T4 Ribonucleic Acid Ligase Joins Single-Strand Oligo(deoxyribonucleotides)†

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ABSTRACT: T4 RNA ligase joins a 3′-hydroxyl-terminated acceptor oligoribonucleotide to a 5′-phosphate-terminated donor oligoribonucleotide. An analogous reaction with single-strand DNA oligonucleotides would be useful for the synthesis of defined sequences of DNA because it would eliminate the need to synthesize complementary sequences to form the duplex substrates required by DNA ligase. We have studied the model reaction

$$dA(pdA)_5 + [5'\text{-}^{32}P](pdT)_4pdCp \rightarrow dA(pdA)_5[3'\rightarrow 5'\text{-}^{32}P]pdT(pdT)_3pdCp$$

and have obtained 40–60% yields at equimolar concentrations (100 μM to 1 mM) of the two substrates. Higher yields have been obtained when acceptor concentrations in excess of those of the donor are used. The use of a 5′-hydroxyl, 3′-hydroxyl terminated acceptor and a 5′-phosphate, 3′-phosphate terminated donor limits the reaction to a unique product. The 3′-phosphate-terminated donor was prepared by using RNA ligase to add a single deoxyribonucleoside 3′,5′-bisphosphate donor to an oligo(deoxyribonucleotide) acceptor [Hinton, D. M., Baez, J. A., & Gumport, R. I. (1978) *Biochemistry 17*, 5091]. The DNA oligomer joining reaction requires low concentrations of ATP and an ATP regenerating system, Mn^{2+}, high levels of nuclease-free RNA ligase (30 μM), and incubation for several days at 17 °C. The product of the reaction was characterized by its resistance to alkaline phosphatase, degradation by micrococcal nuclease to the expected product [3′-^{32}P]dAMP, and mobility during high-pressure liquid chromatography on RPC-5. The joining of several other deoxyoligomers was also demonstrated. We anticipate that this reaction of RNA ligase will contribute to its usefulness as a reagent for the synthesis of DNA of defined sequence.

T4 RNA ligase catalyzes the formation of a phosphodiester bond between a 5′-phosphate of a donor oligoribonucleotide and a 3′-hydroxyl group of an acceptor oligoribonucleotide (Silber et al., 1972). The reaction requires ATP and may be either intramolecular (Silber et al., 1972) or intermolecular (Walker et al., 1975; Kaufmann & Kallenbach, 1975). The intermolecular reaction can be limited to the formation of a unique product by the use of appropriately protected RNA substrates and has, therefore, become an important tool in the synthesis of oligoribonucleotides of defined sequences (Sninsky et al., 1976; Ohtsuka et al., 1976, 1978; Uhlenbeck & Cameron, 1977; Kikuchi & Sakaguchi, 1978; Meyhack et al., 1978).

We have been examining RNA ligase with the objective of developing it to a point of similar utility with DNA substrates. We found that a single 2′-deoxynucleoside 3′,5′-bisphosphate donor could be added to an oligo(deoxyribonucleotide) in high yields, thereby providing an enzymatic method for the stepwise synthesis of deoxyoligomers (Hinton et al., 1978; Hinton & Gumport, 1979). The single deoxyribonucleotide addition reaction is the analogue of that described for RNA substrates using RNA ligase (England & Uhlenbeck, 1978; Kikuchi et al., 1978).

This report describes the development of reaction conditions which allow donor oligo(deoxyribonucleotides) to join to deoxyoligomer acceptors. Single-strand oligo(deoxyribonucleotides) have previously been joined with RNA ligase but in low yields (Sugino et al., 1977). We will describe conditions which allow yields as high as 60% with equimolar concentrations of acceptors and donors. We anticipate that the ability of RNA ligase to join single-strand oligo(deoxyribonucleotides) will be useful for the enzymatic synthesis of DNA of defined sequence because it obviates the synthesis of a template strand to properly abut the ends of the oligomers for the subsequent joining step with DNA ligase.

