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T4 RNA Ligase Joins 2′-Deoxyribonucleoside 3′,5′-Bisphosphates to Oligodeoxyribonucleotides†

Deborah M. Hinton, Julio A. Baez, and Richard I. Gumport*

ABSTRACT: T4 RNA ligase catalyzes the ATP-dependent addition of a single 2′-deoxyribonucleoside 3′,5′-bisphosphate to the 3′-hydroxyl of an oligodeoxyribonucleotide. The bisphosphate is joined to the deoxyoligomer by a 3′→5′ phosphodiester bond and the product, which is terminated by a 3′-phosphate, is one nucleotide longer than the substrate. Bisphosphates of dAdo, dCyd, dGuo, dThd, and dUrd are donors and oligodeoxyribonucleotides with dA, dC, dG, dT, or dU 3′ termini act as acceptors. The preferred residue for both donor and acceptor is dCyd. Deoxyoligomers from 3 to 12 residues in length are active as acceptors. To obtain good yields, high concentration of enzyme, long incubation time at low temperature, and manganous rather than Mg(II) ion are required. Under optimal conditions, yields calculated with respect to deoxyoligomer converted to product vary from 40 to greater than 95%. The turnover number of the enzyme for DNA joining is extremely low but, because the preparation is nearly free of DNases, there is less than 3% degradation of substrate or product after 6 days of reaction. We anticipate that this reaction will serve as the basis for a method for the stepwise enzymatic synthesis of DNA of defined sequence.

RNA ligase from bacteriophage T4-infected *Escherichia coli* catalyzes the ATP-dependent formation of a 3′→5′-phosphodiester bond between one oligoribonucleotide with a 3′-hydroxyl group (the acceptor molecule) and another with a 5′-phosphate (the donor molecule) (Silber et al., 1972). The reaction may be either intramolecular (Silber et al., 1972) or intermolecular (Walker et al., 1975; Kaufmann & Kallenbach, 1975). The intermolecular reaction has proved useful for the synthesis of oligoribonucleotides of defined sequence (Walker et al., 1975; Ohtsuka et al., 1976; Sninsky et al., 1976; Uhlenbeck & Cameron, 1977).

The enzyme also uses DNA as a substrate (Snopek et al., 1976) but the reported yields of the intermolecular reaction with oligodeoxyribonucleotides were too low to be useful for practical syntheses (Sugino et al., 1977). We have been developing RNA ligase as a DNA synthesis reagent and have found reaction conditions that allow high yields with single-strand deoxyoligomers (McCoy & Gumport, unpublished results) and, in addition, have demonstrated that the donor molecule can be a single nucleotide, if it is a 2′-deoxyribonucleoside 3′,5′-bisphosphate (pdNp).[1] It is the single nucleotide addition reaction of RNA ligase with DNA substrates that is the subject of the present report. A preliminary report of this work has appeared (Hinton et al., 1978). This reaction is the analogue of the single ribonucleoside bisphosphate addition to oligoribonucleotides (England & Uhlenbeck, 1978; Kikuchi et al., 1978) and to large RNAs (England & Uhlenbeck, personal communication) and is expected to be similarly useful

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[1] Abbreviations following the IUPAC-IUB Commission on Biochemical Nomenclature recommendations [*J. Mol. Biol. 55,* 299 (1971), and *Proc. Natl. Acad. Sci. U.S.A. 74,* 2222 (1977)] are used throughout. The one-letter abbreviations for simple nucleotides will be used. For example, pdAp represents 2′-deoxyadenosine 3′,5′-bisphosphate. Other abbreviations used: Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; BAP, bacterial alkaline phosphatase; DTT, dithiothreitol; BSA, bovine serum albumin.

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for the synthesis and modification of DNA.

Materials and Methods

Materials. The sodium salt of pdTp and dUrd, dAdo, dGuo, dCyd, dCp, dTp, dA(pdA)$_4$, (pdA)$_2$, (pdA)$_3$, (pdA)$_4$, (pdA)$_{12}$, (pdT)$_4$, (pdT)$_5$, dT(pdT)$_7$(pdC)$_2$, and (pdT)$_8$pdG were purchased from P-L Biochemicals, Inc. ATP was obtained from Sigma Chemical Co. and [γ-^{32}P]ATP was prepared by the method of Glynn & Chapell (1964). Pyrophosphoryl chloride (tetrachloropyrophosphate) was a gift of J. R. Barrio and M. R. Barrio. Bacterial alkaline phosphatase (BAP) C, BAP F, micrococcal nuclease, spleen phosphodiesterase, pancreatic DNase I, and snake venom phosphodiesterase were purchased from Worthington Biochemical Corp. Polynucleotide kinase lacking 3′-phosphatase activity was isolated from *Escherichia coli* infected with *PseT* 1 T4 (Cameron et al., 1978). The absence of the 3′-phosphatase activity permitted the isolation of nucleoside 3′,5′-bisphosphates in high yields. DE 81, Whatman no. 1 and 3 MM paper were obtained from Whatman, Inc. DEAE-Sephadex A-25 was purchased from Pharmacia Inc.

