Characterization of a Baculovirus Enzyme with RNA Ligase, Polynucleotide 5′-Kinase, and Polynucleotide 3′-Phosphatase Activities*

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The end-healing and end-sealing steps of the phage T4-induced RNA restriction-repair pathway are performed by two separate enzymes, a bifunctional polynucleotide 5′-kinase/3′-phosphatase and an ATP-dependent RNA ligase. Here we show that a single trifunctional baculovirus enzyme, RNA ligase 1 (Rnl1), catalyzes the identical set of RNA repair reactions. Three enzymatic activities of baculovirus Rnl1 are organized in a modular fashion within a 694-amino acid polypeptide consisting of an autonomous N-terminal RNA-specific ligase domain, Rnl1-(1–385), and a C-terminal kinase-phosphatase domain, Rnl1-(394–694). The ligase domain is itself composed of two functional units. The N-terminal module Rnl1-(1–270) contains essential nucleotidyltransferase motifs I, IV, and V and suffices for both enzyme adenylation (step 1 of the ligation pathway) and phosphodiester bond formation at a preactivated RNA-adenylate end (step 3). The downstream module extending to residue 385 is required for ligation of a phosphorylated RNA substrate, suggesting that it is involved specifically in the second step of the end-joining pathway, the transfer of AMP from the ligase to the 5′-PO₄ end to form RNA-adenylate. The end-healing domain Rnl1-(394–694) consists of a proximal 5′-kinase module with an essential P-loop motif (404GSGKS408) and a distal 3′-phosphatase module with an essential acylphosphatase motif (560DLDGT564). Our findings have implications for the evolution of RNA repair systems and their potential roles in virus-host dynamics.

RNA ligases join 3′-OH and 5′-PO₄ RNA termini via a series of three nucleotidyl transfer steps. (i) RNA ligase reacts with ATP to form a covalent ligase-(lysyl-N)-AMP intermediate plus pyrophosphate, (ii) AMP is transferred from ligase-adenylate to the 5′-PO₄ RNA end to form an RNA-adenylate intermediate (AppRNA), and (iii) ligase catalyzes attack by an RNA 3′-OH on the RNA-adenylate to seal the two ends via a phosphodiester bond and release AMP (1–4). Bacteriophage T4 RNA ligase 1 (Rnl1) is the founding member of the RNA ligase family (1). The function of Rnl1 in vivo is to repair a break in the anticodon loop of Escherichia coli tRNA by triggering phage activation of a host-encoded anticodon nuclease, PrrC (5). Depletion of tRNA₅⁹⁸ by PrrC blocks phage protein synthesis and arrests the infection before it can spread. However, the bacteriophage T4 enzymes polynucleotide 5′-kinase/3′-phosphatase (Pnkp) and Rnl1 repair the broken tRNAs and thereby thwart the host defense mechanism.

T4 Rnl1 exemplifies a distinct subfamily of RNA ligases with a narrow phylogenetic distribution and signature structural elements that are essential for ligase activity (6). The Rnl1-like enzymes include the tRNA ligases of fungi and a putative RNA ligase/polynucleotide kinase encoded by the Autographa californica nucleopolyhedrovirus (AcNPV), the prototypal baculovirus. The predicted AcNPV ORF86 gene product is a 694-aa polypeptide composed of an N-terminal segment that resembles T4 Rnl1 and C-terminal segment homologous to T4 Pnkp (7, 8). Deletion of the ORF86 gene had no apparent effect on the ability of AcNPV to replicate in cultured Sf21 cells. Finner analysis revealed no abnormalities in viral protein production or virus-induced shut-off of the synthesis of host cell proteins during infection with the orf86Δ virus (8). The inessential nature of the putative RNA ligase for AcNPV replication is not surprising given that the majority of available sequenced baculovirus genomes do not encode an RNA ligase homolog. Indeed the only baculovirus RNA ligase homolog in GenBank™ is a putative Rnl/Pnkp of Rachiplusia ou multiple nucleopolyhedrovirus, which is >99% identical in primary structure to the AcNPV RNA ligase.

The inessentiality of the baculovirus ORF86 gene for replication in a laboratory cell culture model echoes the findings that the RNA repair activities of bacteriophage T4 Rnl1 and Pnkp are inessential in standard laboratory strains of E. coli used to study T4 replication, which lack the prr locus encoding the cellular anticodon nuclease. However, Rnl1 and Pnkp are essential for phage replication in host strains that carry the prr operon (9). Given the similarities between the putative RNA repair enzymes of T4 and AcNPV, it is sensible to speculate that some baculovirus strains may have acquired an RNA repair system to contend with an RNA-damaging antiviral pathway that exists in their natural ecological niche. Broadly speaking, RNA-based “immune” responses to viral infection are common in animal virology as exemplified by the key role of double-stranded RNA, RNase L, and the RNA-activated protein kinase PKR in the interferon response pathway (10). Animal viruses have devised diverse strategies to thwart RNA-triggered host restriction events, especially those involving PKR. Perhaps eukaryotic DNA viruses have taken a page from bacteriophage in this regard.

A major caveat to this line of thought is that there is no evidence as yet that the AcNPV ORF86 gene product possesses any of the activities imputed to it on the basis of amino acid sequence comparisons. Not only is it critical to know whether
the protein has strand-sealing or end-healing capacity, it is just as important to know the specificity of these activities, e.g. whether the baculovirus protein is capable of repairing RNA, DNA, or both. Is the primary structure similarity between the baculovirus and T4 proteins functionally relevant, and, if so, is it instructive with respect to enzyme mechanism and enzyme evolution? Here we address these questions via biochemical characterization of the AcNPV ligase and its component domains.

