Here we report that bacteriophage T4 RNA ligase 2 (Rnl2) is an efficient catalyst of RNA ligation at a 3′-OH/5′-PO₄ nick in a double-stranded RNA or an RNA-DNA hybrid. The critical role of the template strand in approximating the reactive 3′-OH and 5′-PO₄ termini is underscored by the drastic reductions in the RNA-sealing activity of Rnl2 when the duplex substrates contain gaps or flaps instead of nicks. RNA nick joining requires ATP and a divalent cation cofactor (either Mg or Mn). Neither dATP, GTP, CTP, nor UTP can substitute for ATP. We identify by alanine scanning seven functionally important amino acids (Tyr-5, Arg-33, Lys-54, Gln-106, Asp-135, Arg-155, and Ser-170) within the N-terminal nucleotidyltransferase domain of Rnl2 and impute specific roles for these residues based on the crystal structure of the AMP-bound enzyme. Mutational analysis of 14 conserved residues in the C-terminal domain of Rnl2 identifies 3 amino acids (Arg-266, Asp-292, and Glu-296) as essential for ligase activity. Our findings consolidate the evolutionary connections between bacteriophage Rnl2 and the RNA-editing ligases of kinetoplastid protozoa.

Bacteriophage T4 encodes two RNA strand-joining enzymes, RNA ligase 1 (Rnl1) and RNA ligase 2 (Rnl2), that exemplify different branches of the RNA ligase family (1). The function of Rnl1 in vivo is to repair a break in the anticonod loop of Escherichia coli tRNA⁹Glu triggered by phage activation of a host-encoded anticonod nuclease (2). Rnl1-like ligases are few in number, and they have a relatively narrow phylogenetic distribution that is limited, as far as we know, to bacteriophages, fungi, and baculoviruses (3–9). T4 Rnl2 typifies a separate branch (1) that includes virophage KVP40 Rnl2 (10), the RNA-editing ligases (RELs) of Trypanosoma and Leishmania (11–13) (Fig. 1), putative RNA ligases encoded by certain eukaryotic viruses, and putative RNA ligases encoded by many species of archaea (1). Thus, the Rnl2-like ligases are present in all three phylogenetic domains. The function of T4 Rnl2 during phage infection is unknown.

RNA ligases join 3′-OH and 5′-PO₄ RNA termini through a series of three nucleotidyl transfer steps (3, 14–16). Step 1 is the reaction of ligase with ATP to form a covalent ligase-(lysyl-N)-AMP intermediate and pyrophosphate. In Step 2, the AMP is transferred from ligase-adenylate to the 5′-PO₄ RNA end to form an RNA-adenylate intermediate (AppRNA). In Step 3, attack by an RNA 3′-OH on the RNA-adenylate seals the two ends via a phosphodiester bond and releases AMP. Biochemical characterization of T4 and KVP40 Rnl2 revealed an interesting effect of ATP whereby reaction of Rnl2 with a 5′-PO₄ single-stranded 18-mer RNA in the presence of ATP resulted in the accumulation of high levels of AppRNA and scant RNA end sealing (1, 17). This ATP-trapping effect is likely caused by dissociation of Rnl2 from newly formed AppRNA, followed immediately by re-adenylation of Rnl2, which precludes the third step of the strand-joining pathway.

The biochemical properties of the phage Rnl2 enzymes raised questions about the kinds of reactions they might catalyze in a cellular milieu where ATP is present at millimolar concentrations (10). One possibility is that Rnl2 functions to adenylate RNA ends in vivo. The resulting 5′ AppRNA terminus could potentially influence the stability of host or phage-derived RNAs and, in the case of mRNAs, affect their efficiency of translation. Note that ATP-dependent synthesis of AppRNA by Rnl2 is reminiscent of the capping of eukaryotic mRNA by GTP-RNA guanylyltransferase. The analogy between Rnl2 and capping enzymes is underscored by: (i) the presence of shared primary structure motifs I, III, IIIa, IV, and V that define the covalent nucleotidyltransferase superfamily (18, 19) (Fig. 1); (ii) concordance of site-directed mutational analyses at homologous residues of capping enzymes and T4 Rnl2, consistent with a shared constellation of catalytic residues (1, 17, 20); and (iii) similarity in the tertiary structures of the N-terminal nucleotidyltransferase domains of eukaryotic capping enzymes and T4 Rnl2 (21–23).

In a second scenario, we speculated that the biological function of Rnl2 is indeed ATP-dependent RNA strand joining and that Rnl2 either recognizes a specific RNA structure, from which it would not dissociate after the RNA adenylation step, or it requires a partner protein that anchors it to RNA adenylylase to promote completion of the sealing step (10). Studies of the kinetoplastid RNA-editing ligases (which we classify as Rnl2-like enzymes) do indicate that the RELs prefer to join RNA termini that are splinted together by a bridging RNA template strand (24, 25). It is notable that the specificity for RNA versus DNA as the template bridge differed between recombinant REL and the REL present in the native “editosome” complex, suggesting that editosome components associated with REL can alter its substrate preference (25). We report here that T4 Rnl2 displays a vigorous ATP-stimulated RNA-sealing activity when the reactive 3′-OH and 5′-PO₄ RNA termini are opposed at a nick in a doubled-stranded (ds) RNA or an RNA-DNA hybrid. Rnl2 activity is reduced sharply, and then abolished, when the reactive RNA ends are separated incrementally by 1-, 2-, or 3-nucleotide gaps. Activity is also inhibited when the RNA ends protrude as flaps from the template strand.

