Enzymatic Breakage and Joining of Deoxyribonucleic Acid

VIII. HYBRIDS OF RIBO- AND DEOXYRIBONUCLEOTIDE HOMOPOLYMERS AS SUBSTRATES FOR POLYNUCLEOTIDE LIGASE OF BACTERIOPHAGE T4*

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SUMMARY

Polynucleotide ligase of bacteriophage T4, in contrast to the DNA ligase of Escherichia coli, catalyzes the joining of oligomers of deoxyribonucleotides hydrogen-bonded to complementary strands of either polydeoxyribonucleotides or polynucleotides. Hybrid molecules in which oligomers of ribonucleotides are hydrogen-bonded to complementary strands of polydeoxyribonucleotides can also serve as substrates for the T4 enzyme. The rate of joining of oligomers in hybrid molecules of this type is only 1 to 2% of that observed when both strands are polynucleotides. No activity has been detected when both strands are polynucleotidetides. The products of these reactions have been characterized by sedimentation and end group analysis; in all cases joining occurs through phosphodiester bond formation.

It had been shown previously that the introduction of an amber mutation into phage T4 could suppress an amber mutation in the ligase gene. In these studies it is shown that the double mutant, T4rIIamH39X, carrying an amber mutation in the ligase gene, does not have detectable levels of T4 ligase in the nonpermissive host. Similarly, T4rIIaA80, carrying a temperature-sensitive mutation in the ligase gene, can grow at the nonpermissive temperature, despite retaining its temperature-sensitive ligase. Both enzymes catalyze the synthesis of phosphodiester bonds by esterification of a 5'-phosphoryl group to a 3'-hydroxyl group of DNA chains which have been properly aligned in a double helical structure. Furthermore, the reactions catalyzed by the two enzymes involve the formation of both enzyme- and DNA-adenylate intermediates.

An interesting difference between the E. coli and phage-induced ligases is their cofactor requirement. Whereas the E. coli ligase requires DPN⁺ (3, 9), the phage-induced ligase uses ATP (5, 7). This report describes a second difference between the two enzymes: the ability to catalyze the joining of hybrid molecules. The E. coli ligase cannot catalyze the joining of oligomers of ribo- or deoxyribonucleotides when they are present in duplex hybrid molecules (10), whereas the experiments described here show that the T4 ligase can do so. In the present studies we have used several homopolymer pairs of the type shown in Fig. 1. For example, both the T4 ligase and the E. coli ligase (2) can use the d(A)ₜ₋ₜ d(T)ₜ homopolymer pair as substrate (Fig. 1a). However, only the T4 ligase can effect joining of oligo d(T) (Fig. 1b) or oligo (A) (Fig. 1c) in the d(T)ₜ₋ₜ (A)ₜ homopolymer pair. The joining of oligomers in these homopolymer pairs is effected through a mechanism of chain slippage as shown by Olivera and Lehman (10) for the E. coli ligase. A temperature-sensitive ligase purified from a mutant of T4, previously shown to have a mutation in the structural gene for the ligase (11), has been a useful tool in establishing the fact that a single enzyme is responsible for all of these reactions.

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FIG. 1. The joining of oligomers in homopolymer duplexes by T4 polynucleotide ligase. T4 polynucleotide ligase can catalyze the joining of oligomers of deoxyribonucleotides hydrogen-bonded to polydeoxyribonucleotides (a) or to polyribonucleotides (b). It can also catalyze the joining of oligomers of ribonucleotides hydrogen-bonded to polydeoxyribonucleotides (c), although it has not been established whether the linkage is 3',5' or 2',5'.

EXPERIMENTAL PROCEDURE

Nucleic Acids and Nucleotides

Unlabeled nucleotides, salmon sperm DNA, and DNA of phage T7 were obtained as described previously (12). γ-32P-ATP of high specific radioactivity was prepared by a modification (13) of the method of Glynn and Chappell (14). Polyuridylic acid and polyadenylic acid were purchased from Miles Chemical Company. Polydeoxythymidylic acid was obtained from Biopolymers, Inc. Poly d(A-T) was prepared by the method of Schachman et al. (15). Unless otherwise noted, concentrations of polynucleotides are expressed as equivalents of nucleotide phosphorus.

