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' phosphorylated 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' phosphohyl group of the donor. A large amount of DNA adenylate accumulates during the reaction and the dependence of joining and adenylation 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.

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 to 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 DNA (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 (5). 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.

** MATERIALS AND METHODS

Nucleotides and Nucleic Acids—The concentration of nucleic acids are expressed in terms of 5' termini. Preparations of dT18 through chain length 18, ApApA, GpGpG, and dTTP' were purchased from Collaborative Research, and of dT18, dT19, and dC19 were gifts of Drs. E. Tolles, T. Tullius, and R. D. Wells of the University of Wisconsin. 5'-rrPPrA2 was prepared as described by Silber et al. (1). Oligodeoxyribonucleotides were dephosphorylated in a reaction mixture (0.5 ml) containing 50 mM Tris/HCl, pH 8.3, 40 mM oligonucleotide, and 5 units of bacterial alkaline phosphatase. After 20 min at 37°, the mixture was brought to 5 mM in EDTA and 0.15 M in guanidine hydrochloride, heated at 37° for 15 min, neutralized, and purified by Sephadex G-25 or Sephadex G-50 chromatography. 5'-P-Labeled oligodeoxyribonucleotides were prepared in a reaction mixture (0.5 to 1.0 ml) containing 50 mM Tris/HCl, pH 7.5, 5 mM potassium phosphate, 10 mM MgCl2, 2 mM dithiothreitol, 50 μM of bovine plasma albumin, 20 μM phosphorylated oligonucleotide, 0.2 mM γ-32PATP (1 to 3 x 109 cpm/μmol), and 200 units of polynucleotide kinase. After 30 min at 37°, an additional 50 units of enzyme was added and incubation was continued for 30 min. The mixture was heated at 100° for 2 min and the products were purified by Sephadex G-50 chromatography. Poly(rA) was purchased from Miles Laboratories Inc., and 3',5'-OH rA20 was prepared by successive treatment with micrococcal nuclease and bacterial alkaline phosphatase (1). The dinucleotide A'5'pp(5')A was obtained from P-L Biochemicals Inc. 'H- and 'C-labeled nucleotides were from New England Nuclear, and 32P was from Schwarz Mann. Poly(dA) was prepared by first treating with micrococcal nuclease and bacterial alkaline phosphatase (1). The dinucleotide A5'pp(5')A was obtained from P-L Biochemicals Inc. 'H- and 'C-labeled nucleotides were from New England Nuclear, and 32P was from Schwarz Mann. Poly(dA) was prepared by first treating with micrococcal nuclease and bacterial alkaline phosphatase (1). The dinucleotide A5'pp(5')A was obtained from P-L Biochemicals Inc. 'H- and 'C-labeled nucleotides were from New England Nuclear, and 32P was from Schwarz Mann. Poly(dA) was prepared by first treating with micrococcal nuclease and bacterial alkaline phosphatase (1).