Received for publication, March 3, 2004, and in revised form, April 5, 2004
Published, JBC Papers in Press, April 13, 2004, DOI 10.1074/jbc.M402394200
We exploited the newly defined optimal nicked substrate to conduct a mutational analysis of Rnl2, focusing on amino acids that are conserved in vibriophage Rnl2 and the protozoan RELs (Fig. 1). We have identified seven functionally important amino acids within the N-terminal nucleotidyltransferase domain of Rnl2 and suggest specific roles for these residues based on the available crystal structure of the AMP-bound protein. In addition, mutational analysis of 14 conserved residues in the C-terminal domain identifies 3 amino acids as essential for ligase activity. Our findings underscore the evolutionary links between Rnl2 and the RNA-editing ligases. We suggest that templated RNA repair is an ancient phenomenon that is probably not limited presently to kinetoplastid protozoa.

**EXPERIMENTAL PROCEDURES**

**T4 Rnl2**—Missense mutations were introduced into the RNL2 gene by PCR using the two-stage overlap extension method as described previously (1, 17). The PCR products were digested with NdeI and BamHI and inserted into pET16b (Novagen). The inserts of the mutant pET-RNL2 plasmids were sequenced completely to exclude the acquisition of unwanted changes during amplification and cloning. The pET-RNL2 plasmids were transformed into E. coli BL21(DE3). Wild-type Rnl2 and mutated versions thereof were produced as follows (except as noted below). Cultures (200 ml) of E. coli BL21(DE3)pET-RNL2 were grown at 37 °C in Luria Bertani medium containing 0.1 mg/ml ampicillin until the A$_{600}$ reached 0.45. The cultures were adjusted to 0.4 mM isopropyl-$b$-D-thiogalactoside, and incubation was continued at 37 °C for 4 h. Cells were harvested by centrifugation, and the pellet was stored at $-80^\circ$C. All subsequent procedures were performed at 4 °C.
Thawed bacteria were resuspended in 10 ml of buffer A (50 mM Tris-HCl (pH 7.5), 0.25 mM NaCl, 10% sucrose), Lysozyme and Triton X-100 were added to final concentrations of 50 µg/ml and 0.1%, respectively. The lysates were sonicated to reduce their viscosity, and insoluble material was removed by centrifugation. The soluble extracts were applied to 1-mL columns of nickel-nitrilotriacetic acid-agarose (Qiagen, Chatsworth, CA) that had been equilibrated with buffer A containing 0.1% Triton X-100. The columns were washed with 5 ml of the same buffer and then eluted stepwise with 2-ml aliquots of 50, 100, 200, and 500 mM imidazole in buffer B (50 mM Tris-HCl (pH 8.0), 0.25 mM NaCl, 10% glycerol, 0.05% Triton X-100). The polypeptide compositions of the column fractions were monitored by SDS-PAGE. Wild-type and mutant Rnl2 were recovered predominantly in the 200-mM imidazole eluates, which typically contained 2–4 mg of protein. The Rnl2 preparations were stored at -80 °C. Protein concentrations were determined with the BioRad dye reagent using bovine serum albumin as the standard.

Rnl2 mutants R33A, D135A, and R155A were insoluble when produced by isopropyl-β-D-thiogalactoside induction at 37 °C as described above. Solubility was improved by varying the induction method as follows. The cultures were grown at 37 °C until the A600 reached ~0.45. Then they were placed on ice for 30 min, after which they were adjusted to 0.4 mM isopropyl-β-D-thiogalactoside and 2% (v/v) ethanol and then incubated at 17 °C for 20 h with continuous shaking. Wild-type Rnl2 was also produced using this protocol. The wild-type and mutant proteins were purified from soluble extracts by nickel-agarose chromatography as described above.

**RESULTS**

**ATP-stimulated Sealing of a Nicked dsRNA Substrate by T4 Rnl2.** A nicked dsRNA substrate was prepared by annealing two 5'-32P-labeled synthetic 24-mer RNA oligonucleotides with overhanging complementarity. The strands were designed to form a 12-bp duplex with complementary 12-nucleotide 5'-tails (Fig. 2C) that can self-assemble into an extended duplex containing staggered 3'-OH/5'-PO4 nicks on both strands. Rnl2 was incubated with 100 nM of the annealed 5'-32P-labeled RNA strands in the presence of Mg and ATP. The reaction products were analyzed by gel electrophoresis under denaturing conditions. As shown in Fig. 2A, Rnl2 generated a mixed ladder of multimers of the 24-mer illustrated 5'-tailed duplexes can form extended concatemers with potentially ligatable nicks at staggered 24-nt intervals on both strands. The structures of the annealed component 24-mer strands of the nicked dsRNA and nicked dsDNA substrates are illustrated with the 5'-32P-labeled indicated by "p" and the 12-bp segments of complementarity highlighted in shaded boxes. The 2-30 5'-tails of the strands are themselves complementary so that the illustrated 5'-tailed duplexes can form extended concateners with potentially ligatable nicks at staggered 24-nt intervals on both strands. The 32P-labeled nicked dsDNA or 5'-32P-labeled nicked dsRNA ends as indicated and increasing amounts of T4 Rnl2 (62.5, 125, 250, 500, or 1000 fmol, increasing from left to right in each titration series) were incubated for 15 min at 22 °C. The 32P-labeled products were resolved by PAGE and visualized by autoradiography. Rnl2 was omitted from control reaction mixtures shown in lanes marked –. The products of a reaction mixture containing the dsDNA substrate and 1 unit of T4 DNA ligase are shown in the rightmost lane (T4 DNL). B, ATP requirement. Reaction mixtures (10 µl) containing 50 mM Tris acetate (pH 6.5), 40 mM NaCl, 5 mM DTT, 1 mM MgCl2, 1 mM ATP, 1 pmol of either 5'-32P-labeled nicked dsRNA or 5'-32P-labeled nicked dsDNA ends as indicated and increasing amounts of T4 Rnl2 (62.5, 125, 250, 500, or 1000 fmol, increasing from left to right in each titration series) were incubated for 15 min at 22 °C. The 32P-labeled products were resolved by PAGE and visualized by autoradiography. The 32P-labeled nicked dsDNA substrates were stored at -80 °C. Protein concentrations were determined with the BioRad dye reagent using bovine serum albumin as the standard.

