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Identification of the Guanylyltransferase Region and Active Site in Reovirus mRNA Capping Protein λ2*

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The 144-kDa λ2 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 autoguanylylated λ2-GMP intermediate. The smaller size of RNA capping guanylyltransferases from other organisms suggested that the λ2-associated guanylyltransferase would be only a part of this protein. Limited proteinase K digestion of baculovirus-expressed λ2 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 autoguanylylated λ2, mutation of lysine 226 to alanine caused only a partial reduction in guanylyltransferase activity at the autoguanylylation step. Alanine substitution for other lysines within the amino-terminal region of λ2 identified lysine 190 as necessary for autoguanylylation and lysine 171 as an important contributor to autoguanylylation. 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 ^{m7N}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 λ3 core protein (2, 3). Genetic and/or biochemical analyses indicate that the λ1 and μ2 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 λ1 (7). The λ2 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 λ2 (9, 10). Generation of this covalent bond (called "autoguanylylation" 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 λ2 pentameric spike (12, 13). Both of the methyltransferase activities appear to reside in λ2 as indicated by the finding that only the λ2 protein in cores is covalently labeled with the methyl donor *S*-adenosyl-L-methionine after incubation and UV cross-linking (14). Thus, λ2 is thought to catalyze the last three of the four reactions required for cap 1 formation on reovirus mRNA.

The 144-kDa λ2 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 σ1 in virions (17, 18). A multidomain structure for the λ2 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 λ2 protein is likely to be required for its RNA guanylyltransferase activity.

For most RNA guanylyltransferases, a K*X*DG 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 K*X*(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 λ2 protein is ^{226}KPTNG. 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 λ2. 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 λ2 residue that forms the phosphoamide bond with GMP used a combination of proteolysis and chemical cleavage of reovirus virions autoguanylylated with [^{32}P]GMP to show that a lysine residue in λ2 is the site of covalent linkage to GMP (10). Immunoblot analysis of proteolyzed, [^{32}P]GMP-labeled reovirus particles with λ2 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 autoguanylylation activity (27). To extend characterization of the reovirus RNA guanylyltransferase, we expressed recombinant λ2 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 λ2. 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 λ2 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 autoguanylylation 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′-GGAATTCCGCGGCCGCAAAATGGCGAACGTTTGGGG*C*GTG, and the reverse primer 5′-GGGGATCCGGGACAGTGAGTTACAGAGG. 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 λ2-encoding sequences of the T3D L2 gene (15). Both of these sequences have guanosines at positions 1838–1840 compared with thymidines in the sequence deposited in GenBank™ (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 λ2.

Isolation of Soluble λ2—*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 × *g* for 15 min. The supernatant was used to purify λ2 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% bromphenol 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.

Autoguanylylation Analysis—Protein was incubated with 5 μCi of [α-^{32}P]GTP (DuPont) in 50 mM Tris pH 8.0, 10 mM $MgCl_2$, 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 autoguanylylation, the ratio of radiolabel to protein abundance was determined relative to the wild type rλ2 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 phosphate cojugate 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 NH_4HCO_3 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 λ2 (rλ2)—rλ2, 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 μg/ml proteinase K (17 μg/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 an 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 rλ2—To generate mutant K226A, a two-primer method was utilized (39, 40). The L2-specific primer GTGCATTATGATGCGCCAACGAATGGTCATCACTATCA*C*CT*A*GGTACTTTG 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 *Sca*I 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

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[1] The abbreviations used are: T3D, type 3 Dearing; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; rλ2, recombinant λ2.

ABI Prism dye terminator cycle sequencing ready reaction kit (Applied Biosystems). The mutant L2 sequence was subcloned into the pFastBacI-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 λ2. 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; GTCGCAGCTGGTCGTGCGTATCTGCAGA-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 pFastBacI-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 λ2. Automated DNA sequencing for all mutants was performed at the University of Wisconsin Biotechnology Center DNA Sequencing Facility (Madison, WI).