Materials and Methods

Materials. The oligonucleotides[1] $(pdA)_2$, $(pdA)_3$, $(pdA)_4$, $(pdA)_6$, $(pdA)_8$, $(pdA)_{12}$, $(pdT)_8$, and $(pdT)_8pdG$ were purchased from P-L Biochemicals, Inc.; $rA(prA)_3$ and $(pdC)_6$ were obtained from Collaborative Research; $dT(pdT)_3$ was from Ash Stevens, Inc., Detroit, MI. Phosphocreatine, spermine, ATP, creatine kinase (Type 1), and myokinase (Grade V) were purchased from Sigma Chemical Co. Myokinase was dialyzed in 50 mM Hepes, pH 8.0, 1 mM DTT, and 10 mM NaCl and stored in 50% glycerol at −20 °C. Bacterial alkaline phosphatase C (BAP C), BAP F, micrococcal nuclease, spleen phosphodiesterase, pancreatic DNase I, and snake venom phosphodiesterase were obtained from Worthington Biochemical Corp. Polynucleotide kinase lacking 3′-phosphatase activity was isolated from *Escherichia coli* infected with *PseT* 1 T4 (Cameron et al., 1978). [γ-^{32}P]ATP was prepared by the method of Glynn & Chapell (1964), and pdCp was prepared from dC and pyrophosphoryl chloride (Barrio et al., 1978) and purified as previously described (Hinton et al., 1978). RPC-5 chromatography media was a gift of N. J. Leonard, and Whatman No. 1 and 3MM papers were obtained from Whatman, Inc.

Purification of RNA Ligase. DNase-free RNA ligase was

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[1] Abbreviations following the IUPAC–IUB Commission on Biochemical Nomenclature (1971, 1977) recommendations are used throughout. One letter abbreviations for oligonucleotides will be used. For example, $dA(pdA)_3$ and dA_4 both represent d(A-A-A-A) while $(pdA)_3pdAp$ and pdA_3dAp represent d(pA-A-A-Ap). Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BAP, bacterial alkaline phosphatase; DTT, dithiothreitol; Me_2SO, dimethyl sulfoxide.

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purified from T4-infected *E. coli* as described (McCoy et al., 1979). The enzyme used in this study was greater than 90% pure as judged by electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate. Enzyme concentration is expressed as moles per liter by using a value of M_r 43 000 (McCoy et al., 1979) with acid-precipitable protein being determined by the method of Lowry et al. (1951) using lysozyme as a standard (Aune & Tanford, 1969).

Paper Chromatography of Oligonucleotides. Descending paper chromatography was carried out by using the following solvents: (I) 1-C_3H_7OH–concentrated NH_3–water (55:10:35); (II) 1 M $NH_4CH_3CO_2$–95% C_2H_5OH (70:30); (III) 1 M $NH_4CH_3CO_2$–95% C_2H_5OH (30:75). Whatman 3MM paper was prepared as described (Hinton et al., 1978).

Preparation of Oligo(deoxyribonucleotides). Most acceptor oligonucleotides terminated with 5′-hydroxyl groups were prepared from their 5′-phosphate homologues by treatment with BAP (Hinton et al., 1978).

The 3′,5′-bisphosphate donor $(pdT)_4pdCp$ was prepared in two steps by using the single-addition reaction of RNA ligase with $dT(pdT)_3$ and pdCp (Hinton et al., 1978) followed by treatment with ATP and polynucleotide kinase. The RNA ligase reaction (10 μL) contained 50 mM Hepes, pH 8.3, 10 mM $MnCl_2$, 20 mM DTT, 25 μg/mL BSA, 10% Me_2SO, 2.0 mM $dT(pdT)_3$, 5.0 mM pdCp, 0.5 mM ATP, 5 mM phosphocreatine, 120 units/mL creatine phosphokinase, 170 units/mL myokinase, and 21 μM RNA ligase and was incubated at 17 °C for 4 days. The oligonucleotide product $(dTp)_4dCp$ was separated from pdCp by gel filtration through a Sephadex G-50 (fine) column (1.0 × 100 cm) in 50 mM triethylammonium bicarbonate (TEABC), pH 7.5, and from $dT(pdT)_3$ by subsequent chromatography on Whatman 3MM paper in solvent I. After elution from the paper with H_2O and concentration by evaporation under vacuum at below 30 °C, the oligonucleotide (2.0 mM) was incubated at 37 °C for 30 min in a mixture (50 μL) containing 20 mM ATP, 60 units/mL *PseT* 1 polynucleotide kinase, 50 mM Tris-HCl, pH 7.5, 50 μg/mL BSA, 10 mM DTT, and 10 mM $MgCl_2$. The product, $(pdT)_4pdCp$, was purified by paper chromatography in solvent I, eluted with water, and concentrated by evaporation under vacuum.

