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Polynucleotide Ligase-Catalyzed Joining of Deoxyribo-oligonucleotides on Ribopolynucleotide Templates and of Ribo-oligonucleotides on Deoxyribopolynucleotide Templates*,†

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Abstract. T4 polynucleotide ligase efficiently catalyzes the head-to-tail joining of the ribo-oligoadenylates, r-$(pA)_8$ and r-$(pA)_{10}$, in the presence of high molecular weight deoxypolythymidylate. The enzyme also catalyzes the joining of deoxy-oligothymidylates, e.g., d-$(pT)_{10}$, in the presence of ribopolyadenylate. The enzyme failed to bring about the joining of r-$(pA)_{10}$ when poly r-U was used as the template, although a slow formation of the expected activated intermediate from r-$(pA)_{10}$ was detected.

Introduction. The polynucleotide ligase from bacteriophage T4 and the *Escherichia coli* ligase are known to repair single-stranded breaks in double-stranded DNA.[1–5] The action of the enzyme involves the formation of a phosphodiester linkage between a 5′-phosphomonoester group and a 3′-hydroxyl group when these are present at the site of a single-stranded break. In work reported from this laboratory, the chain lengths of the deoxyribo-oligonucleotides required for the enzyme-catalyzed joining reaction have been found to be quite small.[6,7]

Because of the obvious interest in joining of ribopolynucleotides and also in joining of deoxyribo-oligonucleotides on ribonucleotide templates, several groups have previously tested the DNA-ligases for their capacity to bring about such joining reactions. These attempts were evidently uniformly unsuccessful.[4b,6] With our own continued interest in these enzymes, we have reexamined the specificity of the T4 polynucleotide ligase with respect to the nature of the polynucleotides. We report in the present paper that, under slightly altered reaction conditions, the enzyme can bring about the joining of ribo-oligonucleotides on deoxyribopolynucleotide templates as well as the joining of deoxyribo-oligonucleotides on long ribopolynucleotide templates.

Materials and Methods. Enzymes: T4 polynucleotide kinase and ligase were isolated, in cooperation with Dr. R. Kleppe, from *E. coli* cells infected with T4 *am* N82. The enzymes were purified according to the procedures of Richardson and his coworkers[2b,8] except that both streptomycin and protamine sulfate steps were used. The specific activities of the enzyme preparations were comparable to those reported previously.[2b,8] One unit of T4 polynucleotide ligase is defined as the amount which catalyzes the transformation of 1 pmol of ^{32}P-5′-phosphoryl termini of d-T_{10} to a form insusceptible to bacterial alkaline phosphatase, in one minute, using the assay conditions described

previously.[6] In addition, samples of T4 polynucleotide ligase prepared by the original procedure,[2b] as well as a sample kindly provided by Dr. C. C. Richardson, were also tested in the joining experiments. All three preparations gave identical results. Bacterial alkaline phosphatase, electrophoretically purified and free of ribonuclease activity, was obtained from Worthington Biochemical Corporation.

Poly- and oligo-nucleotides: Poly d-A and poly d-T were prepared from poly dA·poly dT by separation of the strands in an alkaline cesium chloride density gradient.[9] Poly r-A and poly r-U were products of Miles Chemical Company. d-T_8 and d-T_{10} were synthesized chemically as previously described.[10] Short chain deoxyadenylates, d-A_8 and d-A_{10}, were prepared by Dr. H. Weber by controlled micrococcal nuclease degradation of poly d-A, followed by treatment with bacterial alkaline phosphatase and chromatography on a DEAE-cellulose column in the presence of 7 M urea. Samples of r-A_6, r-A_8, and r-A_{10} were generously donated by Dr. T. Terao. The oligonucleotides were phosphorylated at the 5'-hydroxyl end according to the published procedure using T4 polynucleotide kinase.[8] [γ-^{32}P]ATP and [γ-^{33}P]ATP were prepared according to the published procedure,[11] their specific activity being approximately 5 Ci/mmol. The phosphorylated oligonucleotides were separated from the excess of ATP by means of gel filtration on Sephadex G-50 columns as described previously.[6]

