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Identification of the Guanylyltransferase Region and Active Site in Reovirus mRNA Capping Protein $\lambda 2$ *

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The 144-kDa $\lambda 2$ protein of mammalian reovirus catalyzes a number of enzymatic activities in the capping of reovirus mRNA, including the transfer of GMP from GTP to the 5' end of the 5'-diphosphorylated nascent transcript. This reaction proceeds through a covalently autoguanlylated $\lambda 2$ -GMP intermediate. The smaller size of RNA capping guanylyltransferases from other organisms suggested that the $\lambda 2$ -associated guanylyltransferase would be only a part of this protein. Limited proteinase K digestion of baculovirus-expressed $\lambda 2$ was used to generate an amino-terminal M_r 42,000 fragment that appears to be both necessary and sufficient for guanylyltransferase activity. Although lysine 226 was identified by previous biochemical studies as the active-site residue that forms a phosphoamide bond with GMP in autoguanlylated $\lambda 2$, mutation of lysine 226 to alanine caused only a partial reduction in guanylyltransferase activity at the autoguanlylation step. Alanine substitution for other lysines within the amino-terminal region of $\lambda 2$ identified lysine 190 as necessary for autoguanlylation and lysine 171 as an important contributor to autoguanlylation. A novel active-site motif is proposed for the RNA guanylyltransferases of mammalian reoviruses and other *Reoviridae* members.

Mammalian reovirus, a multisegmented double-stranded RNA virus in the family *Reoviridae*, replicates in the cytoplasm of the eukaryotic host cell. The reovirus core particle can produce $m^{7N}GpppG^{m2'}O_pC(pN)_n-OH$ (cap 1) plus-strand RNA from each genomic double-stranded RNA segment *in vitro* (1), indicating that it contains all of the enzymes necessary for *de novo* synthesis of capped mRNA. The RNA polymerase itself is likely to be the $\lambda 3$ core protein (2, 3). Genetic and/or biochemical analyses indicate that the $\lambda 1$ and $\mu 2$ core proteins have nucleoside triphosphate phosphohydrolase activity, possibly associated with an RNA helicase (4–6). The γ phosphate of the newly transcribed mRNA is thought to be removed by the RNA triphosphate phosphohydrolase activity of $\lambda 1$ (7). The $\lambda 2$ core

protein is the reovirus RNA guanylyltransferase, which adds a GMP moiety via a 5'-5' linkage to the 5'-diphosphorylated mRNA (8). This transfer reaction occurs through a covalent intermediate, a phosphoamide bond between the GMP of the donor GTP and a lysine of $\lambda 2$ (9, 10). Generation of this covalent bond (called "autoguanlylation" in this paper) is followed by GMP transfer from the enzyme to an acceptor, usually the 5'-diphosphorylated mRNA, although the GMP can be alternatively transferred to a 5'-triphosphorylated RNA or a di- or triphosphorylated nucleoside (11). The resulting product is then sequentially methylated by RNA nucleoside-7-*N*- and 2'-*O*-methyltransferases, yielding the cap 1 mRNA (1) that is released through the channel formed by the $\lambda 2$ pentameric spike (12, 13). Both of the methyltransferase activities appear to reside in $\lambda 2$ as indicated by the finding that only the $\lambda 2$ protein in cores is covalently labeled with the methyl donor *S*-adenosyl-L-methionine after incubation and UV cross-linking (14). Thus, $\lambda 2$ is thought to catalyze the last three of the four reactions required for cap 1 formation on reovirus mRNA.

The 144-kDa $\lambda 2$ protein (15), encoded by the reovirus L2 gene, appears to contain multiple domains. The proposed guanylyltransferase active site (lysine 226) is near the amino terminus (10). There is an *S*-adenosyl-L-methionine-binding site that appears to span residues 827 and 829 (14, 16). A carboxyl-terminal M_r 25,000 region is expendable for capping functions but is implicated in anchoring the reovirus cell attachment protein $\sigma 1$ in virions (17, 18). A multidomain structure for the $\lambda 2$ protein is also consistent with what is known for other capping enzymes. The vaccinia virus capping enzyme that catalyzes the first three reactions required for cap 1 formation is a heterodimer composed of two subunits encoded by separate genes (19–21). By biochemical analysis of proteolytic products, the capping enzyme is separable into a region with RNA triphosphate phosphohydrolase and guanylyltransferase activity and a region with RNA nucleoside-7-*N*-methyltransferase activity (22, 23). The *Saccharomyces cerevisiae* capping enzyme is a complex of two separate gene products (24), one having RNA triphosphate phosphohydrolase and the other having RNA guanylyltransferase activity (25). These two examples suggest that capping enzymes can be multifunctional and that the guanylyltransferase region may be separated biochemically (vaccinia virus) or genetically (yeast) from the rest of the protein. In the case of the *Chlorella* virus PBCV-1, the RNA guanylyltransferase is a 330-amino acid monofunctional enzyme (26). The small size of this guanylyltransferase and the guanylyltransferase regions of the other capping enzymes suggests that only a portion of the 144-kDa $\lambda 2$ protein is likely to be required for its RNA guanylyltransferase activity.

For most RNA guanylyltransferases, a KXDG active-site motif has been proposed based on sequence comparisons (27–29). For the RNA guanylyltransferases of vaccinia virus, *S. cerevisiae*, and baculovirus, the identity of the active site has been

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confirmed by site-directed mutagenesis (27, 30, 31). For the PBCV-1 RNA guanylyltransferase, crystallographic analysis indicates that the active-site lysine interacts with the substrate GTP and that the other residues of the consensus motif interact with the RNA or nucleotide acceptor (32, 33). The RNA guanylyltransferases, as well as RNA and DNA ligases, are members of the RNA/DNA nucleotidyltransferase superfamily (27–29), enzymes that mediate nucleotidyl transfer to RNA or DNA via a covalent intermediate. The active-site motif for this entire superfamily is KX(D/N)G (27–29). The RNA guanylyltransferases of members of the family *Reoviridae* lack sequences that precisely match this consensus motif (15, 34). For example, the sequence of the proposed active site in the reovirus λ 2 protein is ²²⁶KPTNG. This sequence is similar to the nucleotidyl transferase superfamily motif, but the alignment is disrupted by insertion of a proline residue after the lysine in λ 2. The proposed guanylyltransferase active site for the other family *Reoviridae* members rotavirus (KPTGN) and bluetongue virus (KLTGN) is based on the proposed reovirus active site (34).

