Polynucleotide 3’-terminal Phosphate Modifications by RNA and DNA Ligases

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Background: Classic RNA ligases join 5’ pRNA to RNA 3’ OH.

Results: Thermophilic RNA ligases were able to modify ssDNA or RNA with a 3’-phosphate.

Conclusion: Thermophilic ligases form RNA 2’,3’-cyclic phosphate and ssDNA 3’pp5’ A.

Significance: Thermophilic RNA ligases duplicate the enzymatic function of RtcA and may have an important role in nucleic acid 3’-phosphate biology in addition to conventional ligation of 5’ pRNA.

RNA and DNA ligases catalyze the formation of a phosphodiester bond between the 5’-phosphate and 3’-hydroxyl ends of nucleic acids. In this work, we describe the ability of the thermophilic RNA ligase MthRnl from Methanobacterium thermoautotrophicum to recognize and modify the 3’-terminal phosphate of RNA and single-stranded DNA (ssDNA). This ligase can use an RNA 3’ p substructure to generate an RNA 2’,3’-cyclic phosphate or convert DNA3’ p to ssDNA 3’,5’ pp5’ A. An RNA ligase from the Thermus scotoductus bacteriophage TS2126 and a predicted T4 Rnl1-like protein from Thermovibrio ammonificans, TVa, were also able to adenylate ssDNA 3’ p. These modifications of RNA and DNA 3’-phosphates are similar to the activities of RtcA, an RNA 3’-phosphate cyclase. The initial step involves adenylation of the enzyme by ATP, which is then transferred to either RNA 3’ p or DNA 3’ p to generate the adenylated intermediate. For RNA 3’,5’ pp5’ A, the third step involves attack of the adjacent 2’ hydrogen to generate the RNA 2’,3’-cyclic phosphate. These steps are analogous to those in classical 5’ phosphate ligation. MthRnl and TS2126 RNA ligases were not able to modify a 3’ p in nicked double-stranded DNA. However, T4 DNA ligase and RtcA can use 3’-phosphorylated nicks in double-stranded DNA to produce a 3’-adenylated product. These 3’-terminal phosphate-adenylated intermediates are substrates for deadenylation by yeast 5’ Deadenylase. Our findings that classic ligases can duplicate the adenylation and phosphate cyclization activity of RtcA suggests that they have an essential role in metabolism of nucleic acids with 3’-terminal phosphates.

Although 5’-phosphorylated oligonucleotides are common intermediates in nucleic acid biochemistry, terminal 3’-phosphorylated DNA and RNA can also be produced through a variety of chemical and biochemical reactions. Examples of RNases that generate a 3’-phosphate include RNase A, RNase I, and the self-cleaving ribozymes. Alkaline hydrolysis of RNA is a common chemical method used to generate RNA with a 3’-phosphate. DNase II, which is involved in the degradation of DNA during apoptosis, generates a DNA 3’ p cleavage product. Common nucleases, such as micrococcal nuclease, generate DNA and RNA breaks with 3’-phosphate or 2’,3’-cyclic phosphate (1). When topoisomerase I is trapped by inhibitors, it can be removed from DNA by tyrosyl-DNA-phosphodiesterase 1 (Tdp1), leaving a DNA 3’-phosphate end. Topoisomerase I has also been shown to cleave at a single ribonucleotide embedded in duplex DNA, giving rise to DNA nicks with a ribo-2’,3’-cyclic phosphate end. Tdp1 can convert phosphoglycolate to phosphate termini on 3’ overhangs of DNA double strand breaks (2). Bleomycin, neocarzinostatin, and ionizing radiation can also induce DNA damage with 3’-phosphate ends. After 5-methylcytosine removal, the Arabidopsis DNA glycosylase/lase ROS1 cleaves the DNA backbone, and the product has a single-nucleotide gap flanked by 3’- and 5’-phosphate termini. For a more comprehensive review on 3’-terminal phosphate formation and repair, refer to Refs. 3–5.