**ATP-stimulated Sealing of a Nicked dsDNA Substrate by T4 Rnl2.** A nicked dsDNA substrate was prepared by annealing two 5'-32P-labeled synthetic 24-mer RNA oligonucleotides with overhanging complementarity. The strands were designed to form a 12-bp duplex with complementary 12-nucleotide 5'-tails (Fig. 2C) that can self-assemble into an extended duplex containing staggered 3'-OH/5'-PO4 nicks on both strands. Rnl2 was incubated with 100 nM of the annealed 5'-32P-labeled RNA strands in the presence of Mg and ATP. The reaction products were analyzed by gel electrophoresis under denaturing conditions. As shown in Fig. 2A, Rnl2 generated a mixed ladder of multimers of the 24-mer strands. The yield of ligated products was saturated in the range of Rnl2 concentrations employed in this titration experiment (6–100 nM). Equivalent concentrations of Rnl2 were incapable of joining a nicked dsDNA substrate of identical sequence and complementarity (Fig. 2, A and C). A control reaction confirmed
**Fig. 3. DNA-templated ligation of nicked RNA.** A, reaction mixtures (10 µl) containing 50 mM Tris acetate (pH 6.5), 40 mM NaCl, 5 mM DTT, 1 mM MgCl₂, 1 mM ATP, 1 pmol of either 5'-32P-labeled nicked dsRNA substrate (as depicted in Fig. 2C) or 5'-32P RNA-labeled nicked dsRNA-DNA hybrid (shown here below panel B), and increasing amounts of Rnl2 (3.90, 7.81, or 15.6 fmol, left to right in each titration series) were incubated for 15 min at 22 °C. B, a reaction mixture (60 µl) containing 50 mM Tris acetate (pH 6.5), 40 mM NaCl, 5 mM DTT, 1 mM MgCl₂, 6 pmol of RNA-labeled nicked dsRNA-DNA hybrid, and 94 fmol of Rnl2 was incubated at 22 °C. Aliquots (10 µl) were withdrawn at the times specified above the lanes and quenched immediately with formamide/EDTA. The structure of the RNA-DNA hybrid is illustrated with the 5'-32P label of the RNA strand indicated by "p" and the 12-bp segments of complementarity are shaded. The 12-nt 5'-tails of the RNA and DNA strands are complementary so that the 5'-tailed duplexes can form extended concatemers with potentially ligatable RNA nicks at 24-nt intervals. C, reaction mixtures (10 µl) containing 50 mM Tris 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), 40 mM NaCl, 5 mM DTT, 1 mM MgCl₂, 100 µM ATP, 1 pmol of RNA-labeled nicked dsRNA-DNA hybrid, and 15.6 fmol of Rnl2 were incubated for 10 min at 22 °C.

**Fig. 4. Nucleotide and divalent cation requirements for DNA-templated RNA ligation.** A, reaction mixtures (10 µl) containing 50 mM Tris acetate (pH 6.5), 40 mM NaCl, 5 mM DTT, 100 µM ATP, 1 pmol of RNA-labeled nicked dsRNA-DNA hybrid, 15.6 fmol of Rnl2, and either no added metal (--) or 1 mM of the indicated divalent cation (MgCl₂, MnCl₂, CaCl₂, CuCl₂, or ZnCl₂) were incubated for 10 min at 22 °C. B, reaction mixtures (10 µl) containing 50 mM Tris acetate (pH 6.5), 40 mM NaCl, 5 mM DTT, 1 mM MgCl₂, 1 pmol of RNA-labeled nicked dsRNA-DNA hybrid, 15.6 fmol of Rnl2, and either no added metal (--) or 1 µM of each of either ATP, GTP, CTP, UTP, or dATP as specified were incubated for 10 min at 22 °C. C, reaction mixtures (10 µl) containing 50 mM Tris acetate (pH 6.5), 40 mM NaCl, 5 mM DTT, 1 mM MgCl₂, 1 pmol of RNA-labeled nicked dsRNA-DNA hybrid, and either no added ATP (--) or 0, 0.01, 0.1, or 1 mM ATP as specified were incubated for 10 min at 22 °C. A control reaction lacking Rnl2 is shown in lane --.

**Fig. 5. Requirement for alignment of the RNA ends at a nick versus a flap.** Reaction mixtures (10 µl) containing 50 mM Tris acetate (pH 6.5), 40 mM NaCl, 5 mM DTT, 1 mM MgCl₂, 100 µM ATP, increasing amounts of Rnl2 (1.95, 3.90, 7.81, or 15.6 fmol, from left to right in each titration series), and 1 pmol of either 32P RNA-labeled nicked RNA-DNA hybrid, RNA-labeled 1-nt 5' flap RNA-DNA hybrid, RNA labeled 1-nt 5' flap RNA-DNA hybrid, or RNA-labeled 1-nt 3' and 5' flap RNA-DNA hybrid were incubated for 10 min at 22 °C. The reaction products were analyzed by PAGE and visualized by autoradiography (the two gels were exposed to the same film for the same amount of time). The structures of the 5' and 3' flap substrates are illustrated with the 5'-32P label of the RNA strand indicated by ○.
plotted as a function of input Rnl2. The extent of ligation of the nicked and flap substrates is quantified. The extents of ligation of the nicked and flap substrates are as a function of time. Quantification as described under

Resulting DNA

mer RNA oligonucleotide with overlapping complementarity. The hybrid duplex containing staggered 3'-OH/5'-PO4 nucleotide 5'-tails that promote self-assembly into an RNA-DNA hybrid duplex with complementary 12-mer RNA strand to produce a hairpin product that is comprised a perfectly spaced series of n-mer RNA-splinted by either an RNA or DNA template strand (Fig. 3A). The irregular mobility of the dsRNA ligation products was analyzed by PAGE and visualized by autoradiography (the two gels were exposed to the same film for the same amount of time). B, the structures of the gapped substrates are illustrated with the 5'-PO4 label of the RNA strand indicated by •.

