Bacteriophage T4 RNA Ligase

REACTION INTERMEDIATES AND INTERACTION OF SUBSTRATES*

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Bacteriophage T4 RNA ligase catalyzes the ATP-dependent ligation of a 5'-phosphoryl-terminated nucleic acid donor to a 3'-hydroxyl-terminated nucleic acid acceptor. We have identified adenylylated DNA and RNA reaction intermediates in which the AMP moiety is attached by a pyrophosphate bond to the 5'-phosphoryl group of the donor. A large amount of DNA-adenylate accumulates during the reaction and the dependence of joining and adenylylation on chain length are similar. The adenylylated donor is joined by ligase to an acceptor in the absence of ATP, and AMP is released stoichiometrically in this reaction. The acceptor is not only a substrate in the reaction but also a cofactor for adenylylation of the donor; in the absence of a 3'-hydroxyl group the activated intermediate does not form. The activated DNA need not join to the acceptor that initially stimulated activation but can also join to another acceptor. This process of acceptor exchange has proven useful for promoting the cyclization of small DNA substrates and the synthesis of DNA co-polymers.

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Hurwitz and co-workers discovered that bacteriophage T4 induces the formation of a novel enzyme, RNA ligase, which catalyzes the ATP-dependent ligation of single-stranded RNA (1). The enzyme is quite versatile. While the predominant reaction is intramolecular yielding a cyclic product (1), several groups have shown that RNA ligase also catalyzes end to end joining of two RNA chains (2-6). We demonstrated that DNA is also a substrate. RNA ligase catalyzes the formation of circular DNA, linear DNA dimers, and RNA-DNA and DNA-RNA block co-polymers (7, 8). There appears to be little base specificity in the reaction since a variety of homopolymers and fragments of RNA and DNA are substrates (1-10). The enzyme prefers relatively short nucleic acids with the optimum chain length being about 20 (Refs. 1 and 10 and this report). RNA ligase is abundant in T4-infected cells and has been extensively purified in high yield (6, 7).

RNA ligase differs from DNA ligase in two main respects. It efficiently joins single-stranded nucleic acids in the absence of a complementary strand and it has a high activity with RNA substrates (1, 11). The freedom of the reaction from a complementary strand has attracted considerable interest in the enzyme as a reagent for the synthesis of defined sequence nucleic acids (2, 4, 6-8). We are also interested in the mechanism of the RNA ligase reaction. Cranston et al. showed that the first step in the reaction is the formation of an adenylyl-enzyme intermediate as also occurs with DNA ligase (9, 11). An adenylylated RNA intermediate was not demonstrated, although some evidence for its existence has been obtained (3). During our studies of the ligation of DNA chains we uncovered this adenylylated substrate. This report describes the isolation and identification of the adenylylated DNA and RNA intermediates of the RNA ligase reaction and the interplay between the 3'-hydroxyl and 5'-phosphoryl termini of the substrates.
Preparation of 3'-Blocked Oligonucleotides—The 3' terminus of 15'-32P-32P-A from was blocked by oxidation with a 5-fold excess of sodium metaperiodate (J. T. Baker Chemical Co.) for 20 min at room temperature (13). Excess glucose was added to consume remaining oxidant and incubation was continued for 15 min. The oxidation product was purified by Sephadex G-50 gel filtration. DNA substrates were blocked by oxidation by addition of sodium metaperiodate (14). The reaction mixture (0.5 ml) contained 0.1 M sodium cacodylate, pH 7.0, 0.1 M potassium phosphate, pH 7.0, 1 mM MgCl2, 1 mM 2-mercaptoethanol, 6 mM dGTP, 20 μM 5'-32P-labeled oligonucleotide, and 40 units of terminal deoxynucleotidyltransferase. After 60 min at 37°C, an additional 20 units of enzyme were added and incubation was continued for 60 min. The mixture was heated to 100°C for 2 min and the product was purified by Sephadex G-50 chromatography.