Most RNA and DNA repair reactions, including polymerization or ligation, require a free terminal 3’-hydroxyl and cannot act on 3’-phosphorylated polynucleotides. Until recently, our understanding of the removal of 3’-phosphates and their derivatives from modified 3’ ends was thought to be performed mostly by non-specific phosphomonoesterases (phosphatases and enzymes containing phosphatase activity) or by nucleotide excision. Most phosphatases cannot remove a 2’,3’-cyclic phosphate group from the 3’ end of RNA. The exceptions are the multifunctional plant and fungal tRNA ligases that convert a RNA 2’,3’-cyclic phosphate to RNA 2’-phosphate, followed by phosphorylation of the 5’ end and then ligation to generate an unconventional 2’-phosphate-3’,5’-phosphodiester bond at the splice junction (6). Bacteria and archaea contain a 2’,5’ RNA ligase that can seal 2’,3’-cyclic phosphate and 5’-hydroxyl of RNA. Recently, a chloroplast 2’,5’ RNA ligase that can also use cyclic phosphates as a substrates was found (7). T4 polynucleotide kinase, T4 PNK, is another multifunctional enzyme that has 2’,3’-cyclic phosphodiesterase activity that generates a terminal 3’-phosphate that is then removed by its phosphatase. The enzyme is also able to phosphorylate the 5’ end of the RNA or DNA (8).

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2 The abbreviations used are: PNK, polynucleotide kinase; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; FAM, carboxyfluorescein; AnP, Antarctic phosphatase.
However, there are other emerging biochemical pathways for dealing with 3′-terminal phosphate: its activation, modification, and direct involvement in ligation. A major discovery was the identification of RtcB ligase as a noncanonical GTP-dependent RNA ligase that joins the 3′-phosphate of a donor RNA to 5′-hydroxyl of acceptor RNA. In recent studies, the mechanism of 3′-phosphate ligation of RNA (9–11) has been well described (12–14). The 3′-phosphate in this ligation is directly involved in the reaction. It is first activated by the transfer of GMP from the RtcB-GMP complex to form the 3′-guanylated intermediate RNA3 pp5′G. This intermediate is then ligated to the 5′-hydroxyl of an RNA acceptor to form a phosphodiester bond or to the adjacent 2′-OH to form a cyclic phosphate. Moreover, recent studies showed potential for this activity to be involved in DNA repair (15, 16).

Terminal 3′-phosphates are also substrates for ATP-dependent 3′-phosphate cyclase (RtcA) (9, 17). RtcA converts 3′-terminal phosphate on RNA to 2′,3′-cyclic phosphate using a tripartite reaction similar to the mechanism of polynucleotide ligases (18). First (Reaction 1), the RtcA protein self-adenylates by reacting with ATP. Second (Reaction 2), the adenyl group is transferred to 3′-terminal phosphate of RNA, creating an activated RNA3 pp5′A intermediate. Finally (Reaction 3), the phosphate is internally ligated to the adjacent 2′-hydroxyl with loss of AMP.

\[
\text{Enz} + \text{ATP} \rightarrow \text{Enz-p5′A} + \text{pp} \\
\text{REACTION 1}
\]

\[
\text{Enz-p5′A} + \text{RNA 3′p} \rightarrow \text{RNA 3′pp5′A} \\
\text{REACTION 2}
\]

\[
\text{Enz} + \text{RNA3′ pp5′A} \rightarrow \text{RNA 2′3′p} > \text{p} \\
\text{REACTION 3}
\]

The resulting 2′,3′-cyclic phosphate can serve as a substrate for RtcB ligase or certain plant and fungal tRNA ligases that, additionally, have cyclic phosphodiesterase activity (6, 19). When the 2′-hydroxyl of the 3′-terminal ribose is blocked by methylation or the terminal sugar is 2′-deoxyribose (Reaction 4), the intermediate nucleic acid 3′ pp5′A intermediate accumulates (9).

\[
\text{Enz-p5′A} + \text{DNA 3′p} \rightarrow \text{DNA 3′pp5′A} \\
\text{REACTION 4}
\]

Although the chemistry among traditional ATP-dependent ligases is identical, they have diverse specificities with respect to polynucleotide substrates. Most RNA ligases prefer single-stranded RNA, but T4 Rnl2 can also recognize and repair nicks in double-stranded RNA (21). Some thermostable RNA ligases do not strongly discriminate between RNA and single-stranded DNA (ssDNA) (22, 23); they are able to ligate either substrate. Most DNA ligases are specific for dsDNA, although *Chlorella virus* DNA ligase, in addition, recognizes and ligates DNA/RNA hybrids where nicked DNA was splinted with RNA (24). Recently it has been found that RtcA, in addition to 3′-phosphate modification, can also adenylate 5′-terminal phosphates of RNA and DNA (25). The discovery that RtcA had activity on either the 3′- or 5′-phosphate of RNA or DNA prompted us to investigate the possibility that classical RNA ligases, which require a 5′-phosphate for ligation, could modify RNA with a 3′-phosphate.