A previous effort to identify the λ 2 residue that forms the phosphoamide bond with GMP used a combination of proteolysis and chemical cleavage of reovirus virions autoguanylated with [³²P]GMP to show that a lysine residue in λ 2 is the site of covalent linkage to GMP (10). Immunoblot analysis of proteolyzed, [³²P]GMP-labeled reovirus particles with λ 2 peptide-specific antibodies indirectly identified lysine 226 as the residue to which GMP is likely attached. A similar approach involving proteolysis and chemical cleavage identified the active-site lysine of the vaccinia virus guanylyltransferase (35), and the identity of the vaccinia virus active-site lysine was subsequently confirmed by the analysis of site-directed mutants for autoguanylation activity (27). To extend characterization of the reovirus RNA guanylyltransferase, we expressed recombinant λ 2 and analyzed its properties. Utilizing a biochemical approach, we localized a region necessary and sufficient for guanylyltransferase activity to an amino-terminal M_r 42,000 fragment of λ 2. Since this region contains lysine 226, we generated mutant K226A by alanine substitution. Analysis of the mutant indicated that lysine 226 is not necessary for λ 2 guanylyltransferase activity. Alanine substitution for other lysines in the M_r 42,000 region identified lysine 190 as the probable site of covalent GMP linkage and lysine 171 as important for autoguanylation activity. Based on these findings, we propose a novel active-site motif for the RNA guanylyltransferases of mammalian reoviruses and other *Reoviridae* family members.

EXPERIMENTAL PROCEDURES

Recombinant Baculovirus Containing the Reovirus Type 3 Dearing (T3D)¹ L2 Gene—The cloned L2 gene of reovirus T3D is flanked by *Pst*I sites (36). For the purposes of subcloning, the L2 gene was amplified using Vent DNA polymerase (New England Biolabs), the forward primer 5'-GGAATTCGCGGCCGCAAAATGGCGAACGTTTGGGGC-GTG, and the reverse primer 5'-GGGGATCCGGGACAGTGAGTTAC-AGAGG. The forward primer was designed to introduce *Eco*RI and *Not*I restriction sites (underlined) 5' to the start codon and a silent mutation at the wobble position of codon 6 of L2 (italic). The reverse primer was designed to introduce a *Bam*HI site (underlined) 3' to the L2 stop codon. The resulting DNA product was blunt end-ligated into the *Sma*I site of the pBluescript SK(+) vector (Stratagene). The clone was subjected to automated DNA sequencing at the Howard Hughes Medical Institute and Harvard Medical School Biopolymer Facility (Boston, MA) and found to have no differences from the published sequence for the λ 2-encoding sequences of the T3D L2 gene (15). Both of these sequences have guanines at positions 1838–1840 compared with thymidines in

the sequence deposited in GenBankTM (accession no. J03488). At the amino acid level, the cloned and published sequences have a glycine rather than a phenylalanine at amino acid 609. The L2 gene was cut from pBluescript-L2 vector at the *Bam*HI sites (one from the reverse primer, one from pBluescript) and cloned into the *Bam*HI site of the pFastBacI vector (Life Technologies). Maximum efficiency DH10Bac competent cells were transformed with the pFastBacI-L2 construct, and recombinant bacmid was isolated according to the manufacturer's protocol (Life Technologies). Recombinant baculovirus (AcMNPV.T3DL2) was generated by transfection of *Spodoptera frugiperda* 21 (*Sf*21) with recombinant bacmid. Two clones of recombinant virus were propagated and shown to overexpress λ 2.

Isolation of Soluble λ 2—Trichoplusia ni insect cells (High Five, Invitrogen) were infected with AcMNPV.T3DL2 at 2 plaque-forming units/cell. At 48 h postinfection, the cells were harvested, washed with phosphate-buffered saline, and suspended in 25 mM Tris, pH 8.0, 100 mM NaCl with 4% complete protease inhibitor mixture (Roche Molecular Biochemicals). Cells were lysed by shearing using a syringe fitted with a 25-gauge needle. Insoluble protein was pelleted by centrifugation at 12,000 \times g for 15 min. The supernatant was used to purify λ 2 further.

Polyacrylamide Gel Electrophoresis (PAGE)—For SDS-PAGE, protein samples were mixed with one-third volume of Laemmli loading buffer (3% SDS, 9% β -mercaptoethanol, 375 mM Tris, pH 8.0, 30% sucrose, and 0.004% bromophenol blue) and heated at 100 °C for 2 min. For native PAGE, 10% discontinuous 0.75-mm gels, running buffer, and loading buffer were prepared by omitting the SDS and β -mercaptoethanol. The native gels were run for 1.5 h at 15 mA. Molecular weight standards for both gel systems were purchased from Sigma.

Autoguanylation Analysis—Protein was incubated with 5 μ Ci of [³²P]GTP (DuPont) in 50 mM Tris pH 8.0, 10 mM MgCl₂, and 2 mM dithiothreitol (DTT) for 30 min at room temperature. The reaction was terminated by the addition of 50 mM EDTA. The protein was resolved free of noncovalently bound radiolabel by SDS-PAGE. Relative protein abundance was determined with a laser densitometer (Molecular Dynamics). The relative amount of radiolabeling of each protein was determined by PhosphorImager analysis (Molecular Dynamics). To calculate the percentage of autoguanylation, the ratio of radiolabel to protein abundance was determined relative to the wild type λ 2 control sample included on each gel.

Immunoblot Analysis—Protein was electroblotted from SDS-polyacrylamide gels to nitrocellulose (Bio-Rad) in 25 mM Tris, 192 mM glycine, pH 8.3. Binding of monoclonal antibody 7F4 (37) was detected using goat anti-mouse IgG alkaline phosphatase conjugate and color developers 5-bromo-4-chloro-3-indoyl phosphate *p*-toluidine salt and *p*-nitro blue tetrazolium chloride (Bio-Rad). The molecular weight standards (kaleidoscope prestained) were purchased from Bio-Rad.