**EXPERIMENTAL PROCEDURES**

Rnl1 RNA Ligase—A DNA fragment containing the AcNPV ORF66 gene (hereafter RNL1) was amplified by PCR from a Bacioid DNA (Invitrogen) with oligonucleotide primers designed to introduce an NdeI restriction site at the translation start codon and a BamHI site 3' of the stop codon. The PCR product was digested with NdeI and BamHI and inserted into pET16b (Novagen) to generate the expression plasmid pET-RNL1. C-terminal truncation mutants RNL1-(1–394), RNL1-(1–280), and RNL1-(1–270) were generated by PCR amplification using antisense primers that introduced a translation stop codon in lieu of the codons for Pro<sup>394</sup>, Gly<sup>395</sup>, or Pro<sup>377</sup> and a BamHI restriction site immediately 3' of the new stop codon. The N-terminal deletion mutant RNL1-(394–694) was amplified by PCR using a sense strand primer that introduced a methionine codon and an NdeI site in lieu of the codons for Ser<sup>393</sup>. Alanine substitution mutations were introduced by PCR using the two-stage overlap extension method (11). The truncated and/or mutated RNL1 DNAs were digested with NdeI and BamHI and then inserted into pET16b. The inserts of the wild-type and mutant RNL1 plasmids were sequenced completely to exclude the acquisition of unwanted changes during amplification and cloning.

Wild-type and mutant pET-RNL1 plasmids were transformed into *E. coli* BL21(DE3). Cultures (1 liter) of *E. coli* BL21(DE3) pET-RNL1 were grown at 37 °C in Luria-Bertani medium containing 0.1 mg/ml ampicillin until the A<sub>600</sub> reached ~0.6. The cultures were chilled on ice for 30 min, adjusted to 0.1 mM isopropyl-<i>L</i>-thiogalactopyranoside and 2% ethanol, and then incubated at 17 °C for 16 h with continuous shaking. Cells were harvested by centrifugation, and the pellet was resuspended in 30 ml of buffer A (50 mM Tris acetate (pH 7.5), 0.5 mM DTT, 0.05 mM NaCl, 10% sucrose). Lysozyme, phenylmethylsulfonyl fluoride, and Triton X-100 were added to final concentrations of 1 mg/ml, 1 mM, and 0.1%, respectively. The lysates were sonicated to reduce viscosity, and insoluble material was removed by centrifugation. The soluble extracts were applied to 1 ml columns of nickel-nitrirotiacetic acid-agarose (Qiagen) that had been equilibrated with buffer A. The columns were washed with 8 ml of the same buffer and then eluted stepwise with 4-ml aliquots of 25, 50, and 200 mM imidazole in buffer B (50 mM Tris-HCl (pH 8.0), 0.5 mM NaCl, 0.5 mM MgCl<sub>2</sub>, 30 mM ATP, and Rnl1 as specified) were incubated for 30 min at 30 °C. The reactions were quenched by adding 5 μl of 80% formamide, 100 mM EDTA. The products were analyzed by electrophoresis through an 8% polyacrylamide gel containing 7M urea in 45 mM Tris borate, 1 mM EDTA. The ligations products were visualized by autoradiography of the gel and quantified with a PhosphorImager.

**Sealing of Formed RNA Adenylylation—** Reaction mixtures (10 μl) containing 50 mM Tris acetate (pH 7.0), 5 mM DTT, 5 mM MgCl<sub>2</sub>, 0.2 pmol of 5'-32P-labeled 18-mer RNA (pRNA), 50 μM ATP, and Rnl1 as specified were incubated for 15 min at 30 °C. The reactions were quenched by adding 5 μl of 80% formamide, 100 mM EDTA. The samples were analyzed by electrophoresis through an 18% polyacrylamide gel containing 7% urea in 45 mM Tris borate, 1 mM EDTA. The ligation products were visualized by autoradiography of the gel and quantified with a PhosphorImager.

**Glycerol Gradient Sedimentation—** Aliquots of Rnl1-(1–385) (150 μg), Rnl1-(1–385) (150 μg), and Rnl1-(394–694) (200 μg) were mixed with catalase (100 μg), BSA (100 μg), and cytochrome <i>c</i> (100 μg) in 300 μl of 50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 2 mM DTT, 2 mM EDTA, 0.1% Triton X-100, 10% glycerol. The mixtures were applied to a 4.8-ml 15–30% glycerol gradient containing 50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 2 mM EDTA, 2 mM DTT, 0.1% Triton X-100. The gradients were centrifuged in a Beckman SW50 rotor at 50,000 rpm for 16 h at 4 °C. Fractions (∼0.2 ml) were collected from the bottom of the tube. Aliquots (10 μl) of even numbered gradient fractions were analyzed by SDS-PAGE.

**Polynucleotide Kinase Assay—** Reaction mixtures (10 μl) containing 50 mM Tris acetate (pH 5.0), 5 mM DTT, 5 mM MgCl<sub>2</sub>, 1 mM [γ<sup>32</sup>P]ATP, 100 pmol of a synthetic 5'-hydroxyl-terminated oligoribonucleotide (5'-UUUAUCAUUUCCGACC), and Rnl1 as specified were incubated for 30 min at 30 °C. The reactions were quenched with formamide/EDTA, and the products were analyzed by electrophoresis through an 18% polyacrylamide gel containing 7% urea in 45 mM Tris borate, 1 mM EDTA. The radiolabeled oligonucleotide products were visualized by autoradiography of the gel and quantified with a PhosphorImager.

**Polynucleotide 3'-Phosphatase Assay—** Reaction mixtures (25 μl) containing 0.2 mM Tris-acetate (pH 6.0), 5 mM DTT, 5 mM MgCl<sub>2</sub>, and a synthetic 3'-phosphate-terminated oligodeoxyribonucleotide (dTTPA-ATCAATTGCGACCCp), and Rnl1-(394–694) as specified were incubated for 1 h at 30 °C. The reactions were quenched by adding 1 ml of malachite green reagent (purchased from BIORAD Research Laboratories, Plymouth Meeting, PA). Release of phosphate was determined by measuring A<sub>620</sub> and interpolating the value to a phosphate standard curve (13).