Assay conditions: The reaction mixtures for the joining reactions contained the following components: 66 mM Tris, pH 7.6; 6 mM $MgCl_2$; 0.066 mM ATP; 10 mM dithiothreitol. The concentration of ligase, poly-, and oligo-nucleotides are in the legends to the figures. The amount of ligase added varied from 70 to 400 units/ml. The total volume of the reaction mixture was usually 0.1 ml. Before addition of the ligase, the reaction mixtures were kept at 37°C for 10 min, then for 30 min at 12°C. Unless otherwise stated, the temperature during the reaction was 12°C.

The kinetics of the reactions were followed by withdrawing 5-μl aliquots at various times and treating them with 50 μl of 0.1 M Tris, pH 8, that contained 0.4 mM mononucleotide, usually d-pT or d-pC. To this mixture, 2 μl of bacterial alkaline phosphatase (2 mg/ml) was added and the mixture was incubated at 70°C for 30 min. At the end of this period, the whole reaction mixture was applied to a DEAE-paper strip which was developed in 7 M urea–0.3 M ammonium formate for 2 hr. The strips corresponding to each aliquot were then scanned for radioactivity. The phosphatase-resistant material stayed at the origin while P_i moved away. The radioactivity present at the origin and in P_i was measured in a liquid scintillation counter as described previously.[6]

Characterization of the joined products: The joined oligonucleotides were separated from the starting material by gel filtration. Immediately before the material was loaded on the column, EDTA was added to the reaction mixture to a concentration of 0.03 M and the solution was heated to 100°C for 3 min to inactivate the ligase and to denature the duplexes. On the Sephadex G-50 columns used, the joined product was excluded whereas the starting material was included. Both the product peak and the peak of starting material were tested for resistance towards the phosphomonoesterase. In all cases good agreement with the kinetic data was found. Nearest-neighbor analyses were carried out on both the joined deoxyribo- and ribo-oligonucleotides. In the case of deoxyribo-oligonucleotides the sample was hydrolyzed enzymatically with micrococcal and spleen phosphodiesterase as previously described.[12] Ribo-oligonucleotides were hydrolyzed in 0.4 M KOH for 16–18 hr at 37°C and the sample was neutralized with the ion exchange resin Dowex 50 in its pyridinium salt form. The mononucleotides were separated in solvent system A: ammonium sulfate (60 g)—0.1 M sodium phosphate, pH 6.8 (100 ml)—*n*-propanol (2 ml) and by paper electrophoresis in 0.05 M citrate buffer (pH 3.5) using 35 V/cm across the paper strip.

Results. Joining of short riboadenylates on poly d-T: Figure 1 shows the kinetics of joining of short riboadenylates, r-^{32}P-A_6, r-^{32}P-A_8, and r-^{32}P-A_{10} using poly d-T as template. As is seen under the conditions used in these experiments r-^{32}P-A_8 and r-^{32}P-A_{10} were joined at a rate two to three times greater than the

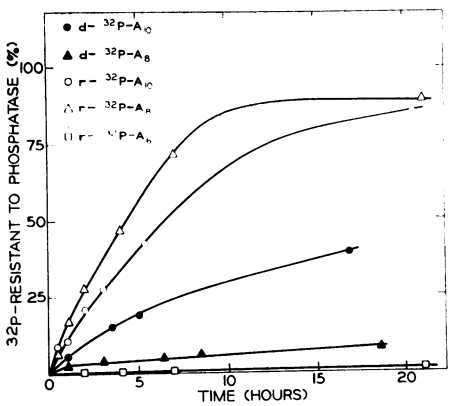
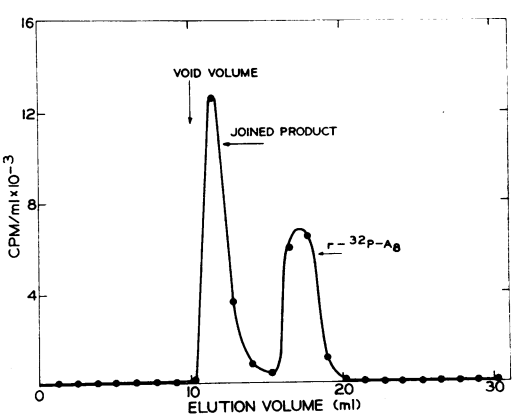