[5′-^{32}P]$(pdT)_4pdCp$ was prepared by incubation of the 5′-hydroxyl homologue (50 μM) with 10 μM [γ-^{32}P]ATP (200 Ci/mmol) and 60 units/mL *PseT* 1 polynucleotide kinase followed by purification as described above.

The internally labeled oligonucleotide $dT(pdT)_3$[3′→5′-^{32}P]$pdT(pdT)_3pdCp$ ([^{32}P]pdT_8dCp) was prepared by using the oligonucleotide joining reaction of RNA ligase. The acceptor $dT(pdT)_3$ was incubated at 1 mM with 100 μM [^{32}P]pdT_4dCp, 100 μM ATP, 40 μM RNA ligase, and the other reaction components as described above. The product, [^{32}P]pdT_8dCp, was treated with BAP to remove the 3′-terminal phosphate and purified by paper chromatography in solvent I. Unlabeled $dT(pdT)_7pdC$ was prepared from $(pdT)_8pdC$ by treatment with BAP and subsequent purification by paper chromatography as described.

Assay of the Joining Reaction. Reaction mixtures (10 μL) contained 70 mM Hepes, pH 8.0, 20 mM DTT, 25 μg/mL BSA, 10 mM $MnCl_2$, 10 mM $MgCl_2$, ATP, the donor $(pdT)_4pdCp$, the acceptor $dN(pdN)_n$, 3 mM phosphocreatine, 120 units/mL creatine kinase, 170 units/mL myokinase, and RNA ligase. The concentrations of nucleotide components and RNA ligase are given in the legends to the table and figures. The reaction mixtures were incubated at 17 °C, and 1-μL aliquots were removed for analysis. Samples not treated with BAP were added to a tube containing 9 μL of 10 mM Tris-HCl, pH 8.0, and 5 mM EDTA and held on ice until applied to paper. Samples to be treated with BAP were added to 9 μL of a solution containing 0.97 M Tris-HCl, pH 8.7, 0.1 M NaCl, 0.01 M $MgCl_2$, and 0.2 mg/mL BAP C and immediately incubated at 65 °C for 1 h. All samples were spotted on Whatman No. 1 paper and developed for 16–20 h in solvent II. The chromatograms were scanned for radioactivity on a Packard Model 7201 radiochromatogram scanner. The radioactivity in each peak and in background areas of the chromatogram was quantified by using a scintillation counter. The yields of product were calculated as the ratio of the radioactivity in the product peak to the total radioactivity and were based upon the limiting substrate. Some radioactivity precipitated in the reaction mixtures containing BAP and was identified as $^{32}P_i$ by its inability to adsorb to charcoal. The amount of this radioactivity was determined by counting the tube (after the liquid sample plus a 4-μL rinse of H_2O had been spotted onto the paper) and correcting for the different efficiency of Cherenkov counting of ^{32}P in the tube as compared to the samples on paper. This radioactivity, which represented as much as 20% of the total, was included in the total for each chromatogram when yields were calculated so as to more accurately express the amounts of oligonucleotide product formed.

Product Characterization. Nearest-neighbor analyses of reaction products were performed as described (Wells et al., 1967) upon material eluted from Whatman No. 1 chromatograms. The chromatogram pieces were washed with absolute ethanol and eluted several times with either H_2O (for the adenylylated intermediate) or 0.2 M TEABC, pH 8.0 (for the product). Combined eluates were concentrated by vacuum evaporation and desalted by application to Whatman No. 1 paper, drying, and repeated washing with absolute ethanol. The oligonucleotides were again eluted with water or 0.2 M TEABC, concentrated by evaporation, and dried from methanol. After treatment with micrococcal nuclease and spleen phosphodiesterase, some samples were adjusted to pH 8.0 and incubated with BAP C (0.1 mg/mL) at 65 °C for 1 h. The nucleotide products of degradation were isolated after adsorption and elution from charcoal as described (Greenfield et al., 1975) and identified by paper chromatography on Whatman No. 1 in solvent III.