Fig. 6. Characteristics of the DNA-templated RNA ligation reaction. A, kinetics. The data from the experiment in Fig. 3B were quantified as described under “Results.” The extent of ligation is plotted as a function of time. B, the data from the experiments in Fig. 5 were quantified. The extents of ligation of the nicked and flap substrates are plotted as a function of input Rnl2.

tained saturation at ≈1.6 nM Rnl2 (Fig. 2B). The instructive finding was that dsRNA ligation by Rnl2 was dependent on added ATP (Fig. 2B). Simple comparison of the Rnl2 titration profiles with and without ATP indicated that the nicked RNA ligase activity was stimulated at least 16-fold by ATP (Fig. 2B). The trace levels of ligated product in the absence of ATP are attributable to the presence of preadenylated Rnl2-AMP in the recombinant enzyme preparation (1). The stimulation of dsRNA nick ligation by ATP contrasts sharply with the ATP inhibition of ligation of single-stranded 18-mer RNA substrates reported previously for T4 and KVP40 Rnl2 (1, 10, 17).

DNA-templated Ligation of Nicked RNA—A nicked dsRNA-DNA substrate was prepared by annealing a 5'-32P-labeled 24-mer DNA oligonucleotide to an unlabeled 24-mer DNA oligonucleotide with overlapping complementarity (Fig. 3B). The annealed strands form a 12-bp duplex with complementary 12-nucleotide 5'-tails that promote self-assembly into an RNA-DNA hybrid containing staggered 3'-OH/5'-PO4 RNA nicks on one strand. (Note that the DNA strand of the RNA-DNA hybrid contains staggered 3'-OH/5'-OH nicks, which cannot be sealed by polynucleotide ligases.) Increasing concentrations of Rnl2 were reacted with 100 nM of the annealed 5'-32P RNA-labeled RNA-DNA hybrid in parallel with the dsRNA substrate. Rnl2 displayed similar activity in sealing 3'-OH/5'-PO4 RNA ends that were splinted by either an RNA or DNA template strand (Fig. 3A). The salient difference was that the ladder of sealed 32P-labeled RNAs generated with the RNA-DNA hybrid substrate comprised a perfectly spaced series of n-mers, with n values ranging from 2–12. In contrast, the ladder of sealed 32P-labeled RNAs generated with the dsRNA substrate was spaced irregularly (Fig. 3A). The irregular mobility of the dsRNA ligation products may reflect the fact that both component RNA strands contain reactive 5'-PO4 termini that, when situated at the ends of the substrate, can be joined by Rnl2 to the 3'-OH terminus of the complementary RNA strand to produce a hairpin product that migrates aberrantly during electrophoresis. In the case of the RNA-DNA hybrid substrate, the 5'-OH of the DNA strand cannot be ligated to the 3'-OH of the labeled RNA strand and, although it is conceivable that the 3'-OH of the DNA strand could be joined to the 5'-PO4 of the RNA strand, in practice T4 Rnl2 is not proficient in ligating a substrate containing a deoxynucleotide at the 3'-OH position (10). To evaluate whether Rnl2 might be better able to ligate DNA strands if they were splinted by an RNA template, we prepared a nicked DNA-RNA duplex by annealing a 5'-32P-labeled 24-mer DNA oligonucleotide to an unlabeled 24-mer RNA oligonucleotide with overlapping complementarity. The resulting DNA-RNA hybrid duplex contains potentially ligatable staggered 3'-OH/5'-PO4 DNA nicks on one strand and nonligatable 3'-OH/5'-OH RNA nicks on the other strand. We were unable to detect any DNA strand joining with this nicked DNA-RNA substrate, even at Rnl2 concentrations that were saturating for sealing of the nicked RNA-DNA hybrid (data not shown).

A kinetic analysis of the reaction of 100 nM nicked RNA-DNA hybrid with 1.6 nM Rnl2 is shown in Fig. 3B. The evolution of the n-mer ladder as a function of time was consistent with a distributive mechanism of sealing of a substrate containing multiple ligatable nicks, i.e., a dimer 48-mer product of a single sealing event predominated at early times, prior to the appearance of higher order ligated RNAs. The extent of ligation was