FIG. 1.—Kinetics of T4 ligase-catalyzed joining of various short-chain ribo- and deoxyoligo-adenylates in the presence of poly d-T. The concentration of oligonucleotides(^{32}P) was 0.3 μM and that of poly d-T was 3 μM (as based on mononucleotide); 70 units/ml of ligase were used. Other conditions as described in *Methods*.

corresponding deoxyribo-oligonucleotides. Longer annealing time, however, resulted in an increase in the rate of the joining of d-^{32}P-A_8 and d-^{32}P-A_{10}, whereas little or no change was observed with ribo-oligonucleotides. One possible explanation for this observation is that upon mixing poly d-T and short-chain deoxyadenylates triple-stranded structures are formed which are slowly converted to double-stranded duplexes.[4,13] The extent of triple-strand formation may be less in the case of the riboadenylates. The rate of joining of the riboadenylates could be further enhanced by increasing the concentration of the ligase. Control experiments showed that this joining was completely dependent on both the template poly d-T and ATP as is to be expected for a reaction catalyzed by T4 polynucleotide ligase.

Ribopolyadenylates formed in the joining reactions were separated from the starting material by gel filtration on Sephadex G-50 as described in the preceding section. A typical elution pattern is shown in Figure 2. The radioactivity in the first peak was 67% resistant to phosphatase while the succeeding peak (r-^{32}P-A_8) was 100% sensitive to the phosphatase. Before it was loaded on the column, 40% of the label in the reaction mixture was resistant to phosphatase. From the amount of phosphatase-resistant radioactivity in the first peak, it was calcu-

FIG. 2.—Fractionation of ligase reaction mixture of r-^{32}P-A_8 and poly d-T on a Sephadex G-50 column (33 × 1 cm). A joining reaction, which had reached 40% resistance to phosphatase with respect to ^{32}P, was stopped by adding EDTA to a final concentration of 0.03 M. It was then heated to 100°C for 3 min, cooled, and applied to a Sephadex G-50 column. The column had been equilibrated with 0.1 M triethylammonium bicarbonate buffer and was eluted with the same buffer at 4°C.



lated that the average chain length of the joined material was 24 nucleotides, i.e., three r-^{32}P-A_8 molecules had joined end-to-end. For further characterization, products of the above reaction were hydrolyzed in alkali. The radioactive products of hydrolysis were adenosine-2′(3′) phosphate and adenosine-2′(3′),5′-diphosphate [2′(3′)pAp]. An example of the separation pattern obtained in solvent system A for several samples is shown in Figure 3. The ratio of 2′- to

