteins—displayed wild-type levels of AdoHcy-stimulated cross-linking to m7GpppA-(pA)n (Figure 11). In contrast, the H682A-Y683A double mutant, which was inactive in cap methylation, and the Y683A and Y683S single mutants, which displayed <1% of the specific methyltransferase activity of the wild-type enzyme, were all virtually inert with respect to photo-cross-linking to capped RNA (Figure 10). The H682A mutant, which had ~4% the specific activity of wild-type methyltransferase, yielded only trace amounts of the UV photoproduct in the presence or absence of AdoHcy (Figure 11). Thus, inability to UV cross-link to the RNA cap structure correlated with loss of catalytic function in cap methylation.

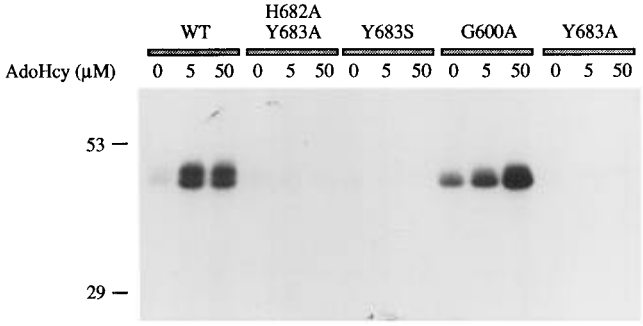

FIGURE 10: Mutational effects on UV cross-linking of the methyltransferase domain to the RNA cap. cross-linking reaction mixtures (30 μL) contained 70 fmol of ^{32}P-labeled m7G**p**pppA(pA)n, 1.4 pmol of the wild-type, H682A/Y683A, Y683S, G600A, or Y683A D1(498-844)/D12 proteins, and either 0, 5, or 50 μM AdoHcy. Irradiation was for 15 min; the nuclease P1 digests were analyzed by SDS−PAGE. An autoradiogram of the gel is shown.

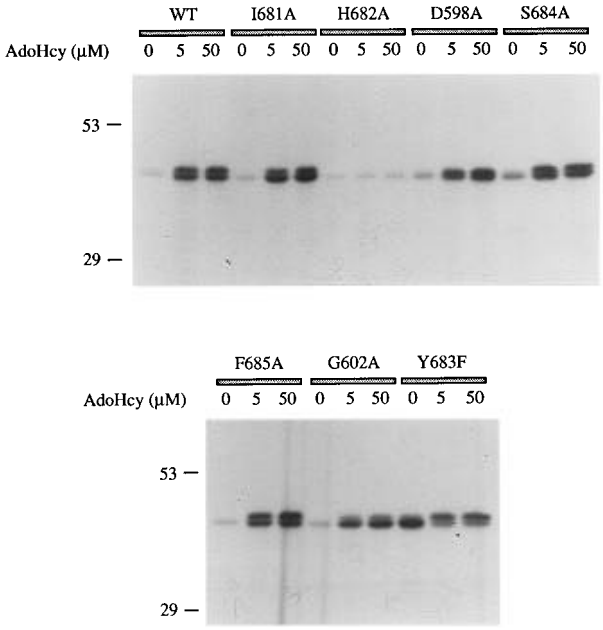

FIGURE 11: Mutational effects on UV cross-linking to the cap structure. cross-linking reaction mixtures (30 μL) contained 70 fmol of ^{32}P-labeled m7G**p**ppA(pA)n, 1.4 pmol of the wild-type or mutant D1(498−844)/D12 proteins as indicated, and either 0, 5, or 50 μM AdoHcy. Irradiation was for 15 min; the nuclease P1 digests were analyzed by SDS−PAGE. An autoradiogram of the gel is shown.