**RESULTS**

Recombinant Baculovirus Rnl1—The 694-aa baculovirus Rnl1 protein was produced in *E. coli* as a His<sub>10</sub>-tagged fusion and purified from the crude soluble bacterial extract by adsorption to nickel-agarose and elution with buffer containing imidazole. SDS-PAGE analysis showed that the protein preparation consisted of an ~80-kDa polypeptide corresponding to full-length His<sub>10</sub>-Rnl1 plus one of smaller polypeptides (60, 45, and 35–36 kDa) representing His<sub>10</sub>-tagged N-terminal proteolytic fragments (Fig. 1A, lane 1–694<sup>a</sup>). A preparation that consisted predominantly of the intact Rnl1 polypeptide was obtained by extraction of recombinant protein from the insoluble pellet with buffer containing 1 M NaCl, and subsequent nickel-agarose chromatography of the 1 M salt extract (Fig. 1A, lane 1–694<sup>a</sup>). The adenylytransferase activity of recombinant Rnl1-(1–694) (step 1 of the ligation pathway) was evinced by label transfer from 50 μM [α<sup>32</sup>P]ATP to the 83-kDa Rnl1 polypeptide to form a covalent enzyme-adenylate adduct (Fig. 1B). The 60-, 45-, and 35–36-kDa N-terminal fragments of Rnl1-(1–694<sup>a</sup>) were also active in adenylylate transfer. It was notable that the extent of autoadenylylation by the intact full-sized Rnl1 protein was lower than that of the 35–36-kDa fragments, taking into account the relative amounts of the polypeptides in the Rnl1 preparation (compare lanes 1–694<sup>a</sup> in Fig. 1, A and B). A partial explanation for this anomaly emerged from an analysis of the pH dependence of the adenylytransferase activity of the
Baculovirus RNA Ligase

Rnl1-(1–694) preparation. Whereas full-length Rnl1 and the 60- and 45-kDa fragments displayed optimal activity at pH 5.5–7.0, the 35–36-kDa fragments had a distinctive alkaline pH optimum with maximum adenylylation at pH 8.5–9.5 (Fig. 1C).

An Autonomous N-terminal Ligase Domain, Rnl1-(1–385)—Truncated proteins Rnl1-(1–385), Rnl1-(1–280), and Rnl1-(1–270) were produced in E. coli as Hisj0 fusions and purified from soluble bacterial extracts by nickel-agarose chromatography. SDS-PAGE analysis revealed the presence of serially truncated Rnl1 polypeptides of the expected size. The Rnl1-(1–385) preparation also contained a minor 35–36-kDa doublet that comigrated with the 35–36-kDa proteolytic fragments seen in the Rnl1-(1–694) preparation (Fig. 1A). The Rnl1-(1–280) polypeptide comigrated with the 35-kDa proteolytic fragment, whereas the Rnl1-(1–270) polypeptide migrated slightly faster than the 35-kDa proteolytic fragment (Fig. 1A). We surmise that baculovirus Rnl1 contains protease-accessible sites close to amino acids 280 and 385 that may correspond to domain boundaries.

The recombinant N-terminal proteins Rnl1-(1–385), Rnl1-(1–280), and Rnl1-(1–270) all had adenylyltransferase activity (Fig. 1B). A detailed characterization of the adenylylation reaction of Rnl1-(1–385) is shown in Fig. 2. Formation of the enzyme-adenylate complex at pH 6.5 required a divalent cation cofactor, which could be either magnesium or manganese (Fig. 2A). The extent of adenylylation was similar at the optimal cofactor concentrations of 10 mM magnesium or 2.5 mM manganese. Calcium was 10% as effective as manganese or magnesium in supporting ligase adenylylation; zinc was inactive (Fig. 2A). The extent of ligase-AMP formation increased with ATP concentration in the range of 0.2–3.1 mM and reached a plateau at 12.5 mM ATP (Fig. 2B). Half-maximal adenylylation was attained at ~3 mM ATP. The pH profile of the adenylylation reaction is shown in Fig. 2C. When the reactions were performed in the presence of 10 mM MgCl2, enzyme-adenylate formation was optimal at pH 6.5–7.0 and declined precipitously at alkaline pH. At 1.25 mM MgCl2, the bell-shaped pH profile was shifted 1 pH unit to the right such that activity was optimal at pH 7.5–8.0 (Fig. 2C). From the data in Fig. 2C, we estimated that 25% of the input Rnl1-(1–385) molecules were labeled in vitro with 32P-AMP.

Enzyme adenylylation is the first of three nucleotidyl transfer steps in the polynucleotide ligase pathway (4). Can the recombinant baculovirus Rnl1 protein seal RNA ends like its T4 homolog Rnl1? Reaction of Rnl1-(1–385) with a 5’-32P-labeled 18-mer RNA oligonucleotide and magnesium in the presence of 50 mM ATP resulted in the formation of a new radiolabeled RNA migrating ~1.5 nucleotides faster than the input 18-mer pRNA strand (Fig. 3A). This 32P-labeled product, which was resistant to alkaline phosphatase (not shown), is a covalently closed 18-mer circle formed by intramolecular ligation of the 5’-PO4 and 3’-OH termini of the substrate strand. The 18-mer circle formed by the baculovirus enzyme migrated identically to the ligated circles formed by T4 Rnl1 and T4 Rnl2 (not shown) (6, 14). The yield of circular RNA is plotted in Fig. 3B as a function of input Rnl1-(1–385). More than 95% of the input pRNA was sealed at saturating enzyme. From the slope of the titration curve, we calculated that 34 molecules of RNA were sealed per molecule of input enzyme (estimated turnover number of 1.1 min−1). Omission of ATP from the ligation reaction mixture reduced strand joining activity by a factor of 50 (Fig. 3B). The residual ATP-independent activity is attributable to the presence of preadenylated ligase in the enzyme preparation. From the titration curve of the ATP-independent circularization reaction, we calculated that 66% of the Rnl1-(1–385) preparation was catalytically competent ligase-AMP.