Cleveland Digestion of λ2—An equal volume of 2× sample buffer (250 mM Tris, pH 6.8, 1% SDS, 20% glycerol, 0.002% bromphenol blue) was added to $[^{32}P]$GMP λ2 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 $MgCl_2$, 150 mM NaCl) containing no added protease or 50 or 200 μg/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 3×10^{13} particles/ml were digested with 200 μg/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/cm^3) 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

rλ2 Expression in Insect Cells—A recombinant baculovirus (AcMNPV.T3DL2) was constructed to express T3D λ2 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 λ2 from reovirus particles and was specifically detected by immunoblot analysis using a monoclonal antibody (7F4) (37) that recognizes a carboxyl-terminal epitope of λ2 (18). Based on these observations, we concluded that the M_r 140,000 protein is rλ2. Maximal expression of rλ2 was observed at 48–72 h postinfection with 1–2 plaque-forming units of AcMNPV.T3DL2/cell, and approximately half of the expressed rλ2 was soluble (data not shown).

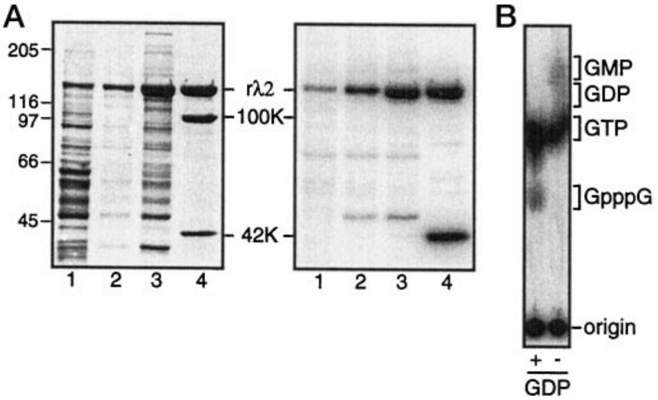


FIG. 1. **Guanylyltransferase activity of rλ2.** rλ2 was assayed for autoguanylylation (*A*) and GMP transfer (*B*) activities. *A*, a Tris-glycine-SDS-8% polyacrylamide gel was used to resolve the protein products of the autoguanylylation reaction. *Lane 1*, soluble lysate of High Five insect cells infected with AcMNPV.T3DL2. *Lane 2*, a rλ2-containing fraction eluted from the HiTrap Q column. *Lane 3*, rλ2 after further concentration by 40% ammonium sulfate precipitation. *Lane 4*, an another preparation of rλ2, 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 rλ2 (144 kDa) and M_r 100,000 (*100K*) and 42,000 (*42K*) cleavage fragments are indicated. The apparent molecular weights of the protein standards (× 10^3) are indicated at the *left*. *B*, $[^{32}P]$GMP-rλ2 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 followed by PhosphorImager analysis. Both samples contained a residual amount of $[^{32}P]$GTP that remained from the autoguanylylation 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.

rλ2 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). rλ2 eluted from the column between 425 and 490 mM NaCl. To remove additional protein contaminants and to concentrate rλ2, the eluted fractions were precipitated with ammonium sulfate. Since rλ2 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 rλ2 was then precipitated in the presence of 40% ammonium sulfate and resuspended in 50 mM Tris, pH 8.0. Although rλ2 can represent up to 95% of the total resuspended protein, Fig. 1*A*, *lanes 2* and *3*, illustrates the results of a typical purification. The oligomeric state of rλ2 was determined by sizing chromatography using a Superose 12 column (Amersham Pharmacia Biotech). rλ2 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 rλ2 expressed in HeLa cells from a recombinant vaccinia virus (11).

rλ2 Has Guanylyltransferase Activity—The λ2 guanylyltransferase acts via a covalent intermediate (9). rλ2 was assayed for autoguanylylation activity by incubating the protein with [α-^{32}P]GTP and detecting covalent labeling after SDS-PAGE and PhosphorImager analysis (Fig. 1*A*). The label was liberated by acid hydrolysis (data not shown), as expected for a

