The joining of duplex DNA at base-paired ends by bacteriophage T4 DNA ligase was confirmed using either a synthetic duplex decamer or restriction endonuclease fragments of CoE1 DNA as substrates. The reaction was not linearly dependent on enzyme concentration but increased markedly at high enzyme concentrations. Although T4 RNA ligase did not catalyze this blunt end joining, it markedly stimulated the DNA ligase reaction particularly at low DNA ligase concentrations. The apparent $K_m$ for the decamer was 50 $\mu$M in the presence or absence of RNA ligase. In the presence of RNA ligase, T4 DNA ligase had about the same turnover number for blunt end and cohesive end joining. The joining of duplex DNA at base-paired ends was proven by several techniques including restriction endonuclease cleavage of the products. The products of the ligation reaction using restriction enzyme fragments were mostly linear oligomers but included some circular duplexes. Escherichia coli DNA ligase in the presence or absence of RNA ligase did not catalyze blunt end joining. RNA ligase only moderately affected the joining of cohesive ends by T4 DNA ligase or E. coli DNA ligase and did not itself catalyze this reaction.

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 single-stranded RNA (2–7). Single-stranded DNA is also a substrate. The enzyme catalyzes the formation of circular DNA, linear DNA dimers, and RNA-DNA and DNA-RNA block co-polymers (8–10). RNA ligase differs from all known DNA ligases (11) in two main respects. It has a high activity with RNA substrates and it efficiently joins single-stranded nucleic acids in the absence of a complementary strand. There is one reported exception to the generalization that DNA ligase only joins together polynucleotides held in juxtaposition by a complementary strand. Preparations of DNA ligase from T4-infected cells have been reported to join duplex DNA substrates intermolecularly at base-paired ends (12–14). This reaction, designated as blunt end joining, has not only mechanistic implications but is valuable for constructing chimeric DNA molecules (15, 16). However, the efficiency of T4 DNA ligase-catalyzed blunt end joining has been somewhat variable. One possibility is that T4 DNA ligase preparations have variable contamination with RNA ligase and that this reaction is marked by RNA ligase. RNA ligase itself does not catalyze the reaction. The products of the reaction are mostly linear oligomers but include circular duplexes.

**MATERIALS AND METHODS**

Enzymes—Bacteriophage T4 RNA ligase was purified from T4 amNF-1 (gene 43) infected Escherichia coli B essentially as described previously (8), except that Sephadex G-100 chromatography was repeated after the hydroxylapatite step. The preparation is physically homogeneous as judged by gel electrophoresis in the presence of sodium dodecyl sulfate (Fig. 1). It was free of DNA ligase, RNase, and DNA endonuclease activities but contained a small amount of an exonuclease which digested single-stranded DNA from the 3'-hydroxyl terminus. After 1 to 2 days at 15°C in the presence of 60 to 120 $\mu$g/ml of RNA ligase no digestion of the duplex substrates used in this report was detected by gel electrophoresis. Polynucleotide kinase and T4 DNA ligase from T4 amNF-2-infected E. coli B were purified by the method of Panet et al. (17). One of the two T4 DNA ligase preparations used was a gift from R. Agarwal of the University of Chicago. Since both preparations had identical properties, they are not distinguished in the text. The T4 DNA ligase was physically homogeneous (Fig. 1) and had no detectable DNase. amRNA ligase activity even at 10 times the enzyme concentration used in the standard blunt end joining reaction. Purified E. coli DNA ligase (18) was the gift of I. R. Lehman of Stanford University. The restriction endonucleases HpaII from Haemophilus aegyptius and AluI from Arthrobacter luteus were purified by the method of P. Green. Restriction endonuclease HpaII from Haemophilus parainfluenzae was purchased from New England Bio Labs. The recognition.
Ligation of Duplex DNA at Base-paired Ends

30 min at 30°, an additional 50 units of kinase were added and incubation continued for 30 min. EDTA was added to 20 mM and the DNA was purified by Sephadex G-50 (superfine) gel filtration. The restriction enzyme fragments were prepared in a 0.15-ml reaction mixture containing 6 mM Tris/HCl, pH 7.6, 6 mM MgCl₂, 6 mM 2-mercaptoethanol, 50 μg/ml of bovine serum albumin, 50 mM CoEl DNA, and 15 units of enzyme. The incubation was carried out at 37° for 8 to 12 h until digestion was complete as monitored by 1.4% agarose gel electrophoresis in the presence of ethidium bromide. The DNA was extracted successively with phenol and ether and ethanol precipitated against 50 mM Tris/HCl, pH 8.0, and 1.0 mM EDTA. To prepare 32P-labeled fragments, the DNA was first treated with 1 unit of bacterial alkaline phosphatase for 30 min at 65° before the phenol extraction and dialysis. The 5° terminal were then phosphorylated with γ-32P[ATP as described above. [H]Poly[d(A-T)] and [5-32P]Ado were prepared enzymatically (1, 23).