We have found previously that the RNA ligase from the thermophile *Methanobacterium thermoautotrophicum*, MthRnl, is very efficient in adenylation of oligonucleotides with a 5′-phosphate (26). We discovered that MthRnl has the ability to convert RNA3′p to RNA 2′,3′ cyclic phosphate and also adenylate ssDNA3′p. We extended this study to investigate the modification of 3′-phosphorylated nicked DNA substrate with RNA and DNA ligases from bacteria and archaea and bacterial and archaeal phases as well as from an eukaryotic virus.

**EXPERIMENTAL PROCEDURES**

**Enzymes**

*RNA Ligases*—Ligase from *M. thermoautotrophicum* (MthRnl), the 5′-DNA adenylation kit, and its K97A mutant thermostable 5′AppDNA/RNA ligase were from New England Biolabs (26, 27). Two genes codon-optimized for *Escherichia coli* expression, an RNA ligase from *Thermus scotoductus* bacteriophage TS2126 (22) (GenBank™ accession no. CQ796353) and a predicted T4 Rnl1-like protein from *Thermovibrio ammonificans* (TVa) (28) (GenBank™ accession no. ADU96140), were synthesized (Celtek Genes) and cloned into the pET16b vector (Novagene) in-frame with a His10 N-terminal tag. The resulting constructs were expressed and purified to near homogeneity essentially as described previously for MthRnl (27). T4 RNA ligase 1 (T4 Rnl1) and T4 RNA ligase 2 (T4 Rnl2) were from New England Biolabs.

The *RtcA* gene was amplified from genomic DNA of *E. coli* K-12 strain NEB Turbo (New England Biolabs) using the PCR primers CATCATATGAAAAAGGTAGATTGCGC (forward) and TTGGATCCTCATTCAATGCTCACCC (reverse) with Ndel and BamH I restriction sites, respectively (underlined). The PCR product was cloned, expressed, and purified as described above for archaeal RNA ligases.

*DNA Ligases*—Phage T4, T3, and T7 DNA ligases and the *Chlorella* virus DNA ligase (PBCV1) SplintRTM were from New England Biolabs. Antarctic phosphatase (AnP), T4 PNK, and yeast 5′-Deadenylase were from New England Biolabs.

*Substrates*—The oligonucleotides used in this study were synthesized by Integrated DNA Technologies. The 3′-phosphate donor substrates were as follows: CTGAGGCAATCATATT (pDNA17p); FAM-CTGAGGCAATCATATT-p (FAM-DNA17p); FAM-CUGAU GGCAACUAUAU-p (FAM-RNA17p); and FAM-CUGAU GGCAACUAUAA (DNA17, OMe)p. FAM is carboxyfluorescein. The 5′-phosphate donor substrate was pCTGAGGCAATCATATT-NH2 (pDNA17-NH2). Double-stranded DNA substrate with a single-strand 3′-phosphate break was created by annealing three DNA oligonucleotides: FAM-CTGAGGCAATCATATT-p (FAM-DNA17p), CATGTGAGCTACATTTTCTTCC (DNA25), and GACACTGGTGGTTATACATCGGCGGGAAGTAAAGG (DNA42). Annealing was performed in 40 μl of solution containing 15 μM FAM-DNA17p, 15 μM DNA25, and 16.5 μM DNA42 (1:1:1.1 ratio) in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5) by heating the mixture at 90 °C for 3 min and leaving...
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it on a heat block that was turned off (Van Waters and Rogers) for 2 h to reach room temperature. Completion of the annealing reaction was confirmed by Banl restriction endonuclease (the recognition site is underlined) digestion at 25 °C for 90 min. After boiling for 2 min, the reaction analyzed on a 15% urea-Tris borate-EDTA denaturing polyacrylamide minigel (Invitrogen).

**Assays**

Terminal phosphate modifications of oligonucleotides with RNA and DNA ligases or RtcA, which are labeled as 1′′enzymes in Fig. 1, were performed in a 10-μl volume. The reactions contained 5–10 pmol of either 3′- or 5′-terminal phosphorylated substrate, 10 pmol of enzyme, 500 μM ATP in buffer containing 10 mM BisTris propane HCl (pH 7.0), 10 mM Mg2+, and 1 mM DTT in 200-μl PCR tubes. The reactions were incubated in an S1000 Thermal Cycler (Bio-Rad) for 30 min at 65 °C for thermophilic RNA ligases or 25–37 °C for other enzymes, followed by heat inactivation at 90 °C for 3 min before next step analysis. All parameters that are different from the standard reaction conditions are indicated in the figure legends.