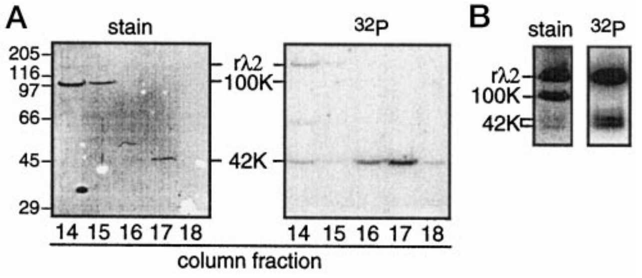


FIG. 2. **Autoguanylylation activity of rλ2 protein fragments generated by limited proteinase K digestion.** *A*, rλ2 and its proteolytic products were separated by sizing chromatography, autoguanylylated in the presence of [α-^{32}P]GTP, and resolved on a Tris-glycine-SDS-10% polyacrylamide gel. The 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 λ2 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 rλ2 and the M_r 100,000 (*100K*) and 42,000 (*42K*) cleavage fragments are indicated. The apparent molecular weights of the protein standards (× 10^3) are indicated at the *left*. *B*, proteinase K-digested rλ2 was resolved on a Tris-glycine 10% polyacrylamide gel (native). Gel lanes containing the protein were excised and equilibriated 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 μCi 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 autoguanylylation activity.

GMP transfer was analyzed by incubating GDP with [^{32}P]GMP-rλ2, produced by the autoguanylylation 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. 1*B*). In this experiment, a radiolabeled spot that co-migrated with GpppG was present only when the reaction contained GDP, indicating that rλ2 can transfer its bound GMP to GDP. Thus, rλ2 expressed in insect cells from recombinant baculovirus exhibits full guanylyltransferase activity with GDP as the acceptor, consistent with the observation that rλ2 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 λ2 was not required for guanylyltransferase activity (18). The observation in this study that purified rλ2 undergoes proteolysis during storage to generate M_r 42,000 and 100,000 fragments (Fig. 1*A*) 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. 1*A* and data not shown). Autoguanylylation analysis of the cleavage products indicated that the M_r 42,000 fragment contains the site for the phosphoamide bond with GMP (Fig. 1*A*). The M_r 100,000 fragment contains the carboxyl-terminal epitope recognized by monoclonal antibody 7F4 (Fig. 2*A*), and its amino-terminal sequence, SDTPSPVQWL, is identical to that of λ2 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 rλ2. 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 λ2 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 λ2 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. 2*A*). Individual fractions were then assayed for autoguanylylation 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 autoguanylylation activity. This result was confirmed by *in situ* autoguanylylation of the M_r 42,000 fragment in a 10% native gel, which resolved the products of proteinase K proteolysis of rλ2 (Fig. 2*B*). These observations indicated that the M_r 42,000 fragment alone is necessary and sufficient for autoguanylylation.

The low concentration of λ2 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 λ2, indicating that the cleaved protein was as active at GMP transfer as intact rλ2. 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 λ2 Autoguanylylation Activity—A mutated λ2 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 λ2. The mutant protein exhibited autoguanylylation activity (Fig. 4*A*), with the amount of mutant-associated radiolabel being 13.6 ± 2.6% of wild type. Time course analysis showed that the mutant had a relative rate of autoguanylylation indistinguishable from wild type rλ2 (Fig. 4*B*).

To confirm that the active site of the K226A mutant was located in the same approximate region as in wild type rλ2, 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 rλ2 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 λ2 GMP Transfer Activity—To rule out the possibility that the K226A mutant can undergo autoguanylylation at an alternative site in a manner nonproductive for GMP transfer, the mutant rλ2 protein was assayed for its ability to transfer [^{32}P]GMP to GTP, generating

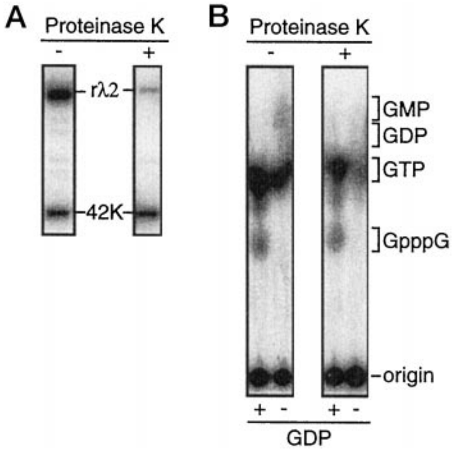


FIG. 3. **GMP transfer activity of rλ2 protein fragments generated by limited proteinase K digestion.** Full-length rλ2 was left untreated (−) or digested with proteinase K (+), after which it was subjected to autoguanylylation in the presence of [α-^{32}P]GTP. *A*, the amount of radiolabeled rλ2 and M_r 42,000 (*42K*) protein was determined by PhosphorImager analysis of 10 μl of each purified fraction resolved on a Tris-glycine-SDS-10% polyacrylamide gel. 30% of the radiolabel in the untreated rλ2 fraction was associated with the M_r 42,000 fragments, indicating that 30% of the full-length rλ2 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 rλ2. Both samples contained a residual amount of [^{32}P]GTP that remained from the autoguanylylation reaction after a Sephadex-50 sizing column. The markers GMP, GDP, GTP, and GpppG were located using a long wave UV lamp.