Enzyme Reactions—The standard assay of T4 DNA ligase measured the conversion of [5-32P]Ado to a phosphatase-resistant form (19) and of DNA ligase the conversion of [3H]poly[d(A-T)] to an enzyme-release III resistant form (23). [32P]Ado catalyzed joining of the decamer or the CoEl DNA restriction enzyme fragments contained in 10 to 20 μl, 50 mM Tris/HCl, pH 7.8, 10 mM MgCl₂, 20 mM dithiothreitol, 1 mM ATP, 2 to 10 μM DNA (the precise concentration is indicated in the figure legends), and enzyme. The reactions with the same substrates using E. coli DNA ligase contained in 10 to 20 μl, 50 mM Tris/HCl, pH 7.8, 5 mM MgCl₂, 10 mM dithiothreitol, 60 μM NAD, 10 mM (NHL)₂SO₄, 5 μM DNA, and enzyme. If the reaction also contained T4 DNA ligase, 60 μM ATP was added. The extent of blunt end ligation was monitored either by conversion of [5-32P]DNA to a phosphatase-resistant form or by gel electrophoresis.

Nearest Neighbor Analysis (25) of Products of Ligation—The ligase reaction mixture (10 μl) was diluted to 50 μl with 50 mM Tris/HCl, pH 9.0, heated at 65° for 2 min, and after addition of CoElC to 6 to 10 μl treated with 15 units of micrococcal nuclease at 37° for 30 min. After addition of 5 μl of 1M ammonium formate, pH 6.0, 5 mM potassium phosphate and 0.1 unit of spleen phosphodiesterase, the incubation was continued for 30 min at 37°. To ensure complete digestion, another 0.1 unit of spleen phosphodiesterase was added and the reaction continued for 30 min at 37°. A portion of the digest was analyzed by polyethyleneimino-cellulose thin layer chromatography (26).

Analysis of 5°-Terminal Nucleotide of Decamer—The reaction mixture (50 μl) contained 50 mM Tris/HCl, pH 8.0, 5 mM MgCl₂, 100 μM 5°-32P-labeled decamer, and 200 μg/ml of DNAase I. After 30 min at 37°, the pH was raised to 9.0 with glycine buffer and venom phosphodiesterase was added to 0.2 mg/ml. The incubation was continued at 37° for 30 min. The digest was analyzed by polyethyleneimino-cellulose thin layer chromatography.

Gel Electrophoresis—Electrophoresis of proteins through 15% polyacrylamide gels containing 0.1% sodium dodecyl sulfate was carried out as described (27). Electrophoresis of restriction enzyme fragments in a 15% polyacrylamide gel was performed as described (28), except sometimes a slab gel (0.2 x 20 x 20 cm) was used. After electrophoresis at 50 mA for 4 h at 20°, the gel was examined by direct illumination from a short wavelength ultraviolet light source and photographed using Polaroid 55 P/N film and a Kodak 25A red filter. The 15% polyacrylamide slab gel (0.2 x 20 x 40 cm) used to resolve the decamer reaction products, contained 50 mM Tris/borate, pH 8.3, 1 mM EDTA, 15% acrylamide, and 2% bisacrylamide. The gels were run at 50 mA for several hours before applying the 20- to 30-μl sample containing 10% sucrose, 0.01% bromophenol blue, 0.01% xylene cyanol FF, and 1 mM EDTA. After the run at 50 to 100 mA the DNA bands were detected by autoradiography.

Electron Microscopy—The procedure was a modification of that of Westmoreland et al. (29). DNA at about 0.6 μg/ml was spread over a water hypophase in a solution containing 0.1 M Tris/HCl, pH 8.5, 0.01 M EDTA, 50 μg/ml of cytochrome c, 2 μM urea, and 50% formamide. The DNA was stained with uranyl acetate and shadowed with Pt/Pd (80:20) before viewing.

RESULTS

Ligation of Duplex Decamer by T4 RNA and DNA Ligases—In order to test whether RNA ligase catalyzes the joining of duplex DNA at base-paired ends or influences this reaction sequence and cut site, indicated by a, for these restriction nucleases are: HaeIII, 5'G-G-T-C-C; AliI, 5'A-G-T-C-T; and Hpol II, 5'C-A-C-G-G (19). E. coli exonuclease III was obtained from Miles and pancreatic DNase I and RNase A were from Worthington Biochemicals. The RNase was heated at 90° for 10 min with 5% sodium acetate, pH 5.0, before use. The other enzymes used have been described (8, 10).