RPC-5 Chromatography. Substrates and reaction mixtures were diluted to 0.5 mL with 10 mM Tris-HCl, pH 7.5, and 0.1 M KCl and applied to a 0.5-mL RPC-5 column (0.2 × 17 cm) equilibrated with the same buffer. Chromatography was at 200–220 psi, and the A_{254} was continuously recorded. Fractions (0.6 mL) were collected and monitored for ^{32}P by Cherenkov counting. After the column was washed with 5–10 mL of equilibration buffer, the elution was with a 100-mL gradient of 0.1–0.5 M KCl in 0.01 M Tris-HCl, pH 7.5. The concentration of KCl was determined by conductivity measurements using a Radiometer CDC-114 cell.

Results

Joining of dA_6 and pdT_4dCp and Product Characterization. RNA ligase catalyzed the joining of $dA(pdA)_5$ and [5′-^{32}P]$(pdT)_4pdCp$ to form the undecanucleotide $dA(pdA)_5$-[3′→5′-^{32}P]$pdT(pdT)_3pdCp$. Chromatography of an aliquot of a reaction mixture at zero time showed that greater than 95% of the radioactivity migrated as a single peak ([5′-^{32}P]$(pdT)_4pdCp$) and the remainder migrated with P_i (Figure 1A). After treatment of an identical aliquot with BAP, all the radioactivity was converted to P_i (Figure 1B). Neither of these controls revealed any contaminating radioactivity

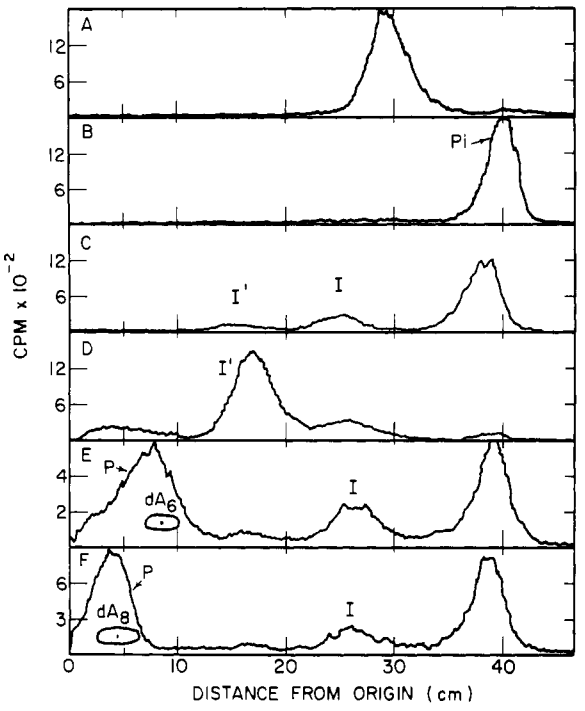

FIGURE 1: Chromatographic analysis of the RNA ligase reaction. The reaction of 250 μM [5′-^{32}P](pdT)$_4$pdCp, 250 μM dA(pdA)$_5$, 100 μM ATP, the ATP regeneration system, and 28 μM RNA ligase was incubated at 17 °C for the indicated times. (A) Aliquot taken before incubation (0 h); (B) as in (A) but with BAP digestion; (C) reaction in which acceptor dA(pdA)$_5$ was omitted (24 h); (D) complete reaction containing 1 mM ATP rather than 100 μM (5 days); (E) complete reaction (7 days); (F) complete reaction in which the acceptor dA(pdA)$_7$ was substituted for dA(pdA)$_5$ (7 days). Reaction mixtures were analyzed by paper chromatography as described under Materials and Methods. P represents the joined product, I the adenylylated intermediate, and I′ the unknown derivative of I.

migrating more slowly than the donor. When the labeled donor was incubated with RNA ligase and ATP in the absence of dA(pdA)$_5$ acceptor, chromatography of an aliquot treated with BAP revealed two peaks resistant to BAP (Figure 1C). The major peak (I) contained 21% of the total radioactivity and the minor peak (I′) contained 3%. This result together with the subsequent nearest-neighbor analysis of these materials indicates that in the absence of acceptor the donor reacts with ATP to yield the expected adenylylated reaction intermediate (Kaufmann & Littauer, 1974; Sninsky et al., 1976; Ohtsuka et al., 1976; Sugino et al., 1977). The formation of the adenylylated intermediate (I) in the absence of acceptor is contrary to the results of Sugino et al. (1977), who described an absolute requirement for acceptor and termed this requirement acceptor activation. Both I and I′ contain the same adenylylated 5′-phosphate group characteristic of intermediate (data below). I is likely to be the expected intermediate [rA-5′pp5′-dT(pdT)$_3$pdC after BAP treatment]. The structure of I′ is not known but may involve a 3′ modification. When high concentrations of ATP are used, I′ becomes the major reaction product and little undecanucleotide is formed even though acceptor is present (Figure 1D). The kinetics of the formation of I′ suggests that it is a product arising from I. Incubation of a complete reaction mixture containing [5′-^{32}P](pdT)$_4$pdCp, ATP, the acceptor dA(pdA)$_5$, RNA ligase, and an ATP regeneration system followed by digestion with BAP resulted in a new peak of radioactivity (Figure 1E) corresponding to 50% of the donor being joined to the acceptor. The product migrated more slowly than either the adenylylated intermediate or I′, indicating that a larger product containing acceptor had been formed. Incubation of a complete reaction

