One or more proteins in mammalian reovirus core particles mediate two RNA methylation activities, (guanosine-7-N)-methyltransferase and (guanosine-2'-O)-methyltransferase, that contribute to forming the 5' cap 1 structure on viral mRNA. We used UV irradiation to identify core proteins that bind S-adenosyl-L-methionine (SAM), the methyl-group donor for both methyltransferases. A [methyl-3H]SAM-binding site was observed among the reovirus λ proteins; was shown to be specific by competition with low levels of S-adenosyl-L-homocysteine, the product of methyl-group transfer from SAM; and was subsequently localized to protein λ2. λ2 mediates the guanylyltransferase reaction in cap formation and was previously proposed to mediate one or both methylation reactions as well. SAM binding was demonstrated for both λ2 in cores and λ2 expressed in insect cells from a recombinant baculovirus. Using three different methods to cleave λ2, a binding site for SAM was tentatively localized to a central region of λ2, between residues 792 and 1100, which includes a smaller region with sequence similarity to the SAM-binding pocket of other methyltransferases. Alanine substitutions at positions 827 and 829 within this predicted binding region greatly reduced the capacity of baculovirus-expressed λ2 protein to undergo UV cross-linking to SAM but had no effects on either the guanylyltransferase activity of this protein or its conformation as judged by partial proteolysis, suggesting that one or both of these residues is essential for SAM binding. Based on these findings, we propose that the two methyltransferase activities involved in mRNA capping by reovirus cores utilize a single SAM-binding pocket within a central region of λ2.

The first of four enzymes for forming the 5’ cap 1 structure (m7-N-Gppp m2'-O-GpC[pN]n-OH) on reovirus mRNA is an RNA triphosphate phosphohydrolase (1–3). This enzyme removes the γ phosphate from the 5'-terminal nucleotide of the nascent mRNA, a guanosine in all 10 reovirus mRNAs, generating a diphosphorylated terminus that serves as substrate for the RNA guanylyltransferase. The reovirus protein that mediates this activity in cores remains unproven; however, two proteins, λ1 and μ2, have been shown to influence the ion and temperature dependence of NTP hydrolysis by cores (4, 5), and a recombinant λ1 protein expressed in yeast has been shown to act in vitro as both NTPase (6) and RNA triphosphatase (7).

The next enzyme to act in cap formation is the RNA guanylyltransferase. The RNA guanylyltransferase activity mediated by core protein λ2 (8–10) was the focus of recent work in our lab. Previous work associated sequences near the N terminus of λ2 with this activity, particularly lysine 226, at which covalent linkage to GMP was found to occur as part of the catalytic mechanism exhibited by this and related enzymes (9, 11). We have recently shown that a 40-kDa N-terminal proteolytic fragment of λ2 can mediate both steps in the guanylyltransferase reaction: covalent linkage to GMP using GTP as substrate and transfer of GMP to an acceptor molecule, GDP or GTP in our experiments.

Lastly, two methyltransferase activities act to form the final cap 1 structure on reovirus mRNAs (3, 12). Both activities appear to be mediated by reovirus-encoded proteins because no host proteins are known to be present in reovirus cores (13). The first is an RNA (guanosine-7-N)-methyltransferase, which uses S-adenosyl-L-methionine (SAM) as substrate for methyl-group transfer to the N-7 position of the RNA guanylyltransferase-added 5’-terminal guanosine (m7-N-GpppGpC[pN]n-OH) (2). The second is an RNA (guanosine-2’-O)-methyltransferase, which uses SAM as substrate for methyl-group transfer to the O-2’ position of the 5’-most RNA polymerase-added nucleotide (m7-N-Gppp m2'-O-GpC[pN]n-OH). For both reactions, S-adenosyl-L-homocysteine (SAH) is formed as the by-product of methyl-group transfer from SAM. The reovirus protein that mediates these methylations has remained unproven, although the literature contains two suggestive findings. First, an observation reported as unpublished data by Seliger et al. (14) indicated that the λ2 protein was the only reovirus core protein that underwent covalent cross-linking to S-azido-S-adenosyl-[3H]methionine. Second, data base sequence comparisons by Koonin (15) revealed that λ2 contains sequences between residues 825 and 888 that bear similarities to the λ2-mediating methyltransferases. Notably, this region of similarity includes...
sequences found in the SAM-binding pocket of methyltransferases for which such information is known (15).