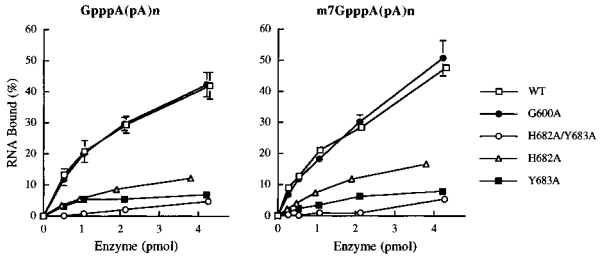

FIGURE 12: Nitrocellulose filter assay for mutational effects on RNA binding. Reaction mixtures (10 μL) containing 50 mM Tris-HCl, pH 7.5, ^{32}P-labeled poly(A) as indicated, and purified D1-(498−844)/D12 proteins were incubated on ice for 10 min. Aliquots (8 μL) of each sample were spotted onto nitrocellulose filters (25 mm diameter, 0.2 μm pore size) that had been wetted with 50 mM Tris-HCl, pH 7.5. The filters were washed under vacuum with 2 mL of 50 mM Tris-HCl, pH 7.5, and the retained radioactivity was determined by liquid scintillation counting. (Left panel) Reaction mixtures contained 16 fmol of unmethylated [^{32}P]GMP-labeled poly(A). The percent of the total labeled RNA retained on the filter was plotted as a function of input protein. The values shown represent the average of three separate titration experiments; standard error bars are shown. Background RNA binding in the absence of added protein (5−8% of input radioactivity) was subtracted from all data points. (Right panel) Reaction mixtures contained 19 fmol of methylated [^{32}P]GMP-labeled poly(A). The percent of the total labeled RNA retained on the filter was plotted as a function of input protein. The values shown represent the average of three separate titration experiments; standard error bars are shown. Background RNA binding in the absence of added protein (2% of input radioactivity) was subtracted from all data points.

The remaining mutant methyltransferases displayed more subtle variations from the wild-type pattern of photoadduct formation. For example, cross-linking of the catalytically active D598A and G602A proteins was stimulated by AdoHcy, yet the photoproduct formed was either exclusively (for D598A) or predominantly (for G602A) the "bottom band" of the ^{32}P-labeled polypeptide doublet (Figure 11). The catalytically defective G600A mutant also yielded a preponderance of the bottom band photoproduct (Figure 10). This effect on the distribution of the photoproducts appeared to be characteristic of mutations within motif I, independent of whether the mutations affected methyltransferase activity. The G600A protein displayed other variant properties in the UV cross-linking reaction. First, the amount of AdoHcy-independent cross-linking was somewhat higher than that observed with the wild-type methyltransferase. Second, the modest stimulation of cross-linking by 5 μM AdoHcy was increased substantially at 50 μM AdoHcy (Figure 10). The increase in cross-linking with increasing cofactor concentration, which was not observed with the wild-type methyltransferase and most of the other mutant proteins, suggests that G600A has reduced affinity for AdoHcy. This would be consistent with the proposal that motif I, which is conserved in most AdoMet-dependent methyltransferases, is involved directly in AdoMet/AdoHcy binding. Yet another variation in cross-linking properties was seen with catalytically active Y683F protein. This mutant displayed relatively high levels of photoadduct formation in the absence of AdoHcy—predominantly the bottom band of the doublet. Addition of AdoHcy elicited little or no further increase in the extent of cross-linking, but caused a marked shift of the photoproduct to the top band (Figure 11). Explanations for this cross-linking behavior are considered under Discussion.

Nitrocellulose Filter Assay of RNA Binding by Wild-Type and Mutant Methyltransferases. UV cross-linking is an inherently indirect method to assay the effects of mutations on the binding of RNA (or any other ligand) to protein. Cross-linking of the D1(498−844) subunit to cap-labeled RNA requires not only that the RNA be bound to the methyltransferase but also that the labeled cap moiety be situated close to a reactive substituent of the protein. Thus, mutations that reduce photoadduct formation may do so by reducing RNA binding or via altering the reactive protein substituent without actually interfering with the binding event. It is easier to interpret mutational effects on RNA cross-linking when an independent assay for RNA binding is available. Hence, we employed a nitrocellulose filter retention assay to examine the binding of the D1(498−844)/D12 methyltransferase domain to the cap-labeled poly(A) ligands that were used in the cross-linking studies.