Thin Layer Chromatography—Samples were spotted directly onto polyethyleneimine-cellulose with fluorescent indicator (Sigma or EM Science). The sheet was placed vertically into a sealed tank with 0.5 M NH₄HCO₃ as the developer. Developing time was 1.5–2 h. The nucleotide and cap analog standards were purchased from Amersham Pharmacia Biotech.

Proteinase K Limited Proteolysis of Recombinant λ 2 (λ 2)— λ 2, concentrated by ammonium sulfate precipitation and resuspended in 50 mM Tris, pH 8.0, was diluted with an equal volume of buffer and incubated on ice for 5 min. The protein was then placed at 4 °C and digested with a 20% volume of 100 μ g/ml proteinase K (17 μ g/ml final concentration) for 10 min. The protease was inactivated with 1 mM phenylmethylsulfonyl fluoride (Sigma).

Amino-terminal Sequencing—The proteolytic products were transferred from a SDS-polyacrylamide gel to polyvinylidene fluoride paper (Applied Biosystems) (38), and the M_r 100,000 protein was excised from the blot. Amino acid analysis and amino-terminal sequencing by Edman degradation were performed at the University of Michigan Protein and Carbohydrate Structure facility (Ann Arbor, MI).

Generation of Mutant λ 2—To generate mutant K226A, a two-primer method was utilized (39, 40). The L2-specific primer GTGCATTATGAT-GCGCCAACGAATGGTCATCACTATCACTTAGGTACTTTG was used, since the underlined nucleotides cause a missense mutation that changes lysine 226 to alanine. The italicized nucleotides indicate silent mutations that generate an *Avr*II restriction site. A second primer, CAGAATGACTTGGTTGACCACTCACCAGTCACAG, was used, since the underlined nucleotides remove the *Scal* restriction site of the plasmid's ampicillin resistance gene, enabling selection for mutants. The pBluescript-L2 clone was the template for site-directed mutagenesis. Clones were screened for the L2 gene mutation by digestion with *Avr*II. The missense mutation was confirmed by DNA sequencing using the

¹ The abbreviations used are: T3D, type 3 Dearing; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; λ 2, recombinant λ 2.

ABI Prism dye terminator cycle sequencing ready reaction kit (Applied Biosystems). The mutant L2 sequence was subcloned into the pFastBac1-L2 plasmid using the unique restriction sites *Mlu*I (nucleotide 77) and *Nsi*I (nucleotide 1820). The fidelity of the subcloned region was confirmed by automated DNA sequencing. Two independent bacmid clones were isolated. The presence of the missense mutation in each clone was confirmed by sequencing bacmid DNA. Each bacmid clone was used to produce recombinant baculovirus (AcMNPV.T3DL2.K226A) by transfection of *Sf*21 cells. Mutant protein generated from each recombinant baculovirus stock was expressed and purified as described for wild type λ 2. The presence of the mutation was confirmed by sequencing the recombinant baculovirus DNA from nucleotides 537–827 of the L2 gene.

Due to the sequence context sensitivity of the two-primer method, the alanine substitution mutants K44A, K89A, K94A, K171A, K190A, and K197A were generated using the Quik Change site-directed mutagenesis kit (Stratagene). Completely complementary mutagenic primers were utilized to create each missense mutation. The primers corresponding to the sense strand were as follows: GGGAGGGAACCGTGG-GCACCTCTGCGTAATC for K44A; GGGAGCGATTCATGAGAGAGG-CGCTGCGTGTGC for K89A; GAAGCTGCGTGTGCTAGCGTATGAA-GTATTGCGC for K94A; GTCGCAGCTGGTCTGCGTATCTGCAGAT-TGG for K171A; GATCCTCCATTATTTGCGGCAGACCTGTCAGATT-ATGC for K190A; and GACCTGTCAGATTATGCTGCAGCATTCTAC-AGTGACAC for K197A. The underlined nucleotides cause the individual alanine substitutions. To generate each mutant clone, the pBluescript-L2 clone was the template for amplification. In brief, 50 ng of template was amplified with either 125 or 500 ng of each complementary primer pair under the following conditions: 1 cycle at 94 °C for 30 s followed by 16 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 DNA sequencing. Each mutant L2 sequence was subcloned into the pFastBac1-L2 plasmid using the unique restriction sites *Mlu*I (nucleotide 77) and *Nde*I (nucleotide 622). The identity of the subcloned region was confirmed by DNA sequencing. Two independent bacmid clones were isolated. The presence of the individual mutations in each clone was confirmed by sequencing the bacmid DNA. Each bacmid was used to produce recombinant baculovirus by transfection of *Sf*21 cells. Mutant protein generated from each recombinant baculovirus stock was expressed and purified as described for wild type λ 2. Automated DNA sequencing for all mutants was performed at the University of Wisconsin Biotechnology Center DNA Sequencing Facility (Madison, WI).

Cleveland Digestion of λ 2—An equal volume of 2 \times sample buffer (250 mM Tris, pH 6.8, 1% SDS, 20% glycerol, 0.002% bromphenol blue) was added to [³²P]GMP λ 2 protein (41). The mixture was boiled for 2 min and cooled to room temperature. One-third of the mixture was added to a one-tenth volume of virion buffer (10 mM Tris, pH 7.5, 10 mM MgCl₂, 150 mM NaCl) containing no added protease or 50 or 200 μ g/ml chymotrypsin (Sigma) in virion buffer, respectively. The reactions were placed at 37 °C for 30 min. One-tenth volumes of β -mercapthoethanol (Bio-Rad) and 20% SDS were added to each reaction. The reactions were boiled for 2 min and resolved on a 5–20% gradient SDS-polyacrylamide gel. The protein-associated radiolabel in the dried gel was visualized by PhosphorImager analysis.

Generation of Reovirus Core Particles—Cores of reovirus T3D were purified as described (14). In brief, virions at a concentration of 3 \times 10¹³ particles/ml were digested with 200 μ g/ml α -chymotrypsin for 1.5 h at 37 °C. The chymotrypsin was inactivated with 1 mM phenylmethyl sulfonyl fluoride. To purify cores, digests were loaded onto a preformed CsCl gradient (ρ = 1.30–1.55 g/cm³) and subjected to equilibrium centrifugation. The particles were dialyzed into virion buffer.