Enzymes

Polynucleotide ligase of phage T4 was the concentrated phosphocellulose fraction (1820 units per mg of protein) purified as previously described (16). Polynucleotide ligase of phage T4tsA80 was the phosphocellulose fraction (1200 units per mg of protein) purchased from Miles Chemical Company. DNA ligase of E. coli (Fraction V) was the generous gift of Dr. I. R. Lehman. Polynucleotide kinase was isolated from E. coli infected with bacteriophage T4. Enzyme purification, assay, and definition of units of activity are as previously described (17). Bacillus subtilis exonuclease was purified according to the method of Kerr, Chien, and Lehman (18). Electrophoretically purified, RNase-free bacterial alkaline phosphatase was purchased from Schwarz BioResearch. Micrococcal nuclease, pancreatic DNase, and phosphodiesterase from the venom of Crotalus adamanteus were products of Worthington. The 5'-nucleotidase of C. adamanteus venom was purified and assayed as described elsewhere (19).

End Group Analysis

Radioactive labeling of the 5'-hydroxyl end groups of polynucleotides with γ-32P-ATP in the polynucleotide kinase reaction was used to determine the average chain length of the polymers. The enzyme assays, including incubation with bacterial alkaline phosphatase to remove 5'-phosphomonoesters, were carried out as previously described (13).

DNA Preparations

The specific activity of the labeled oligomers described below ranged from 1 to 20 × 10^6 cpm per pmole of end-groups.

DNA with Internal 5'-32P-Phosphomonoesters—T7 DNA displaying 5'-32P-phosphomonoesters (ligase substrate) at the site of single strand breaks was prepared and characterized as before (13). The preparation used in these studies had 30% of its phosphomonoesters located at internal sites.

5'-32P-oligo-d(A-T)—5'-32P-oligomers of d(A-T) copolymer, a substrate for DNA ligase, were prepared as described by Olivera, Scheffler, and Lehman (20). Poly d(A-T), approximately 2500 nucleotides per chain, was first degraded with pancreatic DNase to smaller oligonucleotides. The reaction mixture (3.5 ml) contained 0.24 mm poly d(A-T), 0.1 M Tris-HCl buffer (pH 7.6), 6.6 mM MgCl₂, and 4 units (0.4 μg) of pancreatic DNase. After incubation for 20 min at 30°, 50 μmoles of EDTA (pH 8.0) were added and the reaction mixture was placed in a water bath at
80° for 10 min. Subsequently the reaction mixture was cooled to 4° and dialyzed against two changes of 0.01 M Tris-HCl buffer (pH 8.0)-0.05 mM NaCl. The oligo d(A-T) was then dephosphorylated by incubation with phosphatase at 65°, and the 5′ termini were radioactively labeled in the polynucleotide kinase reaction as previously described for labeling single strand breaks in T7 DNA (13). Removal of phosphatase and γ-32P-ATP was accomplished in the following way. A column (3 × 0.8 em²) was formed from 12 ml of a methylated albumin Kieselguhr suspension prepared and washed according to the method of Sueoka and Cheng (21). The column was operated at 25°-25°. The reaction mixture containing the 5′-32P-oligo d(A-T) was diluted 10-fold with 0.15 M NaCl-0.015 M sodium citrate and applied to the adsorbent. The adsorbent was washed with 50 ml of 0.2 M NaCl-0.05 M Tris-HCl buffer (pH 7.7). The oligo d(A-T) was then eluted with 0.4 M NaCl-0.05 M Tris-HCl buffer (pH 6.7). When necessary, air pressure was used to maintain a flow rate of 4 to 6 ml per min. Approximately 90% of the oligo d(A-T) applied to the adsorbent was recovered in a volume of 10 ml. The eluate was dialyzed against 50 mM NaCl-10 mM Tris-HCl buffer (pH 7.9). The average chain length of the 5′-32P-oligo d(A-T) was 290 nucleotides.

5′-32P-oligo d(T)—Poly d(T), 3000 nucleotides per chain, was degraded to oligomers in a manner similar to that described above for poly d(A-T). The reaction mixture (1.0 ml) contained 0.27 μmole of poly d(T), 30 mM Tris-HCl buffer (pH 8.0), 3 mM MgCl₂, and 50 μg of pancreatic DNase. After 25 min at 20°, 5 μmole of EDTA (pH 8.0) were added and the reaction mixture was placed in a boiling water bath for 2 min. After cooling the reaction mixture in an ice bath, 10 μmole of MgCl₂ were added. The oligo d(T) was incubated first with phosphatase and then with polynucleotide kinase as described previously for the labeling of single strand breaks in T7 DNA (13). The reaction mixture was diluted and adsorbed to methylated albumin Kieselguhr as described above for 5′-32P-oligo d(A-T). The adsorbent was washed with 50 ml of 0.15 M NaCl-0.015 M sodium citrate, followed by 50 ml of 0.4 M NaCl-0.05 M Tris-HCl buffer (pH 6.7). The oligo d(T) was eluted with 0.9 M NaCl-0.05 M Tris-HCl buffer (pH 6.7) and dialyzed as described above. The average chain length of the 5′-32P-oligo d(T) was 220 nucleotides with an over-all recovery of 90%.