Identification of the protein(s) that mediates methyltransferase reactions by reovirus cores is important for understanding how the capping enzymes are arranged in these particles. A confounding observation from previous work is that recombinant \( \lambda 2 \) protein (\( \lambda 2 \)) expressed from a vaccinia virus vector does not exhibit methyltransferase activity, possibly because it remains monomeric in solution (10) and does not adopt the pentameric structure observed for \( \lambda 2 \) in cores (16). Having confirmed ourselves that \( \lambda 2 \) expressed from a baculovirus vector remains monomeric in solution and also fails to mediate methyltransferase activity,\(^1\) we turned to an alternative approach to address this potential activity of \( \lambda 2 \). In this study, we used UV cross-linking in combination with protein fragmentation and site-directed mutagenesis to localize a binding site for \( ^{3} \)H-SAM to a central region of \( \lambda 2 \) sequence, overlapping that predicted by Koonin (15). Our results strongly suggest that \( \lambda 2 \) mediates at least one of the two methylation reactions in mRNA capping by reovirus cores.

**EXPERIMENTAL PROCEDURES**

**Purification of Virus Particles—**Virions of reovirus T1L or T3D were purified as described (17). Virion concentrations were determined from the relationship \( I.0_{A_{260}} = 2.1 \times 10^{12} \) virions/ml (18). Cores of reovirus T3D were prepared by digesting virions at a concentration of \( 3 \times 10^{10} \) particles/ml with 200 \( \mu \)g/ml \( \alpha \)-chymotrypsin (Sigma) for 1.5 h at 37 °C in virion buffer (10 mM Tris, pH 7.5, 10 mM MgCl\(_2\), 150 mM NaCl). Cores of reovirus T1L were chymotrypsin-digested as described (19). The chymotrypsin was inactivated with 1 mM phenylmethylsulfonyl fluoride (Sigma). To purify cores, digests were loaded onto a preformed CsCl gradient (\( p = 1.30-1.55 \) g/cm\(^3\)) and subjected to equilibrium centrifugation. The particles were dia lyzed into virion buffer. Modified cores were generated as described in Luongo et al. (20). In brief, \( 1 \times 10^{12} \) cores of reovirus T3D in virion buffer were heated to 52 °C for 30 min, cooled to 37 °C, and treated with 200 \( \mu \)g/ml chymotrypsin for 10 min. Following treatment, the chymotrypsin was inactivated with 1 mM phenylmethylsulfonyl fluoride, and the modified cores were isolated following equilibrium centrifugation.

**UV Cross-linking—**Reactions were UV irradiated using a modification of the protocol of Ahola et al. (21). In brief, 20-\( \mu \)l reactions containing the protein sample and 1.7 \( \mu \)M of S-adenosyl-\( \lambda \)-methionine (\( ^{3} \)H-SAM) (80 Ci/mmol; DuPont) in 50 mM Tris-HCl, pH 7.5, 2 mM dithiothreitol, and 2 mM EDTA were pipetted into 0.5-ml microfuge tubes. The reactions were incubated on ice while being irradiated for 30 min with 254-nm UV light in a Stratalinker 2400 cross-linker (Stratagene, La Jolla, CA) at a distance of 24 cm from the sample. UV cross-linking was followed by treatment with 37 °C for 16 h. The proteins were resolved on a 10% SDS-polyacrylamide gel and visualized as described above for UV cross-linked protein.

**Generation of Mutant \( \lambda 2 \)—**The \( \lambda 2 \) gene cloned into the BamHI site of pBluescript (Stratagene) was the template for site-directed mutagenesis utilizing the Quik Change site-directed mutagenesis kit (Stratagene). As indicated by sequence analysis, the underlined nucleotides missense mutations that change both aspartate 827 and glycine 829 to alanine. In addition, these two alterations remove a BgIII restriction site at nucleotide 2491. In brief, 50 ng of template was amplified with either 125 or 500 ng of each primer under the following conditions: 20 cycles at 94 °C for 30 s, 55 °C for 1 min, and 68 °C for 14 min followed by one cycle at 15 °C for 5 min. Mutant clones were identified by screening for the loss of the BgIII site. The presence of the mutations were confirmed by sequencing both DNA strands from nucleotides 1804 to 3157 using the ABI Prism dye terminator cycle sequencing ready reaction kit (Applied Biosystems). No other sites in the \( \lambda 2 \) coding sequence and genomic DNA were identified.