Nucleotides and Nucleic Acids—The duplex, self-complementary, decamer,

5'C-C-G-A-A-T-T-C-G-G


(thereafter referred to as decamer), was synthesized chemically and provided by R. Scheller and K. Ikakura of the California Institute of Technology. CoEl DNA was prepared from E. coli strain JC411 (obtained from D. Henski of the University of California) as described (20) except that the material was treated with 50 μg/ml of RNase I for 30 min at 37° before the CoEl ethidium bromide gradient and the remainder was relaxed CoEl DNA. Plasmid PM40 DNA was prepared as described (21). [γ-32P]ATP was synthesized by the method of Glynn and Chappell (22) or purchased from New England Nuclear Co.

Chemicals—Acrylamide, bisacrylamide, and N,N,N',N'-tetramethylenediamine were purchased from Bio-Rad Laboratories. Agarose (type II) and ethidium bromide were from Sigma Chemical Co. Polyethyleneimino-cellulose plates were obtained from Brinkmann Instruments, Inc.

Ligase Substrates—The 5° terminal of the duplex decamer were phosphorylated in a 0.1-ml reaction mixture containing 70 mM Tris/HCl, pH 8.0, 15 mM 2-mercaptoethanol, 10 mM MgCl₂, 0.25 mM [γ-32P]ATP (4 to 10 x 10⁶ cpm/mmol), 40 μM decamer, 50 μg/ml of bovine serum albumin, and 50 units of polynucleotide kinase. After

* The concentration of polynucleotides is expressed in terms of total nucleotides.
reaction by T4 DNA ligase, we first used the synthetic decamer, \(5'\)-\(^{32}P\)-labeled


as a substrate. Assays were routinely carried out at 15\(^\circ\)C or less to maintain the duplex structure of the molecule. As monitored by the production of phosphatase-resistant \(^{32}P\), RNA ligase in contrast to T4 DNA ligase did not catalyze joining of the decamer at 15\(^\circ\)C (Fig. 2). No blunt end joining was obtained with RNA ligase at assay temperatures from 8-37\(^\circ\)C and with varying amounts of enzyme up to 120 \(\mu\)g/ml (data not shown). However, the addition of RNA ligase at 60 \(\mu\)g/ml to reactions containing T4 DNA ligase stimulated joining at all levels of DNA ligase tested (Fig. 2). The stimulation was most pro-

![Graph](attachment://graph.png)

**Fig. 2.** Dependency of decamer ligation on T4 DNA ligase concentration in the presence or absence of RNA ligase. The reaction mixtures contained 10 \(\mu\)M \(^{32}P\)-labeled decamer, the indicated amounts of T4 DNA ligase, and either no RNA ligase (○) or 60 \(\mu\)g/ml of RNA ligase (●). After 60 min at 15\(^\circ\)C, phosphatase-resistant \(^{32}P\) was measured.

![Image](attachment://image.png)

**Fig. 3.** Dependency of decamer ligation on T4 DNA ligase concentration in the presence or absence of RNA ligase. The reaction mixtures contained 3 \(\mu\)M \(^{32}P\)-labeled decamer, 60 \(\mu\)g/ml of RNA ligase in Lanes 1 and 6 to 10, and T4 DNA ligase at the following concentrations: 0.25 \(\mu\)g/ml in 2 and 6; 0.75 \(\mu\)g/ml in 7; 2.5 \(\mu\)g/ml in 3 and 8; 7.5 \(\mu\)g/ml in 4 and 9; and 25 \(\mu\)g/ml in 5 and 10. After a 2-h incubation at 12\(^\circ\)C, the reaction products were displayed by electrophoresis through a 15% polyacrylamide gel; an autoradiogram of the gel is shown. The symbols Ori, XC, and BPB designate the position of the origin and of xylene cyanole FF and bromphenol blue references, respectively. The light band just behind the band with the highest mobility is an impurity in the decamer. As judged from the molecular weight of a series of external references (and excluding the impurity in the decamer) the molecular weight of the DNA was 10, 20, 30, 40, etc., base pairs in order of decreasing mobility. The references included a HpaII endonuclease digest of phage \(\Phi\) replicative form DNA (30) and a 21 base pair DNA segment containing the operator for the lactose operon (15).
Ligation of Duplex DNA at Base-paired Ends

b. 