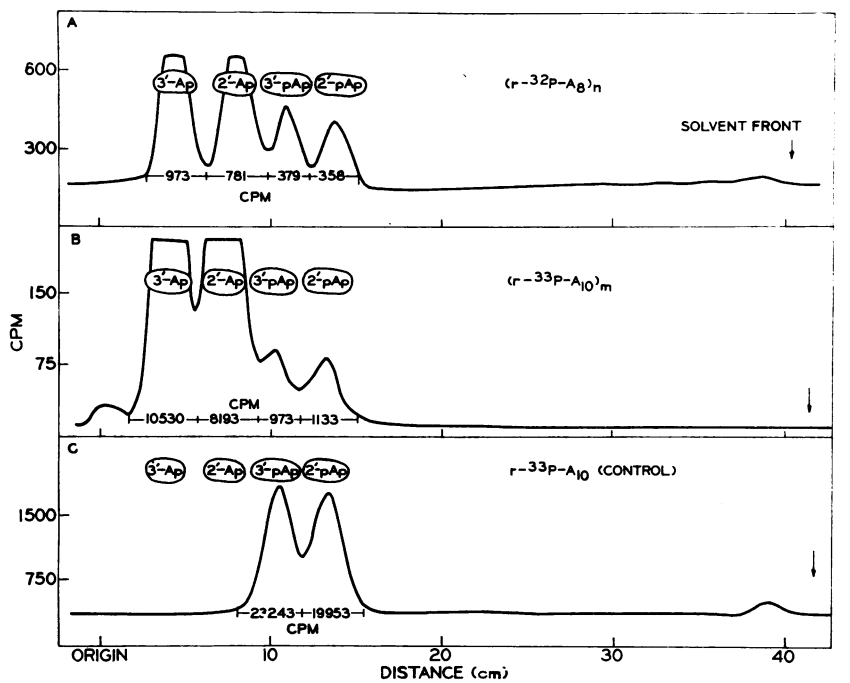


FIG. 3.—Radiochromatographic analyses of products formed on alkaline hydrolysis of T4 ligase-joined r-^{32}P-A_8, r-^{33}P-A_{10}, and of r-^{33}P-A_{10} (*control*). Products were separated in the solvent system: ammonium sulfate (60 g)–0.1 M sodium phosphate, pH 6.8 (100 ml)–*n*-propanol (2 ml). The samples were not treated with phosphatase before alkaline hydrolysis.

3′-nucleotides was approximately 1:1. Upon electrophoresis, samples *A* and *B* gave only two radioactive spots, corresponding to 2′(3′)Ap and 2′(3′)pAp. Sample *C* (the *control*) gave one spot, corresponding to 2′(3′)pAp. The ratio of mono- to di-phosphates was found to be in good agreement with that to be expected from the phosphatase resistance assay. When the joined product was treated with phosphatase before alkaline hydrolysis, only 2′- and 3′-Ap were found.

Joining of oligothymidylates on poly r-A: The kinetics of joining of d-^{32}P-T_8 and d-^{32}P-T_{10}, with poly r-A as the template, are shown in Figure 4. The joining of d-^{32}P-T_{10} proceeds rapidly, reaching a plateau after approximately 2 hr. The rate of joining of d-^{32}P-T_8, on the other hand, was very low. For comparison, the kinetics of joining of d-^{32}P-T_8 and d-^{32}P-T_{10} with poly d-A as the template

are also included in Figure 4. The rate of joining of d-^{32}P-T_{10} with poly r-A as the template is about 20 times lower than when poly d-A is the template. Longer annealing times did not significantly change these rates. The joined product of d-T_{10} was separated from the starting materials on a Sephadex G-50 column and subjected to nearest-neighbor analysis. In the phosphatase-treated material, radioactivity was only found in the region corresponding to d-Tp.

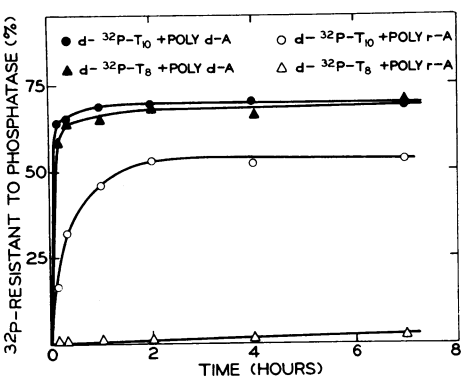


FIG. 4.—Kinetics of enzymatic joining of d-^{32}P-T_8 and d-^{32}P-T_{10} with poly r-A and poly d-A as templates. The concentration of oligonucleotides was 0.16 μM (^{32}P) and that of poly d-A and poly r-A 1.6 μM (as based on mononucleotide); 70 units/ml of T4 ligase was used. Other conditions as described in *Methods*.