Retention of GpppA(pA)n or m7GpppA(pA)n on the nitrocellulose filter was proportional to the amount of methyltransferase included in the binding reaction (Figure 12). From a double-reciprocal plot of the data, we estimated a dissociation constant of 220 nM for the binding of the D1-(498−844)/D12 heterodimer to poly(A). This value agrees quite well with the 210 nM K_m for GpppA(pA)n as a substrate in the cap methylation reaction catalyzed by the native D1/D12 capping enzyme (Martin & Moss, 1976) and is only slightly higher than the dissociation constant of 100 nM determined for the binding of the D1(498−844)/D12 heterodimer to a nonhomopolymeric RNA transcript synthesized by T7 RNA polymerase (Higman et al., 1994).

It is worth emphasizing that the filter binding assay reflects protein−RNA interactions with the polynucleotide chain that are not specific to the 5′ cap structure of the poly(A) ligand. For example, unlike the UV cross-linking reaction, which is cap-specific and inhibitable by cap analogs, the retention of cap-labeled poly(A) on nitrocellulose by D1(498−844)/D12 was unaffected by the addition of 1 mM m7GpppA or GpppA (data not shown). This was consistent with earlier studies showing (i) that the D1/D12 capping enzyme bound

with equal affinity to RNAs containing 5′-triphosphate, 5′-capped, or 5′-hydroxyl termini and (ii) that this binding was not competed by m7GpppG (Luo & Shuman, 1993). We note also that the retention of m7GpppA(pA)n on nitrocellulose by D1(498−844)/D12 was unaffected by 50 μM AdoHcy (not shown).

Mutational effects on RNA binding were tested for those mutant proteins that had a catalytic defect in cap methylation and/or UV cross-linking. The H682A/Y683A double mutant bound with much lower affinity to poly(A) than did the wild-type enzyme (Figure 12). Indeed, binding of H682A-Y683A to m7GpppA(pA)n in the nitrocellulose filter assay was virtually nil at the levels of input protein (1.4 pmol) used to test UV cross-linking to the RNA cap structure. This result indicates that the failure to cross-link may be attributed to a general defect in RNA binding caused by H682A-Y683A mutation. The Y683A single mutation also reduced RNA binding, albeit not quite as severely as the double mutation; Y683A bound m7GpppA(pA)n about one-sixth as well as the wild-type protein (Figure 12B). The H682A mutant bound to m7GpppA(pA)n ∼40% as well as the wild-type protein (Figure 12B). These results implicate the conserved HY dipeptide as a component of the RNA binding site on the methyltransferase. With respect to the H682A mutation, the defects in catalysis and UV cross-linking were more severe than the observed effect on general RNA binding, which suggests an additional role of this residue in cap recognition or the recognition of AdoMet/AdoHcy. The binding of the G600A mutant protein to poly(A) was indistinguishable from that of the wild-type enzyme (Figure 12). Apparently, the G600A mutation exerts its 20-fold effect on catalysis at a reaction step other than general RNA binding.

DISCUSSION

The RNA (guanine-7)-methyltransferase domain of vaccinia virus mRNA capping enzyme is composed of the carboxyl portion of the D1 subunit heterodimerized with the D12 subunit (Cong & Shuman, 1992; Mao & Shuman, 1994; Higman et al., 1994). The D1 component, comprising residues 498−844, has a low intrinsic methyltransferase activity, which is stimulated by heterodimerization with D12. Because the catalytic center of the methyltransferase domain is situated within D1(498−844), we have focused on this protein for structure−function analysis by mutagenesis. The effects of alanine substitution mutations at eight residues of D1(498−844) were analyzed in the context of the heterodimeric methyltransferase domain. The fact that all of the mutated D1(498−844) proteins analyzed in this study were able to heterodimerize when coexpressed with the D12 subunit provided some reassurance that the mutations did not globally alter the D1(498−844) protein structure. We identified three amino acid residues involved in cap methylation. Alanine substitutions of the vicinal positions His-682 and Tyr-683 reduced methyltransferase activity to 4% and 0.05% of the activity of the wild-type enzyme; alanine substitution at Gly-600 reduced activity to 4% of the wild-type protein. In contrast, alanine substitutions at five other positions—Asp-598A, Gly-602, Ile-681, Ser-684, and Phe-685—were well tolerated.