Enzymes—Micrococcal nuclease and spleen phosphodiesterase were purchased from Worthington Biochemical Corp. and the latter enzyme was further purified (16). Crotalus atrox nucleotidyl pyrophosphatase, type III, and bacterial alkaline phosphatase, type IIIS, were obtained from Sigma Chemical Co. Alkaline phosphatase was also purified according to the method of Simpson et al. (17). Pronase was purchased from Calbiochem and autodigested for 1 h at 10 mg/ml in 20 mM Tris/HCl, pH 7.5, 1 mM EDTA before use. Terminal deoxynucleotidyltransferase was obtained from Grand Island Biological Co. Bacillus subtilis DNA polymerase III was prepared according to Low et al. (18). Polyomavirus kinase and T4 DNA ligase were purified by the method of Panet et al. (19). The latter enzyme, a gift of Dr. K. Agarwal of this university, was free of RNA ligase activity, as measured by the [5-32P]PAP assay, and had a low DNA ligase activity (less than 0.01 pmol of bonds broken/unit of RNA ligase), and DNA endonuclease activity (less than 0.01 pmol of nucleotides released/unit of RNA ligase), and DNA endonuclease activity (less than 0.01 pmol of bonds broken/unit of RNA ligase activity). Assays of T4 DNA ligase used the procedure of Gupta et al. (20) with standard buffer. The DNA ligase assay conditions, with 0.12 unit of RNA ligase under standard reaction conditions, were identical since the curve in Fig. 1 was linear at 0.1 to 0.6 unit of enzyme. After 30 min at 37°C, the reaction mixture was heated for 2 min at 100°C, and then after removal of the adenylate moiety by treatment with acid or periodate then base, whereas this protocol does not remove the phosphate moiety of the joined product. By this assay, the adenylylated intermediate was differentiated from joined product by its sensitivity to phosphatase after destruction of the pyrophosphate linkage either by reaction with 1 N HCl for 15 min at 100°C (Method I) or by successive treatment with 5 mM sodium metaperiodate for 20 min at 25°C and 0.25 M lysine, pH 8.0, for 30 min at 4°C (Method II) (13, 21). The results were corrected for the small amount of the joined product (generally 5%) which was broken down by acid treatment. In experiments where RNA acceptors were present, the phosphatase-resistant material was purified by Sephadex G-50 gel filtration. Joint molecules made with an RNA acceptor were estimated from the amount of 2'(3')-rAMP generated by alkaline hydrolysis. Paper chromatography with Solvent I was used in the analysis. To determine the presence of RNA-DNA co-polymer and activated intermediate, the phosphatase- and alkali-resistant products were purified by Sephadex G-50 chromatography and analyzed by Method I or II above. The DNA-DNA joined product was also characterized by a nearest neighbor analysis following the method of Josse et al. (22).

RESULTS

Isolation of Oligo(dT)-Adenylate Intermediate—We showed previously that incubation of 5'-32P-[PATp]dA-dN, with RNA ligase yields predominantly a circular product (7). A second species resistant to bacterial alkaline phosphatase is also formed which we identify here as the 5'-adenylated substrate. These two phosphatase-resistant species can be distinguished easily. The 5'-adenylated substrate is phosphatase-sensitive after removal of the adenylate moiety by treatment with acid or periodate then base, whereas this protocol does not remove the 5' phosphohexy terminus of substrate. The 5'-adenylated substrate is blocked at the 3' end by periodate oxidation, was incubated with 0.12 unit of RNA ligase under standard reaction conditions, except 0.6 mM [32P]ATP was used and 300 μM d[A(TpA)], was added. After 2 h at 37°C, the reaction mixture was treated sequentially with 4 units of phosphatase for 20 min at 65°C and 50 μg of pronase for 30 min at 37°C, and the products were purified by Sephadex G-50 chromatography. The doubly labeled material that eluted near the void volume was concentrated by lyophilization and a portion was degraded with 20 μg/ml of RNase A and RNA was further degraded by treatment with 2 units of phosphatase at 37°C for 20 min, and then chromatographed in Solvent II. Material that migrated with an internal A15'ipp'5' A standard was eluted, concentrated by lyophilization, and subjected to electrophoresis at pH 3.5. Material coincident with A15'ipp'5' A was eluted, concentrated, and partially digested with C. atrox nucleotidyl pyrophosphatase. The reaction mixture (0.15 ml) contained 30 mM Tris/HCl, pH 7.5, 7 mM MgCl2, and 0.1 μg of enzyme. After 30 min at 37°C, EDTA was added to 15 mM and the products characterized by electrophoresis.