**Expression of Recombinant \( \lambda 3 \)—**\( \lambda 3 \) was expressed using a recombinant baculovirus system as follows. The reovirus T3D L1 gene, which encodes the \( \lambda 3 \) protein, cloned into the PstI site of pBR322 (22) was obtained from Michael Roner (Florida Atlantic University). The \( L1 \) gene was PCR amplified using the forward primer 5'-CGGGGGTACCGAGCGAGGACGCGGTCGACGATCAGGC-3' and the reverse primer 5'-CGCGGTATTCCATGATCAGGTGACGGC-3'. These primers add KpnI sites (underlined) 5' and 3' of the L1 coding sequence and Xhol and NcoI restriction sites (italicized), respectively, 5' of the L1 coding sequence. The Extend Long Template PCR system (Boehringer Mannheim) was used to amplify the \( L1 \) gene. In brief, buffer 2 supplemented to 5.25 mM MgCl\(_2\) (final) was used in the reaction. The amplification conditions were one cycle at 92 °C for 2 min followed by 10 cycles at
proteins in reovirus particles. A Tris-glycine-SDS 8% polyacrylamide gel was used to separate the protein band containing the major outer capsid proteins in virions. The apparent molecular weights of protein standards (× 103) are indicated at far left. A, a Tris-glycine-SDS 8% polyacrylamide gel was used to resolve the λ band containing λ1, λ2, and λ3 proteins from the other proteins in reovirus particles. B, a phosphate-urea-SDS 7.5% polyacrylamide gel was used to separate the protein band containing λ2 and λ3 from the band containing λ1. The same numbers of coes and virions (3 × 1011) were utilized in the individual reactions.

92 °C for 15 s, 60 °C for 35 s, and 68 °C for 3.5 min followed by 20 cycles at 92 °C for 15 s, 65 °C for 35 s, and 68 °C for 3 min 50 s with an addition of 20 s/cycle followed by one cycle at 68 °C for 7 min. The L1 PCR product was digested with XhoI and KpnI (New England Biolabs) and ligated into the pFastBacI vector digested with the same enzymes. Recombinant bacmid was generated and used to produce recombinant baculovirus by transfection of Sf21 cells. For protein expression, High Five cells were infected with 0.6 plaque forming units of L1 recombinant baculovirus and harvested 62–70 h postinfection. Cells were placed on ice and lysed in a hypotonic buffer (10 mM Tris, pH 7.4, 2.5 mM MgCl2, 0.5% Triton X-100, 100 mM NaCl, 2% complete protease inhibitor mixture (Boehringer Mannheim)). Nuclei and large cellular debris were pelleted at 200 × g for 15 min at 4 °C. The λ3 in the supernatant was partially purified by removal of protein contaminant by pre-treatment with 20% (saturation at 0 °C) ammonium sulfate and precipitation. The protein precipitated by 35% ammonium sulfate contained λ2 and λ3 proteins from the other proteins in reovirus particles. Following electrophoresis in a Tris-glycine-SDS gel (8% acrylamide), blotting to nitrocellulose to improve detection of 3H, and fluorography, radiolabel was strongly detected in the λ protein band (Fig. 1A). In this type of gel, the λ band comprises core proteins λ1 (120 copies/particle), λ2 (60 copies/particle), and λ3 (12 copies/particle). When virions of reovirus T3D were treated in the same protocol, radiolabel was again strongly detected in the λ band and at similar levels as seen with cores (Fig. 1A). The latter result concurs with findings that the capping methyltransferases have nearly full activity in reovirus virions (24). Only minor amounts of radiolabel were associated with the major outer capsid proteins in virions, μ1C and σ3 (600 copies each per virion), providing evidence that the binding of [3H]SAM is specific to one or more of the λ proteins. The same results were obtained with cores and virions of reovirus T1L (data not shown), indicating that this property is not unique to a single reovirus strain.

To identify which λ proteins contain binding sites for SAM, core particles of reovirus T3D were incubated with [3H]SAM, irradiated with UV light to achieve cross-linking, and subjected to electrophoresis in a phosphate-urea-SDS gel (7.5% acrylamide). Such gels provide good separation of the λ1 protein from the λ2 and λ3 proteins, which still comigrate (18). Following fluorography, radiolabel was strongly detected in the λ2/λ3 band but not in the λ1 band (Fig. 1B). When virions of reovirus T3D were treated in the same protocol, radiolabel was again strongly detected in the λ2/λ3 band and at similar levels as observed with cores (Fig. 1B). The same results for cross-linking to [3H]SAM were obtained with cores and virions of reovirus T1L (data not shown). These results indicate that the primary binding sites for SAM identified by UV cross-linking are found in λ2 and/or λ3, and not in λ1, in both particle types of the two reovirus strains.

**RESULTS**

**UV Cross-linking of [3H]SAM to λ2 and/or λ3 Proteins in Reovirus Cores and Virions**—To identify which reovirus core proteins contain binding sites for SAM, core particles of reovirus T3D were incubated with [methyl-3H]SAM and irradiated with UV light (254 nm) to cross-link this molecule to its binding site(s). Following electrophoresis in a Tris-glycine-SDS gel (8% acrylamide), blotting to nitrocellulose to improve detection of 3H, and fluorography, radiolabel was strongly detected in the λ protein band (Fig. 1A). In this type of gel, the λ band comprises core proteins λ1 (120 copies/particle), λ2 (60 copies/particle), and λ3 (12 copies/particle). When virions of reovirus T3D were treated in the same protocol, radiolabel was again strongly detected in the λ band and at similar levels as seen with cores (Fig. 1A). The latter result concurs with findings that the capping methyltransferases have nearly full activity in reovirus virions (24). Only minor amounts of radiolabel were associated with the major outer capsid proteins in virions, μ1C and σ3 (600 copies each per virion), providing evidence that the binding of [3H]SAM is specific to one or more of the λ proteins. The same results were obtained with cores and virions of reovirus T1L (data not shown), indicating that this property is not unique to a single reovirus strain.