Catalytically important residues Gly-600, His-682, and Tyr-683 of the vaccinia D1 protein are strictly conserved in the D1 homologues encoded by Shope fibroma virus and African swine fever virus and in the RNA (guanine-7)-methyltransferase of *Saccharomyces cerevisiae*. The yeast cap methyltransferase is a 436 amino acid polypeptide encoded by the essential *ABD1* gene (Mao et al., 1995). Unlike the vaccinia cap methyltransferase, which is a heterodimer of D1 and D12 components, the yeast ABD1 methyltransferase is apparently a monomer, as judged by the native size of the cap methyltransferase isolated from yeast whole cell extracts (Mao et al., 1995). The effects of mutations of Gly-600 and Tyr-683 in vaccinia D1(498−844) on methyltransferase activity in vitro are consistent with the in vivo lethality of Ala-substitutions at the homologous positions in ABD1 (Mao et al., 1996).

An interesting feature of the cap methylation reaction has emerged from studies of UV cross-linking of the D1(498−844)/D12 heterodimer to the cap moiety of the RNA substrate, namely, that photo-cross-linking of the cap at the active site on the D1(498−844) subunit was strongly stimulated by concurrent occupancy of the nucleotide cofactor site. In order to rationalize these findings, we envision a model of the active site in which occupancy of the cofactor site elicits a conformational change that either (i) enhances the affinity for the cap guanosine at the cap binding site without neccessarily altering the inherent photoreactivity of the protein interface or (ii) alters the protein interface so that a reactive moiety is brought closer to the cap structure *already bound* at the cap site so that it is better poised to form a covalent bond upon excitation of the cap dinucleotide with UV light. (Note that our data do not discriminate between these models, because alternative assays for RNA binding by the methyltransferase that do not rely on photo-cross-linking do not actually measure ligand binding at the cap-site on the protein.)

The cofactor specificity in the cross-linking reaction was acutely dependent on whether the cap-labeled RNA ligand was methylated at the N7 position. Cross-linking to GpppA-(pA)n was stimulated exclusively by AdoMet. A model of the active site in this situation is illustrated in Figure 13A. The methyl donor (AdoMet) and the guanosine methyl acceptor are placed as we envision them at the onset of the chemical step. The methyl group on the positively charged sulfonium compound is oriented toward the reactive nucleophile—the N7 atom of guanosine. The situation at the active site after reaction chemistry has occurred is modeled in Figure 13B. Here, the methyl group has been transferred to the N7 of guanosine, and the base has acquired a positive charge. AdoHcy, which is uncharged, occupies the cofactor site (as drawn in the model, the position of the methyl group on the enzyme has not changed between state A and state B). In this configuration, which applies when m7GpppA(pA)n is incubated with enzyme in the presence of AdoHcy, the cap is poised to cross-link to the enzyme upon UV irradiation. Note that we have attempted to detect reversal of the chemical step, e.g., by prolonged incubation of m7G**p**ppA(pA)n with excess capping enzyme in the presence of high concentrations of AdoHcy, followed by digestion of the RNA with nuclease P1 and analysis of the radiolabeled product by PEI−cellulose thin-layer chromatography. There was no detectable AdoHcy- or enzyme-dependent conversion of m7G**p**ppA to G**p**ppA in this reaction. We conclude that cap methylation is irreversible and that state B in Figure 13 accurately reflects the species at the active site in our standard cross-linking mixtures containing m7G**p**ppA(pA)n and AdoHcy. The state of the