**Analysis of Reaction Products**

To determine which enzymatic modifications, if any, occurred to an oligonucleotide 3′-phosphate, the products were either assayed directly or treated with a second enzyme: 5 units of AnP supplemented with 25 μM Zn2+, 5 units of T4 PNK, or 5 units of yeast 5′Deadenylase. All reactions were incubated at 37 °C for 60 min. Parameters different from the standard reaction conditions are described in the figure legends. The presence of a 2′,3′-cyclic phosphate on RNA can be determined by the differential activity of two phosphatases. T4 PNK has 3′-phosphatase activity and 2′-phosphodiesterase activity and can dephosphorylate 2′,3′-cyclic phosphate. Antarctic phosphatase has 3′-phosphatase activity but is unable to cleave the 2′,3′-cyclic phosphate. If the 3′-phosphate is adenylated, producing RNAAppA or DNAAppA, they are identified by slower mobility on 15% urea-PAGE (Fig. 1A) or, for better resolution, by capillary electrophoresis (fragment analysis). The reaction products were analyzed by gel shift analysis on denaturing 15% urea-PAGE (Fig. 1A) to allow comparison between assays. Treatment of 3′-phosphorylated RNA with MthRnl in the presence ATP resulted in a small shift in band mobility on PAGE (Fig. 1A, compare lanes 4 and 7), which was clearly evident by fragment analysis (Fig. 1B, compare panels 1 and 4).

RESULTS

**Archaea Mth RNA Ligase Has RNA 2′,3′-Phosphate Cyclization Activity—**We expanded our study of the substrate specificity of MthRnl to include RNA 3′-phosphorylated RNA. A 17-nucleotide RNA oligomer labeled with a fluorophore at the 5′ end (FAM-RNA17p) and a derivative where the 3′-terminal ribonucleoside was 2′-O-methylated (FAM-RNA17(OMe)p) were used as substrates for MthRnl. The activity of MthRnl was compared with the known 3′-phosphate-modifying activity of RtcA. After reaction with ligase and digestion with secondary enzymes, as described under "Experimental Procedures," the oligonucleotide products were analyzed by gel shift analysis on denaturing 15% urea-PAGE (Fig. 1A) or, for better resolution, by capillary electrophoresis (fragment analysis). The reaction products were aligned to internal fragment analysis DNA size standards of 15, 20, and 25 nucleotides (Fig. 1B) to allow comparison between assays. Treatment of 3′-phosphorylated RNA with MthRnl in the presence ATP resulted in a small shift in band mobility on PAGE (Fig. 1A, compare lanes 4 and 7), which was clearly evident by fragment analysis (Fig. 1B, compare panels 1 and 4).

Two phosphatases with different specificities were used to characterize the modifications of RNA 3′-phosphate by MthRnl. Treatment with T4 PNK produced a slower migrating band on the gel, but treatment with AnP did not (Fig. 1A, lanes 5 and 6). The use of fragment analysis gave a cleaner separation of these reaction products (Fig. 1B, panels 5 and 6). No change in mobility was observed after AnP treatment (Fig. 1B, compare panels 4 and 5), but, after T4 PNK treatment, a slower migrating band was observed (Fig. 1B, compare panels 4 and 6). Dephosphorylation of the starting FAM-RNA17p oligonucleotide with either AnP or T4 PNK produced a slower migrating band on the gel (Fig. 1A, lanes 2 and 3). This dephosphorylated band has the same mobility as FAM-RNA17p reacted with MthRnl and then treated with T4 PNK (Fig. 1B, panel 6). We concluded that this product was the dephosphorylated oligonucleotide FAM-RNA17. Because the product formed by the reaction of MthRnl with FAM-RNA17p was resistant to dephosphorylation by AnP but was converted to the dephosphorylated oligonucleotide by T4 PNK, we concluded that it was RNA 2′,3′-cyclic phosphate.