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**Competition by SAH and Optimization of [3H]SAM Binding to Reovirus λ Protein(s)**—Before proceeding with other studies to localize the binding site(s), we addressed the specificity of binding to SAM by the λ protein(s) in cores by performing competition experiments with nonradiolabeled SAH, the stable by-product of methyl-group transfer from SAM in the capping methyltransferase reaction (2). The capacity of SAH to inhibit methyltransferase activities in reovirus cores was previously demonstrated (25). In addition, competition by SAH has been used to demonstrate the specificity of binding to SAM by other methyltransferases (26–28). Using SDS-PAGE, electroblotting, and fluorography, we showed that radiolabeling of the λ protein(s) in reovirus T3D cores upon UV cross-linking in the presence of 1 μM [3H]SAM was inhibited by SAH with an IC50 of ∼0.5 μM (Fig. 2A). In contrast, S-adenosyl-l-homocysteine (dextro form of SAH) had little or no effect on cross-linking to [3H]SAM at the highest concentration tested (5 μM) (data not shown), providing further evidence for the specificity of competition by the physiological, laevor form of SAH. These results provide evidence that cross-linking to [3H]SAM reflects the presence of one or more specific binding sites for SAM in λ2 and/or λ3.

In an effort either to compete or to enhance SAM binding to the λ proteins in cores, we tested the effects of GTP and the cap analog GpppG. We hypothesized that GTP binding to the guanylyltransferase region of λ2 (9) or GpppG binding to one or both putative methyltransferase regions in λ2 (Refs. 14 and 15 and this study) might enhance the observed SAM binding activity. Nevertheless, when cross-linking to [3H]SAM was performed in the presence of up to 20 μM GTP (data not shown) or GpppG (Fig. 2B), SAM binding to λ2 and/or λ3 in T3D cores was unchanged, indicating that these compounds do not modulate this activity.

**Localization of [3H]SAM Binding to a 120K N-terminal Portion of λ2 Protein in Modified Cores**—In an attempt to localize the binding site(s) for SAM in reovirus cores, we used modified cores containing A2 protein that lacks 25K of C-terminal sequences due to sequential treatments with mild heat (52 °C) and chymotrypsin (20). After subjecting these modified cores to UV cross-linking in the presence of [3H]SAM, followed by SDS-PAGE and fluorography, we found the 120K N-terminal fragment of λ2 that is present in these particles to be strongly radiolabeled (Fig. 3). Because the complementary, 25K
Chymotrypsin-modified cores (23776 l) that removing the C terminus of polyacrylamide gel. The 120K (predicted fragment II–V) is indicated at far left.

The N-terminal portion of α2 is degraded as the modified cores are made (20), we could not address whether it, too, might contain a SAM-binding site; nonetheless, the available data demonstrate that at least one SAM-binding site is located within the 120K N-terminal portion of α2. The data also indicate that this large N-terminal portion of α2 is sufficient for at least one SAM-binding activity of α2, consistent with previous findings that removing the C terminus of α2 by treatment with heat and chymotrypsin does not reduce the capacity of modified cores to methylate newly transcribed viral mRNAs (20).

The small amount of radiolabel that was seen to migrate at the position of full-length α2 protein after cross-linking with modified cores (Fig. 3) could represent the small amount of uncleaved α2 protein that remains present in these particles and/or the α3 protein, which fully resists cleavage during the heat and chymotrypsin treatments (20). Nonetheless, because radiolabel was mostly associated with the 120K fragment of α2 after cross-linking with modified cores, the findings strongly suggest that any cross-linking of [3H]SAM to α3 that may also have occurred with modified cores or cores was minor compared with that to α2 (see last section of “Results” for additional analysis of α3).