Sequence Comparisons—Sequences for the RNA guanylyltransferase proteins of viruses from the genera Rotavirus (VP3), Orbivirus (VP4), and Phytoreovirus (P5) were obtained from the sequence data bases, and the sequences from each genus were then separately aligned using the program Pileup in GCG 9.1 (Genetics Computer Group). Output from Pileup was displayed using the program Pretty in GCG 9.1 for ease of analysis.

RESULTS

λ 2 Expression in Insect Cells—A recombinant baculovirus (AcMNPV.T3DL2) was constructed to express T3D λ 2 in insect cells (*Sf*21 or High Five). A M_r 140,000 protein was expressed by this virus but not wild type baculovirus (AcMNPV) (data not shown). This protein comigrated with full-length λ 2 from reovirus particles and was specifically detected by immunoblot analysis using a monoclonal antibody (7F4) (37) that recognizes

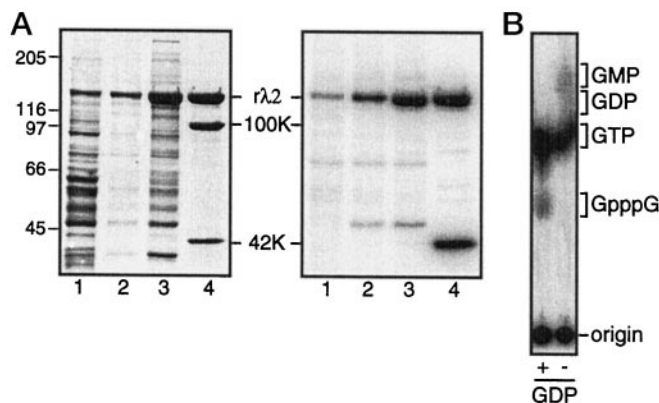


FIG. 1. Guanylyltransferase activity of λ 2. λ 2 was assayed for autoguanlylation (A) and GMP transfer (B) activities. A, a Tris-glycine-SDS-8% polyacrylamide gel was used to resolve the protein products of the autoguanlylation reaction. Lane 1, soluble lysate of High Five insect cells infected with AcMNPV.T3DL2. Lane 2, a λ 2-containing fraction eluted from the HiTrap Q column. Lane 3, λ 2 after further concentration by 40% ammonium sulfate precipitation. Lane 4, an another preparation of λ 2, comparable with that in lane 3 but stored at 4 °C for 5 weeks. The proteins were visualized by Coomassie Brilliant Blue staining (left panel), and the protein-associated radiolabel was visualized by PhosphorImager analysis (right panel). Full-length λ 2 (144 kDa) and M_r 100,000 (100K) and 42,000 (42K) cleavage fragments are indicated. The apparent molecular weights of the protein standards ($\times 10^3$) are indicated at the left. B, [³²P]GMP- λ 2 in 50 mM Tris, pH 8.0, 10 mM MgCl₂, and 2 mM DTT was incubated with 0.5 mM GDP (+) or an equal volume of water (–) for 30 min at room temperature. The reaction products were analyzed by thin layer chromatography followed by PhosphorImager analysis. Both samples contained a residual amount of [³²P]GTP that remained from the autoguanlylation reaction even after a Sephadex-50 sizing column was used to remove it. The markers GMP, GDP, GTP, and GpppG were located using a long wave UV lamp.

a carboxyl-terminal epitope of λ 2 (18). Based on these observations, we concluded that the M_r 140,000 protein is λ 2. Maximal expression of λ 2 was observed at 48–72 h postinfection with 1–2 plaque-forming units of AcMNPV.T3DL2/cell, and approximately half of the expressed λ 2 was soluble (data not shown).

λ 2 was purified by anion exchange chromatography of the soluble lysate using a HiTrap Q column (Amersham Pharmacia Biotech). Soluble lysate was loaded on the column in 50 mM Tris, pH 8.0, 200 mM NaCl buffer using fast protein liquid chromatography (Amersham Pharmacia Biotech). λ 2 eluted from the column between 425 and 490 mM NaCl. To remove additional protein contaminants and to concentrate λ 2, the eluted fractions were precipitated with ammonium sulfate. Since λ 2 in the eluted fractions precipitated between 30 and 40% (saturation at 0 °C) ammonium sulfate, protein contaminants were precipitated by pretreating the fractions with 20% ammonium sulfate. The λ 2 was then precipitated in the presence of 40% ammonium sulfate and resuspended in 50 mM Tris, pH 8.0. Although λ 2 can represent up to 95% of the total resuspended protein, Fig. 1A, lanes 2 and 3, illustrates the results of a typical purification. The oligomeric state of λ 2 was determined by sizing chromatography using a Superose 12 column (Amersham Pharmacia Biotech). λ 2 eluted between the β -amylase (M_r 200,000) and carbonic anhydrase (M_r 29,000) markers (Sigma), suggesting that it is mostly monomeric (data not shown), consistent with the state of λ 2 expressed in HeLa cells from a recombinant vaccinia virus (11).

λ 2 Has Guanylyltransferase Activity—The λ 2 guanylyltransferase acts via a covalent intermediate (9). λ 2 was assayed for autoguanlylation activity by incubating the protein with [³²P]GTP and detecting covalent labeling after SDS-PAGE and PhosphorImager analysis (Fig. 1A). The label was liberated by acid hydrolysis (data not shown), as expected for a