5′-32P-oligo d(A) and 5′-32P-oligo (A)—Poly d(A), 3000 nucleotides per chain, and poly (A), 1000 nucleotides per chain, were partially hydrolyzed by incubation with micrococcal nuclease as described by Olivera and Lehman (2). The hydrolysis products were then incubated with alkaline phosphatase as described above in order to remove 3′-phosphoryl groups. End group labeling was carried out in the methylated albumin Kieselguhr column as described above for 5′-32P-oligo d(A-T). The adsorbent was washed with 50 ml of 0.15 M NaCl-0.015 M sodium citrate, followed by 50 ml of 0.4 M NaCl-0.05 M Tris-HCl buffer (pH 6.7). The oligo d(A) was eluted with 0.9 M NaCl-0.05 M Tris-HCl buffer (pH 6.7) and dialyzed as described above. The average chain length of the 5′-32P-oligo d(A) and 5′-32P-oligo (A) was 200 and 120, respectively.

5′-32P-oligo (U)—Poly (U), 1300 nucleotides per chain, was partially hydrolyzed by incubation with micrococcal nuclease as described above for poly d(A-T) and dialyzed against two changes of 0.01 M Tris-HCl buffer (pH 8.0)-0.05 mM NaCl. The oligo d(A-T) was then dephosphorylated by incubation with phosphatase at 65°, and the 5′ termini were radioactively labeled in the polynucleotide kinase reaction as previously described for labeling single strand breaks in T7 DNA (13). Removal of phosphatase and γ-32P-ATP was accomplished in the following way. A column (3 × 0.8 em²) was formed from 12 ml of a methylated albumin Kieselguhr suspension prepared and washed according to the method of Sueoka and Cheng (21). The column was operated at 25°-25°. The reaction mixture containing the 5′-32P-oligo d(A-T) was diluted 10-fold with 0.15 M NaCl-0.015 M sodium citrate and applied to the adsorbent. The adsorbent was washed with 50 ml of 0.2 M NaCl-0.05 M Tris-HCl buffer (pH 7.7). The oligo d(A-T) was then eluted with 0.4 M NaCl-0.05 M Tris-HCl buffer (pH 6.7). When necessary, air pressure was used to maintain a flow rate of 4 to 6 ml per min. Approximately 90% of the oligo d(A-T) applied to the adsorbent was recovered in a volume of 10 ml. The eluate was dialyzed against 50 mM NaCl-10 mM Tris-HCl buffer (pH 7.9). The average chain length of the 5′-32P-oligo d(A-T) was 290 nucleotides.

**Ligase Assays**

The ligase assays used in these studies measure the conversion of 5′-32P-phosphomonoesters of oligonucleotides into a form which is resistant to the action of phosphatase. T₄ Polynucleotide Ligase—T₄ polynucleotide ligase activity was measured in the standard reaction mixture (16) containing either nicked T₇ phage DNA or the synthetic polymers as substrate. When polydeoxyribonucleotides were used as substrates, the standard assay procedure, previously described (16), was used.

When polyribonucleotides or hybrid molecules were used as substrates, a modification of the assay procedure described by Olivera and Lehman (2) was used. The polyribonucleotides were incubated for 20 min in the standard T₄ ligase reaction mixture. The reaction mixture was placed in a boiling water bath for 2 min and then cooled in an ice bath. After the addition of 1 unit of bacterial alkaline phosphatase, incubation was continued at 65° for 30 min. The reaction mixture was then chilled to 0°, and to it were added 50 μg of salmon sperm DNA, followed by 0.1 ml of a solution containing 1 mM Pi, 1 mM PPi, and bovine serum albumin (1 mg per ml). Then 0.2 ml of a Norit solution (20% packed volume) and 2 ml of cold 1 x trichloroacetic acid containing 0.02 mM Pi were added in succession. The suspension was mixed, and after 5 min at 0° the Norit was collected by filtration through a glass filter (Whatman GF/C glass paper, 2.4-cm diameter). The Norit was washed with eight 2-ml portions of 0.05 M HCl-0.02 M Pi. The filters were dried and the radioactivity was determined in a low background, thin window gas flow counter (Nuclear-Chicago model 1105).