The pRNA ligation reaction of Rnl1-(1–385) was optimal in Tris acetate buffer at pH 7.0; activity declined to ~20% of the peak value at pH 9 and was abolished at pH ≤5 (Fig. 4A). RNA ligation required a divalent cation cofactor; activity was proportional to the magnesium concentration in the range of 0.6–2.5 mM, peaked at 2.5–10 mM, and declined at 20 mM magnesium (Fig. 4B). Manganese (5 mM) also supported ligase activity, whereas calcium, cadmium, cobalt, copper, and zinc did not (Fig. 4C). The extent of RNA ligation increased with ATP concentration from 1.3 to 33 mM and plateaued at 50 mM (Fig. 4D). A $K_m$ of 10 mM ATP was calculated from a double reciprocal plot of the data (not shown). Neither GTP, CTP, UTP, ATP, nor NAD$^+$ (at 50 mM concentration) satisfied the requirement of baculovirus RNA ligase for a high energy cofactor (Fig. 4E). Whereas Rnl1-(1–385) readily catalyzed circularization of an 18-mer pRNA strand, it was unable to seal a 5’-32P-labeled DNA oligonucleotide even under conditions of enzyme excess (not shown).

Shortening the pRNA substrate to 15, 12, or 9 nucleotides...
Reaction mixtures (10 μl) containing 50 mM Tris acetate (pH 6.5), 5 mM DTT, 5 mM MgCl₂, 50 μM ATP, 1 pmol of 5'-32P-labeled 18-mer RNA, and 0.15, 0.31, 0.625, 1.25, 2.5, or 5 ng of Rnl1-(1–385) (increasing from left to right) were incubated for 30 min at 30 °C. The extent of ligase-AMP formation is plotted as a function of ATP concentration.

**Fig. 2.** Characterization of the adenylyltransferase activity of Rnl1-(1–385). A, divalent cation dependence. Reaction mixtures (10 μl) containing 50 mM Tris acetate (pH 6.5), 5 mM DTT, 5 mM MgCl₂, 50 μM ATP, 1 pmol of 5'-32P-labeled 18-mer RNA, and 0.15, 0.31, 0.625, 1.25, 2.5, or 5 ng of Rnl1-(1–385) (increasing from left to right) were incubated for 30 min at 30 °C. Rnl1-(1–385) was omitted from a control reaction (lane —). The products were resolved by PAGE and visualized by autoradiography. The species corresponding to the input pRNA and Rnl1-(1–385) by interpolation to the internal standard phosphodiester standards. His10-Rnl1-(1–385) (calculated to be a 47-kDa polypeptide) sedimented as a single discrete peak on the “light” side of BSA (Fig. 6A). A plot of the S values of the three standards versus fraction number yielded a straight line (not shown), and an S value of 3.5 was determined for Rnl1-(1–385) by interpolation to the internal standard curve. We surmise that the ligase domain of Rnl1 is a monomer in solution.

An Autonomous N-terminal Adenylyltransferase Domain That Lacks pRNA Ligase Activity—Rnl1-(1–280) and Rnl1-(1–270) were tested in parallel with Rnl1-(1–385) for strand joining activity with a 5'-32P-labeled single-stranded 18-mer pRNA substrate. The instructive findings were that the Rnl1-(1–280) and Rnl1-(1–270) proteins were defective in the composite pRNA ligation reaction (Fig. 7A). The specific activities of Rnl1-(1–280) and Rnl1-(1–270) in pRNA ligation were 1.4 and 2% of the specific activity of Rnl1-(1–385). These results show that the segment of Rnl1 from aa 280–385 is essential for pRNA ligation but not for the isolated ligase adenylylation step (Fig. 1B). Characterization of the adenylyltransferase activity of Rnl1-(1–270) showed that the yield of enzyme-AMP was optimal at pH 7.5–9.5; activity declined to 15% of the peak value at pH 6.5 and was virtually nil at pH ≤5.0 (data not shown). Rnl1-(1–270)-AMP complex formation required a divalent cation cofactor. At pH 8.5, magnesium and manganese supported optimal activity at 5–10 and 0.6–5 mM concentrations, respectively. Calcium (5–10 mM) was 30% as effective as magnesium, and zinc was inactive (data not shown). Rnl1-(1–270)-AMP formation was proportional to ATP concentration in the range of 0.2–6.2 μM and plateaued at 25 μM ATP. Half-saturation was attained at ≈5 μM ATP (data not shown).

Rnl1-(1–270) was sediemented through a 15–30% glycerol gradient with catalase, BSA, and cytochrome c as internal standards. His10-Rnl1-(1–270) (calculated to be a 34-kDa polypeptide) sedimented as a single discrete peak midway between BSA and cytochrome c (Fig. 6B). An S value of 2.9 was calculated for Rnl1-(1–270), suggesting that the N-terminal adenylyltransferase domain of Rnl1 is a monomer.

**Fig. 3.** RNA ligase activity of Rnl1-(1–385). A, reaction mixtures (10 μl) containing 50 mM Tris acetate (pH 7.0), 5 mM DTT, 5 mM MgCl₂, 50 μM ATP, 1 pmol of 5'-32P-labeled 18-mer RNA, and 0.15, 0.31, 0.625, 1.25, 2.5, or 5 ng of Rnl1-(1–385) (increasing from left to right) were incubated for 30 min at 30 °C. Rnl1-(1–385) was omitted from a control reaction (lane —). The products were resolved by PAGE and visualized by autoradiography. The species corresponding to the input pRNA and Rnl1-(1–385) by interpolation to the internal standard phosphodiester standards. His10-Rnl1-(1–385) (calculated to be a 34-kDa polypeptide) sedimented as a single discrete peak midway between BSA and cytochrome c (Fig. 6B). An S value of 2.9 was calculated for Rnl1-(1–270), suggesting that the N-terminal adenylyltransferase domain of Rnl1 is a monomer.