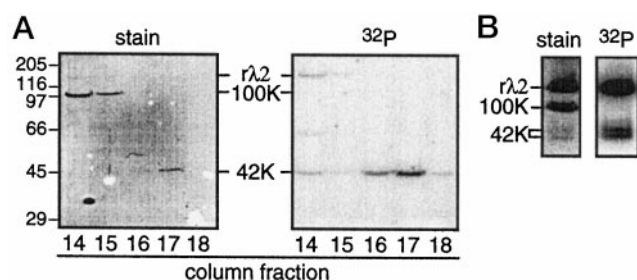


FIG. 2. Autoguanlylation activity of $r\lambda 2$ protein fragments generated by limited proteinase K digestion. A, $r\lambda 2$ and its proteolytic products were separated by sizing chromatography, autoguanlylated in the presence of [α - 32 P]GTP, and resolved on a Tris-glycine-SDS-10% polyacrylamide gel. The proteins were visualized by Coomassie Brilliant Blue staining (left panel), and the protein-associated radiolabel was visualized by PhosphorImager analysis (right panel). Protein containing the carboxyl-terminal portion of $\lambda 2$ was identified using monoclonal antibody 7F4 for immunoblot analysis of a parallel gel containing unlabeled protein (data not shown). The column fractions analyzed (fractions 14–18) are indicated below each panel. Full-length $r\lambda 2$ and the M_r 100,000 (100K) and 42,000 (42K) cleavage fragments are indicated. The apparent molecular weights of the protein standards ($\times 10^3$) are indicated at the left. B, proteinase K-digested $r\lambda 2$ was resolved on a Tris-glycine 10% polyacrylamide gel (native). Gel lanes containing the protein were excised and equilibrated with 50 mM Tris, pH 8.0, 10 mM $MgCl_2$, and 2 mM DTT for 15 min at room temperature. The excised lanes were then incubated with the same buffer containing 5 μ Ci of [α - 32 P]GTP for 30 min. Unbound radiolabel was removed by incubation with 50 mM Tris, pH 8.0, 10 mM EDTA with multiple changes of buffer. The protein was visualized by Coomassie Brilliant Blue staining (left panel). The protein-associated radiolabel was visualized by PhosphorImager analysis (right panel). The identity of the protein products indicated at the left was determined by cutting the individual protein bands from an unstained portion of the gel, boiling for 2 min in Laemmli loading buffer, and resolving on a Tris-glycine-SDS-10% polyacrylamide gel followed by Coomassie Brilliant Blue staining or PhosphorImager analysis. The M_r 42,000 fragment migrated as a single band on the denaturing gel (data not shown).

phosphoamide bond between GMP and the protein. Thus, the recombinant protein has autoguanlylation activity.

GMP transfer was analyzed by incubating GDP with [32 P]GMP- $r\lambda 2$, produced by the autoguanlylation reaction and purified from free GTP by a Sephadex G-50 sizing column (Amersham Pharmacia Biotech). The reaction products were resolved by thin layer chromatography and visualized by PhosphorImager analysis (Fig. 1B). In this experiment, a radiolabeled spot that co-migrated with GpppG was present only when the reaction contained GDP, indicating that $r\lambda 2$ can transfer its bound GMP to GDP. Thus, $r\lambda 2$ expressed in insect cells from recombinant baculovirus exhibits full guanylyltransferase activity with GDP as the acceptor, consistent with the observation that $r\lambda 2$ expressed in HeLa cells from a recombinant vaccinia virus has guanylyltransferase activity (11).

Localization of the Guanylyltransferase Domain by Limited Proteolysis—Previous work indicated that the carboxyl-terminal M_r 25,000 region of $\lambda 2$ was not required for guanylyltransferase activity (18). The observation in this study that purified $r\lambda 2$ undergoes proteolysis during storage to generate M_r 42,000 and 100,000 fragments (Fig. 1A) enabled further localization of the guanylyltransferase region. Although proteolysis of the purified protein with storage was most likely due to contaminating insect cell proteases, similar protein products could be generated in approximately equimolar amounts by controlled proteinase K treatment (Fig. 1A and data not shown). Autoguanlylation analysis of the cleavage products indicated that the M_r 42,000 fragment contains the site for the phosphoamide bond with GMP (Fig. 1A). The M_r 100,000 fragment contains the carboxyl-terminal epitope recognized by monoclonal antibody 7F4 (Fig. 2A), and its amino-terminal sequence, SDTP-SPVQWL, is identical to that of $\lambda 2$ beginning at serine 388 (15).

The predicted masses of the proteolytic fragments are 43,974 and 100,108 daltons, close to the apparent molecular weights based on SDS-PAGE. Thus, mild proteinase K treatment cleaves within a hypersensitive region near amino acid 388 of $r\lambda 2$. Similarly sized cleavage products were also detected after mild treatment with thermolysin or elastase (Ref. 14; data not shown). Because the amino terminus of virion-associated $\lambda 2$ is known to be blocked (42), amino-terminal sequencing of the M_r 42,000 protein was not attempted.

Two putative GTP-binding motifs (residues 893–899 and 1029–1032) that might be necessary for guanylyltransferase activity (15) are present in the M_r 100,000 fragment. To determine if the guanylyltransferase activity of $\lambda 2$ is dependent on these motifs or any other region of the M_r 100,000 protein, the M_r 42,000 and 100,000 fragments were separated using a Superose 12 gel filtration column (Fig. 2A). Individual fractions were then assayed for autoguanlylation activity, and the presence of the M_r 100,000 product in each fraction was assayed by immunoblot analysis. Fractions 16 and 17, containing the M_r 42,000 fragment without detectable M_r 100,000 fragment, retained autoguanlylation activity. This result was confirmed by *in situ* autoguanlylation of the M_r 42,000 fragment in a 10% native gel, which resolved the products of proteinase K proteolysis of $r\lambda 2$ (Fig. 2B). These observations indicated that the M_r 42,000 fragment alone is necessary and sufficient for autoguanlylation.

The low concentration of $\lambda 2$ fragments in the Superose 12 fractions made it difficult to analyze them for GMP transfer activity. Since the proteolytic products do not remain associated in solution (see above), the proteolyzed mixture was analyzed for GMP transfer activity instead of the individual fractions (Fig. 3). GDP was used as the GMP acceptor in this experiment, yielding GpppG as the product of GMP transfer. The proteolytic products transferred 98% of the GMP relative to untreated $\lambda 2$, indicating that the cleaved protein was as active at GMP transfer as intact $r\lambda 2$. Thus, the M_r 42,000 fragment appears to be sufficient for full guanylyltransferase activity with GDP as the acceptor.

Lysine 226 Is Not Necessary for $\lambda 2$ Autoguanlylation Activity—A mutated $\lambda 2$ protein with an alanine substitution at position 226 (K226A) was expressed in insect cells and was purified according to the same protocol used for wild type $\lambda 2$. The mutant protein exhibited autoguanlylation activity (Fig. 4A), with the amount of mutant-associated radiolabel being $13.6 \pm 2.6\%$ of wild type. Time course analysis showed that the mutant had a relative rate of autoguanlylation indistinguishable from wild type $r\lambda 2$ (Fig. 4B).