For the assays with homopolymer substrates, the reaction mixtures contained equimolar amounts of the two complementary homopolymers. The 5′-32P-oligonucleotides were present at a concentration of 0.5 to 1.5 μmoles of termini per reaction mixture. One unit of ligase is defined as the amount of enzyme which catalyzes in 20 min the conversion of 1 μmole of 32P-phosphomonoesters into a form insusceptible to the action of phosphatase.

E. coli DNA Ligase—The DNA ligase (polynucleotide joining enzyme) of E. coli was assayed according to the method of Olivera and Lehman (2) with the modification that each reaction mixture was made 10 mM in (NH₄)₂SO₄.

**Sedimentation Analysis**

Zone sedimentation of polydeoxyribonucleotides in alkalai was carried out at 4° in linear concentration gradients of 5 to 20% (w/v) sucrose in 0.7 M NaCl-0.3 mM NaOH-0.001 mM EDTA in the Spinco type SW50.1 swinging bucket rotor.

Zone sedimentation of polyribonucleotides was carried out in linear concentration gradients of 5 to 20% (w/v) sucrose in 0.7 M NaCl-0.02 mM potassium phosphate buffer (pH 7.6)-6% formamide in the Spinco type SW50.1 swinging bucket rotor. The polyribonucleotides were denatured prior to sedimentation in neutralized 12% formamide (Mallinkrodt, 37% analytical reagent) according to the method of Freifelder and Davison (22). The collection and analysis of fractions were carried out as previously described (23).

* I. R. Lehman, personal communication.
Polynucleotide ligase activity on ribo- and deoxyribopolymers

Activity was measured in the standard T4 ligase assay with each of the substrates listed. The homopolymer substrates were added in equimolar (nucleotide) amounts. The concentration of 5'-32P-phosphomonoesters in each reaction mixture was 3 \( \mu \)M, except in the case of nicked T7 DNA, for which the concentration was 0.5 \( \mu \)M. The extent of the reaction was measured in reaction mixtures containing 0.2 unit of T4 polynucleotide ligase. An additional 0.2 unit of enzyme, added to the incubation mixture after 20 min, followed by incubation for an additional 20 min, did not increase the extent of joining. The extent of joining is expressed as the percentage of 32P-labeled phosphomonoesters in each substrate which could be converted to a phosphatase-resistant form. Nicked DNA refers to T7 DNA containing single strand breaks.

| Substrate                  | Activity | Extent |
|---------------------------|----------|--------|
| Nicked T7 DNA             | 1,820    | 34     |
| Oligo d(A-T)              | 462      | 70     |
| Poly d(A) + oligo d(T)    | 13,800   | 95     |
| Poly (A) + oligo d(T)     | 280      | 93     |
| Poly d(T) + oligo (A)     | 160      | 77     |
| Poly d(A) + oligo (U)     | <10      | <5     |
| Poly (U) + oligo (A)      | <0.01    | <1     |
| Poly (A) + oligo (U)      | <0.01    | <1     |

Other Methods

Hydrolysis of Polynucleotides—Polynucleotides were enzymatically hydrolyzed to either nucleoside 3'-monophosphates or nucleoside 5'-monophosphates, as previously described (16). Polyrribonucleotides were hydrolyzed to nucleoside 2'- and 3'-monophosphates by incubation in 0.3 N NaOH at 37° for 16 hours. Nucleoside 5'-monophosphates were identified by their susceptibility to 5'-nucleotidase (16). Nucleoside 3'-monophosphates were identified by chromatography on Whatman No. 3MM paper in a solvent system (24) consisting of 95% ethanol-l ammonium acetate saturated with sodium tetraborate (70:30, v/v).

Bacteria and Bacteriophage—E. coli O11' is a strain permissive for amber mutants. The nonpermissive strain was E. coli ER22, a B strain deficient in endonuclease I. T4amH39X (25) is a mutant of phage T4 bearing an amber mutation in gene 30, the structural gene for the ligase (11). T4teA80 is a mutant of phage T4 bearing a mutation in gene 30 which leads to the synthesis of a temperature-sensitive ligase (11). The double mutants, T4rIamH39X and T4rIteA80, were prepared by crossing the single mutants in a mixed infection and isolating progeny from the infection which showed defects in both alleles (26, 27). The ligase-negative amber mutant of phage T7, T7am13, was isolated from a hydroxylamine-mutagenized stock.