Purification of RNA Ligase. T4 RNA ligase was purified from *E. coli* cells infected with T4 *am* E4314 (gene 43 mutant) by a modification of the method described by Walker et al. (1975) using Sephadex G-150 in place of Sephadex G-75. In addition, the enzyme was further purified by gradient elutions from columns of hydroxylapatite and DEAE-cellulose and the remaining DNase activity removed by chromatography on two Affi-gel Blue columns (manuscript in preparation). The enzyme was greater than 90% pure and migrated as a polypeptide of M_r = 43 000 upon NaDodSO$_4$ gel electrophoresis. One unit of RNA ligase exchanges 1 nmol of $^{32}PP_i$ into a charcoal adsorbable form in 30 min at 37 °C in a mixture (50 μL) containing 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes), pH 7.5, 10 mM $MgCl_2$, 0.25 mM DTT, 1 mM ATP, and 0.04 mM $^{32}PP_i$ (50 cpm/pmol). This assay is a modification of that of Silber et al. (1972). The specific activity of the enzyme used in this study was 38 400 U/mg. Acid precipitable protein was determined using lysozyme as a standard (Lowry et al., 1951).

Paper Chromatography of Nucleotides and Oligonucleotides. The following solvents were used to develop paper chromatograms: (I) 1.89 g of NH_4HCO_2 and 1.23 mL of HCO_2H per 100 mL; (II) 1-C_3H_7OH: concentrated NH_3: water (55:10:35); and (III) 100 mL of 0.1 M Na_2HPO_4, pH 6.8, to which is added 60 g of $(NH_4)_2SO_4$ and 2.3 mL of 1-C_3H_7OH. Whatman 3 MM paper was prepared by washing with 10% CH_3CO_2H for 24 h, solvent II for 24 h, and finally water for 24 h.

Synthesis of Nucleotides. Deoxyribonucleoside bisphosphates were prepared from the appropriate 2′-deoxyribonucleoside and pyrophosphoryl chloride (Barrio et al., 1978). The products were purified by chromatography on columns of DEAE-Sephadex A-25 (bicarbonate) by elution with linear gradients of triethylammonium bicarbonate, pH 7.5. The fractions containing pdNp were pooled, dried by evaporation under vacuum at less than 35 °C, and desalted through repeated solution and evaporation of spectral grade methanol. The sodium salts were formed by precipitation from methanol with NaI in acetone. The precipitates were washed extensively with acetone followed by diethyl ether and were dried under vacuum over P_2O_5.

[5′-^{32}P]pdTp and [5′-^{32}P]pdCp were synthesized in a mixture (50 μL) containing 50 mM Tris-HCl, pH 7.5, 50 μg/mL bovine serum albumin (BSA), 10 mM dithiothreitol (DTT), 10 mM $MgCl_2$, 1.0 mM dTp or dCp, 10 μM [γ-^{32}P]ATP (2.2 × 10^4 cpm/pmol), and 60 U/mL *PseT* 1 polynucleotide kinase. The reaction mixture was incubated at 37 °C for 30 min and the products were purified by elution at 7.5 mL/h from a DEAE-Sephadex A-25 (acetate) column (0.9 × 13.5 cm) with a 150 mL gradient of 0.04–1.0 M triethylammonium acetate (pH 5.8 for pdTp, and pH 4.7 for pdCp). The fractions containing pdNp were pooled and dried by evaporation. The concentrate was desalted by application to Whatman 3 MM paper, drying, and repeated washing with absolute ethanol. The product was eluted with water and a final yield of 90% was obtained. Ado-5′PP5′-dThd was prepared and purified as previously described (England et al., 1977).

Preparation of Oligodeoxyribonucleotides. Acceptor 3′,5′-dihydroxyl oligodeoxyribonucleotides dN(pdN)$_n$ were prepared from their 3′-hydroxyl 5′-phosphate homologues by incubation at 65 °C for 4 h in a solution (200 μL per 0.1 μmol of substrate) containing 100 mM Tris-HCl, pH 8.0, 50 mM $MgCl_2$, and 63 U/mL BAP F. All 3′,5′-dihydroxyl oligodeoxyribonucleotides were purified by chromatography on Whatman 3 MM paper in solvent II and desalted as described for the [5′-^{32}P]pdNp's. Nucleotide concentrations were determined by UV spectrophotometry using reported extinction coefficients (Janik, 1971).

The ^{32}P-labeled acceptor dT(pdT)$_3$[3′→5′-^{32}P]pdT was synthesized in a 10-μL reaction mixture containing 50 mM Hepes, pH 8.3, 10 μg/mL BSA, 20 mM DTT, 10 mM $MnCl_2$, 2.0 mM dT(pdT)$_3$, 250 μM [5′-^{32}P]pdTp (10^3 cpm/pmol), 500 μM ATP, and 42 μM RNA ligase. The mixture was incubated at 17 °C for 168 h, and the products were purified by chromatography on Whatman 3 MM in solvent II followed by elution from a Sephadex G-50 fine column (0.9 × 100 cm) with 50 mM triethylammonium bicarbonate, pH 7.5. The dT(pdT)$_3$[3′→5′-^{32}P]pdTp product peak was pooled and concentrated by evaporation. The dihydroxyl acceptor was purified and desalted after treatment with BAP F as described.