Fig. 7. Requirement for alignment of the RNA ends at a nick versus a gap. A, reaction mixtures (10 μl) containing 50 mM Tris acetate (pH 6.5), 40 mM NaCl, 5 mM DTT, 1 mM MgCl2, 100 μM ATP, increasing amounts of Rnl2 (1.95, 3.90, 7.81, or 15.6 fmol, from left to right in each titration series), and 1 pmol of either 32P RNA-labeled nicked RNA-DNA hybrid, RNA-labeled 1-nt gap RNA-DNA hybrid, RNA-labeled 2-nt gap RNA-DNA hybrid, or RNA-labeled 3-nt gap RNA-DNA hybrid were incubated for 10 min at 22°C. The reaction products were analyzed by PAGE and visualized by autoradiography.
quantified by scanning the gel with a Fujix imaging apparatus. The radioactivity signal was determined for each n-mer (e.g. n = 1 for the input substrate strand, n = 2 for linear dimer, etc.) that was visualized. The radioactivity for individual n-mers in each reaction was summed, and each n-mer species was expressed as the fraction of the total. The amount (in fmol) of 5'-32P-labeled 24-mer comprising each labeled n-mer was determined by multiplying this fraction by the known amount of input 24-mer substrate strands. Each linear n-mer was generated as a consequence of “n minus 1” ligation events. Thus, the amount (in fmol) of ligation required to form each n-mer product was calculated for each n-mer by the equation: fmol,ligation = fmol,24-mer × [(n-1)/n]. The total amount of strand ligation (fmol of 5'-ends joined) for each reaction was then determined by summing the ligation events for each n-mer product in the ladder. A plot of ligation versus reaction time is shown in Fig. 6A. The initial rate of reaction was 11 fmol of ends ligated/fmol of enzyme/min. 67% of the available 5'-32P-labeled RNA ends were sealed after 15 min. Thus, Rnl2 acts catalytically and efficiently in sealing RNA nicks.

Rnl2 sealing of the nicked RNA-DNA hybrid substrate was optimum at pH 6.0–7.0 in 50 mM Tris acetate buffer (Fig. 3C). Activity was virtually nil at pH ≤5.0 but persisted at alkaline pH up to 9.0. Nicked RNA ligation required a divalent cation cofactor, which could be magnesium or manganese, but not calcium, copper, or zinc (Fig. 4A). Nicked RNA ligation in the absence of added ATP was 2% of the activity in the presence of 10 μM ATP; neither GTP, CTP, UTP, nor dATP could substitute for ATP in stimulating the ligation reaction (Fig 4B). Joining of nicked RNA by Rnl2 was remarkably efficient with respect to ATP utilization insofar as the yield of ligated product was saturated at 0.1 μM ATP, which was equivalent to the concentration of 32P-labeled RNA strands included in the reaction (Fig. 4C). Reducing ATP to 0.01 μM elicited a proportional reduction in the extent of ligation (Fig. 4C). The standard ligation reaction mixtures contained 40 mM NaCl contributed by the enzyme and RNA substrate solutions. The effect of increasing ionic strength on RNA ligation efficiency was gauged by supplementing the reactions with NaCl to final concentrations of 65, 90, 140, 240, 340, or 540 mM NaCl. Activity was unaffected up to 65 mM NaCl but was reduced by 36% and 97% at 90 and 240 mM NaCl, respectively. Rnl2 activity was undetectable at ≥340 mM NaCl (data not shown).

**Requirement for Alignment of the RNA Ends at a Nick versus a Flap**—The DNA strand of the RNA-DNA hybrid was altered by removing 1 nucleotide in the center such that, upon annealing to the 24-mer 32P-labeled RNA strand, either the 3'-OH RNA end or the 5'-PO4 side (Fig. 5). Rnl2 was much less effective in sealing a 1-nucleotide flap on the 3'-OH side than it was in joining at a perfectly aligned nick and was nearly inert in sealing a 1-nucleotide flap on the 3'-OH side (Fig. 5). We then further altered the DNA strand of the RNA-DNA hybrid by removing 2 central nucleotides so that annealing to the 24-mer 32P-labeled RNA strand yielded 1-nucleotide flaps on both the 3'-OH RNA and the 5'-PO4 RNA ends (Fig. 5). Rnl2 displayed minimal activity on the dou-
ble-flap substrate, comparable with that seen with the 3’ flap alone. Quantitation of the titration data revealed that Rnl2-specific activities on the 5’-flap, 3’-flap, and double-flap substrates were 11, 0.3, and 1% of the specific activity on the nicked substrate, respectively (Fig. 6B). We conclude that Rnl2 ligase activity is impeded by protruding nucleotides and is especially sensitive to a flap at the 3’-OH terminus.

Requirement for Alignment of the RNA Ends at a Nick versus a Gap—The DNA strand of the RNA-DNA hybrid was altered by adding 1, 2, or 3 deoxyadenosine nucleotides in the center of the oligonucleotide such that, upon annealing to the 24-mer 32P-labeled RNA strand, the 3’-OH RNA end was separated from the 5’-PO4 end by a 1, 2-, or 3-nt gap (Fig. 7B). Rnl2 was much less effective in sealing across the 1-nucleotide gap than it was in joining at a perfectly aligned nick (Fig. 7A, top panel). Activity was minimal at a 2-nt gap and undetectable at a 3-nt gap. Rnl2-specific activities on the 1-, 2-, and 3-nt gapped substrates were 10, 1.6, and <0.1% of the specific activity on the nicked substrate, respectively (not shown). We surmise that Rnl2 requires exact approximation of the reactive termini at a nick stabilized by the template strand.

Structure-guided Mutational Analysis of the N-terminal Domain of T4 Rnl2—T4 Rnl2 provides an excellent model for structural and mechanistic analysis of the Rnl2/REL class of RNA-joining enzymes. There is primary structure similarity between Rnl2 and the kinetoplastid RELs through the entire length of the Rnl2 protein (Fig. 1). Many of the conserved positions localize to the N-terminal adenylyltransferase domain (spanning amino acids 1–249 and demarcated by the arrowhead in Fig. 1), which includes nucleotidyltransferase motifs I, III, IIIa, IV, and V that are conserved in DNA ligases and mRNA capping enzymes. Previous mutational analysis of Rnl2 pinpointed 12 individual amino acids that are essential for Rnl2-catalyzed circularization of an 18-nucleotide single-stranded pRNA substrate (indicated by vertical bars in Fig. 1) (1, 17); these include at least one conserved residue in each of the nucleotidyltransferase motifs. A crystal structure of the Rnl2 adenylyltransferase domain with AMP bound at the active site revealed a shared fold and catalytic mechanism for RNA ligases, DNA ligases, and mRNA capping enzymes and provided plausible atomic explanations for the observed mutational effects on Rnl2 activity (23). For example, essential resi-
idues Lys-35, Asn-40, Arg-55, Glu-99, Phe-119, Glu-204, Lys-225, and Lys-227 are implicated directly in the nucleotidyl transfer reaction via direct or indirect atomic contacts to AMP (23). Other essential residues appeared to play noncatalytic structural roles.