Differentiation of Activated Intermediate and Joined Product—The total amounts of adenylated substrate and true joined products were measured by resistance to bacterial alkaline phosphatase. The adenylated intermediate was differentiated from joined product by its sensitivity to phosphatase after destruction of the pyrophosphate linkage either by reaction with 1 N HCl for 15 min at 100°C (Method I) or by successive treatment with 5 mM sodium metaperiodate for 20 min at 25°C and 0.25 M lysine, pH 8.0, for 30 min at 4°C (Method II) (13, 21). The results were corrected for the small amount of the joined product (generally 5%) which was broken down by acid treatment. In experiments where RNA acceptors were present, the phosphatase-resistant material was purified by Sephadex G-50 gel filtration. Joint molecules made with an RNA acceptor were estimated from the amount of 2'(3')-rAMP generated by alkaline hydrolysis. Paper chromatography with Solvent I was used in the analysis. To determine the presence of RNA-DNA co-polymer and activated intermediate, the phosphatase- and alkali-resistant products were purified by Sephadex G-50 chromatography and analyzed by Method I or II above. The DNA-DNA joined product was also characterized by a nearest neighbor analysis following the method of Josse et al. (22).

Chromatography and Electrophoresis—Paper chromatography and electrophoresis used Whatman No. 3MM paper. The pH 8.0 sodium acetate buffer for gel filtration was 0.05 M sodium citrate, pH 3.5. The solvents for chromatography were 1-propanol/concentrated NH4OH/H2O, 63/1 (Solvent I), and isobutyl alcohol/nitric acid/H2O, 100/0.1 (Solvent II). Homochromatography of oligonucleotides used the Kleid et al. (23) modification of the method of Brownlee and Sanger (24). Two-dimensional chromatography on cellulose thin layer plates (Eastman) was performed essentially by the method of Khorana and Vizvoly (25); this procedure resolves cyclic and linear oligonucleotides. Two-dimensional electrophoresis on cellulose thin layer plates (Whatman) was performed essentially by the method of Olmsted (26). The samples were obtained from Pharmacia Fine Chemicals. The buffer for gel filtration was 50 mM triethylammonium bicarbonate, pH 8.0; fractions were lyophilized and resuspended in water.

Isolation of Oligo(dT)-Adenylate Intermediate—We showed previously that incubation of 5'-32P-[PATp]dA-dN, with RNA ligase yields predominantly a circular product (7). A second species resistant to bacterial alkaline phosphatase is also formed which we identify here as the 5'-adenylated substrate. These two phosphatase-resistant species can be distinguished easily. The 5'-adenylated substrate is phosphatase-sensitive after removal of the adenylate moiety by treatment with acid or periodate then base, whereas this protocol does not remove the 5' phosphohexy terminus of substrate. The 5'-adenylated substrate was for all the dT, substrates tested between 6 and 30 nucleotides long (Fig. 1). The chain length dependence for adenylation and true joining were similar but not identical since the curve in Fig. 1 for adenylation is displaced toward smaller substrates. For the poorly ligated substrates, dT, and dT, the adenylated substrate was the predominant phosphatase-resistant species. The structures of the two phosphatase-resistant species were corroborated by measurement of sensitivity to the exonuclease of Bacillus subtilis DNA polymerase III. The circular joined products are nucleo-
adenylylated structure. The RNA and DNA ligase intermediates have the same 5'-phosphatase-resistant after acid treatment. We conclude that ligase, 70% of the 32P was converted to a form which was

The joining of the doubly labeled substrate to acceptor and the release of 32PAMP were coordinate and stoichiometric (Fig. 3). The formation of a phosphodiester bond between donor and acceptor was confirmed by a nearest neighbor analysis; the molar ratio of 32P to 31P which was phosphatase-sensitive after periodate plus base treatment.