Attempted joining of r-^{33}P-A_{10} using poly r-U as the template: Several attempts were made to join r-^{33}P-A_{10} using ribopolyuridylate as the template. Under conditions similar to those used above for the joining of ribo-oligoadenylates in the presence of poly d-T, slow formation of a phosphatase-resistant product was observed. The amount of product after 50 hr at 12°C was approximately 22%. However, gel filtration on Sephadex G-50 showed that this material eluted at approximately the same position as the starting material. Furthermore, after treatment with phosphatase, followed by alkaline hydrolysis, only one radioactive spot was observed. Its characteristics were as follows: (1) it traveled in solvent A with an R_f of 0.09, the R_f of 3′-and 2′-Ap were 0.11 and 0.20, and that of 3′-pAp and 2′-pAp were 0.27 and 0.34; (2) its electrophoretic mobility was similar to that of 2′(3′)pAp. These data suggest that the enzymatic reaction led to the activation of ^{33}P-A_{10} to form the pyrophosphate AMP-*O*-^{33}P-A_{10}. On alkaline treatment, the latter was hydrolyzed to form AMP-*O*-^{33}P-A-2′(3′)p. Pyrophosphate intermediates of this type have been demonstrated in reactions of *E. coli* ligase[14] as well as the T4 ligase.[15]

Comments. The results presented show that the T4 polynucleotide ligase can catalyze the joining of ribo-oligonucleotides on deoxyribo-templates and deoxyribo-oligonucleotides on ribo-templates. The rate of joining of d-^{33}P-A_{10} on poly d-T as template increased several times after prolonged preincubation. A similar observation was made by Olivera and Lehman[4] with the *E. coli* ligase-catalyzed joining of short d-A chains on long d-T templates. The explanation for this observation put forward by the authors was the conversion of a kinetically favored triple-stranded structure to a thermodynamically favored double-strand form, the latter being used by the enzyme. In other experiments of the present study, prolonged preincubation had no effect on the rate of joining.

An enhancement in the rate of joining of riboadenylates on poly d-T and deoxythymidylates on poly r-A was observed with increased enzyme concentrations. Earlier reports[4b,6] that neither the T4 nor the *E. coli* ligase was able to join ribo-oligonucleotides or use ribo-templates could be explained by the lower enzyme concentrations employed. In this study, the T4 ligase concentration used was 20–50 times higher than those employed previously. In the present work, no experiments were carried out with the *E. coli* ligase.

No conclusion can be drawn from the present study as to whether the observed properties of the T4 ligase have any biological significance. However, the present findings suggest that T4 ligase could be used as a tool in the synthesis of large ribo-oligonucleotides of known sequence by joining smaller fragments on deoxyribo-templates of complementary sequence; likewise; the enzyme could be employed for synthesizing large deoxyribo-oligonucleotides on ribo-templates. Work is currently in progress in this laboratory to test these possibilities.

Abbreviations: d-T_8 and homologs refer to thymidine oligonucleotides containing free 5′- and 3′-hydroxyl end groups. Similarly, d-A_8 and homologs and r-A_6 and higher homologs, respectively, refer to deoxyadenosine and adenosine oligonucleotides containing free 5′-OH and 3′-OH end groups. The presence of ^{32}P or ^{33}P to the left of the oligonucleotide abbreviation indicates the presence of a labeled 5′-phosphoryl end group with oligonucleotide. The linkage of AMP to form a pyrophosphate bridge with the 5′-phosphate end group of, e.g., r-^{33}P-A_{10} is shown by the structure AMP-*O*-^{33}P-A_{10}.

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