Extracts of E. coli and of E. coli infected with phages T4, T7, and mutants of these phages were prepared as described previously (11, 28). Protein was determined by the method of Lowry et al. (29).

4 Y. Masamune and C. C. Richardson, unpublished results.

T4 Ligase—Purified preparations of T4 polynucleotide ligase catalyze the joining of oligomers hydrogen-bonded to their complementary polynucleotides, as well as effecting the repair of single strand breaks in DNA (Table I). Three substrates that have been used extensively to characterize the E. coli ligase and the phage induced ligase are: DNA containing single strand breaks (13, 16), oligo d(T) hydrogen-bonded to poly d(A) (2, 10), and oligomers of d(A-T) (20). While the single strand breaks in DNA are fixed in position as a result of the unique base sequence in naturally occurring DNAs, chain slippage can displace the breaks from this original location in the other two substrates (10, 20). Oligomers of d(A-T) differ further from natural DNA in that they can form single stranded, circular molecules which can be joined in the ligase reaction (20).

As shown in Table I, all of the above substrates are joined by the purified T4 ligase. Poly d(A)-oligo d(T) is joined 50-fold more rapidly than oligo d(A-T). In this experiment the reduced rate of joining observed with nicked T7 DNA should not be compared with rates observed with the other substrates, since the concentration of internal phosphomonoesters (single strand breaks) is significantly lower than the concentration of 1.5 nm required for half-maximal velocity (16).

When poly (A)-oligo d(T) or poly d(T)-oligo (A) was incubated with T4 ligase, joining of the oligomers was observed (Table I). Since the hybrid polymers contain saturating concentrations of termini, as measured with nicked T7 DNA (16), the rate of joining can be compared to that obtained with poly d(A)-oligo d(T). The oligomers of deoxyribonucleotides in these hybrid molecules are joined at approximately 2% of the rate observed when both strands consist of deoxyribonucleotides. The oligomers of ribonucleotides are joined at approximately 1% the rate of poly d(A)-oligo d(T). Although the rates observed with the hybrid molecules are greatly reduced compared to the deoxyribonucleotide substrates, the reaction can be carried to completion by either prolonged incubation or by the addition of excess enzyme.

The rate and the extent of joining of oligo d(A) in the presence of poly d(T) are much less than those observed with poly d(A)-oligo d(T). Similarly, no activity was observed when oligo (U) was incubated in the presence of poly d(A). As described below,
Polynucleotide ligase activity in extracts of Escherichia coli infected with T4*  

Extracts of E. coli ER22 infected with phase T4r+ were prepared and assayed for T4 ligase activity in the standard assay containing each of the substrates listed below. *P made susceptible to phosphatase was determined as described under "Experimental Procedure."

| Substrate                  | Activity (mmoles/20 min/mg protein) |
|----------------------------|-------------------------------------|
| Nicked T7 DNA              | 1.6                                 |
| Oligo d(A-T)               | 0.25                                |
| Poly d(A) + oligo d(T)     | 4.7                                 |
| Poly (A) + oligo d(T)      | 0.08                                |
| Poly d(T) + oligo (A)      | 0.07                                |

Polynucleotide ligase activity in extracts of E. coli infected with T7  

E. coli ER22, a nonpermissive host for amber mutants, was infected with either T7am29 or T7am13. Extracts were prepared and assayed for T7 ligase activity in the standard T4 ligase assay containing each of the substrates listed below. *P made susceptible to phosphatase was determined as described under "Experimental Procedure." T7am29 has an amber mutation in gene 3, the structural gene for an endonuclease (28). T7am13 has an amber mutation in the structural gene for T7 polynucleotide ligase (see Footnote 4). The values in parentheses are the activities of T7 ligase in E. coli O117, the permissive host, infected with T7am13.

| Substrate                  | Extract T7am29 | T7am13 |
|----------------------------|---------------|-------|
| Oligo d(A-T)               | 0.1           |       |
| Poly d(A) + oligo d(T)     | 4.1           | <0.1  (0.5) |
| Poly (A) + oligo d(T)      | 0.03          |       |
| Poly d(T) + oligo (A)      | 0.22          | <0.001 (0.1) |

there is evidence that these two homopolymer pairs may exist as triple stranded structures.