FIG. 5. Dependency of decamer joining on RNA ligase concentration in the presence of a constant amount of T4 DNA ligase. The reaction mixtures incubated at 15° contained 10 nM 5'-32P-labeled decamer, either 2.5 μg/ml of T4 DNA ligase (a) or 10 μg/ml of T4 DNA ligase (b), and RNA ligase at 6 μg/ml (●), 15 μg/ml (△), 30 μg/ml (○), 60 μg/ml (▲), 90 μg/ml (●), or 120 μg/ml (▲). The controls contained T4 DNA ligase alone (□) or 60 μg/ml RNA ligase alone (x). At the indicated times, phosphatase-resistant 32P was measured.

FIG. 6. Decamer ligation by T4 DNA ligase or T4 RNA and DNA ligases: effect of substrate concentration. The 10-μl reaction mixtures incubated at 15° contained 5'-32P-labeled decamer at the indicated concentrations and either 2.5 μg/ml of T4 DNA ligase (○) or 2.5 μg/ml of T4 DNA ligase and 60 μg/ml of RNA ligase (▲). The velocity (v) of the reaction was measured by the production of phosphatase-resistant material during the early portion of the reaction where products increased linearly with time.

nounced at relatively low DNA ligase levels, below 3 μg/ml, where the increment in the rate of ligation reached 20-fold.

Analysis of the reaction products by polyacrylamide gel electrophoresis confirmed that true ligation was obtained (Fig. 3). The 20-mer, 30-mer, 40-mer, 50-mer, 60-mer, 70-mer, and 80-mer joined products were resolved and most of the products were larger than the 80-mer. The stimulation of the reaction by RNA ligase is most clearly shown by comparison of Lane 2 (0.25 μg/ml of DNA ligase alone) with Lane 6 (0.25 μg/ml of DNA ligase plus 60 μg/ml of RNA ligase). Two additional experiments proved that true blunt end joining was catalyzed by T4 DNA ligase and by the mixture of the two ligases. First, after digestion of the reaction products with micrococcal nuclease and spleen phosphodiesterase, 90% of the 32P was recovered as Gp, as expected from the 3'-terminal G in the decamer sequence. Digestion of the 5'-32P-labeled decamer substrate with pancreatic DNAase and snake venom phosphodiesterase generated only [32P]pC. Second, blunt end ligation of the decamer will generate the recognition sequence for HaeIII restriction nuclease at the join. After digestion of the ligated material with this enzyme the starting decamer should be recovered. As shown by the polyacrylamide gel electrophoresis patterns in Fig. 4, virtually all the joined products were digested by HaeIII nuclease to material which migrates identically with the decamer substrate.

To determine how much RNA ligase was needed to promote blunt end joining, the amount of this enzyme was varied and two fixed levels of T4 DNA ligase were employed. With 2.5 μg/ml of T4 DNA ligase, only 1% of the 32P was rendered phosphatase-resistant after 2 h at 15° (Fig. 5a). The addition of 60 μg/ml of RNA ligase stimulated joining about 4-fold and maximum stimulation was achieved at 60 μg/ml of RNA ligase (Fig. 5b). At the indicated times, phosphatase-resistant 32P was measured.

FIG. 7. Co-purification of poly[d(A-T)] cyclization and blunt end joining activities of T4 DNA ligase by hydroxylapatite chromatography. DNA ligase (750 units) purified by the method of Panet et al. (17) was applied to a hydroxylapatite column (0.9 x 3 cm) in 20 mM potassium phosphate, pH 6.8, 10 μM ATP, and 5 mM 2-mercaptoethanol and eluted with a 60-ml linear 0.2 to 0.5 M potassium phosphate, pH 6.8, gradient containing 10 μM ATP, 10% glycerol, and 5 mM 2-mercaptoethanol. One-milliliter fractions were collected and assayed for poly(dA-T) cyclization (○) and joining of 5'-32P-labeled decamer (▲). The latter 10-μl assay contained 4.5 μM 5'-32P-labeled decamer and 2 μl of each fraction; incubation was for 18 h at 15°.

FIG. 8. Escherichia coli DNA ligase does not catalyze joining of the decamer. The reaction mixtures incubated at 15° contained 5 μM 5'-32P-labeled decamer, 60 μM ATP, 60 μM NAD, and either 50 μg/ml of E. coli ligase (△), 60 μg/ml of RNA ligase (△), 50 μg/ml of E. coli DNA ligase and 60 μg/ml of RNA ligase (▲), 10 μg/ml of T4 DNA ligase (□), or 60 μg/ml of RNA ligase and 10 μg/ml of T4 DNA ligase (●). At the indicated times, phosphatase-resistant 32P was measured.

restriction nuclease at the join. After digestion of the ligated material with this enzyme the starting decamer should be recovered. As shown by the polyacrylamide gel electrophoresis patterns in Fig. 4, virtually all the joined products were digested by HaeIII nuclease to material which migrates identically with the decamer substrate.