To further confirm that the MthRnl product is RNA 2′,3′-cyclic phosphate, we performed the same reactions with RNA 3′-phosphate cyclase from E. coli, RtcA. These reactions were performed under identical conditions as the MthRnl reaction but at a lower temperature to match the temperature optimum of RtcA (25). Fragment analysis revealed that the product generated by RtcA (Fig. 1B, panel 13) migrated identically to the MthRnl reaction product (Fig. 1B, panel 4). The RtcA reaction product of FAM-RNA17p was resistant to digestion by AnP but sensitive to digestion by PNK. This is identical to what was observed with the MthRnl reaction products (Fig. 1B, compare panels 5 and 6 and 14 and 15).

When the 3′-terminal 2′-OMe analog FAM-RNA17(OMe)p was used as a substrate, MthRnl produced a slower migrating band that was approximately one nucleotide larger than the starting material on a 15% denaturing urea-PAGE gel (Fig. 1A, compare lanes 11 and 14). This product was not sensitive to T4
PNK or AnP phosphatases. The oligo showed no change in mobility by PAGE after treatment with either phosphatase (Fig. 1A, compare lanes 12 and 13 to lane 11), and no change was observed by fragment analysis (Fig. 1B, panels 10–12). RtcA treatment of the 2'-OMe substrate also generated a peak of the same mobility as the sample treated with MthRnl and was resistant to both phosphatases (Fig. 1B, panels 16–18). These results demonstrate that MthRnl and RtcA have the same 3'-phosphate adenylation activity, producing RNA(2'OMe)3'pp5'A as a product (29). MthRnl and RtcA both produced the same 2',3'-cyclic phosphate from FAM-RNA17p and the same RNA(2'OMe)3'pp5'A product from the 2'-O-methylated derivative. These results suggest that MthRnl and RtcA share the same mechanism for the cyclization of 3'-phosphorylated RNA oligonucleotides through a RNA3'pp5'A intermediate, followed by internal esterification with the adjacent 2'-hydroxyl. MthRnl,

**FIGURE 1.** RNA 3'-terminal phosphate modifications with MthRnl. *A*, gel shift analysis of archaeal RNA ligase (MthRnl) reaction products of either FAM-RNA17p (column I) or FAM-RNA17(OMe)p (column II) followed by phosphatase treatment with either AnP or PNK. Lanes 1, 7, 8, and 14 (input) were control reaction mixtures without enzymes. Additional controls included incubation with only AnP (lanes 2 and 9) or only PNK (lanes 3 and 10). The reactions treated with MthRnl (lanes 4–6 and 11–13) were further treated with either AnP (lanes 5 and 12) or with T4 PNK (lanes 6 and 13). The reaction conditions and buffers are described under "Experimental Procedures." The products were analyzed on urea-PAGE and visualized with SYBR Gold staining. Positions of oligonucleotide markers are indicated on the left. *B*, fragment analysis (capillary electrophoresis) of 3'-phosphate modifications produced by various ligases (1st enzyme, right panel) of either FAM-RNA17p (column I) or FAM-RNA17(OMe)p (column II), followed by phosphatase treatment with either AnP or PNK (2nd enzyme, right panel). A dashed line indicates no enzyme treatment. The reactions described in *A* are presented in panels 1–12. As a positive control, 10 pmol of FAM-RNA17p or FAM-RNA17(OMe)p were treated with RtcA (panels 13 and 16). These products were additionally treated with AnP (panels 14 and 17) or T4 PNK (panels 15 and 18). The T4 RNA ligase 1 and 2 reactions with FAM-RNA17p or FAM-RNA17(OMe)p were incubated at 25 °C for 2 h in the buffer supplied by the manufacturer (panels 19–26). Some reactions were also treated with AnP (panels 20, 22, 24, and 26). Substrates and corresponding products are indicated at the bottom and are color-coded. Substrates are shown in red, the products of primary enzymes and unreactive substrates of secondary enzymes are shown in green, and the products of secondary enzymes are shown in blue. The DNA standards are not colored. *C*, schematic of the enzymatic reactions in *A* and *B*.
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similar to RtcA, also did not produce any detectable ligation product in the reaction of FAM-RNA17p with free 5’-hydroxyl of various RNA substrates in the presence of ATP, GTP, Mg\(^{2+}\), or Mn\(^{2+}\) (data not shown).