Here we conducted an alanine scan of 13 positions located in the N-terminal domain of Rnl2 that were chosen on the basis of their conservation in other Rnl2/REL family members, their hydrophilic character, and/or their positions in the Rnl2 fold suggestive of either internal structural roles or contributions to a putative surface RNA-binding site (see below). The mutated residues are indicated by question marks over the alignment in Fig. 1. The Y5A, E29A, R33A, K54A, E63A, Q106A, K107A, D135A, Y136A, E139A, R155A, and R221A proteins were produced in E. coli as His10-tagged fusions and purified from soluble bacterial extracts by Ni-agarose chromatography. The 42-kDa Rnl2 polypeptide was the predominant species detected by SDS-PAGE, and the extents of purification were comparable for mutant and wild-type Rnl2 (Fig. 8).

The adenylyltransferase activity of the Rnl2/Ala proteins was assayed by label transfer from [α-32P]ATP to the Rnl2 polypeptide to form the covalent Rnl2/AMP intermediate (Fig. 8). The E29A, E63A, K107A, Y136A, E139A, and R221A mutants displayed near wild-type adenylyltransferase activity, i.e. the yield of mutant Rnl2/AMP was within a factor of two of the extent of adenylation of wild-type Rnl2 produced under the same conditions. Five mutants displaying significant defects in adenylation were: R33A (3% of the wild-type control), D135A (4%), R155A (16%), and S170A (8%). The Y5A mutation had a modest effect on adenylation (39% of the wild-type control).

Each of the mutants was assayed for RNA ligase activity with the nicked RNA-DNA hybrid substrate. The E29A, E63A, K107A, Y136A, E139A, and R221A mutants displayed wild-type or near wild-type strand-sealing activity, whereas mutants Y5A, R33A, K54A, Q106A, D135A, R155A, and S170A were either unreactive or severely impaired in RNA sealing (Fig. 9). The relative activities of the Rnl2-Ala mutants are quantified in Fig. 10A. The retention of activity of the E29A, E63A, K107A, Y136A, E139A, and R221A mutants displayed in Fig. 9. The relative activities of the Rnl2-Ala mutants are quantified in Fig. 10A. The retention of activity of the E29A, E63A, K107A, Y136A, E139A, and R221A mutants displayed wild-type or near wild-type strand-sealing activity, whereas mutants Y5A, R33A, K54A, Q106A, D135A, R155A, and S170A were either unreactive or severely impaired in RNA sealing (Fig. 9). The relative activities of the Rnl2-Ala mutants are quantified in Fig. 10A. The retention of activity of the E29A, E63A, K107A, Y136A, E139A, and R221A mutants displayed wild-type or near wild-type strand-sealing activity, whereas mutants Y5A, R33A, K54A, Q106A, D135A, R155A, and S170A were either unreactive or severely impaired in RNA sealing (Fig. 9). The relative activities of the Rnl2-Ala mutants are quantified in Fig. 10A. The retention of activity of the E29A, E63A, K107A, Y136A, E139A, and R221A mutants displayed wild-type or near wild-type strand-sealing activity, whereas mutants Y5A, R33A, K54A, Q106A, D135A, R155A, and S170A were either unreactive or severely impaired in RNA sealing (Fig. 9). The relative activities of the Rnl2-Ala mutants are quantified in Fig. 10A. The retention of activity of the E29A, E63A, K107A, Y136A, E139A, and R221A mutants displayed wild-type or near wild-type strand-sealing activity, whereas mutants Y5A, R33A, K54A, Q106A, D135A, R155A, and S170A were either unreactive or severely impaired in RNA sealing (Fig. 9). The relative activities of the Rnl2-Ala mutants are quantified in Fig. 10A. The retention of activity of the E29A, E63A, K107A, Y136A, E139A, and R221A mutants displayed wild-type or near wild-type strand-sealing activity, whereas mutants Y5A, R33A, K54A, Q106A, D135A, R155A, and S170A were either unreactive or severely impaired in RNA sealing (Fig. 9). The relative activities of the Rnl2-Ala mutants are quantified in Fig. 10A. The retention of activity of the E29A, E63A, K107A, Y136A, E139A, and R221A mutants displayed wild-type or near wild-type strand-sealing activity, whereas mutants Y5A, R33A, K54A, Q106A, D135A, R155A, and S170A were either unreactive or severely impaired in RNA sealing (Fig. 9). The relative activities of the Rnl2-Ala mutants are quantified in Fig. 10A. The retention of activity of the E29A, E63A, K107A, Y136A, E139A, and R221A mutants displayed wild-type or near wild-type strand-sealing activity, whereas mutants Y5A, R33A, K54A, Q106A, D135A, R155A, and S170A were either unreactive or severely impaired in RNA sealing (Fig. 9). The relative activities of the Rnl2-Ala mutants are quantified in Fig. 10A. The retention of activity of the E29A, E63A, K107A, Y136A, E139A, and R221A mutants displayed wild-type or near wild-type strand-sealing activity, whereas mutants Y5A, R33A, K54A, Q106A, D135A, R155A, and S170A were either unreactive or severely impaired in RNA sealing (Fig. 9).
Waters are depicted as red spheres. Potential hydrogen-bonding interactions are depicted by dashed lines. B, ribbon diagram of the fold of the Rnl2 adenylyltransferase domain. α-helices are colored cyan, β-strands are red, and connecting polypeptide is yellow. AMP and surface residues Lys-54 and Glu-106 are shown in bond representation. A network of ionic interactions between Arg-33, Asp-135, Arg-155, and Glu-139 that tethers several secondary structure elements at the base of the Rnl2 structure is highlighted in the box. The atomic contacts of these residues are shown in detail in panel C. The images were generated with SETOR (38).