To determine how much RNA ligase was needed to promote blunt end joining, the amount of this enzyme was varied and two fixed levels of T4 DNA ligase were employed. With 2.5 μg/ml of T4 DNA ligase, only 1% of the 32P was rendered phosphatase-resistant after 2 h at 15° (Fig. 5a). The addition of 6 μg/ml of RNA ligase stimulated joining about 4-fold and maximum stimulation was achieved at 60 μg/ml of RNA ligase with both 2.5 and 10 μg/ml of DNA ligase (Fig. 5). At this saturating level of RNA ligase there are about 7 times as many 5' ends of substrate as RNA ligase molecules.

The apparent Kₘ for the decamer was about 50 μM in reactions catalyzed by either 25 μg/ml of DNA ligase or 2.5 μg/ml of DNA ligase and 60 μg/ml of RNA ligase (Fig. 6). This value is 2 orders of magnitude higher than the Kₘ of 0.6 μM measured for nick sealing by T4 DNA ligase with substrates of...
Ligation of Duplex DNA at Base-paired Ends

3991
dT<sub>10</sub> poly(dA) (31) and HpaII restriction nuclease fragments (see below). This is consistent with the enzyme having a higher affinity for nicks than base-paired ends. In contrast, the measured turnover number of blunt end joining in the presence of RNA ligase of 0.8 mol/min/mol of T4 DNA ligase at 15°C is comparable to the turnover number for nick sealing (Ref. 31 and below).

It seems unlikely that contaminating enzymes in the ligase preparations play a role in these reactions. The proteins are physically homogeneous as determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Fig. 1). No cross-contamination of the ligases was detected by sensitive assays. When the purified DNA ligase was fractionated by hydroxylapatite chromatography, the ratio of blunt end joining to nick sealing activities was constant across the peak (Fig. 7). The two activities of DNA ligase also were copurified by Sephadex G-100 chromatography (data not shown).

Sgaramella showed that *Escherichia coli* DNA ligase does not catalyze the joining of phage P22 double-stranded DNA at base-paired ends (14). This enzyme also did not lead to a significant ligation of the decamer even in the presence of RNA ligase (Fig. 8). In the control with the T4 ligases, 95% of the 32P was rendered phosphatase-resistant. The amount of *E. coli* and T4 DNA ligases used in this experiment catalyzed the cyclization of [3H]poly[d(A-T)] at the same rate. The addition of the *E. coli* enzyme to the T4 ligases or T4 DNA ligase alone did not inhibit blunt end joining. The lack of blunt end joining was observed with various concentrations of ATP, *E. coli* DNA ligase, and T4 RNA ligase.

Joining of *HaeIII* or *AluI* Restriction Enzyme Fragments—

*HaeIII* and *AluI* restriction enzyme fragments generate DNA fragments with base-paired ends. In order to study ligation of molecules larger than a decamer, the joining of ColEl DNA treated with either nuclease was measured by agarose gel electrophoresis. The results are the same as obtained with the decamer. DNA ligase at high levels catalyzed blunt end joining and RNA ligase stimulated markedly the DNA ligase activity, particularly at low levels of the latter enzyme. In Fig. 9, the ligation of the *HaeIII* nuclease-generated fragments is shown after 1, 6, and 20 h at 15°C. The gel patterns after 6 h show most clearly the stimulation of the reaction by RNA ligase: with 2.5 µg/ml of T4 DNA ligase no joining is apparent (Lane c), whereas the addition of RNA ligase to this amount of DNA ligase resulted in extensive joining of the fragments (Lane e). After 20 h at 15°C, some joining was observed with DNA ligase alone (Lanes c and d) but none with RNA ligase alone (Lane b) nor with *E. coli* DNA ligase (data not shown); with both T4 ligases all the fragments participate in joining. In a control reaction, the addition of 60 µg/ml of T4 polynucleotide kinase to 2.5 µg/ml of T4 DNA ligase (Lane g) did not alter the pattern obtained with DNA ligase alone (Lane c). Treatment of the RNA and bromide staining of the DNA is shown and the origin is at the top of the figure.