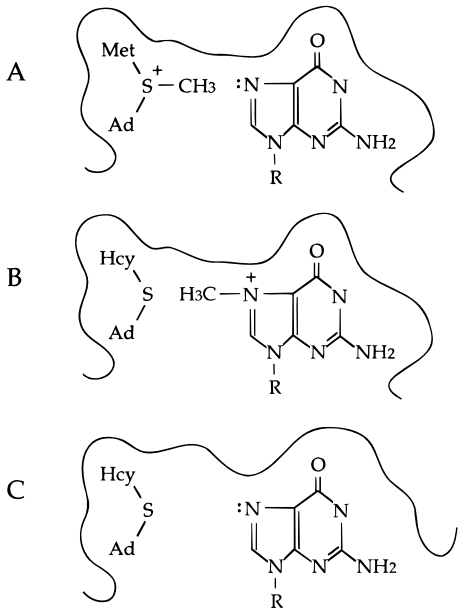

FIGURE 13: Model for the binding of the cap and the nucelotide cofactor at the active site of vaccinia cap methyltransferase. The model in (A) depicts the methyl donor AdoMet [shown as the sulfonium ion with adenosine (Ad) and methionine (Met) substituents] and the methyl acceptor (N7 of the cap guanosine) in the context of a hypothetical protein interface at the active site on the methyltransferase. The structures of the reaction products, AdoHcy and N7-methylguanosine, are depicted at the active site in (B). Although the reaction mechanism is not known, we presume for the purpose of the model that it occurs by direct attack of N7 on the methyl group. Shown in (C) is a model of the site when AdoHcy and unmethylated cap guanosine are bound. In this state, the cap moiety is poorly reactive in UV cross-linking to the protein; hence, the contour of the protein interface is depicted differently in state C than in states A and B.

reactive ligands in cross-linking reactions containing G**p**ppA-(pA)n and AdoMet cannot be assigned because the cap becomes methylated during the incubation.

An instructive finding was that D1(498−844) was not stimulated to cross-link to unmethylated cap-labeled RNA in the presence of AdoHcy. The state of the active site in this case is modeled in Figure 13C. Photo-unreactive state C differs from photo-reactive state B in two respects: (i) state C has an unoccupied "methyl site" on the enzyme; and (ii) state C lacks a positive charge at the active site. We propose that one or both of these parameters—occupancy of a putative methyl site or the presence of a positive charge at either the cofactor or the cap site—triggers the conformational change proposed above that leads to enhancement of cross-linking to the cap.

One might imagine that steric hindrance between the methyl group on the 5′ cap guanosine and the methyl group on AdoMet might disfavor simultaneous occupancy of the active site by these molecules. Yet, we observed experimentally that cross-linking to m7GpppA(pA)n was stimulated by AdoMet. Either the enzyme is capable of binding simultaneosuly to AdoMet and m7GpppA(pA)n or else the AdoMet preparation is contaminated with AdoHcy or another compound capable of allosteric activation. AdoMet is unstable at pH 7.5, decomposing primarily to methylthioadenosine (Parks & Schlenk, 1958). Methylthioadenosine could, in principal, be accommodated at the active site without steric hindrance by m7GpppA(pA)n.

Allosteric effects of AdoMet or AdoHcy on enzyme binding to the methyl acceptor have been described for several other nucleic acid methyltransferases. Examples are as follows: (i) binding of *Eco*RII DNA cytosine methyltransferase to azacytosine-containing DNA was stimulated by AdoMet and AdoHcy (Friedman, 1986); (ii) AdoHcy enhanced target site binding by *Msp*I and *Hha*I DNA cytosine methyltransferases and increased discrimination between correct and incorrect target sequences (Dubey & Roberts, 1992; Klimasauskas & Roberts, 1995); (iii) binding of AdoMet to *Eco*KI DNA adenine methyltransferase elicited a conformational change in the protein and a 5-fold enhancement of binding to its specific DNA target site (Powell et al., 1993).