Phosphodiester Formation at a Preadenylated RNA 5' End—A preadenylated RNA substrate (AppRNA) was used for analysis of step 3 of the ligation pathway in isolation. Formation of a phosphodiester at the activated 5' end by Rnl1-(1–385) was evinced by the appearance of a sealed circular RNA prod-
uct, the yield of which was proportional to the amount of input enzyme (Fig. 7B). 80–90% of the AppRNA substrate was converted to circular RNA at saturating enzyme. From the slope of the titration curve, we calculated that Rnl1-(1–385) circularized 4.8 fmol of AppRNA/fmol of input enzyme. The remarkable findings were that Rnl1-(1–280) and Rnl1-(1–270) were 35 and 31% as active in AppRNA ligation as Rnl1-(1–385) despite being only 1–2% as active as Rnl1-(1–385) in overall pRNA ligation. Thus, the functional groups required for catalysts of RNA phosphodiester bond formation reside within the N-terminal adenylyltransferase domain of Rnl1. We infer that the inability of the 270/280-aa N-terminal adenylyltransferase/AppRNA ligase domain to catalyze pRNA ligation stems from a specific requirement for the segment from 280–385 for the second step of the ligation pathway, transfer of AMP from the ligase to the $5'$$\text{PO}_4$ end to form AppRNA.

**Identification of Individual Essential Amino Acids in the Adenylyltransferase Domain**—The N-terminal adenylyltransferase domain of baculovirus Rnl1 resembles the homologous portion of bacteriophage T4 Rnl1 (Fig. 8D). Gait and colleagues (15, 16) mapped the site of covalent adenylation of T4 Rnl1 to Lys99 and demonstrated the essential role of Lys99 in catalysis by site-directed mutagenesis. The active site lysine of T4 Rnl1 is located within a conserved sequence element (K$\text{X}$DG, motif I) that defines a superfamily of covalent nucleotidyltransferases, which includes DNA ligases and mRNA capping enzymes (17). DNA ligases and capping enzymes have a common tertiary structure composed of five conserved motifs (I, III, IIIa, IV, and V) that contain amino acid functional groups responsible for nucleotide binding and catalysis (18–23). Perusal of the aligned primary structures of T4 and baculovirus Rnl1 highlights potential counterparts in Rnl1 of nucleotidyltransferase motifs IV (EG$\phi$H/$\phi$H/$\phi$H/$\phi$H) and V ($\phi$K$\phi$K$\phi$H$\phi$K where $\phi$ is a hydrophobic side chain) (Fig. 8D). To delineate whether any of the conserved amino acids are functionally relevant for baculovirus Rnl1, we performed an alanine scan of five positions of the ligase domain.
The targeted residues included Lys103 and Asp105 in motif I, Glu238 in motif IV, and Lys252 and Lys254 in motif V. The five mutants were expressed in bacteria and purified from soluble lysates by nickel-agarose chromatography (Fig. 8A). The proteins were assayed for ATP-dependent pRNA ligation and autoadenylylation as a function of increasing enzyme concentration, and the specific activities were normalized to that of wild-type Rnl1-(1–385). Mutational effects on activity are summarized in Fig. 8B. We found that the motif I mutant K103A was catalytically inert in overall pRNA ligation and in the isolated step 1 reaction. This result is consistent with the prediction that the motif I lysine is the active site nucleophile for AMP transfer. The motif IV mutant E238A and the motif V mutants K252A and K254A were also effectively inert in overall pRNA ligation and severely defective in ligase adenylylation (thereby explaining their inability to perform the composite pRNA ligation reaction).

The D105A mutant was 0.9% as active as wild-type enzyme in overall pRNA ligation, although it retained appreciable enzyme adenylylation activity (15% of the wild-type value). The inference from this result is that Asp105 plays a critical role in downstream steps of the RNA ligation pathway. We examined this issue via kinetic analysis of pRNA ligation by the D105A mutant under conditions of enzyme excess (Fig. 8C). The predominant product at all times up to 30 min was a novel species migrating 1 nucleotide step slower than the input 18-mer RNA. This species corresponds to the RNA-adenylate intermediate (AppRNA), which is normally not detected during the RNA ligation reaction of wild-type Rnl1-(1–385). AppRNA was visible at 10 s and accumulated steadily from 10 s to 15 min, concomitant with decay of the 18-mer pRNA substrate. AppRNA comprised 62% of the total RNA at the 15-min time point. Ligated circular RNA accumulated slowly at late times to an extent of 33% of the total labeled RNA. These findings provide kinetic evidence that the strand-joining reaction of baculovirus RNA ligase proceeds through an RNA-adenylate intermediate and that the D105A mutation selectively impacts the sealing step (step 3) of the pathway.

The mutant proteins were also tested for activity in ligating the preadenylated AppRNA substrate. All of the mutations resulted in isolated step 3 defects with specific activities in the range of 2–11% of the wild-type value (Fig. 8B). Taken together, these results highlight the importance of conserved residues in motifs I, IV, and V and identify baculovirus Rnl1...
as a member of the ligase/capping enzyme nucleotidyltransferase superfamily.