The adenylylated substrate is a true reaction intermediate and not a dead-end reaction product. When the 32P- and 31P-labeled adenylylated dT T was incubated with RNA ligase and 3',5'-OH rA, but in the absence of ATP, 5'-32PAMP was released (Fig. 3). The addition of ATP has the expected effect of diminishing the release of 32PAMP, since adenylylated enzyme cannot catalyze ligation of the adenylylated substrate. The joining of the doubly labeled substrate to acceptor and the release of 32PAMP were coordinate and stoichiometric (Fig. 3). The formation of a phosphodiester bond between donor and acceptor was confirmed by a nearest neighbor analysis; the product of alkaline digestion was identified as 2'(3')-32PAMP by chromatography in Solvent II. The RNA ligase intermediate is also a substrate for T4 DNA ligase in the absence of ATP. When 100 pmol of adenylylated [5'-32P]dT T annealed to poly(dA) were incubated for 9 h at 25° with 6 units of DNA ligase, 70% of the 32P was converted to a form which was phosphatase-resistant after acid treatment. We conclude that the RNA and DNA ligase intermediates have the same 5'-adenylylated structure.

Isolation of Adenylylated RNA—While DNA and RNA are about equally efficient donor molecules in the RNA ligase reaction, RNA is generally an order of magnitude better acceptor (7). This probably explains why the cyclization of 5'-32PAMP proceeded efficiently without detectable accumulation of activated intermediate. To demonstrate the intermediate with this substrate, the 3'-end was oxidized by periodate treatment and the poorer DNA acceptor, d[ApA] 4, was added with enzyme and [3H]ATP. After 2 h at 37°, the phosphatase-resistant material was purified by Sephadex G-50 chromatography and treated successively with alkaline phosphatase, and the products were analyzed by chromatography in Solvent II (Fig. 4A). All the 3H migrated with internal A(5')pp(5')A reference, while the 32P migrated with A(5')pp(5')A (from the intermediate), P (from RNA to DNA joined product), and oligo(dA) terminated with rA (from RNA to DNA joined product). The material migrating with A(5')pp(5')A was eluted and subjected to electrophoresis at pH 3.5 (Fig. 4B). All the 32P and 3H were coincident with the internal A(5')pp(5')A reference. When this doubly labeled compound was digested with snake venom pyrophosphatase, both the 3H- and 32P-labeled products migrated with pA. The molar ratio of 32P:AMP to

FIG. 1. Chain length dependence of activation and cyclization of dT T. The standard reaction mixture (20 ml) contained 200 pmol of [5'-32P]dT T and 0.6 unit of RNA ligase. After 1 h at 37°, the extent of adenylylation (□) and joining (●) were measured by Method I described under "Materials and Methods." Under these conditions, the reactions were generally linear for at least 2 h, except the joining of dT T and dT T reached a plateau early in the reaction.

FIG. 2. Isolation of 32P- and 31P-labeled adenylylated dT T by Sephadex G-25 chromatography. The reaction mixture (2 ml) contained 10 μM [5'-32P]dT T (2.3 × 105 cpm/μmol), 0.33 mM [8-3H]ATP (4.5 × 105 cpm/μmol), and 1.2 units of RNA ligase. After 2 h at 37°, the mixture was heated at 100° for 2 min and treated successively with 0.2 mg of bacterial alkaline phosphatase for 20 min at 65° and 0.2 mg of pronase for 90 min at 37°. The products were applied to a column of Sephadex G-25, superfine (0.9 × 30 cm), fractions (0.5 ml) were collected, and the 32P (●) and 31P (□) were measured.

FIG. 3. Coordinate release of AMP from the adenylylated substrate and ligation to acceptor. The three 50-ml reaction mixtures contained 0.7 μM [32P]A(5')pp(5')dT(dT) T and 10 μM d(T[pT]) T, and either 1.2 units of RNA ligase and 1 mM ATP (■); enzyme but not ATP (●, ○); or neither enzyme nor ATP (□). After incubation at 37° for the indicated times, samples were removed and heated at 100° for 2 min. The release of [32P]5'-rAMP from the adenylylated substrate was measured by chromatography in Solvent I (closed symbols) and the extent of joining (□) was measured by the production of phosphatase-resistant 32P after acid treatment.