In control studies no activity (<0.01 unit per mg of protein) was detected in the absence of any of the complementary homopolymers. Furthermore, when the reaction mixture containing poly (A) oligo d(T) was incubated with 0.3 M NaOH at 37°C for 19 hours (conditions sufficient for the complete hydrolysis of the poly (A)), there was no joining of the oligo d(T) (less than 1% of the total *P). During incubation in the standard assay containing 0.3 unit of ligase, no radioactivity in the ribohomopolymers became acid-soluble, either in the presence or absence of ATP and the complementary deoxyribohomopolymers.

When both strands consisted of ribohomopolymers, no joining was observed (Table I). Attempts to obtain joining by replacement of Mg++ by Mn+++ were unsuccessful.

E. coli Ligase—In contrast to the results obtained with the T4 ligase, Olivera and Lehman (10) found that ribohomopolymers could not substitute for deoxyribohomopolymers in the E. coli ligase reaction. By using a more concentrated preparation of E. coli ligase, we have been able to increase the sensitivity of the assay (Table II). In confirmation of their results we were unable to detect any activity (<0.02% of that observed with oligo d(A-T)) of the E. coli ligase on poly d(T) oligo (A), a substrate for the T4 ligase.

Identity of Activities toward DNA and Hybrid Molecules

Similar Reaction Requirements—The requirements for ligase activity with hybrid substrates were essentially the same as those for the T7 DNA substrate (16). No activity was detected in the absence of ATP or Mg++. Mn+++ could replace Mg+++. At an optimal Mn+++ concentration of 10 mm the rate was twice that observed with Mg++.

Activity in Extracts of Phage-infected Cells—Extracts of E. coli infected with T4r+ contain an activity capable of catalyzing the joining of oligomers in hybrid molecules. As shown in Table III, the relative activities on the various substrates closely parallel those found with the enzyme purified 1000-fold. In particular, the hybrid molecules, both poly (A) oligo d(T) and poly d(T)-oligo (A), are joined at 1 to 2% of the rate observed with poly d(A)-oligo d(T), the same rate relationship found with the purified ligase (Table I).

In these studies we also measured ligase activity in extracts of E. coli B infected with T4rII and in a double mutant (T4rIIamH39X) which contains, in addition to the rI1 mutation, an amber mutation in gene 30 (polynucleotide ligase). The rI1 mutation has been shown to suppress the gene 30 mutation in T4 mutants by an as yet unknown mechanism (26, 27, 30). Whereas T4rII induced normal levels of ligase (1.4 units per mg of protein), the double mutant, T4rIIamH39X, failed to induce ligase in the nonpermissive host (0.006 unit per mg of protein). Similar results were obtained with the oligo d(A-T) substrate. Thus, the rI1 mutation does not restore T4 ligase activity.

The E. coli ligase uses DPN+ as a cofactor, whereas the T4 ligase requires ATP. It was of interest to see whether another ligase with an ATP requirement could also use hybrid molecules as substrate. Bacteriophage T7 resembles T4 in that it induces the synthesis of a ligase which uses ATP (7). As shown in Table IV, extracts of cells infected with bacteriophage T7 catalyze the joining of oligomers of ribo- and deoxyribonucleotides in hybrid molecules as well as in the other standard helical deoxypolynucleotide substrates. An amber mutant of T7 phage, T7am13, does not produce an active ligase in E. coli strains lacking an amber suppressor. 4 Extracts of the nonpermissive host infected with this mutant do not catalyze the joining of oligo (A) in the presence of poly d(T), whereas extracts of the infected permissive host catalyze joining at a rate similar to that of extracts of cells infected with wild type phage (Table IV).

Activity of Temperature-sensitive T4 Ligase on Hybrid Substrates

The ligase purified from E. coli infected with T4tsA80 carrying a temperature-sensitive mutation in the structural gene for polynucleotide ligase displays reduced activity on DNA containing single-strand breaks at the nonpermissive temperature as previously shown (11). The T4tsA80 ligase also shows reduced activity on hybrid molecules at elevated temperature (Table V).