1 The abbreviations used are: dTTP', 2',3'-dideoxyribosylthymidine triphosphate; dTMP, 2',3'-dideoxyribosylthymidine monophosphate; dT18, dT19, and dC19 were gifts of Drs. E. Tolles, T. Tullius, and R. D. Wells of the University of Wisconsin.
Preparation of 3'-Blocked Oligonucleotides—The 3' terminus of 15-[32P]rATP 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 at the 5' end by addition of TMTMP residue. The reaction mixture (0.5 ml) contained 0.1 M sodium cacodylate, pH 7.0, 0.1 M potassium phosphate, pH 7.0, 1.0 mM CoCl2, 1.0 mM 2-mercaptoethanol, 6.0 mM dTTP, 20.0 μM 5'-32P-labeled oligonucleotide, and 40 units of terminal deoxynucleotidyl transferase. After 60 min at 37 °, an additional 20 units of enzyme were added, and incubation was continued for 60 min. The mixture was heated to 100 ° 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 nucleotidyle pyrophosphatase, type III, and bacterial alkaline phosphatase, type II, 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/HC1, pH 7.5, 1.0 mM EDTA before use. Terminal deoxynucleotidyl transferase was obtained from Grand Island Biological Co. Bacillus subtilis DNA polymerase III was prepared according to the method of Brownlee and Sanger (24). Polynucleotide kinase and T4 DNA ligase were purified by the method of Panet et al. (18). The latter enzyme, a gift of Dr. K. Agarwal of this university, was free of RNA ligase activity, as measured by the [5'-32P]rA, cyclization assay. RNA ligase was prepared by a modification of our previous procedure (7). The enzyme had a specific activity of 2000 units/mg of protein and was physically homogeneous; 10 μg of enzyme migrated as a single sharp band during polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (19). The preparation was free of DNA ligase (less than 1 pmol of ends joined/unit of RNA ligase). RNase (less than 1 pmol of nucleotides released/unit of RNA ligase), RNase (less than 1 pmol of nucleotides released/unit of RNA ligase), and DNA endonuclease activity (less than 0.01 pmol of bonds broken/unit of RNA ligase) was also absent. A detailed description of the enzyme was, however, a low level of DNA exonuclease activity present which released about 1 pmol of TTP from T34(rA)template for every 100 pmol of [5'-32P]rA, ligated.

Enzyme Reactions—The standard RNA ligase assay measured the conversion of 5'-32P-labelled oligonucleotides to a phosphatase-resistant form. The reaction mixture (20 μl) contained 50 mM Tris/HC1, pH 7.6, 10 mM MgCl2, 20 mM dithiothreitol, 1.0 mM ATP, 10 μM 5'-32P-labelled oligonucleotide, and 0.1 to 0.6 unit of enzyme. After 30 min at 37 °, the reaction mixture was heated for 2 min at 100 ° and then the oxidation product was formed. The reaction mixture was heated for 2 min at 100 ° for 2 min and then chromatographed in Solvent II. The product of [32P]rA, digestion was identified as the 3'-phosphohydroxyl terminated substrate is referred to as the donor while the 3'-hydroxyl terminated substrate is termed the acceptor. Assays of T4 DNA ligase used the procedure of Gupta et al. (20) and the substrate was [5'-32P]rATP, poly(dA). The ligation of DNA-adenylate by T4 DNA ligase employed the same protocol, except ATP was omitted from the reaction. Measurement of nucleoside activity in the RNA ligase preparation used the RNA ligase assay conditions, except the substrates were [32P]labelled nucleic acids at 20 μM in total nucleotide. Exonuclease assays measured the conversion of either [3H]poly(rC) or [3H]poly(rA) to a 0.97% poly(rC) or poly(rA) per hour. The reaction mixture (0.25 ml) contained 20 mM Tris/HC1, pH 7.5, 6.5 mM MgCl2, 3 mM dithiothreitol, 10% glycerol, 0.1 mg/ml of bovine plasma albumin, 0.1 μM [3H]HTMP as an internal standard, and 0.2 unit of enzyme. Samples were removed at 36-min intervals from the 37 ° incubation mixture and spotted on strips of DEAE-cellulose paper (2 x 3 cm). The strips were washed three times with 0.3 M ammonium formate, pH 8.0, twice with 95% ethanol, dried, and counted.