Fig. 9 (top). Joining of *HaeIII* restriction endonuclease fragments by either T4 DNA ligase or T4 DNA and RNA ligases. The 10-µl reaction mixtures incubated at 15°C contained 5 µM ColEl DNA digested with *HaeIII* endonuclease and either no enzyme (a), 60 µg/ml of RNA ligase alone (b), 2.5 µg/ml of T4 DNA ligase alone (c), 1.55 µg/ml of T4 DNA ligase (d), 2.5 µg/ml of T4 DNA ligase and 60 µg/ml of RNA ligase (e and f), or 2.5 µg/ml of T4 DNA ligase and 60 µg/ml of T4 polynucleotide kinase (g). After 1, 6, and 20 h, 1 µl of each of the reaction mixtures was heated in 10 µl of 60 mM Tris/HCl, pH 7.5, 6 mM MgCl₂, at 65°C for 15 min and treated with 1 unit of *HaeIII* nuclease at 37°C for 4 h after addition of 6 mM 2-mercaptoethanol. The ethidium bromide staining of the DNA is shown and the origin is at the top of the figure.

Fig. 10 (bottom). RNA ligase promotion of the joining of *AluI* restriction enzyme fragments. The 10-µl reaction mixtures contained 5 µM ColEl DNA digested with endonuclease *AluI* and either no enzyme (1), 0.5 µg/ml of T4 DNA ligase (2), 0.5 µg/ml of T4 DNA ligase and 60 µg/ml of RNA ligase (3), 1.25 µg/ml of T4 DNA ligase (4), 1.25 µg/ml of T4 DNA ligase and 60 µg/ml of RNA ligase (5), or 60 µg/ml of RNA ligase alone (6). Incubation was for 15 h at 15°C. One microliter of reaction mixtures 1 to 6 was applied to a 1.4% agarose gel. One microliter of Reaction 5 was further treated with endonuclease *AluI* as described in Fig. 9 for cutting by endonuclease *HaeIII* and applied to Lane 7. The ethidium bromide staining of the DNA is shown and the origin is at the top of the figure.
The reaction mixtures contained 5 µM HaeIII or AluI restriction enzyme fragments of CoE1 DNA, and either no enzyme, 12.5 µg/ml of T4 DNA ligase, or 1.25 µg/ml of T4 DNA ligase and 60 µg/ml of T4 RNA ligase. Incubation was for 20 h at 15°C. The agarose gel electrophoresis patterns of the reactions containing HaeIII enzyme fragments are shown in Fig. 9. A portion of the reaction mixtures were viewed by electron microscopy and the distribution in randomly selected fields is shown.

<table>
<thead>
<tr>
<th>DNA substrate, CoE1 DNA digested by</th>
<th>Ligase added</th>
<th>Linear</th>
<th>Circular</th>
<th>Branched</th>
<th>Poorly spread*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HaeIII nuclease</td>
<td>None</td>
<td>138 (100)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HaeIII nuclease</td>
<td>DNA</td>
<td>155 (88)</td>
<td>18 (11)</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>HaeIII nuclease</td>
<td>RNA, DNA</td>
<td>187 (85)</td>
<td>31 (14)</td>
<td>2 (1)</td>
<td>18</td>
</tr>
<tr>
<td>AluI nuclease</td>
<td>None</td>
<td>100 (100)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AluI nuclease</td>
<td>DNA</td>
<td>133 (93)</td>
<td>10 (7)</td>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td>AluI nuclease</td>
<td>RNA, DNA</td>
<td>166 (80)</td>
<td>19 (10)</td>
<td>1 (1)</td>
<td>4</td>
</tr>
</tbody>
</table>

* This class consists of tangled molecules whose shape and length could not be determined.

Fig. 11. Size distribution and shape of ligated HaeIII restriction enzyme fragments. A portion of the reaction mixtures incubated for 20 h displayed in Fig. 9a (no ligase) and Fig. 9c (T4 RNA and DNA ligases) were examined by electron microscopy and the size distribution of DNA molecules in randomly selected fields is shown. Filled bars indicate circular molecules and open bars indicate linear molecules.

DNA ligase-joined products with HaeIII nuclease generated the original substrate fragments (Lane f), showing that true blunt end ligation had occurred. Dramatic promotion of blunt end joining by RNA ligase was also obtained with an AluI nuclease treated CoE1 DNA substrate (Fig. 10) and PMB9 DNA hydrolyzed by this enzyme (data not shown). Also shown in Fig. 10 is the sensitivity of the ligation products to recutting by AluI nuclease (Lane 7).