To address the question whether similar 3’-phosphate modification of RNA was a common property of other RNA ligases, we tested T4 RNA ligases 1 and 2 for activity on FAM-RNA17p and RNA17(2’OMe)p. These reactions were performed with a molar excess of enzyme as in the RtcA assays. As shown in Fig. 1B, panels 19–26, under these conditions, neither T4 RNA ligase 1 nor T4 RNA ligase 2 modified RNA with a 3’-phosphate. After the reaction, the mobility of the oligonucleotide was unchanged. In a previous report, however, T4 RNA ligase 1 has been shown to catalyze 3’-phosphate cyclization at a very slow rate (30).

**Thermophilic RNA Ligases Modify Terminal 3’-Phosphate of ssDNA**—Thermophilic RNA ligases have been shown to catalyze the ligation of 5’-phosphorylated ssDNA substrates in addition to RNA substrates (22, 26). On the basis of our results in the previous section, it was hypothesized that these ligases could also modify terminal 3’-phosphate on ssDNA oligonucleotides through the enzymatic transfer of adenosine monophosphate. This activity has been reported for T4 Rnl1. However, it required several days of incubation (30). We first tested three thermophilic RNA ligases, the archaeal enzyme MthRnl, the bacterial enzyme TVa, and a phage-encoded enzyme, TS2126, with a 3’-phosphorylated DNA 17-mer (ssDNA17p) and analyzed the reaction products by gel shift on denaturing 15% PAGE. As shown in Fig. 2A, all three enzymes formed a product with a shift approximately one nucleotide longer than

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**FIGURE 2.** A single-stranded DNA 3’-terminal phosphate modification by thermophilic RNA ligases. Enzymes and reactions for each experiment are indicated at the tops of the panels. A, gel shift analysis of reaction products of DNA17p with MthRnl, TVa, and TS2126. Reaction mixtures were incubated at 65 °C for 60 min, heat-inactivated at 90 °C for 5 min, and treated with 10 µg of proteinase K at 37 °C for 60 min. The products were analyzed on 15% urea-PAGE and visualized with SYBR Gold staining. Positions of a substrate (ssDNA17p) and an adenylated product (ssDNA17ppA) are indicated on the right. Positions of oligonucleotide markers are indicated on the left. C, control. B, pH dependence of α-[\(^{32}\)P]AMP incorporation into DNA17p using 5 pmol of a DNA17p substrate, 10 pmol of MthRnl under the conditions described for A (except for the indicated variable pH), and supplement with 1 µCi of α-[\(^{32}\)P]ATP. B I, reaction products were treated and separated as described for A. B II, radioactive products visualized by PhosphoScreen scanning. Mr is the oligonucleotide molecular weight marker. C, comparison of 5’- and 3’-phosphorylated ssDNA adenylation with MthRnl. Reactions were performed as described in A using 5 pmol of pDNA17-NH₂ or DNA17p and variable amounts (5,000–0.625 pmol) of enzyme. The molar ratio of substrate to enzyme (S/E) is listed at the bottom of the gel. D, comparison of 5’- and 3’-phosphorylated ssDNA adenylation with RtcA. Reactions were performed and analyzed as described for MthRnl in C but at 37 °C with the variable substrate-to-enzyme ratios indicated at the bottom. E, deadenylation of 3’- and 5’-phosphate-modified ssDNA with yeast 5’ Deadenylase. 5 pmol of DNA17ppA and AppDNA17-NH₂, the products of MthRnl modification described in legend for A, were treated with serially diluted 5’ Deadenylase at 25 °C for 30 min. The products were analyzed by gel electrophoresis as described in A. Variable substrate-to-enzyme ratios are indicated at the bottom. F, K97A MthRnl mutant activity assays using 5 pmol ssDNA17p substrate were performed and analyzed as described for wild-type enzyme in C.
activities were compared with RtcA, we found that both same sequence showed similar activity (Fig. 2).

Even though the ssDNAp substrate used for these assays migration of DNA17p was resistant to both AnP and PNK phosphatase-sensitive, which suggested that the deadenylated product was ssDNA17p (data not shown). Additional confirmation that the product was ssDNA17p was made by treatment with yeast 5′-Deadenylase, which can resolve the 5′-App group to 5′-phosphate (31). The resulting product was approximately one nucleotide shorter (Fig. 2E), migrated identically to the ssDNA17p starting oligonucleotide, and was AnP and PNK phosphatase-sensitive, which suggested that the deadenylated product was ssDNA17p (data not shown). Thus, yeast 5′-Deadenylase can also convert 3′ppA groups to 3′-phosphates, a newly observed activity for this enzyme. 5′Deadenylase appears to be about five times more active on 5′-adenylated DNA compared with 3′-adenylated DNA substrates. The mechanism of DNA 3′-phosphate adenylation by MthRnl is likely the same as 5′-phosphate DNA adenylation because the reaction for both substrates is blocked when the catalytically inactive mutant (K97A) of MthRnl was used (Fig. 2F) (27).