Localization of [3H]SAM Binding to a 55K C-terminal Portion of α2 in Cores by Limited Hydrolysis with Formic Acid—The deduced amino acid sequence of T3D cores (1.2 × 1012 particles/reaction) that had been UV irradiated in the presence of [3H]SAM were incubated with water (F−) or 75% formic acid (F+) and then resolved on a Tris-glycine-SDS 10% polyacrylamide gel. The α protein cleavage fragments were visualized by staining one-third of the gel with Coomassie Brilliant Blue (left panel). Cross-linking of [3H]SAM to protein was demonstrated by fluorography after electroblotting another third of the gel (middle panel). The identity of cleavage fragments containing the C terminus of α2 was demonstrated by immunoblot analysis using the 7F4 monoclonal antibody (20, 30) (right panel). The four hypersensitive sites for acid hydrolysis in α2 are diagrammed in the bottom panel along with the expected cleavage fragments (II–V, III–V, IV–V, and V) that contain the epitope in region V recognized by 7F4. Full-length α2 and the cleavage fragments that appear to contain region V are identified in all three upper panels. The apparent molecular weights of protein standards (× 103) are indicated at far left.

**Fig. 2.** Effect of SAH and GpppG on [3H]SAM binding to α proteins. Reovirus T3D cores (8 × 1013 particles/reaction) were mixed with 1 μM [3H]SAM and various concentrations of unlabeled competitor SAH (A) or cap analog GpppG (B) and then UV irradiated. The core proteins were separated on Tris-glycine-SDS 8% polyacrylamide gels and visualized by Coomassie Brilliant Blue staining of one-half of each gel (left panels) or fluorography of the electroblotted other half of each gel (right panels). The apparent molecular weights of protein standards (× 103) are indicated at far left.

**Fig. 3.** UV cross-linking of [3H]SAM to the 120-kDa α2 fragment of heat and chymotrypsin-modified cores. Cores (C) and heat and chymotrypsin-modified cores (H) (6 × 1013 particles/reaction) were UV irradiated in the presence of [3H]SAM and resolved on a Tris-glycine-SDS 8% polyacrylamide gel. The 120K α2 cleavage product is evident beneath the α protein band in the Coomassie Brilliant Blue-stained half of the gel (left panel) and after fluorography of the electroblotted half of the gel (right panel). The apparent molecular weights of protein standards (× 103) are indicated at far left. The diagram in the bottom panel represents the pattern of α2 cleavage in the modified cores. As shown, the 120K fragment represents the N-terminal portion of α2 (20). The 25K C-terminal portion of α2 (hatched) is not detectable because of its rapid degradation (20).

**Fig. 4.** Limited formic-acid hydrolysis of α protein after UV cross-linking to [3H]SAM. The α proteins of T3D cores (1.2 × 1012 particles/reaction) that had been UV irradiated in the presence of [3H]SAM were incubated with water (F−) or 75% formic acid (F+) and then resolved on a Tris-glycine-SDS 10% polyacrylamide gel. The α protein cleavage fragments were visualized by staining one-third of the gel with Coomassie Brilliant Blue (left panel). Cross-linking of [3H]SAM to protein was demonstrated by fluorography after electroblotting another third of the gel (middle panel). The identity of cleavage fragments containing the C terminus of α2 was demonstrated by immunoblot analysis using the 7F4 monoclonal antibody (20, 30) (right panel). The four hypersensitive sites for acid hydrolysis in α2 are diagrammed in the bottom panel along with the expected cleavage fragments (II–V, III–V, IV–V, and V) that contain the epitope in region V recognized by 7F4. Full-length α2 and the cleavage fragments that appear to contain region V are identified in all three upper panels. The apparent molecular weights of protein standards (× 103) are indicated at far left.

**Table 3.** SDS-PAGE of α2 cleavage fragments. The α2 cleavage fragments were visualized by staining one-third of the gel with Coomassie Brilliant Blue (left panel) and after fluorography of the electroblotted other half of each gel (right panel). The apparent molecular weights of protein standards (× 103) are indicated at far left.
which region of $\lambda_2$ the 80K fragment represents, we subjected it to N-terminal sequencing. The sequence obtained by Edman degradation was leucine-alanine-proline-phenylalanine-proline (data not shown), corresponding to residues 562–567 in the deduced amino acid sequence of the T3D $\lambda_2$ protein (14) and consistent with cleavage by thermolysin (N-terminal to leucine). The calculated mass of a fragment spanning residues 562–1289 (the C terminus of $\lambda_2$) is 80.7 kDa, consistent with the observed Mr (80K).

The findings for localizing SAM-binding sites within either $\lambda_2$ from cores or r$\lambda_2$ using three different methods of proteinase or chemical cleavage can be summarized as follows. The SAM-binding fragments resulting from each of these treatments overlap sequences between positions 792, in the most C-terminal aspartate-proline pair in $\lambda_2$, and $\sim1100$, the site of $\lambda_2$ truncation in cores treated with heat and chymotrypsin. Thus, if $\lambda_2$ were to contain a single site for binding SAM that can be identified by UV cross-linking, this region (amino acids 792 to $\sim1100$) would represent the likely location for this binding site. It is notable that this region includes the SAM-binding motif and adjacent sequences with similarity to known SAM-utilizing methyltransferases (residues 825–888) as described by Koonin (15).