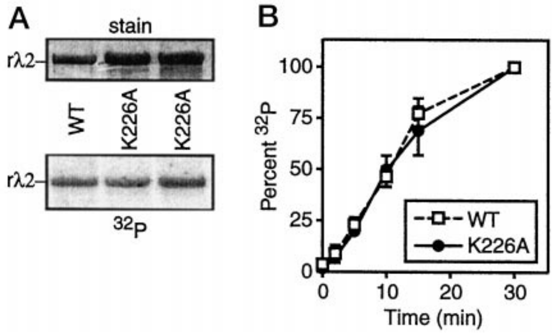


FIG. 4. **Autoguanylylation activity of K226A mutant rλ2.** *A*, HiTrap Q-purified wild type (*WT*) and mutant (K226A) rλ2, expressed from separate clones of recombinant baculovirus, were individually autoguanylylated in the presence of [α-^{32}P]GTP. The proteins (λ2) were resolved on a Tris-glycine-SDS-8% 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 autoguanylylation for wild type (*WT*) and mutant (K226A) rλ2 was determined by measuring the amount of protein-associated radiolabel *versus* incubation time. The autoguanylylation 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.

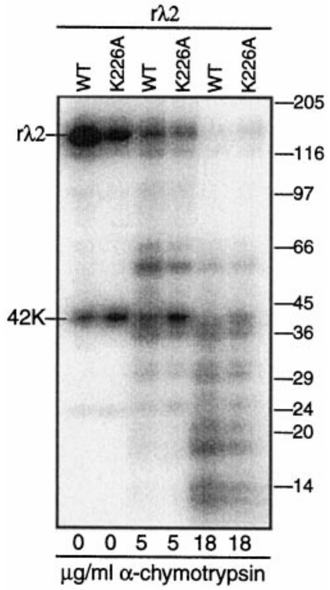


FIG. 5. **Cleveland digestion of wild type and K226A mutant rλ2.** Wild type (*WT*) and mutant (K226A) [^{32}P]GMP-rλ2 was denatured in the presence of 0.5% SDS and digested with various concentrations of chymotrypsin, as by Cleveland *et al.* (41). The proteolytic products were resolved on a 5–20% gradient SDS-polyacrylamide gel, and the protein-associated radiolabel was visualized by PhosphorImager analysis. The chymotrypsin concentrations are indicated *below* the gel. The full-length rλ2 and M_r 42,000 (*42K*) cleavage fragment are indicated at the *left*, and the apparent molecular weights of the protein standards (× 10^3) are indicated at the *right*. This gel is representative of two independent experiments.

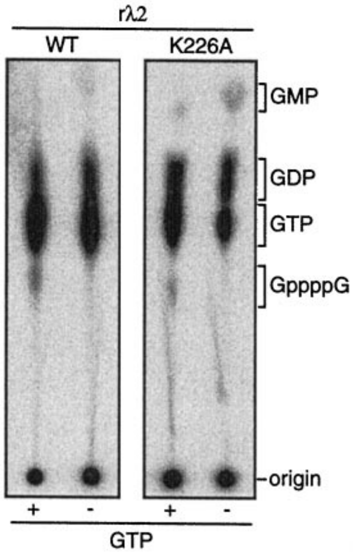
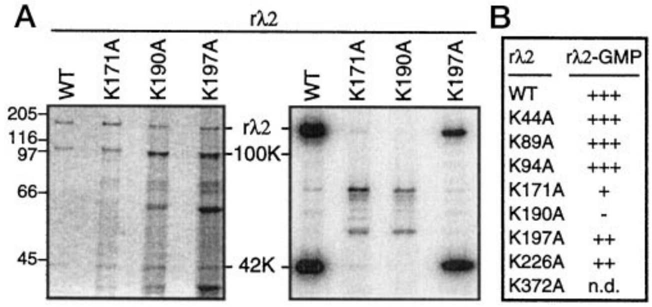