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nucleotide oligomer contained a 6-FAM fluorescent label at the 5′-end (FAM-DNA17p). Completion of the FAM-DNA17p hybridization into the double-stranded form was confirmed by restriction endonuclease BanI digestion, which produced a six-nucleotide-long product (FAM-DNA6) that was detected by a fluorescent scan of a denaturing PAGE (Fig. 3A). Because of the short length of the DNA duplex it was expected that the duplex would melt at the standard reaction temperatures of the thermophilic ligases. Therefore, all reactions with this substrate were performed at 25 °C.

When T4 DNA ligase was tested on the nicked dsDNA substrate, a FAM-labeled product one nucleotide longer than the starting material was observed. This shift is very similar to products produced with MthRnl and RtcA using ssDNA. We concluded that T4 DNA ligase indeed adenylated the 3′-phosphorylated nick (Fig. 3C, lane 2). Neither T3, T7, nor PBCV1 DNA ligases have detectable 3′-adenylation activity in this assay (Fig. 3C, lanes 3–5). In contrast to thermophilic RNA ligases, none of the tested DNA ligases react with 3′-phosphorylated single-stranded DNA (Fig. 3D). DNA ligases are very specific for double-stranded substrates and have very poor activity on ssDNA substrates, indicating the lack of single stranded substrate in the reaction conditions with duplex DNA.

The thermophilic RNA ligases and RtcA were assayed on nicked dsDNA3′p substrate (Fig. 3C, lanes 6–9). RtcA was able to adenylate a 3′-phosphorylated nick in the double-stranded DNA substrate (Fig. 3C, lane 6), a novel activity for this enzyme. A previous report found that RtcA can adenylate a 5′-phosphate at a nick in dsDNA (25). This extends the range of DNA substrates that can be modified by RtcA to include either 5′- or 3′-phosphates on dsDNA or ssDNA (Fig. 2D). With the 3′-phosphorylated nicked dsDNA substrate, neither MthRnl nor TS2126 RNA ligases had significant 3′-adenylation activity despite overnight incubation (Fig. 3C, lanes 7 and 9). Although the lack of adenylation activity on this double stranded substrate was most likely due to substrate specificity, we cannot rule out low activity at low temperature, which was 40 °C below optimal. However, the putative thermophilic RNA ligase from *T. ammonificans*, TVa, was active with respect to the adenylataion of 3′-phosphorylated nicked DNA (Fig. 3C, lane 8). None of the tested ligases, RNA or DNA, displayed any nick-sealing activity on this 3′-phosphorylated dsDNA substrate.

DISCUSSION

We are revising our view of the function of RNA ligases with the discovery that they have unexpected diversity in their choice of phosphate donor substrates. We found that traditional RNA ligases that use a 5′pRNA as a substrate for ligation can also modify either RNA or ssDNA that has a 3′-phosphate. All studied ATP-dependent polynucleotide ligases activate a 5′-phosphorylated nucleic acid substrate by transferring an adenosine monophosphate from an active site lysine to the substrate 5′-phosphate, producing a 5′-adenylated intermediate. We report here that “canonical” ATP-dependent ligases also possess polynucleotide 3′-terminal phosphate adenylation activities, in most cases comparable with their 5′-adenylation activity.

The archaeal RNA ligase MthRnl converts 3′-phosphorylated RNA termini into 2′,3′-cyclic phosphates using the same tripartite mechanism of the classic ligation reaction. The first step of the reaction is the same as for classic ligation. The enzyme is adenylated with ATP at the ε amino group of a catalytic active site lysine. Second, this AMP group is transferred to 3′-terminal phosphate, creating the activated intermediate 3′-adenylated RNA (RNA3′pp1A), where adenosine is joined to the 3′ end of RNA through a diphosphate linkage. In the third step, activated phosphate is attacked by adjacent 2′-hydroxyl, making the intramolecular “ligation” product 2′,3′-cyclic phosphate. When 2′-terminal hydroxyl is blocked by methylation, the reaction stops at the adenylated intermediate in step two of the reaction. The adenylated intermediate is also generated when the 3′-phosphorylated substrate is ssDNA, which does not have a 2′-hydroxyl acceptor. This mechanism is almost identical to the 3′-terminal phosphate cyclization activity described for RtcA (9), except that the catalytic residue in RtcA is histidine and not lysine (18, 32). The identity of the reaction products was determined by mobility on polyacrylamide gel or capillary electrophoresis in comparison with known modifications and also by differential sensitivity to alkaline phosphatase, the phosphatase activity of T4 polynucleotide kinase, and yeast 5′-Deoxyadenylate.