Reduced Capacity of $\lambda_2$ to Bind $[^3H]SAM$ after Alanine Substitutions at Positions 827 and 829—To test the predictions of Koonin (15) regarding binding to SAM by residues near position 830 in $\lambda_2$, as well as our own evidence that SAM is bound in this region, we created mutations in the pBluescript-inserted cDNA copy of T3D L2 (see “Experimental Procedures”) such that the encoded $\lambda_2$ protein contained two amino acid substitutions: alanine 827 (for aspartate in wild type $\lambda_2$) and alanine 829 (for glycine in wild type $\lambda_2$) in the B-B-aspartate-B-glycine-X-glycine motif (where B represents a hydrophobic residue and X represent any residue) shared by many methyltransferases (15). The mutant L2 gene was then used to generate a recombinant baculovirus for expressing the mutant $\lambda_2$ protein in insect cells. Upon demonstrating that the mutant $\lambda_2$ was expressed in these cells, we partially purified it using the same protocol as described for wild type $\lambda_2$. The purified mutant and wild type $\lambda_2$ proteins were then analyzed for SAM binding by incubation with $[^3H]SAM$ and UV cross-linking. The results indicate that the mutant $\lambda_2$ protein is greatly diminished in its capacity to bind SAM (Fig. 6A). By quantitating the amounts of radiolabel associated with the wild type or mutant $\lambda_2$ proteins with the amounts of each protein loaded in the gel lanes, we estimated that the mutant protein was labeled with $[^3H]SAM$ at $\leq4\%$ of the level of wild type. The greatly reduced binding to SAM by the mutant $\lambda_2$ was also demonstrated in a sample to which the 100K fragment of wild type $\lambda_2$ (generated by prior thermolysin treatment) was added prior to cross-linking with $[^3H]SAM$. In this case, the 100K fragment of wild type protein was strongly radiolabeled, and the full-length mutant protein again showed little detectable labeling above background. In contrast, the mutant $\lambda_2$ protein was efficiently radiolabeled by covalent linkage to GM$[^32P]$ (Fig. 6B) as part of the guanylyltransferase reaction mediated by an N-terminal region of $\lambda_2$ (9); was recognized by monoclonal antibody 7F4 (data not shown), which binds to a C-terminal epitope in $\lambda_2$ (20, 30); and exhibited an identical pattern of cleavage upon treatment with thermolysin as the wild type protein (data not shown), indicating that other regions of $\lambda_2$ were not substantially affected by the alanine substitutions at 827 and 829. Because mutations at these two closely spaced positions in $\lambda_2$ so greatly reduced binding to SAM in the cross-linking assay but did not affect other properties, the results provide evidence that a single SAM-binding site in $\lambda_2$, involving residues near
Within the SAM-binding motif of $\lambda 2$, and the protein-associated radiolabel was visualized by PhosphorImager. The covalent linkage to GM[32P] in association with guanylyltransferase recombinant baculovirus. The resulting preparations of $\lambda 3$ might also contain a site was localized between the N terminus and a position near position 830, was identified and analyzed in this study.

No UV Cross-linking to $[3H]SAM$ by $\lambda 3$ Protein—Because $\lambda 2$ and $\lambda 3$ comigrate in phosphate-urea-SDS gels, the results for UV cross-linking of $[3H]SAM$ to proteins in reovirus cores (Fig. 1B) leave open to question whether $\lambda 3$ might also contain a binding site for SAM. Similarly, the small amount of $[3H]SAM$-labeled protein observed in the position of full-length $\lambda 2$ and $\lambda 3$ proteins from cores modified by previous treatment with mild heat and chymotrypsin (Fig. 3) could represent $\lambda 3$ and/or a small amount of uncleaved $\lambda 2$. To address more directly whether $\lambda 3$ might also bind SAM, we used a recombinant form of the T3D $\lambda 3$ protein that was expressed in insect cells from a recombinant baculovirus. The $\lambda 3$ protein was partially purified using a combination of ammonium sulfate precipitation and anion exchange chromatography (see "Experimental Procedures") and was shown to have poly(C)-dependent poly(G) polymerase activity (data not shown) (31). When partially purified preparations of $\lambda 2$ (see above) and $\lambda 3$ were subjected to UV cross-linking in the presence of $[3H]SAM$ and then analyzed by SDS-PAGE, electroblotting, and fluorography, SAM bound to $\lambda 2$ and its 100K fragment (generated by a contaminating proteasome in this protein preparation) but not to $\lambda 3$ (Fig. 7). Thus, the results for baculovirus-expressed $\lambda 2$ and $\lambda 3$ proteins, coupled with the results for proteins in cores, suggest that $\lambda 3$ exhibits little if any binding to $[3H]SAM$ using the UV cross-linking assay.