FIG. 6. **GMP transfer activity of K226A mutant rλ2.** Wild type (*WT*) and mutant (K226A) [^{32}P]GMP-rλ2 in 50 mM Tris, pH 8.0, 10 mM MgCl$_2$, and 2 mM DTT were separately incubated with 0.5 mM GTP (+) or an equal volume of water (−) for 30 min at room temperature. The reaction products were analyzed by thin layer chromatography and quantitated by PhosphorImager analysis. Both samples contained a residual amount of [^{32}P]GTP that remained from the autoguanylylation 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 autoguanylylated protein upon storage prior to sample resolution (11). The markers GMP, GDP, GTP, and GpppG were located using a long wave UV lamp.

GppppG (Fig. 6). By standardizing the amount of radiolabel in the GppppG spot to the amount of [^{32}P]GMP-rλ2 in the reaction, the percentage of GMP transferred was calculated. The percentage of GMP transfer by the mutant (2.7 ± 0.8%) was similar to that of λ2 in reovirus cores (3.4 ± 1.1%). Thus, lysine 226 is neither necessary for rλ2 GMP transfer activity, nor does it affect the efficiency of GMP transfer with GTP as the acceptor.



rλ2	rλ2-GMP
WT	+++
K44A	+++
K89A	+++
K94A	+++
K171A	+
K190A	-
K197A	++
K226A	++
K372A	n.d.

FIG. 7. **Autoguanylylation activity of other rλ2 alanine substitution mutants.** *A*, purified wild type (*WT*) or mutant (K171A, K190A, or K197A) rλ2 proteins were separately autoguanylylated 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 rλ2 (144 kDa) and the M_r 100,000 (*100K*) and 42,000 (*42K*) cleavage fragments are indicated. The identities of the rλ2 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 autoguanylylation 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 rλ2 protein abundance. The apparent molecular weights of the protein standards (× 10^3) are indicated at the *left*. *B*, autoguanylylation activity of each mutant was determined relative to wild type (*WT*) rλ2 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 Autoguanylylation—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 K*X*(D/N)G, the nucleotidyl transferase active-site motif (27–29). Since the autoguanylylation 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 λ2 proteins of different mammalian reovirus isolates.[2]

Each purified mutant protein was assayed for autoguanylylation activity relative to wild type λ2 (Fig. 7*A*). The most amino-terminal mutants (K44A, K89A, and K94A) had approximately wild type levels of activity (Fig. 7*B*). In contrast, mutant K171A had only 0.22 ± 0.07% and K197A had only 7.5 ± 1.8% of the wild type level, and K190A was essentially inactive (0.06 ± 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 autoguanylylation 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 autoguanylylation 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. 7*A*, *left*). From this analysis, as well as from the fact that K190A was the only mutant to be essentially inactive at autoguanylylation, we conclude that lysine 190 is the site for GMP linkage.

DISCUSSION

Our rλ2 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 rλ2 expressed in mammalian cells from a vaccinia virus vector (11), however, we were unsuccessful at demonstrating guanylyltransferase activity with baculovirus-expressed rλ2 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 λ2, rλ2 is hypersensitive to cleavage into complementary M_r 42,000 and 100,000 fragments that dissociate in solution. Retention of both autoguanylylation 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 λ2 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 K*X*DG (27–29, 31). The two DNA ligases of African swine fever virus and the *S. cerevisiae* tRNA ligase have the motif K*X*NG (27, 28). The second reason is that insertion of additional residues may interfere with noncovalent 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 λ2 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 λ2 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 autoguanylylation activity. Lysine 197, like lysine 226, is not necessary for activity but affects the level of autoguanylylation. These two residues may function to stabilize the structure of the active

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[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 autoguanylylation 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 autoguanylylation activity. Based on the severe defect in autoguanylylation 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 λ2 protein is in a sequence context, KDLS, that lacks similarity with the consensus active-site motif (K*X*DG) of the well characterized class of eukaryotic and viral RNA guanylyltransferases (27–29). This lack of similarity suggests that the λ2 active site may be formed via a novel protein fold. Sequences similar to the λ2 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 λ2. Additionally, the consensus sequence K*X*DG is not strictly conserved among the RNA guanylyltransferases of any of these viruses, although K*XX*G 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 λ2 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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