Assay of the Joining Reaction. Reactions (10 μL) were performed in a mixture containing 50 mM Hepes, pH 8.3, 10 mM $MnCl_2$, 20 mM DTT, 10 μg/mL BSA, ATP, the donor pdNp, the acceptor dN(pdN)$_n$, and RNA ligase. The exact concentrations of the nucleotide components and enzyme are given in the legends. The reaction tubes were incubated at 17 °C and 2-μL aliquots removed for assay. Samples not treated with BAP were applied directly to DE 81 paper and developed for 6 h in solvent I. Samples to be treated with BAP were added to 5 μL of a solution containing 0.97 M Tris, pH 8.7, 100 mM NaCl, and 10 mM $MgCl_2$, boiled for 2 min, and added to 2 μL of BAP C at 1 mg/mL in the same buffer. After incubation for 1 h at 65 °C, the entire mixture was spotted on DE 81 paper and developed for 6 h in solvent I. The chromatograms were scanned for radioactivity in a Packard 701 B strip scanner. Radioactivity in product peaks and backgrounds were quantitated using a scintillation counter. The yields of product were calculated as the ratio of radioactivity in the product peak to the total radioactivity and were based upon the limiting substrate. Because of the low solubility of manganese phosphate, radioactivity precipitated during the course of the BAP reaction and the amount was determined by dissolving the precipitate in concentrated HCl. This material was identified as P_i by its inability to adsorb to charcoal and the amount of radioactivity it represented was included in the total when yields were calculated.

Product Characterization. Nearest neighbor analyses of the joined deoxyoligomers were performed as described (Wells et al., 1967) upon material isolated from DE 81 chromatograms by elution with 2 M NH_4HCO_3 and removal of the salt as described for the [5′-^{32}P]pdNp's. The nucleoside monophos-

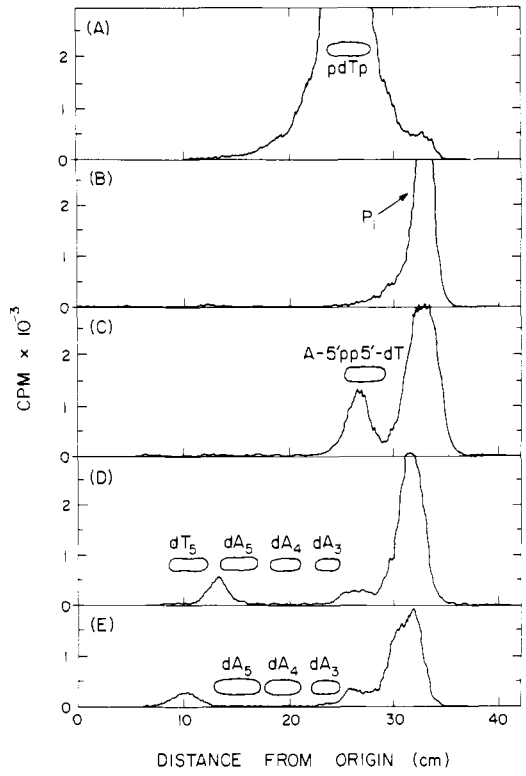

FIGURE 1: Ion-exchange paper chromatography of the single nucleotide addition reaction. The reaction of 2.0 mM [5′-^{32}P]pdTp (10^4 cpm/nmol), 250 μM dA(pdA)$_3$, and 500 μM ATP with 42 μM RNA ligase was incubated at 17 °C for the indicated times and assayed as described in Materials and Methods. (A) Control of [5′-^{32}P]pdTp before incubation; (B) as in A but with BAP digestion; (C) control reaction in which acceptor dA(pdA)$_3$ was omitted (24 h); (D) complete reaction (144 h); and (E) complete reaction using the acceptor dA(pdA)$_4$ in place of dA(pdA)$_3$.

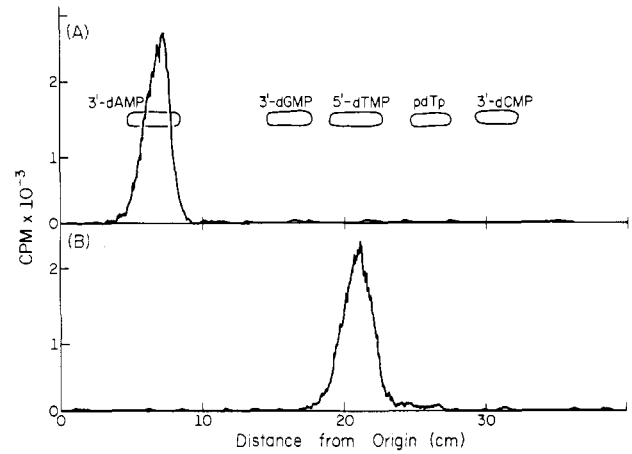

FIGURE 2: Nearest neighbor analysis of product isolated from the chromatogram shown in Figure 1E. Analyses were performed as described in Materials and Methods. (A) Product after incubation with spleen phosphodiesterase and (B) product after incubation with snake venom phosphodiesterase.

phates were isolated after adsorption and elution from charcoal as described (Greenfield et al., 1975) and separated by paper chromatography on Whatman no. 1 in solvent III.