Isolation and Characterization of Oligo(rA)-Adenylate—5'-32P]rATP, blocked at the 3' end by periodate oxidation, was incubated with 0.12 unit of RNA ligase under standard reaction conditions, except 0.5 mM ATP and 300 μM dTPA, was added. After 2 h at 37 °, the reaction mixture was treated sequentially with 4 units of phosphatase for 20 min at 65 ° and 50 μg of pronase for 30 min at 37 °, and the products were purified by Sephadex G-50 chromatography. The doubly labeled material that eluted near the void volume was concentrated by lyophillization and a portion was degraded with pronase (1 mg of enzyme in 300 μg of substrate) for 1 h at 37 °. The reaction mixture was treated with 2 units of phosphatase at 37 ° for 20 min and then chromatographed in Solvent II. Material that migrated with an internal A(5'1ppp5')A standard was eluted, concentrated by lyophillization, and subjected to electrophoresis at pH 3.5. Material coincident with A(5'1ppp5')A was eluted, concentrated and partially digested with C. atrox nucleotidyle pyrophosphatase. The reaction mixture (0.15 ml) contained 30 mM Tris/HC1, pH 7.5, 7.9 mM MgCl2, and 0.1 μg of enzyme. After 30 min at 37 °, 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 mM HC1 for 15 min at 100 ° (Method I) or by successive treatment with 5 mM sodium metaperiodate for 30 min at 25 ° and 0.25 mM l-mylene, pH 8.0, for 30 min at 45 ° (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 reactions where DNA and 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')3'-AMP generated by alkaline hydrolysis. Paper chromatography with Solvent I was used in the analysis. To determine the amount of DNA-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 3.5 chromatography buffer was 0.05 M sodium citrate, pH 3.5. The solvents for chromatography were 1-propanol/concentrated NH4OH/H2O, 63/1 (Solvent 1), and isobutyl alcohol/1 M NaOH/0.1 M EDTA, 100/0.1/0 (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 Vizoso (25); this procedure resolves cyclic and linear oligonucleotides. Neutral sucrose gradient centrifugation was performed using Sepharose 4B. The electrophoresis buffer was 50 mM triethylenammonium bicarbonate, pH 8.0; fractions were lyophillized and resuspended in water.

RESULTS

Isolation of Oligo(dT)-Adenylate Intermediate—We showed previously that incubation of [5'-32P]dTP, 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 32P of the adenylated substrate is phosphatase-sensitive after removal of the adenylyl moiety by treatment with acid or periodate then base, whereas this protocol does not remove 32P from the joined product. By this anomy, the adenylated substrate was formed 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, dT1 and dT2, 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 nucleoside-resistant, whereas the adenylated substrate does not
adenylylated structure. The RNA and DNA ligase intermediates have the same 5'-phosphatase-resistant after acid treatment. We conclude that ligase, 70% of the \( ^{32} \text{P} \) was converted to a form which was not ATP. After 2 h at 37°, the mixture was heated at 100° for 2 min and then treated successively with 0.2 mg of bacterial alkaline phosphatase for 20 min at 65° and 0.2 mg of pronase for 30 min at 37°. The products were applied to a column of Sephadex G-25, superfine (0.9 x 30 cm), fractions (0.9 ml) were collected, and the \( ^{32} \text{P} \) and \( ^{14} \text{C} \) were measured.

The stoichiometry of the adenylylated substrate was determined by analyzing the products of an RNA ligase reaction containing \( ^{32} \text{P} \)ATP and \( ^{32} \text{P} \)dT. The reaction mixture was heated at 100° for 2 min and then treated sequentially with phosphatase and pronase. When this material was filtered through Sephadex G-25, portions of the \( ^{32} \text{P} \) and \( ^{14} \text{C} \) were excluded (Fig. 2). The stoichiometry of the AMP and dT, moieties of the adenylylated substrate was calculated to be 1.1 from the ratio of \( ^{32} \text{P} \) to \( ^{14} \text{C} \) 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 \( ^{32} \text{P} \) and \( ^{14} \text{C} \)-labeled adenylylated dT, was incubated with RNA ligase and 3',5' OH rA\(_{30}\), but in the absence of ATP, 5',3'[^32]C]-AMP was released (Fig. 3). The addition of ATP has the expected effect of diminishing the release of \( ^{32} \text{C} \)AMP, since adenylylated enzyme cannot catalyze ligation of the adenylylated substrate. The joining of the doubly labeled substrate to acceptor and the release of \( ^{32} \text{C} \)AMP were coordinate and stoichiometric (Fig. 3). The formation of a phosphodiester bond between donor and acceptor was confirmed by a nearest neighbor analysis; the AMP and dT, molar ratio of \( ^{14} \text{C} \)AMP to \( ^{32} \text{P} \)AMP was calculated to be 5.1 from the ratio of \( ^{14} \text{C} \) to \( ^{32} \text{P} \) which was phosphatase-sensitive after periodate plus base treatment.