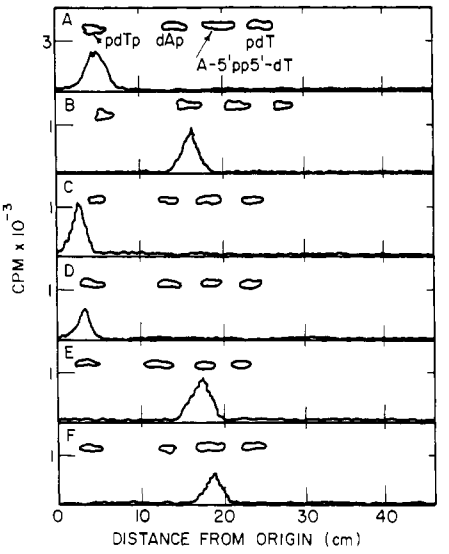

FIGURE 2: Enzymatic characterization of RNA ligase reaction components. Analyses and chromatography were as described under Materials and Methods. (A) The degradation product of donor [5′-^{32}P]pT$_4$Cp; (B) the degradation product of combined oligonucleotide products (P from both parts E and F in Figure 1); (C) the degradation product of the intermediate (I) (from Figure 1C); (D) the degradation product of I′ (from Figure 1D); (E) as for (C) but with an additional incubation with BAP; (F) as for (D) but with an additional incubation with BAP.

mixture containing an acceptor 2 nucleotides longer, dA-(pdA)$_7$, resulted in a product peak which migrated yet more slowly than the product observed with dA(pdA)$_5$ acceptor (Figure 1F). In each case the product migrated in approximately the same position as the unreacted acceptor.

Product oligonucleotides were isolated from chromatograms similar to those shown in Figures 1C–F and analyzed by enzymatic digestion to 3′-phosphate nucleotides. A control analysis of unreacted donor, [5′-^{32}P](pdT)$_4$pdCp, showed that the ^{32}P-labeled degradation product comigrated with pdTp, as expected (Figure 2A). The degradation of the product material indicated as P in parts E and F of Figure 1 resulted in [^{32}P]dAp (Figure 2B), indicating that a phosphodiester bond had been formed between the labeled donor and the dA-containing acceptor. The absence of labeled dTp or dCp (data for dCp not shown) shows that the donor was not degraded by phosphatases or nucleases such that it could serve as an acceptor. When material from either of the peaks indicated as I (parts C and E of Figure 1) was degraded, the ^{32}P-labeled product migrated in a position consistent with the structure rA-5′pp5′-dTp (Figure 2C). The degradation of I′ (Figure 1D) gave identical results (Figure 2D). Further incubation of the degraded intermediate or I′ with alkaline phosphatase resulted in labeled material comigrating with authentic rA-5′pp5′-dT (parts E and F of Figure 2). This result confirms the identity of both of these compounds as forms of the adenylylated intermediate; they probably differ in structure at their 3′ ends since they migrate differently in solvent II (Figure 1).

Enzymatic digestion of donor, intermediate, I′, and product to 5′-phosphate nucleotides with DNase I and venom phosphodiesterase resulted in the formation of ^{32}P-labeled pdT only in each case (data not shown), showing that the labeled phosphate had not been removed from its bond on the 5′ end of the donor. Samples not incubated with nucleases chromatographed at or very near the origin, indicating that mononucleotides were not produced by any other means than the specific nucleases used for analysis in each case. In addition,