The QSt (ratio of activity at 37°C to that at 25°C) for the ligase reaction is well below unity for both hybrid substrates assayed with the tsA80, whereas it is greater than 2 for the same substrates with the wild type ligase. The QSt values for wild type activity are significantly higher than the value of 1.5 determined...
Activity of temperature-sensitive ligase with hybrid substrates

Activity was measured in the standard T4 ligase assay at either 25° or 37° with the two substrates listed. 32P make insusceptible to phosphatase was determined as described under “Experimental Procedure.”

| Substrate          | Enzyme    | Polynucleotide ligase activity | C^ss/   |
|--------------------|-----------|-------------------------------|--------|
| Poly (A) + oligo d(T) | Wild type | nmoles/20 min/ml | 2.1 |
| Poly (A) + oligo d(T) | tsA80    | 0.08             | 0.37 |
| Poly (d(T) + oligo (A) | Wild type | 3.3              | 4.8  |
| Poly (d(T) + oligo (A) | tsA80    | 0.02             | 0.6  |

FIG. 2. Alkaline sedimentation analysis of the poly (A)-5'-32P-oligo d(T) products of the ligase reaction. Poly (A) + oligo d(T) was incubated in the standard T4 ligase reaction either in the presence or absence of T4 ligase (0.3 unit). After incubation for 20 min at 37°, the reaction was terminated by the addition of 5 μmoles of EDTA and 50 μmoles of NaOH. Samples (0.2 ml) of each reaction mixture were layered onto alkaline sucrose gradients. Centrifugation was carried out at 35,000 rpm for 22 hours in the Spinco type SW50.1 rotor at 4°. Three-drop fractions were collected and the radioactivity was measured as described under “Experimental Procedure.”

Characterization of Product

Sedimentation Analysis Previous studies involving the T4 polynucleotide ligase reactions have used zone sedimentation in alkali and an increase in molecular weight of the single strands to show the joining of polynucleotides (16). When poly d(A) + oligo d(T) was incubated with the ligase and then sedimented in alkali, more than 50% of the oligo d(T) was found to sediment faster than the molecules which had not been incubated with ligase (Fig. 2). An even more striking increase in the size of oligo d(T) chains could be observed when they were hydrogen-bonded to poly (A) and analyzed in a similar manner (Fig. 3). More than 90% of the oligo d(T) molecules experienced an increase in sedimentation rate over that found prior to incubation with ligase. In fact, 60% of the oligo d(T) sedimented at a rate 3 to 4 times that of the untreated control samples, corresponding to an increase in molecular weight of 9- to 10-fold. Approximately 10% of the oligo d(T) achieved a size approximately 20-fold greater.

Since the sedimentation analysis of poly d(T)-oligo (A) could not be carried out in alkali, the polymer was denatured by treatment with formaldehyde at 65°, followed by sedimentation through sucrose gradients containing 6% formaldehyde (22). Seventy-five per cent of the product sedimented at a rate at least twice that of the control oligo (A) (Fig. 4). Approximately 30% of the oligo (A) was at least 9-fold larger than the control oligomers.

In the reactions used for product analysis, arbitrary amounts of ligase in large excess were used, in order to assure that the reactions would go to completion. The apparent inability of the ligase to repair completely poly d(A) - oligo d(T) as shown by sedimentation analysis (Fig. 2) was confirmed by analysis of an aliquot of the product in the standard ligase assay. In this instance 68% of the 32P-labeled substrate was resistant to phosphatase after incubation with ligase. However, several subse-
The experiments described in this report show that polynucleotide ligase of phage T4 can catalyze the joining of oligomers of ribo- or deoxyribonucleotides which are properly aligned in duplex hybrid molecules. However, the enzyme activity measured with the hybrid substrates, which in this study consisted exclusively of homopolymers, was only a fraction of that found for substrates in which both strands are deoxyhomopolymers. That the joining of both ribo- and deoxyribopolymers is effected by the same enzyme can be deduced from their identical reaction requirements and the constant ratio of activities during purification. Furthermore, the reaction involving either substrate is thermostable when a purified temperature-sensitive ligase is used.

In these studies the joining of oligomers was measured by the conversion of phosphatase-sensitive $^32P$-phosphomonomoesters to a phosphatase-resistant form. Joining of DNA by both the E. coli (35) and the T4 ligase is mediated by a DNA-adenylate intermediate in which the $^32P$-phosphomonomoester of DNA is linked to the $^32P$-phosphate of AMP.