ATP-dependent DNA ligases and GTP-dependent mRNA capping enzymes contain an additional catalytic motif (motif VI) located 40–120 aa downstream of motif V (24–26). Inspection of the sequence of the baculovirus RNA ligase domain downstream of its motif V highlighted a decapeptide element \(336\text{WKCLAYRCRK}345\) that resembles motif VI of \(Chlorella\) virus capping enzyme (WKYIQGRSDK) and \(Schizosaccharomyces\) pombe capping enzyme (WRFLRFRDDK) (18). The arginine and lysine residues of the RXXX peptide at the distal end of motif VI are essential for capping enzyme guanylyltransferase activity (24, 26). To evaluate the functional relevance of this local sequence similarity, we replaced residues Lys337, Arg342, Arg344, and Lys345 of Rnl1-(1–385) with alanine, purified the recombinant His\(^{10}\)-Rnl1-(1–385)-Ala mutants in parallel with wild-type Rnl1-(1–385), and assayed them for pRNA ligase and adenylyltransferase activities. The specific activities in pRNA ligation in the presence of ATP were calculated from the slopes of the titration curves and normalized to the wild-type values (defined as 100%): K337A, 160%; R342A, 160%; R344A, 30%; and K345A, 160%. The normalized adenylyltransferase activities determined from the titration curves were: K337A, 170%; R342A, 160%; R344A, 61%; and K345A, 200%. Note that the variations in adenylyltransferase specific activity correlated with the differences in the levels of preadenylated ligase in the recombinant protein preparations, which were determined by titration of pRNA ligation activity in the absence of ATP (not shown). The fractions of Rnl1-(1–385)-AMP in the preparations were as follows: wild type, 82% preadenylated; K337A, 70%; R342A, 71%; R344A, 89%; and K345A, 63%. These results indicate that the Lys337, Arg342, and Lys345 side
chains do not play a role in Rnl1 function in vitro. Moreover, as Arg^{342} and Lys^{345} are the residues that would correspond to the essential Arg and Lys side chains of motif VI of the capping enzymes, we surmise that the similarity of the 336WKCLAY-RCRK^{345} peptide to motif VI is spurious. The modest effect of the R344A mutation on pRNA ligation suggests that Arg 344 is not directly involved in catalysis.

An Autonomous C-terminal Kinase-Phosphatase Domain, Rnl1-(394–694)—The primary structure of the C-terminal portion of baculovirus RNA ligase resembles that of T4 Pnkp (Fig. 9A). The 301-aa T4 Pnkp polypeptide consists of an N-terminal polynucleotide 5'-kinase domain and a C-terminal polynucleotide 3'-phosphatase domain (27, 28). The 5'-kinase domain of T4 Pnkp is a member of the classical P-loop

**Fig. 9.** The C-terminal kinase-phosphate domain of baculovirus Rnl1. A, the amino acid sequence of AcNPV Rnl1 from residues 394 to 676 is aligned to the sequence of T4 Pnkp from residues 2 to 286. Gaps are denoted by dashes (-). Positions of side chain identity/similarity are indicated by dots (•). The defining motifs of the kinase (GSGKS) and phosphatase (DXXDK) active sites are highlighted in shaded boxes. B, aliquots (4 µg) of the nickel-agarose preparations of full-length wild-type Rnl1, wild-type Rnl1-(394–694), and the K407A and D560A mutants of Rnl1-(394–694) were analyzed by SDS-PAGE. The Coomassie Blue-stained gel is shown. The positions and sizes (kDa) of marker polypeptides are indicated on the left. C, reaction mixtures (10 µl) containing 50 mM buffer (either Tris-HCl, pH 7.5, or Tris acetate, pH 5.0, as specified), 5 mM DTT, 5 mM MgCl₂, 1 µM [γ³²P]ATP, 100 pmol of 5'-hydroxyl-terminated 18-mer oligoribonucleotide, and 200 ng of either full-length wild-type Rnl1, wild-type Rnl1-(394–694), or mutated versions of Rnl1-(394–694) were incubated for 30 min at 30 °C. The products were analyzed by PAGE and visualized by autoradiography. The phosphorylated 18-mer RNA (pRNA) and ligated 18-mer RNA circle are indicated by arrows on the left. D, reaction mixtures (10 µl) containing 50 mM Tris acetate (pH 5.0), 5 mM DTT, 5 mM MgCl₂, 1 µM [γ³²P]ATP, 1 µg of wild-type Rnl1-(394–694), and 100 pmol of 5'-hydroxyl-terminated 18-mer, 15-mer, 12-mer, or 9-mer oligoribonucleotides as specified were incubated for 30 min at 30 °C. The products were analyzed by PAGE and visualized by autoradiography. WT, wild type.
phosphotransferase superfamily defined by a GXGK(S/T) motif that coordinates the β phosphate of the NTP substrate (29, 30). The Ψ'-phosphatase domain of T4 Pnkp belongs to the DDXXT family of phosphotransferases that act via a covalent acyl-phosphoeyzyme intermediate in which the phosphate is linked to the first aspartate in the DDXXT motif (28–30). Both catalytic motifs are conserved in the C-terminal portion of the baculovirus Rnl1 protein as 404GSGKS408 and 560DLDGT564, respectively (Fig. 9B). The 5'-polynucleotide kinase activity of wild-type Rnl1-(394–694) was demonstrated by the transfer of [γ-32P]P from 1 mM [γ-32P]ATP to the 5'-OH terminus of an 18-mer RNA oligonucleotide to form a 5'-32P-labeled RNA product (pRNA) that was resolved from free ATP by polyacrylamide gel electrophoresis (Fig. 9C). Shortening the 5'-OH RNA substrate to 15, 12, or 9 nucleotides did not diminish the 5'-kinase activity of Rnl1-(394–694) (Fig. 9D). The K407A mutation in the P-loop completely abolished 5'-kinase activity, whereas the D560A mutation in the phosphatase signature motif had no effect (Fig. 9C). Rnl1-(394–694) also used [γ-32P]GTP as the phosphate donor in the RNA 5'-kinase reaction; the GTP-dependent RNA kinase activity was abolished by the K407A mutation (data not shown). These findings confirm that the observed polynucleotide kinase activity is intrinsic to the Rnl1-(394–694) protein and implicate the P-loop lysine in catalysis of phosphoryl transfer.