When the T4 ligase reaction mixtures were examined by electron microscopy, three kinds of DNA molecules were observed. The frequency of each class is summarized in Table I and the length distributions are shown in Figs. 11 and 12. The predominant class representing 89% of well spread molecules were linear duplexes. The average size was much larger than the restriction enzyme fragment starting material and some molecules were larger than CoE1 DNA. Many molecules are clearly the result of multiple ligations. The result is a scrambling of the original fragment sequence in CoE1 DNA. The second class representing 11% of well spread molecules were circular duplexes. The circular products were on the average smaller than the linear products. The previous study of blunt end joining of the much larger P22 DNA failed to reveal circular products (21). The third class consisted of 3 molecules which appeared branched. The small number of these molecules precludes an interpretation of their nature at this time. Neither circular nor branched molecules were detected in the absence of ligase treatment (Table I).

Effect of RNA Ligase on Closure of Nicks—To study the interaction of T4 RNA and DNA ligases with substrates con-
taining single strand interruptions, ColEI DNA digested with HpaII nuclease, which makes staggered cuts, was used as a substrate (Fig. 13). RNA ligase alone did not catalyze joining but DNA ligase did. In contrast to blunt end joining (compare Fig. 2), the rate of joining by T4 DNA ligase was linearly dependent on enzyme concentration and the addition of RNA ligase only moderately stimulated the reaction, about 2-fold. RNA ligase resulted in a similar modest stimulation of the E. coli DNA ligase-catalyzed sealing of the nicks in the substrate. The measured $K_m$ for the reaction with T4 DNA ligase alone was 0.8 $\mu$M and the turnover number was 1.1 mol/min/mol of enzyme at 15$^\circ$. Previous work had shown that RNA ligase also does not catalyze the cyclization of poly[d(A-T)] nor the closure of nicks in oligo(dT):poly(dA) (1, 10) which are good substrates for DNA ligases (11). In assays of poly[d(A-T)] cyclization, 60 $\mu$g/ml of RNA ligase actually inhibited the DNA ligase reaction by about 50%. Reciprocally, a high concentration of T4 DNA ligase, 80 $\mu$g/ml, inhibited the RNA ligase-catalyzed cyclization of RNA by 75%, but had no effect on cyclization of rRNA. The major conclusions are that the strong RNA ligase promotion of DNA joining by low concentrations of T4 DNA ligase seems limited to substrates with base-paired ends and that RNA ligase itself has no detectable nick-sealing activity.

**Discussion**

Sgaramella et al. (12) first reported that T4 DNA ligase catalyzed not only the closure of nicks but also the joining of double-stranded DNA at base-paired ends. The blunt end joining reaction is useful for the construction of several kinds of DNA including chimeric DNA molecules (15, 16). Of the two alternative procedures for making chimeric DNA, one is limited to DNA fragments generated by restriction endonucleases which leave cohesive termini (19) and the other method introduces a variable length poly(dA):poly(dT) sequence into the product (32, 33). In our hands, blunt end joining has been a reliable procedure with several DNA and RNA ligase preparations. Particularly in the presence of RNA ligase, the efficiency of the reaction is high; molecules of all sizes participate in the reaction and many do so repeatedly. The evidence is convincing that true blunt end joining occurred rather than a creation of cohesive ends by a contaminating nuclease or by adventitious hydrogen bonding. After efficient joining of the decamer, AluI nuclease fragments, or HaellIII enzyme fragments, the starting material was almost quantitatively recovered by treatment with the appropriate restriction enzyme (Figs. 4, 9, and 10). The fidelity of the reaction was also shown by the sharp banding pattern of the decamer products on polyacrylamide gels (Figs. 3 and 4) and by the nearest neighbor analysis of the products. Since T4 DNA and RNA ligases are easy to purify and are commercially available, they are attractive reagents for constructing chimeric DNA molecules.

The cyclization by the ligases of duplex DNA with base-paired ends should be a valuable technique. This intramolecular reaction was not observed in the previous study of blunt end joining which employed a very long DNA substrate, phage P22 DNA (14). The frequency of circular molecules in the experiment reported in Table 1 was 11%. Presumably this frequency can be increased by extending the reaction time and amount of ligase and the cyclization of single fragments increased by using lower substrate concentrations. The technique will be limited by the ease of cyclization of very small duplexes. This is not a serious restraint, since it should be possible to use a multistep procedure of cyclizing single-stranded fragments, annealing the complementary strand, and sealing the nick. A single-stranded molecule as small as a hexadeoxyribonucleotide can be cyclized by RNA ligase (9, 10).