Results

Single Addition Reaction and Product Characterization. RNA ligase catalyzed the addition of a single 3′,5′-deoxyribonucleoside bisphosphate donor to the 3′-hydroxyl of a 3′,5′-dihydroxyl acceptor oligodeoxynucleotide through a 3′ to 5′ phosphodiester bond. The ion-exchange chromatograms used to assay the joining reaction are shown in Figure 1. Chromatography of [5′-^{32}P]pdTp alone showed that >95% of the radioactivity comigrated with authentic pdTp and the remainder with P_i (Figure 1A). After a 1-h incubation with BAP at 65 °C, all of the radioactivity was converted to P_i (Figure 1B). These controls indicate the absence of any contaminating radioactivity which migrates as a species of greater molecular weight than the donor. Incubation of [5′-^{32}P]pdTp and ATP with RNA ligase, in the absence of dA(pdA)$_3$, followed by BAP digestion, yielded 17% of the radioactivity (68% conversion of ATP) comigrating with authentic Ado-5′-PP5′-dThd and the remainder as P_i (Figure 1C). This result indicates that, in the absence of acceptor, pdTp reacts with ATP to yield the expected adenylylated reaction intermediate (Kaufmann & Kallenbach, 1975; Sninsky et al., 1976; Ohtsuka et al., 1976; Sugino et al., 1977). Incubation of a complete reaction mixture containing [5′-^{32}P]pdTp, ATP, and dA(pdA)$_3$ with RNA ligase followed by BAP digestion resulted in a new peak of radioactivity (Figure 1D) equivalent to 7.7% of the radioactivity transferred to the acceptor, dA(pdA)$_3$. Since the donor to acceptor concentration ratio was 8:1, this transfer represents a 62% conversion of the acceptor to product. The presence of a product with BAP resistant radioactivity migrating more slowly than dA(pdA)$_3$ on ion-exchange paper suggests that a larger product containing [5′-^{32}P]pdT has been formed. Incubation of a complete reaction mixture containing an acceptor one nucleotide residue longer, dA(pdA)$_4$, yielded a single product peak (60% yield) which migrated more slowly than both dA(pdA)$_4$ and the product of the reaction with dA(pdA)$_3$ (Figure 1E). This chromatography system also demonstrated the near absence of DNase activity in the enzyme preparation. Only one product peak was observed for a given acceptor in this system which is capable of separating oligodeoxyribonucleotides differing by single residues in length (Figures 1D and 1E).

The location of the ^{32}P label in the product peak was determined by isolation of the material from the chromatogram shown in Figure 1E followed by enzymatic digestion. Treatment with spleen phosphodiesterase alone or in conjunction with micrococcal nuclease, an exonuclease and an endonuclease, respectively, which cleave to give 3′-NMP's, resulted in all of the radioactivity comigrating with 3′-dAMP during paper chromatography (Figure 2A). Treatment with snake venom phosphodiesterase alone or in combination with pancreatic DNase I, an exonuclease and endonuclease, respectively, which yield 5′-dNMP's, resulted in all of the radioactivity comigrating with 5′-dTMP (Figure 2B). This nearest neighbor analysis uniquely locates the ^{32}P label between an A and a T residue. In addition, the requirements of spleen phosphodiesterase and snake venom phosphodiesterase for terminal 5′- and 3′-hydroxyls, respectively, indicate that the product, which had been treated with BAP, contained these termini. The isolated product of the pdTp plus dA(pdA)$_4$ reaction was also resistant to alkaline hydrolysis (0.2 M KOH at 37 °C for 18 h), thus eliminating the possibility that the pdTp had added to a ribose hydroxyl of either ATP or to an oligoribonucleotide contaminant in the acceptor. Taken together, these results are all consistent with the structure of the product in Figure 1E being dA(pdA)$_4$[3′→5′-^{32}P]pdT. In addition, nearest neighbor analyses using spleen phosphodiesterase plus micrococcal nuclease and snake venom phosphodiesterase plus pancreatic DNase I were done on the isolated products of the following RNA ligase reactions which will be subsequently presented: [5′-^{32}P]pdTp plus (1) dA(pdA)$_4$, (2) dT(pdT)$_3$, (3) dT(pdT)$_7$dG, or (4) dT-

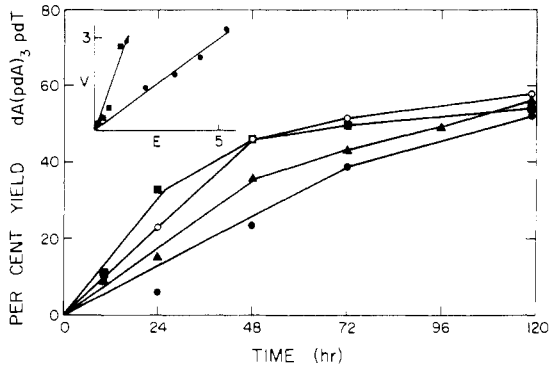

FIGURE 3: Kinetics of product formation for the reaction of 2.0 mM [5′-^{32}P]pdTp, 250 μM dA(pdA)$_3$, and 500 μM ATP. RNA ligase concentration: 21 μM (●), 32 μM (▲), 42 μM (○), and 53 μM (■). Reactions were assayed as described in Materials and Methods and the percent yield was calculated from the percent dA(pdA)$_3$ converted to product. The insert shows the initial velocities (pmol h^{-1} × 10^{-1}) of the reaction of either [5′-^{32}P]pdTp (●) or [5′-^{32}P]pdCp (■) as a function of RNA ligase concentration (μM × 10^{-1}).