To confirm that the active site of the K226A mutant was located in the same approximate region as in wild type $r\lambda 2$, Cleveland digestion (40) was undertaken with α -chymotrypsin on [32 P]GMP-labeled mutant and wild type protein. In addition to the M_r 42,000 fragment generated upon protein storage, a larger, unstable M_r 56,000 fragment characteristic of both chymotrypsin and trypsin (data not shown) digestion is apparent. The fragmentation pattern is identical for the $r\lambda 2$ proteins (Fig. 5), indicating that GMP linkage occurs in the same region of the mutant and wild type proteins. The smallest detectable fragment was approximately M_r 13,000, roughly corresponding to 120 amino acids in size. Hydroxylamine cleavage (35, 43) was unsuccessful in generating defined fragments for further localization of the active site (data not shown).

Lysine 226 Is Not Necessary for $\lambda 2$ GMP Transfer Activity—To rule out the possibility that the K226A mutant can undergo autoguanlylation at an alternative site in a manner nonproductive for GMP transfer, the mutant $r\lambda 2$ protein was assayed for its ability to transfer [32 P]GMP to GTP, generating

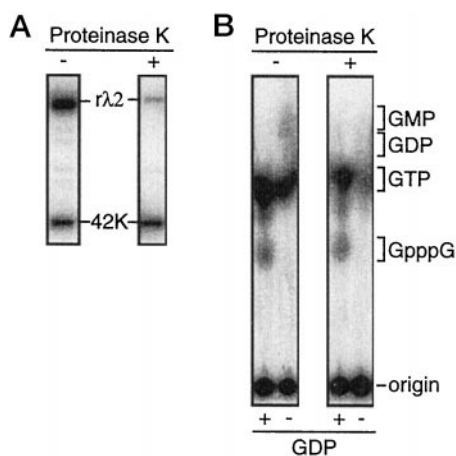


FIG. 3. GMP transfer activity of $r\lambda 2$ protein fragments generated by limited proteinase K digestion. Full-length $r\lambda 2$ was left untreated (-) or digested with proteinase K (+), after which it was subjected to autoguanlylation in the presence of [α - 32 P]GTP. *A*, the amount of radiolabeled $r\lambda 2$ and M_r 42,000 (42K) protein was determined by PhosphorImager analysis of 10 μ l of each purified fraction resolved on a Tris-glycine-SDS-10% polyacrylamide gel. 30% of the radiolabel in the untreated $r\lambda 2$ fraction was associated with the M_r 42,000 fragments, indicating that 30% of the full-length $r\lambda 2$ had been cleaved during storage, most likely by contaminating insect cell proteases. 90% of the radiolabel in the proteinase K-treated fraction was associated with the M_r 42,000 protein. The total amount of protein-associated radioactivity present in the proteinase K-treated fraction was 86% of the amount in the untreated fraction. *B*, each protein fraction in 50 mM Tris, pH 8.0, 10 mM $MgCl_2$, and 2 mM DTT was incubated with 0.5 mM GDP (+) or an equal volume of water (-) for 30 min at room temperature. The reaction products were analyzed by thin layer chromatography and quantitated by PhosphorImager analysis. The amount of radiolabeled GpppG produced by the proteolytic products was 88% of the amount produced by untreated $r\lambda 2$. Both samples contained a residual amount of [32 P]GTP that remained from the autoguanlylation reaction after a Sephadex-50 sizing column. The markers GMP, GDP, GTP, and GpppG were located using a long wave UV lamp.

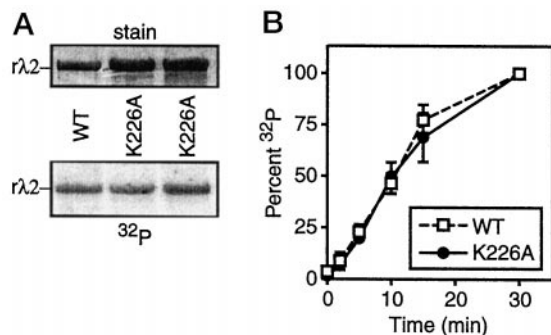


FIG. 4. Autoguanlylation activity of K226A mutant $r\lambda 2$. *A*, HiTrap Q-purified wild type (WT) and mutant (K226A) $r\lambda 2$, expressed from separate clones of recombinant baculovirus, were individually autoguanlylated in the presence of [α - 32 P]GTP. The proteins ($\lambda 2$) were resolved on a Tris-glycine-SDS-8% polyacrylamide gel and visualized by Coomassie Brilliant Blue staining (top panel). The protein-associated radiolabel was visualized by PhosphorImager analysis (bottom panel). The identity of the protein band was confirmed by immunoblot analysis using the monoclonal antibody 7F4 (data not shown). *B*, the rate of autoguanlylation for wild type (WT) and mutant (K226A) $r\lambda 2$ was determined by measuring the amount of protein-associated radiolabel versus incubation time. The autoguanlylation reaction was terminated at various times by the addition of 50 mM EDTA. The proteins were resolved in a Tris-glycine-SDS-8% polyacrylamide gel, and the protein-associated radiolabel was quantitated by PhosphorImager analysis. For comparison, the 30-min time point was set at 100% activity. Each experiment was done in triplicate.