Unpublished data.
B, l-cm strips were counted for "H (O) and 32P (C). The positions of acceptor augments donor activation rather than the alternate acceptor 3'-hydroxyl group in the 5'-phosphoryl terminus.

Figure 4. Identification of A(5')pp(5')A as the product of alkaline plus phosphatase digestion of A(5')pp-adenylate. [3H]ATP was incubated with oxidized [5'-32P]ApApA, d(A[pA]n), and RNA ligase, and the phosphatase-resistant reaction products, representing 8% of the total 32P, were purified by Sephadex G-50 chromatography as described under "Materials and Methods." These were hydrolyzed successively with 0.4 M KOH for 20 h at 37° and 3 units of bacterial alkaline phosphatase for 20 min at 65° and chromatographed in Solvent I. A, 1 cm strips were monitored for "H (O) and 32P (C). The positions of internal A(5')pp(5')A and P are shown. Material migrating with A(5')pp(5')A was eluted and subjected to electrophoresis at pH 3.5. B, 1-cm strips were counted for "H (O) and 32P (C). The positions of A(5')pp(5')A, AMP, ADP, and ATP markers are shown.

[3H]AMP in the A(5')pp(5')A fragment was calculated to be 1.8 and not the expected 1.0. The discrepancy is probably the result of an error in determining specific activity, rather than an impurity in the material, since the ratio remained constant throughout the late steps of the analysis.

Activation of Donor Requires 3'-Hydroxyl-terminated Acceptor—Contrary to our expectation, the activation of a donor required the presence of an acceptor. Alteration of the 3'-hydroxyl terminus by periodate oxidation of an RNA substrate or addition of dTMP to a DNA substrate markedly reduced activation (Table I). The low activation seen with the blocked 3'-hydroxyl terminus or DNA acceptor was provided. The data in Table I show the effect of ApApA, but other acceptors including 3',5'-OH rA, and CpCpC have also been effective. Exchange of acceptors has proven useful not only in cyclizing small substrates, but also in promoting the intermolecular joining of DNA. For example, the rate of joining of dC3-dT7 to dA(pA)11 was increased more than 10-fold by the addition of ApApA (Table II).

**DISCUSSION**

Our current scheme for the RNA ligase-catalyzed reaction is:

\[
E + \text{pppA} + \text{Mg}^{2+} \rightarrow \text{EppA} + \text{PP}_{i} \tag{1}
\]

\[
\text{EppA} + pD \rightarrow A(5')pp(5')D + E \tag{2}
\]

\[
A(5')pp(5')D + R \rightarrow RppD + pA \tag{3}
\]

Sum: \[\text{pppA} + pD + R \rightarrow E_{i} + \text{pppA} + pA + PP_{i}\]

where pD and R refer to the donor and acceptor (receptor) molecules.

The first step of adenylylation of the enzyme was demonstrated by Cranston et al. (9). They found that in the absence of a polynucleotide substrate, the enzyme catalyzed a rapid exchange reaction between ATP and PP, but not between ATP and AMP. In the course of the reaction ATP was hydrolyzed to AMP and PP in amounts equimolar to joining (1). The isolated enzyme-adenylate complex could be dissociated by poly(rA) in

\( \text{Sum, } \text{pppA} + pD + R \rightarrow E_{i} + \text{pppA} + pA + PP_{i} \)

\( \text{Dissociation of the enzyme adenylate by substrate requires } \text{Mg}^{2+} \)
The standard reaction mixture contained 10 μM donor, 0.6 unit of RNA ligase, and where indicated 100 μM ApApA. After 1 h at 37°C, the amount of joined product and adenylylated substrate was measured by Method II described under "Materials and Methods."