If this intermediate accumulated in the reaction, the $^32P$ would also be resistant to phosphatase. Such an intermediate did not accumulate in these reactions, since it was shown that the $^32P$ was incorporated into phosphodiester bond linkage and that the molecular weight of the product was increased over that of the oligomeric substrates. Although it is clear that joining occurs through the formation of phosphodiester bonds, it has not been established whether the linkage is $3', 5'$ or $2', 5'$ in the case of ribooligonucleotides.

Striking differences were observed in the rates of joining with the various polymer-oligomer pairs. For example, we found that the rate and extent of joining of oligo d(A) in the presence of poly d(T) were much less than those observed with poly d(A) and oligo d(T). Furthermore, oligo (U) could not be joined in the presence of poly d(A). These observed differences may reflect the formation of triple stranded structures (36), but precise mixing curves under the conditions of the ligase assay would be necessary to establish this point. It is of interest to note that Cassani and Bolllum (37) found that, when d(A) oligomers are added to poly d(T), a three-stranded structure is produced, and Olivera and Lehman (10) found it necessary first to incubate such a reaction mixture for long periods before it could serve as a substrate for the E. coli ligase. The lack of activity observed with mixtures of poly (A) and oligo (U) may also reflect the formation of triple stranded structures, or it may reflect a requirement that at least one strand be a deoxypolyribonucleotide. In this regard it would be of interest to examine double stranded riboheteroduplexes, preferably natural RNAs.

The ligase of E. coli has greater specificity for DNA substrates than does the phage-induced ligase. Olivera and Lehman (10) reported that ribohomopolymers could not substitute for deoxyribonucleosides in the reaction catalyzed by the E. coli ligase.

We have confirmed this observation in experiments with an even higher concentration of the purified enzyme than that used previously. The difference in specificity for hybrid molecules is the second distinguishing feature between the E. coli and T4 ligase. The other major difference is in their cofactor requirement (8); whereas the E. coli enzyme uses DPN$^+$, the T4 ligase requires ATP. It is possible that these distinguishing properties

6 C. L. Harvey, T. Gabriel, E. M. Wilt, and C. C. Richardson, unpublished results.
are related in that we also find that the ligase of phage T7, which uses ATP, can also use hybrid molecules as substrates. At present the functions in vivo of the phage-induced enzymes are not clear. For example, a mutant of phage T7, deficient in the T7 ligase, shows no abnormality in growth or DNA metabolism. Similarly, although T4r1 phage with a mutation in the T4 ligase gene are unable to synthesize DNA under nonpermissive conditions, the introduction of an rII mutation can suppress the ligase mutation (26, 27, 30). In this report we show that the double mutant, T4rIIamH39X, carrying an amber mutation in the ligase gene, does not have detectable levels of T4 ligase in the nonpermissive host. A second double mutant, T4rIIamH39X, carrying a temperature-sensitive mutation in the ligase gene, can grow at the nonpermissive temperature despite retaining its temperature-sensitive ligase. Therefore, the suppression is not specific for amber mutations, and it does not occur through a mechanism which restores T4 ligase activity.

The difference in specificity could reflect an important difference in the physiological role of the two enzymes. Although in vitro the rate of the reactions with hybrid substrates was low for T4 ligase in comparison to the activity on DNA substrates, this activity may yet be functional in vivo. It is possible, for example, that in regions of the T4 genome where messenger RNA is being transcribed single strand breaks could appear in the messenger RNA or in the DNA strand being transcribed. These breaks may require repair by ligase action. In order to evaluate fully the importance of these newly discovered reactions catalyzed by the T4 phage ligase, it will be necessary to prepare ligase substrates with natural DNA-RNA hybrids and to measure ligase activity on them.

In the synthesis of the gene for an alanine transfer RNA from yeast (38), T4 polydeoxyoligonucleotide ligase has been used to join short segments of nucleotides properly aligned by annealing to a complementary overlapping oligomer. The fact that T4 ligase can catalyze the joining of oligodeoxynucleotides hydrogen-bonded to an RNA strand suggests a possibility for the future. If natural RNA messenger can be isolated in sufficient quantity, then it can be used as the complementary strand to which synthetic oligodeoxyribonucleotides are annealed and subsequently joined by T4 ligase.

Note Added in Proof—While this paper was in press, Kleppe, van de Sande, and Khurana (59) reported results similar to ours. They found that polydeoxynucleotide ligase can catalyze the joining of deoxyribo-oligonucleotides on ribopolydeoxynucleotide templates, and of ribooligonucleotides on deoxyribo-polydeoxynucleotides.

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