**Discussion**

Three different fragmentation methods were used to localize binding sites for SAM in the reovirus $\lambda 2$ protein. In the first, involving C-terminally truncated $\lambda 2$ proteins in modified cores, a site was localized between the N terminus and a position near residue 1100. In the second, involving limited acid hydrolysis of $\lambda 2$ in cores, a site was localized between position 792 and the C terminus. In the third, involving thermolysin cleavage of $\lambda 2$, a site was localized between position 562 and the C terminus. Recognizing that these localizations overlap a region of $\lambda 2$ between positions 792 and ~1100, we hypothesized that one or more binding sites for SAM is located within this specific 35-kDa, central region of the protein. Although it is formally possible that more than one SAM-binding site is contained within this region, no candidate sequences are evident besides those identified by Koonin (15) between positions 825 and 888. Moreover, because the alanine substitutions we introduced at positions 827 and 829 within this region so greatly reduced the capacity of $\lambda 2$ to undergo UV cross-linking to $[3H]SAM$, the findings suggest that a single SAM-binding site involving amino acids near position 830 in $\lambda 2$ has been identified by UV cross-linking in this study. Although it is tempting to state more definitively that $\lambda 2$ contains only the one SAM-binding site, an important caveat is that a second SAM-binding site in $\lambda 2$ may be less efficiently cross-linked to $[3H]SAM$ in our protocol than the site we have studied and may in fact account for the very low level of activity (~4% wild type levels) we found remaining in the alanine substitution mutant. Another possibility is that the alanine substitutions at positions 827 and 829 knocked out two distinct or overlapping SAM-binding sites in $\lambda 2$ at the same time. Thus, whereas we obtained no strong evidence for a second SAM-binding site in $\lambda 2$, we cannot definitively rule out that possibility.

**Region of $\lambda 2$ Sufficient for SAM Binding**—We conceived of other experiments involving expression of $\lambda 2$ or portions of that protein in *Escherichia coli* in an effort to identify the minimal region of $\lambda 2$ that is necessary for SAM binding. The full-length $\lambda 2$ protein and six deletion mutants expressed to date, however, have been found to be mostly insoluble, not readily solubilized, and inactive at SAM binding in the cross-linking assay (data not shown). Similar difficulties with $\lambda 2$ expression in *E. coli* have been described by other investigators (9). Thus, we abandoned that approach for any further experiments in the current study.

On the other hand, the findings from partial proteolysis of intact $\lambda 2$ described in this paper, which suggest that a central region of $\lambda 2$ contains a SAM-binding site, can be considered in light of other findings to suggest that a region from 562 to ~1100 is sufficient for SAM binding. Because the ~25K C-terminal portion of $\lambda 2$ is degraded in generating the 120K N-terminal fragment in cores treated with heat and chymotrypsin (20) and because the 120K fragment can subsequently bind SAM and undergo cross-linking to it (Fig. 3), the ~25K C-terminal portion must be dispensable for at least one SAM-binding activity. In addition, because the 40K N-terminal and 100K C-terminal fragment that can be generated from baculovirus-expressed $\lambda 2$ protein by mild proteolysis with several proteases including thermolysin (Fig. 5) are physically separated according to both native gel electrophoresis and gel filtration chromatography, because the 20K N-terminal portion of the 100K thermolysin-generated fragment is degraded in generating the 80K fragment at later times of thermolysin treatment (data not shown), and because the 80K fragment can...
bind SAM and undergo cross-linking to it after proteolysis (Fig. 5), the N-terminal ~60K region of λ2 must also be dispensable for at least one SAM binding activity. Thus, our data suggest that an ~60-kDa portion of λ2 spanning amino acids 562 to ~1100 constitutes the smallest region of the protein defined to date as being sufficient for SAM binding and UV cross-linking.

Implications for λ2 Function as a Capping Methyltransferase—Biochemical evidence for SAM binding by λ2 supports a general expectation (14, 15) that λ2 mediates one or both of the RNA methylation activities (RNA (guanosine-7-N)-methyltransferase and RNA (guanosine-2′-O)-methyltransferase) in forming a 5′ cap 1 structure on reovirus mRNA. Nevertheless, the failure of rλ2 to exhibit methylation activity (Ref. 10 and data not shown) still limits studies to dissect the putative methyltransferase activities of λ2 at a molecular level. This failure may reflect the incapacity of rλ2 expressed in isolation from other reovirus proteins to adopt the pentameric structure observed for λ2 in cores (10) (see next section).