### Table I

**RNA Ligase Reaction Intermediates**

<table>
<thead>
<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td>Intermedi-</td>
<td>Joined</td>
<td>Intermediate</td>
</tr>
<tr>
<td>pmol</td>
<td>pmol</td>
<td>pmol</td>
</tr>
<tr>
<td>rAB, oxidized</td>
<td>&lt;0.2</td>
<td>160</td>
</tr>
<tr>
<td>dT,,-1,dT,</td>
<td>10</td>
<td>25</td>
</tr>
<tr>
<td>dT,</td>
<td>&lt;0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>dT,,-1,dT,</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
</tr>
</tbody>
</table>

* The 3' terminus was oxidized by periodate treatment as described under "Materials and Methods."

### Table II

**Stimulation of DNA to DNA joining by acceptor RNA**

<table>
<thead>
<tr>
<th>Added acceptor</th>
<th>Alkaline phosphatase-resistant material pmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA (pdA)n</td>
<td>None</td>
</tr>
<tr>
<td>DNA (pdA)n, + ApApA</td>
<td>90*</td>
</tr>
<tr>
<td>DNA (pdA)n, - ApApA</td>
<td>25</td>
</tr>
</tbody>
</table>

*Includes 20 pmol of adenylylated donor and 45 pmol of RNA-DNA co-polymer.

### Figures

**Figure 5.** Identification of a RNA ligase reaction product by nearest neighbor analysis. The reaction mixture (0.2 ml) containing 10 μM (5'-32P)pdA, 100 μM ApApA, and 6 units of RNA ligase was incubated for 2 h at 37°C, heated for 2 min at 100°C, and treated with 0.5 mg of bacterial alkaline phosphatase for 20 min at 65°C. After incubation with 0.3 M KOH for 15 h at 37°C, the products were purified by Sephadex G-50 gel filtration. The products of a micrococcal nuclease and spleen phosphodiesterase digest (B) and an undigested control (A) were analyzed by paper chromatography using Solvent I. The material near the origin in B is probably incompletely digested adenylylated substrate.

The presence of Mg2+ to yield AMP, whereas ATP was generated in the presence of PD (2). We have confirmed the formation of the enzyme-AMP complex with the physically homogeneous enzyme used in this report. After incubation of RNA ligase with [α-32P]ATP and Mg2+, about 60% of the enzyme was adenylylated as measured by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

The second step in the reaction is the transfer of the adenylate moiety to the 5'-phosphoryl end of the donor, as also occurs in the T4 and Escherichia coli DNA ligase-catalyzed reactions (26, 27). The evidence for the structure of this intermediate is:

(a) After incubation with labeled ATP and [5'-32P]DNA or RNA, a covalently joined, doubly labeled molecule was isolated from the RNA ligase reaction mixture by gel filtration;
(b) the AMP moiety of the intermediate was dissociated by reaction with acceptor in the presence of RNA ligase and by treatment with acid or periodate and base; (c) the digestion of [3H, 32P]adenylylated rAB with alkali and phosphatase yielded doubly labeled A(5'ppp5')A as the only product; and
(d) the adenylylated dT10 intermediate generated by RNA ligase was ligated by T4 DNA ligase in the absence of ATP when a complementary poly(dA) strand was added. We conclude that A(5'ppp5')D is a reaction intermediate and not a dead-end product since release of the AMP was coordinate and stoichiometric with joining and this reaction occurred in the absence of ATP. A(5'ppp5')D is shown in the above reaction scheme free of enzyme, but it is not known if it remains enzyme-bound. What was demonstrated was that free enzyme can efficiently catalyze Step 3. While the data do not rigorously establish that the enzyme and substrate adenylates are kinetically significant intermediates on the main reaction path of the joining reaction (11), this seems quite likely.

Cyclization is generally the preferred reaction by orders of magnitude over intermolecular joining. The flexibility of the polynucleotide chain results in a high local concentration of the 3'-hydroxyl near the 5'-phosphoryl of the same molecule thereby favoring the intramolecular reaction. With very short substrates, cyclization is probably limited by the requirement for binding to the same molecule in the enzyme simultaneously at the donor and acceptor sites. The smallest donors and acceptors for intramolecular ligation were 2 to 3 nucleotides long and the smallest cyclizable substrate was 6 nucleotides long (Ref. 9; Fig. 1 and Table I). As the chain length increases beyond a hexamer, proper folding of the nucleic acid and binding to the enzyme is probably facilitated. Beyond the optimum chain length of about 20 nucleotides (Fig. 1), the diminishing local concentration of the acceptor near the donor results in a diminished reaction rate.