GppppG (Fig. 6). By standardizing the amount of radiolabel in the GppppG spot to the amount of [32 P]GMP- $r\lambda 2$ in the reaction, the percentage of GMP transferred was calculated. The percent-

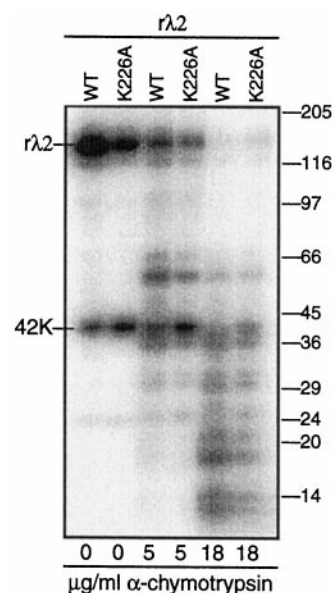


FIG. 5. Cleavage digestion of wild type and K226A mutant $r\lambda 2$. Wild type (WT) and mutant (K226A) [32 P]GMP- $r\lambda 2$ was denatured in the presence of 0.5% SDS and digested with various concentrations of chymotrypsin, as by Cleveland *et al.* (41). The proteolytic products were resolved on a 5–20% gradient SDS-polyacrylamide gel, and the protein-associated radiolabel was visualized by PhosphorImager analysis. The chymotrypsin concentrations are indicated below the gel. The full-length $r\lambda 2$ and M_r 42,000 (42K) cleavage fragment are indicated at the left, and the apparent molecular weights of the protein standards ($\times 10^3$) are indicated at the right. This gel is representative of two independent experiments.

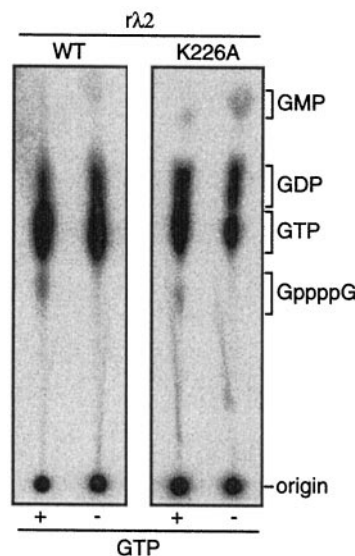


FIG. 6. GMP transfer activity of K226A mutant $r\lambda 2$. Wild type (WT) and mutant (K226A) [32 P]GMP- $r\lambda 2$ in 50 mM Tris, pH 8.0, 10 mM $MgCl_2$, and 2 mM DTT were separately incubated with 0.5 mM GTP (+) or an equal volume of water (-) for 30 min at room temperature. The reaction products were analyzed by thin layer chromatography and quantitated by PhosphorImager analysis. Both samples contained a residual amount of [32 P]GTP that remained from the autoguanlylation reaction after a Sephadex-50 sizing column. For the mutant samples, a small amount of [32 P]GMP is apparent due to the release of GMP from the autoguanlylated protein upon storage prior to sample resolution (11). The markers GMP, GDP, GTP, and GppppG were located using a long wave UV lamp.

age of GMP transfer by the mutant ($2.7 \pm 0.8\%$) was similar to that of $\lambda 2$ in reovirus cores ($3.4 \pm 1.1\%$). Thus, lysine 226 is neither necessary for $r\lambda 2$ GMP transfer activity, nor does it affect the efficiency of GMP transfer with GTP as the acceptor.

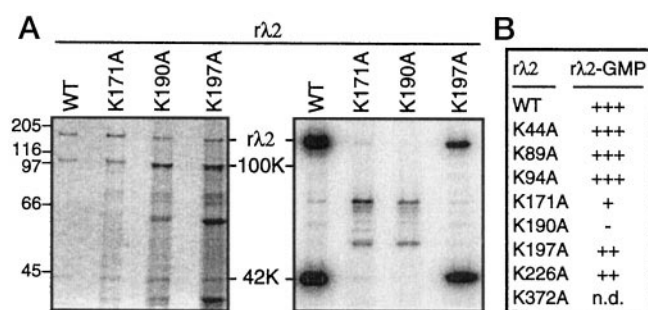


FIG. 7. Autoguanlylation activity of other $r\lambda$ 2 alanine substitution mutants. A, purified wild type (WT) or mutant (K171A, K190A, or K197A) $r\lambda$ 2 proteins were separately autoguanlylated in the presence of [α - 32 P]GTP. The proteins were then resolved on a Tris-glycine-SDS-8% polyacrylamide gel and visualized by Coomassie Brilliant Blue staining (left). The protein-associated radiolabel was visualized by PhosphorImager analysis. An overexposed image is shown at right for visualization of protein radiolabeled at low levels. Full-length $r\lambda$ 2 (144 kDa) and the M_r 100,000 (100K) and 42,000 (42K) cleavage fragments are indicated. The identities of the $r\lambda$ 2 and M_r 100,000 protein bands were confirmed by immunoblot analysis using monoclonal antibody 7F4 (data not shown). The identity of the M_r 42,000 protein band was confirmed by autoguanlylation activity. The radiolabeled M_r 50,000–60,000 protein bands represent co-purifying insect cell proteins (data not shown). They are more abundant in the mutant 171 and 190 samples, because approximately 3-fold more of the respective HiTrap Q fractions were loaded on the gel for these mutants due to lower $r\lambda$ 2 protein abundance. The apparent molecular weights of the protein standards ($\times 10^3$) are indicated at the left. B, autoguanlylation activity of each mutant was determined relative to wild type (WT) $r\lambda$ 2 protein, included on each gel as an internal standard. For each mutant, protein expressed from two separate baculovirus clones were analyzed. Protein abundance was quantitated by densitometry of the Coomassie Brilliant Blue-stained gel. Protein-associated radiolabel was quantitated by PhosphorImager analysis. The relative activity of each mutant is indicated by +++ (approximately wild type level of activity), ++ (2–10% activity), + (0.1–1% activity), and - (<0.1% activity). n.d., not done.

Identification of Lysine 190 as the Site for Autoguanlylation—The amino-terminal M_r 42,000 region contains seven lysine residues in addition to lysine 226 (15). None of these lysines are within the sequence context KX(D/N)G, the nucleotidyl transferase active-site motif (27–29). Since the autoguanlylation site must be contained within the M_r 42,000 region, the first six lysines were mutated individually to alanine, generating the mutants K44A, K89A, K94A, K171A, K190A, and K197A. Lysine 372 was not mutated, because it is close to the site of proteinase K cleavage, making it a less likely site for the phosphoamide bond; moreover, recent L2 sequence analyses indicate that lysines 44 and 372 are not conserved among λ 2 proteins of different mammalian reovirus isolates.²

Each purified mutant protein was assayed for autoguanlylation activity relative to wild type λ 2 (Fig. 7A). The most amino-terminal mutants (K44A, K89A, and K94A) had approximately wild type levels of activity (Fig. 7B). In contrast, mutant K171A had only $0.22 \pm 0.07\%$ and K197A had only $7.5 \pm 1.8\%$ of the wild type level, and K190A was essentially inactive ($0.06 \pm 0.02\%$). Based on the low signal-to-background ratio (1.10 ± 0.02) for K190A, the residual amount of radiolabel with that mutant appears most likely to be due to nonspecific association of 32 P with the abundant protein rather than due to true autoguanlylation activity. A similar residual level of radiolabel association was seen for vaccinia virus guanylyltransferase mutated at the active-site lysine (27).