The reaction mixture (0.2 ml) contained 0.7 FM \( ^{32} \text{P} \)dA(dpT), and 10 pM d[T(pT),l, and either 1.2 units of RNA ligase and 1 mM ATP (A); enzyme but not ATP (B); or neither enzyme nor ATP (C). After incubation at 37° for the indicated times, samples were removed and heated at 100° for 2 min. The release of \( ^{14} \text{C} \)AMP from the adenylylated substrate was measured by chromatography in Solvent I (closed symbols) and the extent of joining (C) was measured by the production of phosphatase-resistant \( ^{32} \text{P} \) after acid treatment.

**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',3'[^32]P]-adenylate proceeds 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[T(pA),l, 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 alkali and phosphatase, and the products were analyzed by chromatography in Solvent II (Fig. 4A). All the \( ^{32} \text{P} \) migrated with internal A(5')pp(5')A reference, while the \( ^{32} \text{P} \) migrated with A(5')pp(5')A (from the intermediate), P, (from RNA to RNA 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 \( ^{31} \text{P} \) and \( ^{31} \text{P} \) were coincident with the internal A(5')pp(5')A reference. When this doubly labeled compound was digested with snake venom nucleotidyl pyrophosphatase, both the \( ^{31} \text{P} \) and \( ^{31} \text{P} \) labeled products migrated with pA. The molar ratio of \( ^{31} \text{P} \)AMP to

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*Unpublished data.*
from the activated intermediate was stoichiometric with joining (Fig. 3). Silver et al. showed that during the reaction ATP was broken down to AMP plus PP, in amounts equimolar to joining (1).

Acceptor Exchange - The addition of ApApA to a reaction containing [5'-32P]dT, led to a large increase in ligation (Table I). Surprisingly, the increment in ligation was not solely the result of synthesis of rA-A-dT, co-polymer but also of augmented cyclization of dT, The stimulation of DNA to DNA joining by addition of an RNA acceptor was shown in several ways. The data will be cited for the experiment with [5'-32P]dT, and ApApA shown in Table I. First, one-fourth of the joined products (3 pmol) was resistant to alkaline hydrolysis; this represents a 25 fold increase in DNA to DNA ligation over that obtained in the absence of ApApA. Second, the ligation reaction products treated successively with alkaline phosphatase were purified by Sephadex G-50 filtration. About one-half was oligo(dT) joined product since 54% of the labeled material was digested to 3'-dTMP by micrococcal nuclease and spleen phosphodiesterase (Fig. 5). The other half was adenylated intermediate since this fraction was sensitive to phosphatase after acid treatment. Third, the ligase product which was resistant to successive treatment by 0.3 N KOH, periodate, base, and phosphatase was shown to be cyclic dT, by two-dimensional cellulose-plate chromatography and by its resistance (80%) to digestion by B. subtilis DNA polymerase III exonuclease.

The simplest conclusion is that although activation requires the presence of the acceptor, the activated donor can join to another acceptor. This phenomenon, which we call acceptor exchange, is widespread. ApApA stimulates the cyclization of dCT, dC-dT, dT, dT, and dT, and other acceptors such as 3',5'-OH rA, and CcC 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 dT, to d(A(pA),) was increased more than 10-fold by the addition of ApApA (Table II).