There were three unanticipated findings in this study. First, we did not expect the large accumulation of activated DNA under standard reaction conditions. With a short substrate such as dT10, much more adenylylated substrate than product is formed; even with dT100, the best DNA substrate we have found, a large amount of intermediate is formed (Fig. 1). In the study of E. coli and T4 DNA ligases, the DNA-adenylate was difficult to demonstrate unless unusual reaction conditions were employed (11). However, RNA is generally a much better substrate for RNA ligase than DNA (7, 8) and adenylylated RNA did not accumulate in the standard reaction.

Therefore, Step 3, the reaction of adenylylated donor with acceptor, is a relatively slower step for DNA than RNA substrates. This conclusion is consistent with the finding that the
one observed exception to the generalization that cyclization is by far the favored reaction is the efficient joining of a DNA donor to an RNA acceptor (7). It is also consistent with the detection of adenylylated RNA when its 3'-terminus was blocked and a DNA acceptor was added. Once all the enzyme is adenylylated, activated DNA or RNA is stable in the reaction mixture since Step 3 requires unadenylated enzyme.

The second unexpected result was that the acceptor plays two roles in the reaction. It is a cofactor for donor activation and a substrate for joining. The major evidence for its cofactor role is that RNA and DNA substrates that do not have a 3’-hydroxyl terminus or are too small to cyclize were not activated unless an acceptor was added. The results with the small substrates dT₆ and dT₇ are particularly revealing since here the 3’-hydroxyl is present but apparently the difficulty in cyclization prevents the attainment of the high local concentration of 3’-hydroxyl groups needed for activation. RNA ligation could have two distinct acceptor sites, a substrate site and a positive effector site, or the same site could be used for both the cofactor and substrate activities—the binding of the donor could require prior binding of the acceptor. We favor, tentatively, the one-site model because it is simpler. Consistent with this model is that some of the requirements for both roles of the acceptor were the same. Aₐ neither stimulated activation nor was joined, but ApAₐ and higher homologues participated in both reactions (Table I). In the absence of additional acceptor, dT₆ and dT₇ were neither activated nor cyclized while dT₁ was (Table I). The strength of this evidence is vitiated somewhat by the small number of acceptors examined. Also, the chain length dependence for activation is not the same for cyclization; with small substrates the acceptor promotes activation much more readily than it is joined (Fig. 1).

In the DNA ligase reaction, the complementary strand serves to juxtapose the 5’-phosphoryl and 3’-hydroxyl that will participate in cyclization; with small substrates the acceptor promotes activation much more readily than it is joined (Fig. 1). In catalysis by RNA ligase, the enzyme itself perhaps serves an analogous function. The donor is not activated irrespective of whether an appropriate acceptor is available but rather the enzyme first promotes a noncovalent joining of donor and acceptor before activation occurs. This mechanism may minimize wasteful turnover of unprotected high energy intermediate. In the reaction catalyzed by E. coli DNA ligase, a d₄TMP-bounded nick is activated about 1000 times less well than joining of the corresponding 3’-hydroxyl-bounded nick (11). Thus the acceptor plays a dual role for both ligases. The stimulation of activation by the acceptor seems less surprising (11). Thus the acceptor plays a dual role for both ligases. The stimulation of activation by the acceptor seems less surprising (11).

Acknowledgments — We are indebted to Drs. E. Selsing and R. D. Wells for gifts of highly purified oligonucleotides and valuable advice.

Addendum — An independent report of an adenylylated intermediate in the RNA ligase reaction was published after submission of this paper. Ohtsuka et al. (28) identified pU₄-adenylate in reactions containing an oligo(dA) acceptor.

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RNA Ligase Reaction Intermediates

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A Sugino, T J Snoper and N R Cozzarelli


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