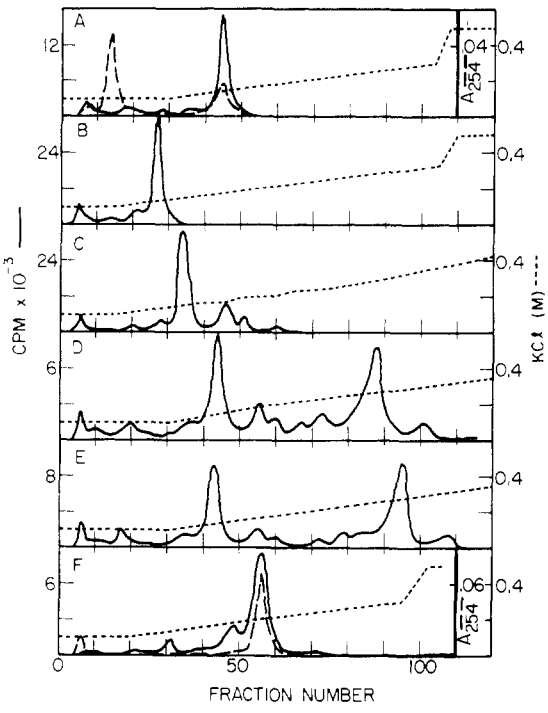

FIGURE 3: RPC-5 chromatography of the RNA ligase reaction. Aliquots of reactions carried out as described in the legend to Figure 1 were separated on RPC-5 columns as described under Materials and Methods. (—) ^{32}P; (– – –) A_{254}; (- - -) KCl concentration. (A) Unreacted dA(pdA)$_5$ and [5′-^{32}P](pdT)$_4$pdCp; (B) reaction in which acceptor and ATP were omitted; (C) reaction in which acceptor only was omitted (corresponding to Figure 1C); (D) complete reaction containing dA(pdA)$_5$ acceptor (corresponding to Figure 1E); (E) complete reaction containing dA(pdA)$_7$ acceptor (corresponding to Figure 1F); (F) chromatography of unlabeled (dTp)$_8$dC and the product of an RNA ligase reaction between dT(pdT)$_3$ and [5′-^{32}P](pdT)$_4$pdCp subsequently treated with BAP and isolated by paper chromatography as described under Materials and Methods.

degradation to 3′-phosphate nucleotides was also performed on the isolated products from other RNA ligase reactions between [5′-^{32}P](pdT)$_4$pdCp donor and the following acceptors: dA(pdA)$_2$, dA(pdA)$_3$, dA(pdA)$_{11}$, dT(pdT)$_7$, dC(pdC)$_5$, and dT(pdT)$_7$pdG. In each case the results indicated the formation of the expected phosphodiester bond between the labeled 5′-phosphate of the donor and the 3′-terminal nucleoside of the acceptor.

The unreacted substrates and the reaction mixtures analyzed by paper chromatography and shown in parts C, E, and F of Figure 1 were also characterized by RPC-5 high-pressure liquid chromatography. Chromatography of the unreacted substrates showed that the acceptor dA(pdA)$_5$ eluted in the 0.1 M KCl wash and the donor [5′-^{32}P](pdT)$_4$pdCp eluted at 0.135 M KCl (Figure 3A). The major peak consisting of ^{32}P-labeled donor constituted 83% of the radioactivity eluted from the column. After the donor was incubated with 28 μM RNA ligase and the ATP regeneration system for 7 days, 76% of the radioactivity eluted at the same salt concentration as it did before incubation, 0.135 M (Figure 3B). This result shows that little degradation (<9%) of the donor occurred during the time required for successful RNA ligase reactions. A reaction mixture containing donor, ATP, and enzymes but no acceptor was incubated for 1 day. Paper chromatography (Figure 1C) showed that 21% of the donor had been converted to the intermediate (I). RPC-5 chromatography of an aliquot of the same reaction mixture showed three new peaks of radioactivity eluting at higher salt concentrations than donor, containing 16, 7, and 2% of the radioactivity at 0.18, 0.195, and 0.23 M KCl, respectively (Figure 3C). The major new peak eluting at 0.18 M KCl corresponds to the adenylylated intermediate; the other minor peaks eluting at 0.195 and 0.23 M KCl may be 3′-modified donor and I′, respectively. A complete reaction mixture containing dA(pdA)$_5$ acceptor and product in 50% yield as determined by paper chromatography (Figure 1D) showed three new peaks of radioactivity eluting at higher salt concentrations than those observed with ATP and donor alone, containing 8, 37, and 3% of the radioactivity at 0.23, 0.26, and 0.295 M KCl, respectively (Figure 3D). The major peak at 0.26 M KCl (37%) probably corresponds to the expected product [^{32}P]dA(pdA)$_5$(pdT)$_4$pdCp. The larger compound at 0.295 M KCl may correspond to 3′-modified product (or the union of acceptor with I′), and the more weakly bound peak at 0.23 M KCl may be the joining product of donor and partially degraded acceptor. This result would indicate that 8% of the total of 48% of label converted to products arose by product degradation or modification of the acceptor. The three new peaks together (48%) correspond to the 50% product identified by paper chromatography. The complete reaction mixture containing the larger dA(pdA)$_7$ acceptor resulted in a major product peak eluting at higher salt concentration than the product with dA(pdA)$_5$ acceptor. This product, consisting of 36% of the radioactivity, eluted at 0.28 M KCl (Figure 3E). The sum of the peaks eluting above 0.23 M KCl was 48% of the total, corresponding to the determination by paper chromatography (Figure 1E) of 50% yield. The peaks (at fraction 55 in each case) which correspond to the intermediate in the two complete reactions and elute at 0.175 M KCl (8% of the radioactivity) (Figure 3D) and 0.17 M KCl (6% of the radioactivity) (Figure 3E) also correspond well in quantity to the peaks of the intermediate shown in paper chromatography of the two reactions, 11 and 8%, respectively (parts D and E of Figure 1).