One possible explanation for the loss of autoguanlylation activity of the K190A and other mutants was misfolding of the protein. This possibility is unlikely given the detection of both

the M_r 100,000 and 42,000 protein products after limited proteolysis (Fig. 7A, left). From this analysis, as well as from the fact that K190A was the only mutant to be essentially inactive at autoguanlylation, we conclude that lysine 190 is the site for GMP linkage.

DISCUSSION

Our $r\lambda$ 2 protein expressed in insect cells from a baculovirus vector has guanylyltransferase activity using either GDP or GTP as GMP acceptor. In contrast to published observations with $r\lambda$ 2 expressed in mammalian cells from a vaccinia virus vector (11), however, we were unsuccessful at demonstrating guanylyltransferase activity with baculovirus-expressed $r\lambda$ 2 using either 5'-diphosphorylated reovirus RNA or poly(A) RNA as acceptor (data not shown). Given this limitation in the activity of the baculovirus-expressed protein, which might be explained by a defect in allowing RNA molecules into the acceptor region of the enzyme, the conclusions we reach about amino acids required for GMP transfer must be considered as tentative with regard to RNA acceptors.

Unlike core-associated λ 2, $r\lambda$ 2 is hypersensitive to cleavage into complementary M_r 42,000 and 100,000 fragments that dissociate in solution. Retention of both autoguanlylation and GMP transfer activities by the M_r 42,000 fragment suggested that it is both necessary and sufficient to act as a guanylyltransferase. The M_r 42,000 region contains the active site originally localized by direct biochemical analysis to the region between amino acids 131 and 266 (10). Further indirect biochemical analysis in the previous study identified lysine 226 as the active-site residue that forms a phosphoamide bond with GMP (10).

The previously identified active-site motif 226 KPTNG in reovirus λ 2 is anomalous for two reasons. First it is not conserved outside the family *Reoviridae*. Of the known nucleotidyl transferases, at least 13 RNA guanylyltransferases, 16 DNA ligases, and one RNA ligase have the motif KXDG (27–29, 31). The two DNA ligases of African swine fever virus and the *S. cerevisiae* tRNA ligase have the motif KXNG (27, 28). The second reason is that insertion of additional residues may interfere with non-covalent bonding of one of the substrates. Based on the structure of the PBCV-1 guanylyltransferase (32, 33) and the ATP-dependent DNA ligase of bacteriophage T7 (44), the lysine in the active-site motif covalently binds GMP or AMP, respectively, and the remaining residues interact with the nucleotide acceptor in RNA for PBCV-1 or the ATP for T7 DNA ligase.

As was done for the vaccinia virus RNA guanylyltransferase (27), we chose to confirm the identity of the active-site lysine in reovirus λ 2 by site-directed mutagenesis. Since alanine substitution has been used to identify the active-site lysine of the RNA guanylyltransferase of *S. cerevisiae* (30) and baculovirus (31), we generated the λ 2 mutant K226A. Based on the demonstrable guanylyltransferase activity of this mutant, we concluded that lysine 226 is not the active-site residue. This conclusion has ramifications for the guanylyltransferases of the other members of the double-stranded RNA virus family *Reoviridae* and brings into question the functional significance of the similar motifs recently identified in the rotavirus and bluetongue virus RNA guanylyltransferases (34).

In addition to lysine 226, the M_r 42,000 region contains seven lysines at positions 44, 89, 94, 171, 190, 197, and 372 (15). A series of six alanine substitution mutants were generated to identify the active-site residue. Lysines 44, 89, and 94 are not necessary for activity, based on mutants K44A, K89A, and K94A having approximately wild type levels of autoguanlylation activity. Lysine 197, like lysine 226, is not necessary for activity but affects the level of autoguanlylation. These two residues may function to stabilize the structure of the active

² M. L. Nibert, T. J. Broering, L. A. Breun, A. M. McCutcheon, S. J. Harrison, and C. L. Luongo, manuscript in preparation.

site or the bound GTP, consistent with K226A having decreased autoguanlylation activity while maintaining wild type levels of GMP transfer. Lysine 171 is likely to be critical for substrate binding, since K171A showed less than 1% of wild type autoguanlylation activity. Based on the severe defect in autoguanlylation of the K190A mutant, lysine 190 is proposed to be necessary for activity and to be the active-site residue for formation of the phosphoamide bond.

Lysine 190 in the reovirus λ 2 protein is in a sequence context, KDLS, that lacks similarity with the consensus active-site motif (KXDG) of the well characterized class of eukaryotic and viral RNA guanylyltransferases (27–29). This lack of similarity suggests that the λ 2 active site may be formed via a novel protein fold. Sequences similar to the λ 2 KDLS sequence were found to be widely conserved among the RNA guanylyltransferases of other *Reoviridae* family members within the genera Rotavirus, Orbivirus, and Phytoreovirus (data not shown) and may suggest that the enzymes from these other viruses share a novel active-site motif with reovirus λ 2. Additionally, the consensus sequence KXDG is not strictly conserved among the RNA guanylyltransferases of any of these viruses, although KXXG motifs are conserved at two positions in the aligned orbivirus sequences and at one position in the aligned rotavirus sequences (data not shown). Our current hypothesis is that the reovirus RNA guanylyltransferase and perhaps also the RNA guanylyltransferases of other viruses in this family represent a distinct class of these enzymes. Clearly, biochemical and mutational analyses, as performed for mammalian reovirus λ 2 in this study, are required to identify the active-site residues in the RNA guanylyltransferases of the other *Reoviridae* family members.

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Identification of the Guanylyltransferase Region and Active Site in Reovirus mRNA Capping Protein $\lambda 2$

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