We have shown previously that substitution of catalytic lysine to alanine (K97A) in the active site of MthRnl abolished the reaction of the enzyme with ATP and, consequently, blocked adenylation of the 5′-phosphate of RNA or ssDNA (27). We also found that the K97A mutant of MthRnl was unable to adenylate the DNA 3′-phosphorylated substrate (Fig. 2E), indicating that the mechanism of 3′-adenylation is likely identical to that of 5′-adenylation. This observation has interesting implications for the mechanism used by MthRnl to recognize an RNA or ssDNA phosphate donor substrate in either the 3′ or 5′ orientation.

The biological role for RNA 2′,3′-cyclic phosphate is not well understood. The wide conservation of RtcA in all life forms suggests that the enzyme is essential. However, *E. coli* strains lacking RtcA are not impaired in growth (33). This may be partially explained by the fact that both RtcA and RtcB have cyclase activity. In the case of *Methanobacteria*, there are three enzymes that have RNA-2′,3′phosphate cyclase activity: MthRnl and homologues of RtcA and RtcB. Although they share this biochemical activity, their biological roles may be diverse. RtcA has cyclase but no ligase activity, and RtcB can ligate RNA3′p but not 5′pRNA, whereas the reverse is true for MthRnl; it is only able to ligate RNA with a 5′-phosphate.

Although MthRnl is called an RNA ligase, it has the ability to modify both RNA and ssDNA substrates. Its activities include adenylation of either the 5′-phosphate or the 3′-phosphate of ssDNA, ligation of either 5′pDNA or 5′pRNA, and RNA 2′,3′-phosphate cyclase activity. An element common to all of these substrates is a terminal phosphate, which must be a major determinant in substrate binding. The ability to ligate either 5′pRNA or 5′p-ssDNA and to adenylate either the 5′ or 3′ end of ssDNA suggests a remarkable flexibility in the active site of the enzyme. Both TVa ligase and TS2126 RNA ligase have also been found to adenylated ssDNA3′p. However, neither T4
RNA ligase 1 nor T4 RNA ligase 2 can modify RNA 3’p (Fig. 1B, panels 19–24). These phage ligases function in concert with T4 PNK, which has a 3’-phosphatase activity that removes the RNA 3’-phosphate and a kinase activity to generate a 5’pRNA, which creates the correct lysine tRNA substrate for ligation (20). It appears that the archaeal Mth RNA ligase has evolved to have a wider range of functions.

We also tested the ability of ligases to adenylate a 3’-phosphate nick in dsDNA. Of the three thermophilic enzymes tested only the TVa ligase from *T. amonificans* was able to adenylate the nicked substrate. The enzyme was identified by sequence similarity to T4 Rnl1 and has not been characterized. We found that the recombinant enzyme has strong 5’ and 3’ adenylation activity. However, actual RNA ligation activity is very weak (data not shown). Of the mesophilic dsDNA ligases tested on this substrate, only T4 DNA ligase was able to adenylate the 3’-phosphate nick. As expected, neither T4 DNA ligase nor any of the other dsDNA ligases are able to adenylate ssDNA3’p. This also served as a control that dsDNA did not melt in reaction conditions. Even though adenylation was observed on DNA substrates with a 3’-phosphate, none of the enzymes were able to ligate these substrates. Only RtcB has been reported to ligate ssDNA 3’-phosphate in a stem loop structure but not a nicked dsDNA substrate (15).

Our study of thermophilic RNA ligases suggests that they have activities beyond RNA ligation. These enzymes are able to modify either the 5’- or 3’-terminal phosphates of RNA or DNA. Their ability to adenylate the 3’-phosphate on DNA should stimulate studies to determine what role, if any, they may have in the repair of damaged DNA. The RNA cyclase activity may have a role in protecting RNA3’p from exonucleases and allowing their repair by ligation. Additional research is required to elucidate the biological role of these 3’-phosphate modifications.

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