The full-length baculovirus Rnl1 protein also catalyzed 5'-phosphorylation of the 18-mer 5'-OH RNA substrate; however, the majority of the 32P-labeled product formed by Rnl1 at pH 7.5 migrated faster than the 18-mer pRNA strand generated by the isolated C-terminal domain (Fig. 9C). This faster species corresponds to a ligated 18-mer RNA circle; an identical species was generated when the unlabeled 18-mer 5'-OH RNA substrate was incubated with [γ-32P]ATP, T4 Pnkp, and T4 Rnl1 (not shown). Thus, the product formed by the C-terminal kinase domain of Rnl1 is efficiently circularized by the N-terminal ligase domain. Further evidence that the ligase domain is responsible for the conversion of newly labeled pRNA to circular RNA emerged when we analyzed the kinase reaction products formed at pH 5.0 (Fig. 9C). The N-terminal ligase component of Rnl1 is virtually inactive at pH 5.0 as shown above in Fig. 4A. In contrast, the C-terminal 5'-kinase component remains active at pH 5.0 as seen in the right panel of Fig. 9C. Inhibition of the ligase activity at pH 5.0 results in production of linear pRNA by full-length Rnl1 instead of the circular RNA seen at pH 7.5 (Fig. 9C). Rnl1-(394–694) and full-length Rnl1 also catalyzed transfer of [32P]P from [γ-32P]ATP to the 5'-OH terminus of a DNA oligonucleotide (not shown). The notable finding was that full-length Rnl1 was unable to circularize the 5'-PO4-terminated 18-mer oligodeoxyribonucleotide product of the kinase reaction (not shown).

The kinase reaction of Rnl1-(394–694) was characterized in detail using an 18-mer 5'-OH RNA substrate. Kinase activity was optimal from pH 4.5 to 7.0 in 50 mM Tris acetate buffer, declined to half of the peak value at pH 4.0, and was abolished at pH 3.0 (data not shown). Activity declined gradually at alkaline pH, e.g. to one-fourth of the peak value at pH 50 mM Tris-HCl, pH 9.0 (data not shown). The divalent cation requirement for the kinase reaction was satisfied by either manganese or magnesium in the range of 1.25–5 mM concentration (not shown). Kinase activity at pH 5.0 in 2 mM MgCl2 increased with ATP concentration in the range of 0.03–0.3 mM and reached a plateau at 0.6–5 mM ATP (Fig. 9C). From the slope of the turnover number of ~1.4 min⁻¹ (not shown).

The polynucleotide 3'-phosphatase activity of Rnl1-(394–694) was demonstrated by the release of P1 from a 5'-OH, 3'-PO4-terminated 18-mer oligodeoxyribonucleotide (Fig. 10). The polynucleotide 3'-phosphatase activity was optimal at pH 6.0 and declined sharply as the pH was either lowered to ≤5 or raised to ≥8.0 (Fig. 10A). The reaction required a divalent cation cofactor; activity was proportional to magnesium concentration in the range of 0–0.3 mM and reached a plateau at 0.6–5 mM (Fig. 10B). Phosphate release was proportional to Rnl1-(394–694) concentration (Fig. 10C); from the slope of the titration curve, we calculated a specific activity of 190 pmol of P1 formed/pmol of enzyme, corresponding to a turnover number of ~3.1 min⁻¹. The amount of P1 generated at saturating Rnl1-(394–694) was 90% of the amount released by incubation of the substrate with alkaline phosphatase, an enzyme that hydrolyzes phosphomonoesters at the termini of polynucleotides but not the internucleotide phosphodiester. The fact that Rnl1-

![Figure 10. Polynucleotide 3'-phosphatase activity of Rnl1-(394–694)](image-url)

A. pH dependence. Reaction mixtures (25 μl) containing 50 mM buffer (either Tris acetate, pH 4.5, 5.0, 5.5, 6.0, 6.5, or 7.0, or Tris-HCl, pH 7.5, 8.0, 8.5, 9.0, or 9.5), 5 mM DTT, 10 mM MgCl2, 3 nmol of 3'-phosphate-terminated 18-mer DNA, and 200 ng of Rnl1-(394–694), and MgCl2 as specified were incubated for 1 h at 30 °C. B. magnesium dependence. Reaction mixtures (25 μl) containing 50 mM Tris acetate (pH 6.0), 5 mM DTT, 5 mM MgCl2, 3 nmol of 3'-phosphate-terminated 18-mer DNA, and either wild-type Rnl1-(394–694) or the K407A or D560A mutants as specified were incubated for 1 h at 30 °C. The extent of P1 release is plotted as a function of input enzyme. WT, wild type.
(394–694) failed to release P from a 5′-OH, 3′-OH-terminated 18-mer oligonucleotide (data not shown) confirmed that only the 3′-PO4 was removed, i.e. that Rnl1-(394–694) did not release any phosphate from internucleotide phosphodiester. The D560A mutation in the DXXD motif abolished the phosphatase activity of Rnl1-(394–694), whereas the K407A mutation in the P-loop had no effect (Fig. 10C). These findings confirm that the observed polynucleotide 3′-phosphatase activity is intrinsic to the Rnl1-(394–694) protein and implicate the Asp560 in the DLDGT motif as the active site nucleophile in phospho-

dry transfer.