These results show that the size of the product reflects that of the acceptor. The final panel of Figure 3 shows the co-chromatography of dT(pdT)$_7$pdC synthesized with RNA ligase and of independently prepared material. An unlabeled preparation of this compound was obtained by phosphatase treatment of commercially available (pdT)$_8$pdC with BAP; the ^{32}P-labeled material was prepared by an RNA ligase reaction between dT(pdT)$_3$ and [5′-^{32}P](pdT)$_4$pdCp followed by treatment with BAP. The exact comigration of the major radioactive compound with the peak of UV-absorbing material (Figure 3F) indicates that the expected intermolecular product was formed in the RNA ligase reaction. The shoulder of labeled material eluting at lower salt may be the intermediate or the product of the partially degraded acceptor dT(pdT)$_2$ and donor that was not resolved from the major product by the paper chromatography. Because RPC-5 chromatography under these conditions is capable of resolving oligo(deoxyribonucleotides) differing in length by one residue, these results show that RNA ligase does not generate a broad distribution of products arising from removal of one or a few residues from the substrates or reactants during the course of the reaction but rather forms one major product peak.

Reaction Kinetics. The kinetics of the reaction between equimolar amounts of dA(pdA)$_5$ and [5′-^{32}P](pdT)$_4$pdCp was examined at three enzyme concentrations (Figure 4). The initial velocity determined from the 1-day time point of each reaction was proportional to the enzyme concentration (Figure 4, insert). The final yields also varied as a function of enzyme concentration, as reported for other RNA ligase reactions (Uhlenbeck & Cameron, 1977). These results are in contrast, however, to observations made under similar conditions with the single nucleoside bisphosphate addition reaction of RNA

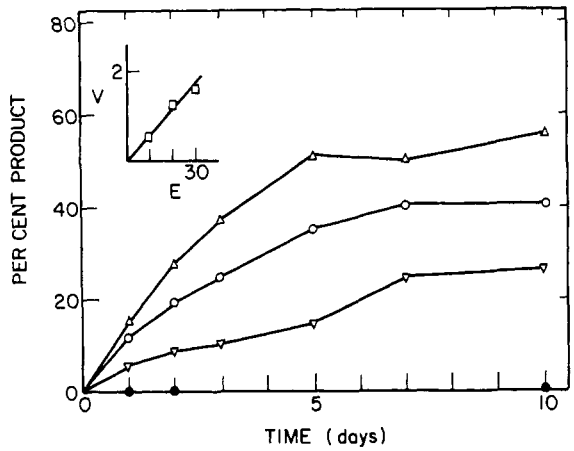

FIGURE 4: Kinetics of the RNA ligase reaction with equimolar substrates as a function of enzyme concentration. Reactions of 250 μM dA(pdA)$_5$ and 250 μM [5′-^{32}P](pdT)$_4$pdCp were with 100 μM ATP, the regeneration system, and the indicated concentrations of RNA ligase: (●) 0; (▽) 10; (○) 20; (△) 30 μM. The insert shows initial velocity (picomoles per hour) as a function of enzyme concentration (micromolarity). Reactions were analyzed as described in Figure 1.