C-terminal domain of Rnl2 is homologous to the C termini of kinetoplastid RELs but is apparently unrelated (at least at the level of primary structure) to the OB domains that flank the nucleotidyltransferase domains of DNA ligases (ATP- and NAD−-dependent) and mRNA capping enzymes (21, 22, 26–28). Available evidence suggests that the polynucleotide substrate specificities of DNA ligases and capping enzymes are dictated by their OB-fold domains (29–34). The C-terminal domain of T4 Rnl2 is essential for ligation of a 5′-PO4 RNA but dispensable for strand sealing when the RNA adenylylation step is bypassed by the use of a preadenylated AppRNA substrate (23). The isolated Rnl2 N-terminal domain retains autoadenylylation activity, albeit with an alkaline-shifted pH optimum of 9.0 compared with the optimum of pH 6.5 observed for full-length Rnl2 (23). Based on these findings, we suggested that the C-terminal domain of Rnl2 might comprise a novel substrate specificity determinant for the Rnl2/REL branch of the polynucleotide ligase family (23).

To map the essential structural features of this domain, we conducted an alanine scan of 14 residues in the C-terminal segment of T4 Rnl2 (indicated by question marks in Fig. 1) that were chosen on the basis of their conservation in other Rnl2/REL family members and (with the exception of Trp-329) their hydrophilic character. The R266A, S272A, K273A, D292A, E295A, E296A, R299A, E300A, D308A, N309A, K314A, K315A, K319A, and W329A mutants were produced in E. coli as His10-tagged fusions and purified from soluble bacterial extracts by Ni-agarose chromatography (Fig. 11). The adenylyltransferase activity of the recombinant Rnl2 proteins was assayed at pH 6.5 and 9.0. Whereas 11 of the mutants displayed wild-type or near wild-type adenylyltransferase activity at pH 6.5, three other mutants, R266A, D292A, and E296A, were defective in Rnl2-AMP formation (Fig. 11). Wild-type Rnl2 displayed weak adenylylation activity at pH 9.0 as did all 11 of the Rnl2-Ala mutants that retained adenylyltransferase activity at pH 6.5. The instructive findings were that the R266A and D292A mutants (but not E296A) displayed a significant gain of adenylyltransferase activity at pH 9.0 compared with wild-type Rnl2. Thus, the single R266A and D292A mutations phenocopied the effects of deleting the C-terminal domain on the pH optimum of the ligase adenylylation reaction.

The 11 C-terminal Ala mutants that retained adenylyltransferase activity also displayed wild-type or near wild-type strand-sealing activity with the nicked RNA-DNA substrate (Figs. 10B and 12). Mutants R266A, D292A, and E296A were either unreactive or severely impaired in RNA sealing (Fig. 12). The defects of these three mutants in overall RNA ligation were consonant with their defects in forming the ligase-adenylate intermediate.

To further evaluate the contributions of Arg-266 and Asp-292 to the RNA ligase reaction, we tested the effects of conservative substitutions. The purity of the conservatively mutated Rnl2 proteins was comparable with wild-type Rnl2 and the corresponding Ala mutants (Fig. 13A). Introduction of lysine at position 266 restored adenylyltransferase activity at pH 6.5, diminished adenylyltransferase activity at pH 9.0 (Fig. 13B), and elicited a significant gain of function in RNA sealing (to ~50% of the wild-type level) compared with the defective R266A mutant (Figs. 10B and 13C). In contrast, a glutamine substitution had no salutary effects relative to alanine. We surmise that positive charge at residue 266 is critical for Rnl2 activity. Replacing Asp-292 with asparagine resulted in a modest gain of adenylyltransferase function at pH 6.5 and diminution at pH 9.0 compared with the D292A protein (Fig. 13B) as well as a partial revival of RNA-sealing activity (Fig. 13C) to
20% of the wild-type level (Fig. 10B). Glutamate was less effective than asparagine in restoring Rnl2 function. We surmise that a carboxylate at position 292 supports optimal activity, that an isosteric amide is suboptimal, and that there is a steric constraint on the size of the main chain to carboxylate the reactive 3'-OH and 5'-PO₄ termini, that an isosteric amide is suboptimal, and that there is a steric constraint on the size of the main chain to carboxylate the reactive 3'-OH and 5'-PO₄ termini.

**DISCUSSION**

Here we have shown that T4 Rnl2 is an efficient catalyst of RNA ligation at a 3'-OH/5'-PO₄ nick template by a bridging polynucleotide strand, which can be either RNA or DNA. The critical role of the template strand in approximating the reactive 3'-OH and 5'-PO₄ termini is underscored by the drastic reductions in the sealing activity of Rnl2 when the duplex substrates contain gaps or flaps instead of nicks. Rnl2 displays “fidelity” in RNA nick joining comparable with that of DNA ligases in sealing nicked duplex DNAs. Like Rnl2, exemplary ATP-dependent DNA ligases are inhibited by displacement of the reactive termini by 1- or 2-nucleotide gaps and are typically more affected by perturbations at the 3'-OH terminus than at the 5'-PO₄ terminus (35–38).