With these precedents in mind, we suggest a model for substrate recognition by the vaccinia capping enzyme in which the methyltransferase domain binds without specificity to the RNA polynucleotide and then captures the 5′ cap terminus at its specific binding site when the nearby AdoMet/AdoHcy binding site is suitably occupied. Structural features of the cap that are required for specific binding at the acceptor site reside primarily in the cap guanosine nucleotide, because the enzyme will methylate GTP, GDP, GMP, and even guanosine, albeit inefficiently compared with capped polynucleotides (Martin & Moss, 1976). The K_m of the vaccinia methyltransferase for GTP is 150−2000 times higher than the K_m for capped poly(A) (Martin & Moss, 1976; Higman et al., 1994). Thus, the engagement of the enzyme on RNA via the "general" RNA binding site significantly enhances affinity for the methyl acceptor. A clear prediction is that elimination of general RNA binding should result in a loss of methyltransferase activity and a defect in cap-specific cross-linking; this was the case for the H682A-Y683A double mutant, which was severely compromised with respect to its ability to bind to poly(A) in the nitrocellulose filter retention assay. [RNA ligands other than poly(A) were not tested.]

The effects of amino acid substitution mutations on UV cross-linking to capped poly(A) were informative for the G600A protein. The mutation of Gly-600 had no impact on general RNA binding, yet reduced methyltransferase activity to 4% of that of the wild-type enzyme. G600A cross-linked to the cap, but required higher concentrations of AdoHcy to achieve maximal photoadduct formation. This suggests that the G600A mutation affects binding of the methyl donor, as might be expected for a mutation within a highly conserved AdoMet binding motif. However, we suspect that this does not entirely account for the G600A catalytic defect, because methyltransferase assays were performed at 50 μM AdoMet—which is well above the 3 μM K_m of the wild-type methyltransferase for the methyl donor (Higman et al., 1994)—and because cap cross-linking was quite robust at 50 μM AdoHcy. This suggests that the G600A mutation also affects the chemical step of transmethylation. Other catalytically significant mutations at His-682 and Tyr-683 abolished or severely reduced cap cross-linking. These were more difficult to interpret, because failure to cross-link in our assay can have multiple causes other than general RNA binding defects, namely: (i) failure to bind AdoHcy, (ii) failure to bind the cap, or (iii) failure to photo-cross-link, despite normal binding of AdoHcy and m7GpppA(pA)n. At least in the case of H682A, the modest effect on general RNA binding was insufficient to account for the marked defects in cap cross-linking and RNA methylation.

The essential His-Tyr motif of the vaccinia methyltransferase is strictly conserved among cap methyltransferases and is implicated by our findings in RNA binding and potentially in the binding of the cap and/or the methyl donor. In the crystal structure of the ternary complex of catecholamine *O*-methyltransferase bound to AdoMet and a catechol analog, a His-Trp dipeptide makes direct contact with AdoMet and with the methyl acceptor (Vidgren et al., 1994). Conceivably, the His-Tyr motif of vaccinia D1 plays a similar role in cap methylation. Our studies show that the aromatic group of Tyr-683 is essential for cap methylation. Replacement of Tyr-683 by phenylalanine had little effect, whereas replacement by serine reduced the specific activity by more than 2 orders of magnitude. The substitution of Phe for Tyr had the effect of conferring the ability to cross-link to the cap structure in the absence of the AdoHcy cofactor (although the addition of cofactor did elicit conversion to the slower migrating constituent of the doublet photoproduct). This "gain-of-function" effect might occur if elimination of the phenolic hydroxyl group at position 683 partially mimics the conformational change elicited by AdoHcy binding to the wild-type methyltransferase, either rendering the mutant enzyme capable of binding to the cap in the absence of cofactor or else rendering it more photoreactive with the cap guanosine in the absence of AdoHcy. (Another possibility is that the aromatic moiety at position 683 is actually a participant in the photo-cross-linking reaction.) Clearly, the shortest path to addressing all of the issues raised by the present study will be to obtain a crystal structure either for the vaccinia methyltransferase domain or for the yeast ABD1 cap methyltransferase.

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