Our results suggest three possible reasons why some investigators have found poor blunt end joining with preparations of T4 DNA ligase active in sealing nicks. First, the degree of contamination of T4 DNA ligase by T4 RNA ligase will sharply influence the reaction (Fig. 2). Second, the blunt end joining reaction with T4 DNA ligase alone is not linearly related to enzyme concentration and requires a large amount of enzyme (Fig. 2). Third, if the high apparent $K_m$ of the reaction obtained with the decamer substrate (Fig. 6) is correct generally, then dilute reactions will show disappointing joining.

Although the mechanism of RNA ligase promotion of blunt end joining is unknown, some alternatives are clearly less likely than others. Since T4 DNA ligase alone but not T4 RNA ligase will catalyze joining of DNA of base-paired ends, the actual phosphodiester bond formation is probably catalyzed by the DNA ligase when a mixture of both enzymes is present. The possibility that RNA ligase adenylates the 5' ends of the DNA which are then joined by DNA ligase is remote. This hypothesis suggests that the adenylated intermediate inhibits its own synthesis, since we detect no significant level of intermediate in the presence of RNA ligase alone. Such inhibition was not observed in the study of adenylolation of single-stranded DNA (10). The possibility that RNA ligase protein nonspecifically protects T4 DNA ligase from dilution inactivation is countered by several arguments. Polynucleotide kinase (Fig. 9), bovine serum albumin, and heat-denatured RNA ligase did not promote blunt end joining by T4 DNA ligase. Some stimulation by RNA ligase was seen at even very high concentrations of T4 DNA ligase and as little as 6 $\mu$g/ml of RNA ligase stimulated blunt end joining. Except at high enzyme concentrations, the reactions were linear with time (Fig. 5). The most persuasive argument is that RNA ligase had only a small effect on the closure of nicks by T4 DNA ligase (and by E. coli enzyme) and in this case, the activity was linearly dependent on enzyme concentration (Fig. 13). The possibility that a contaminant in the ligase preparations is involved in blunt end joining is not definitely ruled out. However, for reasons cited above, it cannot be a nuclease, the ligases are quite pure (Fig. 1), and the activity of DNA ligase in blunt end and cohesive end joining co-purify on hydroxyapatite (Fig. 7) and Sephadex columns. Three feasible models that remain are: (a) RNA ligase competes with DNA ligase for nonproductive binding sites, (b) the two enzymes form a protein-protein complex with enhanced blunt end joining activity, and (c) blunt end joining requires the cooperation of 2 ligase molecules, 1 molecule of either DNA ligase or RNA ligase holds the base-paired ends in juxtaposition and DNA ligase catalyzes phosphodiester bond formation. All these models are consistent with the observed concave curve relating T4 DNA ligase concentration and blunt end joining as opposed to the linear curve for sealing nicks and the larger promotion of blunt end joining by RNA ligase at low DNA ligase concentrations. The interaction of the two enzymes may be manifest in the inhibition by DNA ligase of the RNA ligase catalyzed cyclization of single-stranded DNA.

The efficiency of blunt end joining by the two ligases raises the possibility that this reaction has some physiological role. RNA ligase is present in large amounts in T4-infected cells, and thus maximum promotion by RNA ligase of blunt end joining would be manifest. However, DNA ligase catalyzed cyclization of single-stranded DNA was not stimulated by RNA ligase.
joining is possible. In terms of turnover number, the addition of RNA ligase has the effect of making T4 DNA ligase activity essentially independent of prealignment of the polymers to be joined by a complementary DNA strand. The turnover number at 15°C for joining the duplex decamer of 0.8 mol/min/mol of DNA ligase is similar to the turnover number of joining HpaII nuclease generated cohesive ends of 1.1 mol/min/mol of ligase and the literature value (31) for joining dT10 annealed to poly(dA) of 5 mol/min/mol of enzyme obtained at 20°C with DNA ligase alone. One potential in vivo role of the blunt end joining reaction is the catalysis of illegitimate recombination.

Acknowledgments – We are grateful to Dr. J. Locker for performing the electron microscopy and to Doctors I. R. Lehman, K. L. Agarwal, and K. Itakura for gifts of Escherichia coli DNA ligase, T4 DNA ligase, and the synthetic decamer, respectively.

REFERENCES
Interaction of bacteriophage T4 RNA and DNA ligases in joining of duplex DNA at base-paired ends.

A Sugino, H M Goodman, H L Heyneker, J Shine, H W Boyer and N R Cozzarelli


Access the most updated version of this article at [http://www.jbc.org/content/252/11/3987](http://www.jbc.org/content/252/11/3987)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/252/11/3987.full.html#ref-list-1](http://www.jbc.org/content/252/11/3987.full.html#ref-list-1)