The sealing of nicked duplex RNAs by Rnl2 is analogous to the sealing reactions that occur during RNA-guided mRNA editing in kinetoplastid protozoa. The kinetoplastid REL enzymes have been studied biochemically either in the context of a native mitochondrial editsome complex or as translation products synthesized in vitro in a coupled transcription-translation system (24, 25). Rnl2 displays certain similarities to the RELs with respect to substrate and cofactor specificity, including a requirement for ATP, a common preference for a perfectly paired nicked substrate, and loss of activity when gaps are introduced between the reactive termini. However, there are two noteworthy differences in the properties of Rnl2 and those reported for RELs. First, although the native trypanosome REL activity was reported to seal RNA strands annealed to a complementary DNA template, the isolated REL proteins were unable to do so and specifically required an RNA template strand (25). T4 Rnl2 is clearly not restricted to an RNA-template substrate. Second, although neither the RELs nor Rnl2 can ligate across a 3-nucleotide gap, the RELs are more tolerant of 2-nucleotide gaps than is Rnl2 (24, 25).

We had suggested previously that Rnl2 and the RELs comprise a single family of structurally homologous RNA-joining enzymes defined by several signature residues in the N-terminal nucleotidyltransferase domain and the presence of a unique C-terminal domain module not found in other members of the covalent nucleotidyltransferase superfamily. The C-terminal domain is required for overall RNA ligation and is implicated specifically in the RNA adenylylation step of the RNA-sealing pathway (23). Here we have shown that alanine mutations of residues Arg-266 and Asp-292 in the C-terminal domain inactivate the effects of deleting the C-terminal domain, i.e. they elicit an alkaline shift in the pH optimum of the Rnl2 adenylylation activity and suppress the overall RNA ligation reaction. Loss of the Glu-296 side chain suppresses ligase adenylylation and the composite nick-sealing reaction. The specificity of these effects is underscored by the fact that alanine mutations at 11 other amino acids in the C-terminal domain had no significant effect on Rnl2 activity. Because Arg-266, Asp-292, and Glu-296 are conserved in the trypanosome and Leishmania RELs (Fig. 1), we would predict that these residues play an essential role in RNA editing.

The crystal structure of the adenylyltransferase domain of Rnl2 with bound AMP was used here as a guide for mutational analysis of residues with potential roles in forming or stabilizing the active site. Seven new essential or important residues were thereby identified: Tyr-5, Arg-33, Lys-54, Gln-106, Asp-135, Arg-155, and Ser-170. Of these seven, only Tyr-5 is poised to contact the adenosine ligand in the crystal. Tyr-5 also engages in a hydrogen bond to essential side chain Asp-120 in motif IIIa, which in turn forms salt bridges with both the essential Lys-209 side chain in motif IV and the nonessential Arg-221 side chain flanking motif V (Fig. 14A). Tyr-5 is strictly conserved in the kinetoplastid RELs (Fig. 1). Ser-170, which we show here is essential for Rnl2 activity, donates a hydrogen bond from the Oγ to the essential Glu-34 side chain in motif I (Fig. 14A). Glu-34 also accepts a hydrogen bond from the amide nitrogen of Val-171. These interactions occur on the “back side” of the motif I β strand that forms the active site pocket (Fig. 14A). We suggest that the Glu-34-Ser-170 interaction helps achieve a backbone conformation in motif I suitable for adenylate binding and catalysis; note that the Glu-34 main chain carbonyl oxygen and the Ile-36 amide nitrogen make hydrogen bonds to the adenine base that are likely to account for the ATP specificity of Rnl2 (Fig. 14A). Whereas Glu-34 is strictly conserved in all Rnl2-like ligases, the position corresponding to Ser-170 is either a serine in KVP40 Rnl2 or an asparagine or an arginine in the REL proteins (Fig. 1). Asn and Arg have the potential to donate a hydrogen bond to the motif I glutamate. Arg-33, Asp-135, and Arg-155 (all of which are critical for Rnl2 activity) comprise an interaction network that includes Glu-139. Arg-33 forms a buried bidentate salt bridge with Asp-135, Asp-155 O6i forms a bifurcated salt bridge to Arg-155, and Arg-155 makes a bifurcated ion pair with Glu-139 Oe1 (Fig. 14C). The contact between Arg-155 and Glu-139 is apparently not functionally relevant insofar as the E139A mutation did not affect Rnl2 activity. The contacts between Arg-33, Asp-135, and Arg-155 tether two β strands and an α helix and may thereby stabilize the fold of the nucleotidyltransferase domain (Fig. 14B). Asp-135 and Arg-155 are conserved in all of the Rnl2-like ligases, whereas Arg-33 is found uniquely in T4 Rnl2.

Instructive mutational effects were seen at residues Lys-54 and Gln-106 where alanine substitutions suppressed overall RNA sealing but not the ligase adenylylation step. Lys-54 and Gln-106 are both located on the surface of Rnl2 at distances of 14.8 and 9.3 Å from the AMP phosphate, respectively (Fig. 14B). Thus, neither side chain is in a position to interact with the AMP substrate, which would account for the lack of mutational impact on Rnl2 adenylylation. Lys-54 forms an ion pair with Glu-63 on the protein surface, but this interaction is not relevant to Lys-54 function insofar as the E63A mutation did not suppress ligase activity. The only intramolecular interaction of the Glu-106 side chain is a hydrogen bond between its amide nitrogen and the main chain carbonyl oxygen of Gly-38. We envision that Lys-54 and Gln-106 form part of the RNA binding surface of T4 Rnl2. Lys-54 is conserved in all of the Rnl2-like proteins, whereas Gln-106 is unique to the bacteriophage enzymes.