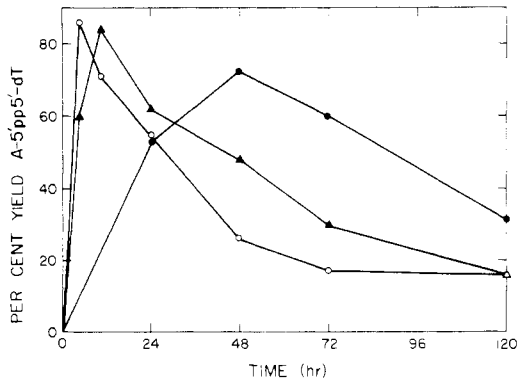

FIGURE 4: Kinetics of intermediate formation for the reaction of 2.0 mM [5′-^{32}P]pdTp, 250 μM dA(pdA)$_3$, and 500 μM ATP. The adenylylated intermediate derivative, A-5′pp5′-dT, was determined as described in Materials and Methods and the text and the percent yield calculated from the percent ATP converted to intermediate. RNA ligase concentration: 21 μM (●), 32 μM (▲), and 42 μM (○).

(pdT)$_7$(pdC)$_2$; and [5′-^{32}P]pdCp plus (1) dA(pdA)$_4$, (2) dT(pdT)$_3$, (3) dT(pdT)$_7$dG, or (4) dT(pdT)$_7$(pdC)$_2$. In each case the results clearly indicated the formation of the expected internucleotide linkage.

Reaction Kinetics. The kinetics of the reaction between [5′-^{32}P]pdTp and dA(pdA)$_3$ and [5′-^{32}P]pdCp and dA(pdA)$_4$ were examined at four enzyme concentrations and the results with pdTp are shown in Figure 3. Although the initial velocities of product formation were proportional to enzyme concentrations for either pdTp or pdCp (Figure 3, insert), the velocity of the reaction with pdCp was approximately 3.5 times that of the one with pdTp at 21 μM enzyme. Under these conditions, all enzyme concentrations with the pdTp donor gave similar yields of approximately 60% at 120 h. The reaction containing pdCp yielded 80% product at the same enzyme concentration after 48 h. These velocities and yields are not the maximum obtainable but rather represent the results of the specific conditions used.

Because the ion-exchange chromatograms used in Figure 1 resolved the dephosphorylated product from the reaction intermediate derivative Ado-5′PP5′-dThd, the rate of formation of the latter could also be followed. Increasing the enzyme concentration from 21 to 42 μM increased both the rate of formation of the intermediate as well as its turnover into product (Figure 4). At 42 μM RNA ligase a yield of 83% adenylylated donor was observed in 4.5 h. At this time less than 5% product deoxyoligomer, equivalent to 0.13 nmol, had been formed (Figure 3). The formation of this amount of intermediate required 4.2 nmol of the total of 5.0 nmol of ATP. After 120 h of reaction the adenylylated intermediate was reduced to 0.83 nmol (20% of its 4.5 h value) and 58% of the possible product has formed. The kinetics and stoichiometry of the reaction reveal that not all of the adenylylated intermediate, which in principle could react, formed product. At 42 μM enzyme, 3.4 nmol of intermediate was turned over after 120 h, while only 1.5 nmol (of a possible 2.5 nmol) of product was formed. Since a maximum of only 0.42 nmol of ATP could be sequestered as adenylylated RNA ligase at this enzyme concentration, it is clear that most of the Ado-5′PP5′-dThd-3′P formed was turned over by a mechanism other than product formation. Either reversal of its formation or a hydrolytic activity could be responsible for this result. We are currently investigating the intermediate turnover.

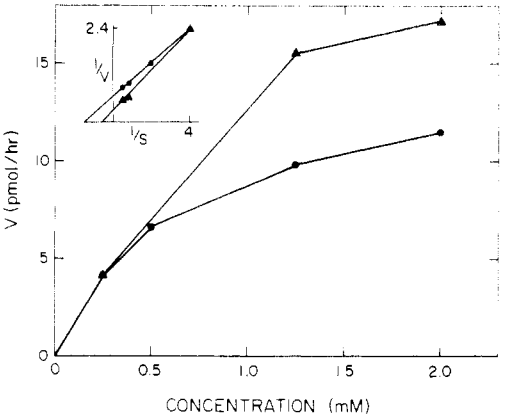

FIGURE 5: Initial velocities of the reaction of [5′-^{32}P]pdTp and dA(pdA)$_4$ as a function of substrate concentrations. Each reaction contained 250 μM ATP and 21 μM RNA ligase and was analyzed as described in Materials and Methods. Constant dA(pdA)$_4$ (250 μM) and variable pdTp (●). Constant pdTp (250 μM) and variable dA(pdA)$_4$ (▲). The insert shows a Lineweaver-Burk plot of the data in h pmol^{-1} × 10^1 vs. mM^{-1}.