Despite the caveat mentioned in the preceding section, an interesting possibility that can be inferred from the evidence for a single SAM-binding site in λ2 is that the same region of λ2 sequence may provide binding to SAM for the two different cap methylation activities in reovirus cores. An example similar to this possibility is seen in the multi-specific DNA methyltransferases of certain bacteriophages. In these enzymes, one polypeptide chain includes a single SAM-binding pocket and a single set of catalytic residues for methylation but multiple DNA-binding sites that allow specific recognition and subsequent methylation of several different DNA sequence motifs (32). Thus, different DNA-binding regions are arranged in those proteins such that each of the different DNA targets can be effectively bound and presented to the same catalytic machinery for methylation of defined bases within them. A similar hypothesis for λ2 would be that there are two different sequence regions in the protein that bind the nascent cap at the 5′ end of reovirus mRNA, one region that binds GpppGp[pN] -OH and presents the base of the terminal guanosine for methylation at the N-7 position and another region that binds m7GpppGp[pN] -OH (cap 0) and presents the sugar of the penultimate guanosine for methylation at the O-2′ position (Fig. 8). Both substrates are methylated by catalytic machinery using the same SAM-binding pocket. Another possibility is that once the first methylation occurs, that is, once m7GpppGp[pN] -OH is generated, this new RNA acceptor may undergo a change in orientation rather than translocating the RNA to a distinct binding site, such that the sugar of the penultimate guanosine is now properly presented for methylation at the O-2′ position. This rearrangement might involve conformational changes in λ2, m7GpppGp[pN] -OH, or both, possibly due to the presence of the (guanosine-7-N)-methyl group. The presence of one or two sites in λ2 for binding RNA acceptors is testable, and such experiments are in progress.

The circumstance suggested in Fig. 8 for λ2, one SAM binding site for the two methylation reactions, has not been described for a capping methyltransferase to date. For example, the (guanosine-7-N)-methyltransferase and (nucleoside-2′-O)-methyltransferase activities of vaccinia virus are mediated by distinct proteins (33, 34). Similarly, the capping methyltransferase encoded by the ABD1 gene of Saccharomyces cerevisiae has been shown to act as a (guanosine-7-N)-methyltransferase but not as a (nucleoside-2′-O)-methyltransferase (27). Thus, a detailed analysis of the SAM binding and methyltransferase activities of λ2 may provide unique insights into this class of enzymes.

Relationship of Findings to Pentameric Structure of Core-bound λ2—The arrangement of λ2 as homopentamers in reovirus cores has been known for some time (35, 36) although visualized at higher resolution only recently by cryoelectron microscopy with (16, 20) or without (37) three-dimensional image reconstruction. The regions of λ2 that are essential for pentamization remain undefined; however, the aforementioned three-dimensional reconstructions suggest that regions at the intermediate radii spanned by λ2 (which extends radially over ~100 Å in reovirus cores) may be most important.1 Although the SAM-binding pocket is functionally constituted by a λ2 monomer, as shown in this study, it is conceivable that residues in λ2 that contribute to catalysis and/or to binding RNA acceptor molecules in association with the SAM-binding pocket (Fig. 8) may be contributed by an adjacent monomer in the core-bound, pentameric λ2. This requirement would provide one possible explanation for why monomeric λ2 does not exhibit methyltransferase activity. A related, though distinct, possibility is that the residues necessary to constitute each active methyltransferase unit are contributed by the same λ2 monomer but that interactions with adjacent monomers in the λ2 pentamer are necessary for permitting these residues to assume their catalytically active conformations. These putative interactions between adjacent λ2 monomers are apparently not

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**Fig. 8.** Model for a single SAM binding pocket associated with putative RNA (guanosine-7-N)- and (guanosine-2′-O)-methyltransferase activities in λ2. SAM is shown in its binding pocket (dark gray rectangle) identified in the central portion of λ2. The methyl group that is transferred to the RNA is indicated on the SAM molecule. Catalytic residues for methyl transfer are represented by the gray oval. The GpppG binding region is identified by a rectangle with straight corners, and the m7GpppG binding region is indicated by a rectangle with rounded corners. The RNA is represented in bold letters. According to the model shown here, λ2 contains two methyltransferase-associated RNA-binding regions for binding either a GpppG or a “m7GpppG RNA terminus. In the upper panel, the methyl group of the bound SAM is transferred (curved arrow) to the N-7 position of the terminal G residue of the RNA bound in the GpppG binding region to generate m7GpppGp[pN] -OH RNA (cap 0) and SAH, both of which are released from λ2. As shown in the lower panel, the m7GpppGp[pN] -OH RNA then binds in the “m7GpppG binding region of λ2 and a second SAM molecule binds in the SAM binding pocket (as shown by the arrow). The methyl group of the bound SAM is transferred (curved arrow) to the O-2′ position of the penultimate G residue to generate m7Gppp m7GpppGp[pN] -OH RNA and SAH, both of which are released from λ2.
required for forming a functional SAM-binding site, however, as we show in this study using monomeric rλ2 obtained from insect cells.

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