Sedimentation Analysis of the Kinase-Phosphatase Domain—The active site motifs of the kinase-phosphatase domain of baculovirus Rnl1 are similar to those of the bifunctional T4 enzyme Pnkp. T4 Pnkp has a distinctive homotetrameric quaternary structure formed via pairs of phosphatase-phosphatase and kinase-kinase homodimer interfaces (27–30). Is this quaternary structure shared by the baculovirus kinase phosphatase? We examined the native size of Rnl1-(394–694) by glycerol gradient sedimentation with internal standards (Fig. 11). The 38-kDa Rnl1-(394–694) polypeptide cosedimented with BSA. Interpolation to a standard curve yielded an apparent S value of 4.3. The gradient fractions were assayed for kinase and phosphatase activity; both activity profiles peaked in fraction 18 coincident with the peak of the Rnl1-(394–694) polypeptide. These results are consistent with a homodimeric structure, although the resolution of the gradient does not exclude a monomeric native structure or a mixture of monomer and dimer. The key point is that there was no peak of the Rnl1-(394–694) protein or activity sedimenting at a position between BSA and catalase that would be consistent with a 150-kDa tetrameric structure akin to that of T4 Pnkp. Thus, the T4 and baculovirus enzymes differ with respect to quaternary structure.

DISCUSSION

The end-healing and end-sealing steps of the phage T4-encoded tRNA repair pathway are performed by two separate enzymes, a bifunctional polynucleotide kinase/phosphatase (Pnkp) and an ATP-dependent RNA ligase (Rnl1). Here we show that a single trifunctional baculovirus enzyme Rnl1 catalyzes the same repertoire of RNA repair reactions. Three enzymatic activities of baculovirus Rnl1 are organized in a modular fashion within a 694-aa polypeptide. Gene fusion is the most plausible scenario for the genesis of the trifunctional baculovirus ligase given the existence in other cellular and viral niches of free standing adenylyltransferase/ligase, 5′-kinase/3′-phosphatase, and 3′-phosphatase enzymes that resemble the baculovirus Rnl1 domains. The physical order of the active sites within the primary structure of baculovirus Rnl1, H2N-(adenylyltransferase/ligase)-(kinase)-(phosphatase)-COOH, echoes the domain organization of the 827-aa yeast tRNA ligase, which consist of an N-terminal ligase domain, a central kinase domain, and a C-terminal 2′,3′-cyclic phosphodiesterase domain (31–33). Whereas the ligase and kinase modules of yeast tRNA ligase are homologous to the T4 and baculovirus enzymes, the cyclic phosphodiester-
The biochemical characterization of the ligase domain of AcNPV Rnl1 reveals both similarities and differences with respect to other covalent nucleotidyltransferase family members. We report here that conserved residues in motifs I (Lys 

[103]) IV (Glu 

[238]), and V (Lys 

[252] and Lys 

[254]) are essential for the overall RNA ligase activity and the ligase adenyllylation step. The corresponding side chains were shown previously to be essential for the activities of T4 RNA ligase 1 (6) and yeast mRNA capping enzymes, and T4 RNA ligase 2 (12, 14), which belongs to a different RNA ligase family than the Rnl1-like enzymes. The lack of a stringent requirement for the motif I residue Asp 

[105] in the first nucleotidyl transfer step of the baculovirus RNA ligase reaction is in accord with mutational data for DNA ligase (21, 36–38), mRNA capping enzyme (39), T4 RNA ligases 1 and 2 (14, 16), and yeast tRNA ligase (33).

We show that the ligase domain Rnl1-(1–385) is itself a composite of two functional units. The N-terminal module Rnl1-(1–270) contains motifs I, IV, and V and suffices for enzyme adenyllylation. (In this respect, baculovirus Rnl1 clearly differs from mRNA capping enzymes and Chlorella virus DNA ligase, which require an OB-fold domain C-terminal to motif V to catalyze enzyme guanylylation and adenyllylation, respectively (25, 26).) Rnl1-(1–270) also suffices for phosphodiester bond formation at a preactivated AppRNA end, indicating that the recognition of the 3′-OH terminus and catalysis of its attack on AppRNA is mediated by amino acids within the 270-aa N-terminal module. The C-terminal module extending to residue 385 is required for ligation of a pRNA substrate; we presume that the C-terminal module is required specifically for the second step of the ligation pathway entailing the transfer of AMP from the ligase to the 5′-PO4 end to form AppRNA.

The C-terminal segment Rnl1-(394–694) is a bifunctional 5′-OH-polynucleotide kinase/polynucleotide 3′-phosphatase domain that resembles T4 Pnpk with respect to the modular organization of its two catalytic sites and its general biochemical properties (27–30, 40–43). The kinase component transfers the γ phosphate from either ATP or GTP to the 5′ end of RNA or DNA oligonucleotides. The reaction depends on a divalent cation cofactor and is abolished by mutation of the conserved P-loop lysine that is also essential for the kinase activity of T4 Pnpk. The phosphatase component of Rnl1-(394–694) hydrolyzes a 3′-PO4 from a DNA oligonucleotide. The phosphatase reaction is metal-dependent and abolished by mutation of the Asp nucleophile of the signature DXDXT motif of the acylphosphatase superfamily. Although neither the kinase nor the phosphatase components of baculovirus Rnl1 are specific for RNA end modification, the fact that the covalently linked ligase component is RNA-specific makes it likely that baculovirus Rnl1 functions in vivo in RNA repair rather than DNA repair.

The baculovirus and T4 Pnpk enzymes differ in their quaternary structures. Whereas T4 Pnpk is a stable homotetramer, the baculovirus Pnpk domain clearly is not. The velocity sedimentation data presented here suggest that the baculovirus Pnpk domain is a homodimer with caveats as noted above. The T4 Pnpk homotetramer is formed by two pairs of kinase-phosphatase homodimer interfaces (29, 30). A dimeric baculovirus Pnpk could form either via single phosphatase-phosphatase or kinase-kinase interfaces or through a pair of intermolecular kinase-phosphatase interfaces. It has been proposed that the tetrameric quaternary structure of T4 Pnpk may facilitate simultaneous binding of the broken 3′ and 5′ termini of tRNA (30), but it remains to be determined whether tetramerization is relevant for T4 Pnpk function in RNA repair.

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