Dependence of Rate on Donor and Acceptor Concentrations. The rate of product formation in the reactions of pdTp or pdCp with dA(pdA)$_4$ showed a dependence on both donor and acceptor concentrations (Figure 5). In the pdTp reaction, an eightfold increase in the pdTp concentration from 0.25 to 2.0 mM tripled the initial velocity, while the same acceptor dA(pdA)$_4$ concentration increase quadrupled the rate. Using these data in a Lineweaver-Burk plot, apparent K_m's of ca. 0.7 and 2.1 mM were calculated for pdTp and dA(pdA)$_4$, respectively (Figure 5, insert). Turnover numbers were determined under these nonsaturating cosubstrate conditions assuming a single active site per polypeptide of M_r = 43 000. Values of 0.2 and 0.07 h^{-1} were calculated for dA(pdA)$_4$ and pdTp, respectively. Similar calculations with data from the pdCp plus dA(pdA)$_4$ reaction yielded apparent K_m's and turnover numbers of 1.8 mM (0.6 h^{-1}) and 1.1 mM (0.4 h^{-1}) for dA(pdA)$_4$ and pdCp, respectively, at 11 μM RNA ligase concentration. These extremely low turnover numbers account for the necessity of long incubation times for this reaction to attain high yields.

The [5′-^{32}P]pdTp donor, dA(pdA)$_3$ acceptor, and ATP concentration were also varied together keeping the concentration ratio constant at 8:1:2, respectively, with 32 μM enzyme. Reactions at 40 μM donor showed no product and 23% intermediate after 192 h. Increasing the donor concentrations to 400 μM, 2.0 mM, or 10 mM resulted in initial velocities of 1.2, 24, and 56 pmol/h and 196-h product yields of 22%, 65%,

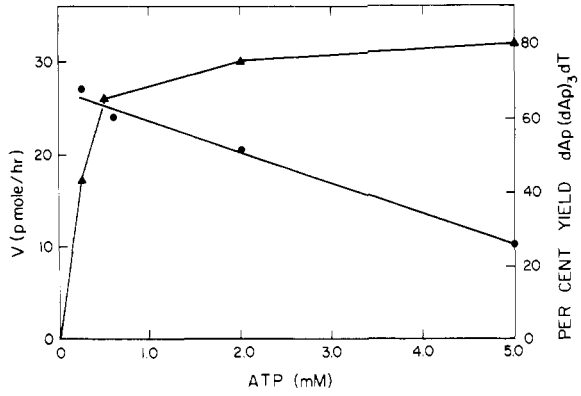

FIGURE 6: Effect of ATP concentration on the initial velocity (●) and yield (▲) of the single nucleotide addition reaction. [5′-^{32}P]pdTp (2.0 mM) and 250 μM dA(pdA)$_3$ were incubated at 17 °C with 42 μM RNA ligase and the reactions assayed as described in Materials and Methods.

TABLE I: Effect of Acceptor Chain Length and Composition upon Initial Velocity.[a]

acceptor	pdTp V_i (pmol/h)	pdCp V_i (pmol/h)
dApdA	0 (42)[b]	0 (11)[b]
dA(pdA)$_2$	10 (32)	22 (11)
dA(pdA)$_3$	18 (32)	23 (11)
dA(pdA)$_4$	15 (32)	29 (11)
dA(pdA)$_{11}$	11 (32)	11 (11)
dA(pdA)$_3$	24 (42)	23 (11)
dT(pdT)$_3$	15 (42)	22 (11)
dT(pdT)$_7$(pdC)$_2$	45 (42)	73 (11)
dT(pdT)$_7$pdG	15 (42)	22 (11)
dU(pdU)$_3$	33 (42)	21 (11)

[a] Each reaction contained 2.0 mM [5′-^{32}P]pdNp, 250 μM dN(pdN)$_n$, and 500 μM ATP and was analyzed as described in Materials and Methods. Points from the linear portion of product versus time curves were used for initial velocity determinations. [b] The numbers in parentheses represent RNA ligase concentrations (μM).

and 75%, respectively. Similar results were obtained with [5′-^{32}P]pdCp and dA(pdA)$_4$. Taken together, these results indicate that increased yields and increased initial rates can be obtained by increasing the donor and acceptor concentrations.

Other Reaction Variables. Low incubation temperatures and the replacement of Mn(II) for Mg(II) both enhanced the single nucleotide addition reaction with DNA substrates. Figure 1D shows the chromatographic analysis of a typical reaction mixture incubated at 17 °C which yielded 62% product in 144 h. When these substrates at the same concentrations were incubated at 37 °C, no product was observed, although a yield of greater than 80% of the adenylylated donor was formed in 24 h. In addition, preliminary experiments showed that low temperature reactions using Mn(II) rather than Mg(II) resulted in a two-fold increase in the initial velocity. We have obtained similar results in the RNA ligase reaction with single-strand deoxyoligomers (McCoy & Gumport, unpublished results).