DISCUSSION

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

\[ E + pppA \xrightarrow{Mg^{2+}} E \cdot pppA \]

(1)

\[ E \cdot pppA + pD \xrightarrow{R} A(5')pp(5')D + E \]

(2)

\[ A(5')pp(5')D + R \xrightarrow{E} RpD + pA \]

(3)

Sum: \[ pppA + pD + RpD + pA \]

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

The first step of adenylylation of the enzyme was demonstrated by Cranstow 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

\[ ^8 \] Dissociation of the enzyme adenylate by substrate requires Mg^{2+} (9), but it is not known if it is required in Step 2 or 3 or both. Steps 2 and 3 are shown as reversible but this has not been demonstrated.
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°, the amount of joined product and adenylylated substrate was measured by Method II described under "Materials and Methods." The second step in the reaction is the transfer of the adenyl-

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<tr>
<td>Intermediate</td>
<td>Joined product</td>
<td>Intermediate</td>
</tr>
<tr>
<td>rA₃</td>
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<tr>
<td>rA₈, oxidized&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.2</td>
<td>0.5</td>
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<td>95</td>
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<sup>a</sup> The 3' terminus was oxidized by periodate treatment as described under "Materials and Methods."

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 the presence of Mg<sup>2+</sup> to yield AMP, whereas ATP was generated in the presence of PD, (9). We have confirmed the formation of the enzyme-AMP complex with the physically homogene-

![Fig. 5](https://example.com/fig5.png)

**TABLE II**

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

The standard reaction mixture contained 0.6 unit of RNA ligase, 10 μM [5'←32P]dC₅-dT, and where indicated each acceptor at 100 μM. After 1 h at 37°, the several possible products were distinguished as described under "Materials and Methods." The dA-dC-dT product had the expected mobility during homochromatography.

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<sup>a</sup> Includes 20 pmo1 of adenylylated donor and 45 pmo1 of RNA-DNA co-polymer.

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 enzyme simultaneously at the donor and acceptor sites. The smallest donors and acceptors for intermo-

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**TABLE I**

**RNA Ligase Reaction Intermediates**

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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 unadenylylated 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 dT$_5$ and dT$_6$ 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 ligase 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. ApA neither stimulated activation nor was joined, but ApApA and higher homologues participated in both reactions (Table I). In the absence of additional acceptor, dT$_5$ and dT$_6$ were neither activated nor cyclized while dT$_7$ 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 as for cyclization; with small substrates the enzyme 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 be joined. 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 d$_2$TMP-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 for DNA ligase since the 3'-hydroxyl is constrained to be adjacent to the 5'-phosphoryl by the nick structure. However, the same juxtaposition can be mediated by RNA ligase itself.

In light of the acceptor requirements for activation, we anticipated that activation and joining would be tightly coupled. Instead, the third unexpected result was acceptor exchange—the addition of an RNA acceptor stimulated activation and joining not only to itself but to other acceptors. The examples of acceptor exchange are the promotion by RNA acceptors of cyclization of various oligonucleotides and the promotion of joining of d$_2$TMP blocked dC$_6$ and dT$_{12-14}$ to d(ApApA)$_2$. A plausible explanation for this phenomenon is that once activation occurs, the acceptor (and perhaps also the activated donor) dissociates from the enzyme. Thus, even though RNA is generally a much better acceptor than DNA, the high local concentration of the DNA ends leads to cyclization or, in the case where cyclization is prevented by modification of the donor 3'-hydroxyl, the high concentration of the best DNA acceptor, oligo(dA), leads to formation of a DNA copolymer. An alternative molecular interpretation for acceptor exchange is that the stimulation of activation by the acceptor takes place at a site distinct from the substrate site. Although we have not investigated it thoroughly, we have not seen acceptor exchange reactions with an RNA donor. Thus with this better substrate, the enzyme-mediated noncovalent joining of donor and acceptor residues may lead immediately to covalent joining.

One reason we initiated the study of RNA ligase was that it might be an attractive reagent for synthesizing DNA of defined sequence. The yield of intermolecular ligation of DNA was distressingly low in initial experiments. The discovery of acceptor exchange has provided a method of increasing the yield by over an order of magnitude (Table II).

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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 pUG$_2$-adenylate in reactions containing an oligo(dA) acceptor.

REFERENCES

Bacteriophage T4 RNA ligase. Reaction intermediates and interaction of substrates.
A Sugino, T J Snoper and N R Cozzarelli


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