The ATP concentration was varied in a series of reactions between 2.0 mM [5′-^{32}]pdTp and 0.25 mM dA(pdA)$_3$ using 32 μM enzyme to determine its effect on the reaction (Figure 6). Although the initial velocities of these reactions decreased linearly as the ATP concentration was increased from 0.25 to 5.0 mM, the yields increased from 40 to 80%. These results

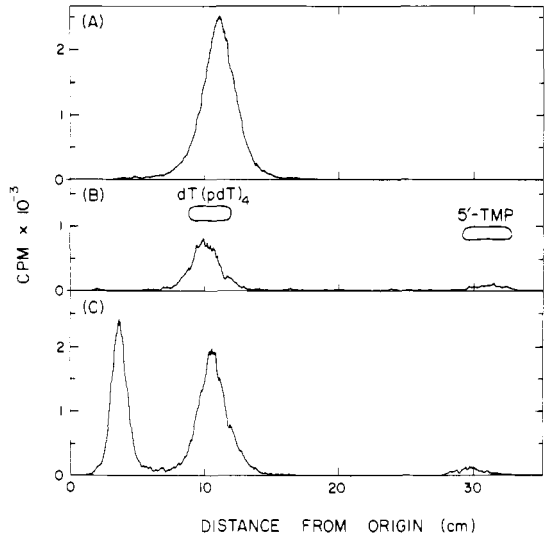

FIGURE 7: Ion-exchange paper chromatography of the single nucleotide addition reaction with dT(pdT)$_3$[3′→5′-^{32}P]pdT (250 μM) and pdUp (2.0 mM). The reaction contained 500 μM ATP and 42 μM enzyme and was incubated at 17 °C for the indicated times. The reaction mixture was assayed as described in Materials and Methods. (A) dT(pdT)$_3$[3′→5′-^{32}P]pdT before incubation; (B) control reaction in which donor was omitted (144 h); and (C) complete reaction (144 h).

show a complex dependence of the reaction upon the ATP concentration and indicate the need to consider these opposing effects when attempting to optimize the reaction for synthetic purposes.

Effects of Acceptor Chain Length and Composition. A series of dA(pdA)$_n$ acceptors was tested to determine the effect of chain length on the reaction (Table I). Although no product was observed after 120 h with the acceptor dApdA and either pdTp or pdCp, the acceptors containing 3, 4, 5, and 12 deoxyadenosine residues reacted with both donors. For each particular donor, the initial velocities in this series varied by no more than 2.5-fold and gave 60–80% yields at 144 h.

Acceptors containing different 3′-terminal deoxyribonucleosides were similarly tested to examine base composition effects upon the reaction (Table I). The series containing 3′-terminal dA, dG, dT, and dU showed only slight variations in initial velocities for a given donor. The dC terminated deoxyoligomer reacted with both pdTp and pdCp at substantially increased initial rates with respect to the other acceptors. The yields obtained with the acceptors shown in Table I varied in the following manner: dU (40–50%) < dA, dG and dT (60–70%) < dC (80–100%). These results indicate that DNA acceptors of chain length greater than 3 and terminated with any common deoxynucleotide can be lengthened successfully with RNA ligase.

Survey of Donors. Bisphosphate donors of dT, dC, dA, dU, and dG were tested with the labeled acceptor dT(pdT)$_3$-[3′→5′-^{32}P]pdT at 42 μM enzyme. A representative reaction with pdUp is shown in Figure 7. Chromatography of the labeled acceptor alone (Figure 7A) and after incubation with RNA ligase (Figure 7B) showed that 93% of the radioactivity comigrated with the marker dT(pdT)$_4$ after 6 days of incubation. The remaining 7% comigrated with 5′-dTMP. The addition of pdUp to the reaction resulted in a 41% yield of the product dT(pdT)$_3$[3′→5′-^{32}P]pdTpdUp after a 6-day incubation with less than 3% of the total radioactivity comigrating with 5′-dTMP (Figure 7C). These results further show the near absence of DNase activity in the RNA ligase preparation. In addition, the lesser degradation in the complete reaction mixture with respect to that lacking donor indicates that

the 3′-phosphate terminated product is more resistant than the unmodified acceptor to the 3′-exonuclease contaminant.

The bisphosphorylated dA, dG, dT, and dU donors gave similar initial velocities of 14, 7, 8, and 16 pmol/h, respectively, while pdCp showed a markedly higher rate of 64 pmol/h. Yields at 144 h were 60, 41, 41, and 63% for the dA, dT, dU, and dC donors, respectively. The donor pdGp gave a 20% yield under these conditions. Increasing the enzyme and ATP concentrations to 50 μM and 1.0 mM, respectively, increased the pdGp yield to 40% in 120 h. These results demonstrate that any of the common deoxynucleoside 3′,5′-bisphosphates can serve as donors in the RNA ligase reaction.

Discussion

Although RNA ligase readily catalyzes the single ribonucleotide addition to oligoribonucleotides (England & Uhlenbeck, 1978; Kikuchi et al., 1978), our results show that the addition of a deoxyribonucleotide to DNA acceptors occurs much less efficiently and requires higher enzyme concentrations and longer reaction times. It is useful to discuss the DNA joining reaction in terms of its probable mechanism. RNA ligase reacts with ATP to form a covalent enzyme–AMP intermediate with the release of PP_i (Cranston et al., 1974). The adenylyl group is subsequently transferred to the 5′-phosphate of the donor deoxyribonucleoside 3′,5′-bisphosphate to form a 5′→5′-phosphoanhydride linkage (Kaufmann & Littauer, 1974; Sninsky et al., 1976; Ohtsuka et al., 1976; Sugino et al., 1977). The formation of the phosphodiester bond in the product presumably occurs through the nucleophilic attack of the 3′-hydroxyl group of the acceptor oligodeoxyribonucleotide upon the activated 5′-phosphate of the donor with the release of AMP. The three steps may be symbolized as follows:

$$\text{pppA} + \text{E} \rightleftharpoons \text{EpA} + \text{PP}_i \quad (1)$$

$$\text{EpA} + \text{pdNp} \rightleftharpoons \text{A-5′pp5′-dNp} + \text{E} \quad (2)$$

$$\text{dN(pdN)}_n + \text{A-5′pp5′-dNp} \xrightarrow{\text{E}} \text{dN(pdN)}_n\text{pdNp} + \text{pA} \quad (3)$$

England & Uhlenbeck have shown that, although pdNp donors are with the exception of pdCp, poorer substrates than their prNp counterparts, either series can be added in good yields to oligoribonucleotides by <5 μM RNA ligase at 37 °C in 1 h (England & Uhlenbeck, 1978). We find that neither prCp nor pdTp adds to $dA(pdA)_4$ under these same conditions even though the adenylylated deoxyribonucleoside bisphosphate intermediate is rapidly formed. These results indicate that the first steps of the reaction involving enzyme, ATP, and deoxyribonucleotide donor readily occur and that the failure to form a phosphodiester bond resides in the final step involving the 3′-hydroxyl of the DNA acceptor. The same conclusion has been reached in the study of single strand deoxyoligomer joining by RNA ligase (Sugino et al., 1977). We find that decreased reaction temperatures promote the reactions of DNA acceptors as has been reported for poor RNA acceptors (England & Uhlenbeck, 1978). This effect may be due to the stabilization of a reactive Michaelis complex between the reactants and the enzyme in the final step of the mechanism. In addition, we find that the substitution of manganous ion for Mg(II) increases the rate of reaction of DNA acceptors. It has been shown that Mn(II) decreases the nucleoside triphosphate specificities of both RNA (Hurwitz et al., 1973) and DNA (Berg et al., 1963) polymerases and a similar effect may be operating with RNA ligase. In spite of the rate-enhancing conditions we have determined, DNA remains a poorer substrate for RNA ligase than RNA. High yields with the worst RNA acceptors can generally be obtained at the lower incubation temperatures in 24 h with <5 μM enzyme (Uhlenbeck, personal communication), whereas practical yields of DNA products require 10–50 μM enzyme and from 2 to 7 days of reaction.

The effects of ATP concentration upon the rate and yield of the reaction can be rationalized in terms of the mechanism. Since by analogy to DNA ligase, free enzyme is probably required for the third step in the reaction (Harvey et al., 1971), high ATP levels lower its rate by sequestering the enzyme in the adenylylated form. However, in the presence of an excess of pdNp over ATP, free enzyme is regenerated by the rapid formation of the adenylylated deoxyribonucleoside bisphosphate intermediate in step 2 (Figure 4). High levels of ATP, therefore, decrease the rate of reaction by lowering the concentration of free enzyme but increase the yield by maintaining a high concentration of the activated donor required in step 3. A ratio of deoxyoligomer to ATP to pdNp of 1:2:8 helps to balance these competing ATP effects.

The single nucleotide addition of RNA ligase with DNA substrates presented here provides a basis for the stepwise synthesis of DNA and for the modification of DNA 3′ termini. The equilibrium constant of the reaction is large and a unique monoextended product bearing a 3′-phosphate is produced. Enzymatic dephosphorylation can be followed by another single nucleotide addition or alternatively a 3′,5′-bisphosphate donor can be prepared by reaction of the product with ATP and polynucleotide kinase. This donor can then be reacted with a dihydroxyl acceptor of three or more nucleotides to extend it in the 5′ direction (McCoy & Gumport, unpublished results). Thus, DNA chains may be synthesized in either direction without a template strand by the block addition of deoxyoligomers as small as trimers and also in the 3′ direction by the addition of pdNp's. Polynucleotide phosphorylase (Gillam et al., 1978) and terminal deoxynucleotidyl transferase (Chang & Bollum, 1971) have been used to synthesize oligodeoxyribonucleotides but these methods suffer from the disadvantages of producing a distribution of addition products and requiring specialized reaction conditions depending upon the particular nucleotides added. RNA ligase gives a single extended product and, although there are differences in substrate efficiencies, yields of 40–95% have been obtained under a single set of reaction conditions by simple variation of the enzyme and ATP concentrations.

Because we have determined reaction conditions which allow DNA to serve as an acceptor, we believe that RNA ligase can probably use DNA substrates in all of the reactions described for RNA acceptors. Compounds of the general structure Ado-5′PP-X should donate their nonadenylyl portions (England et al., 1977) to DNA and base-modified deoxynucleosides (Barrio et al., 1978) should also serve as either donors or acceptors. Preliminary results of joining DNA restriction fragments to pdNp and $rN(prN)_n$ (D. Hinton & R. I. Gumport, unpublished results) indicate that long strands of DNA also serve as either donors or acceptors. In addition, synthesis of the oligonucleotides $rA(prA)_2prC(pdT)_2$ and $dA(pdA)_4prC$ from the reactions of $rA(prA)_2prC$ with pdTp and $dA(pdA)_4$ with prCp, respectively, demonstrates that the synthesis of oligonucleotides containing both deoxy and ribonucleotides in any order is possible using RNA ligase (unpublished results). In summary, the ability of RNA ligase to use DNA acceptors suggests that this enzyme will be useful for the synthesis of DNA of defined sequence as well as for the modification of the 3′ termini of DNA with a wide variety of compounds.

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