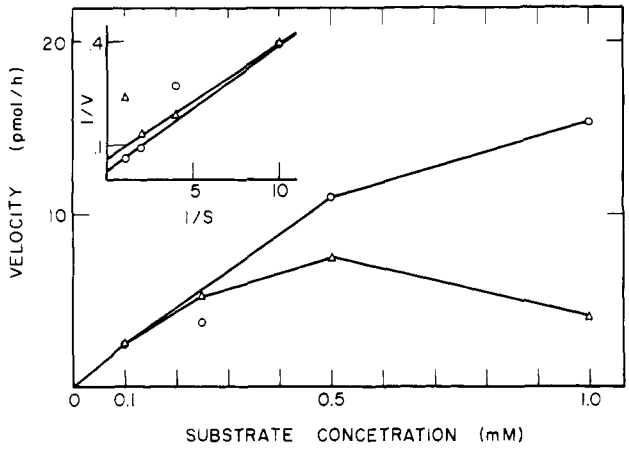

FIGURE 5: Initial velocity of the RNA ligase reaction as a function of substrate concentration. Each reaction contained 100 μM ATP, the regeneration system, and 20 μM enzyme: (○) constant (pdT)$_4$pdCp (100 μM) and variable dA(pdA)$_5$; (△) constant dA(pdA)$_5$ (100 μM) and variable (pdT)$_4$pdCp. The insert shows a Lineweaver–Burk plot of the data in h pmol^{-1} vs. mM^{-1}. Reactions were analyzed as described in Figure 1.

ligase (Hinton et al., 1978), where the yield was independent of enzyme concentration.

Dependence of Rate on Donor and Acceptor Concentrations. The initial velocity of the intermolecular reaction between dA(pdA)$_5$ and [5′-^{32}P](pdT)$_4$pdCp was dependent on both the acceptor and donor concentrations (Figure 5). A 5-fold increase in the acceptor concentration from 0.1 to 0.5 mM resulted in a 4.3-fold increase in the initial velocity; the same donor concentration increase tripled the rate. A further increase of the acceptor concentration to 1.0 mM produced an additional increase in reaction rate, but a similar increase in donor concentration resulted in a decreased reaction velocity, indicating that excessive donor concentrations may inhibit the reaction. The reaction yields, as well as rates, were increased by higher acceptor concentrations. A 10-fold excess of acceptor resulted in an 86% yield with respect to donor after a 10-day incubation compared to 22% at equimolar concentrations (data not shown). Higher concentrations of donor also resulted in increased yields under conditions where its concentration remained less than that of the acceptor. A Lineweaver–Burk plot of the data illustrated in Figure 5 allows

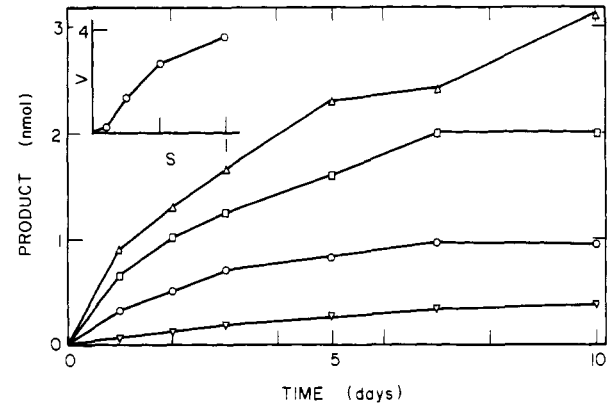

FIGURE 6: Kinetics of the RNA ligase reaction as a function of increasing equimolar substrate concentrations. Each reaction contained 100 μM ATP, the regeneration system, and 20 μM RNA ligase. Both [5′-^{32}P](pdT$_4$)pdCp and dA(pdA)$_5$ were at (▽) 100 μM, (○) 250 μM, (□) 500 μM, and (△) 1 mM. The insert shows initial velocity (picomoles per hour × 10^{-1}) as a function of substrate concentration (millimolarity). Reactions were analyzed as described in Figure 1.

a rough approximation of the apparent K_m of pdT$_4$Cp as 0.5 mM and of dA(pdA)$_5$ as 1.5 mM (Figure 5, insert). These values are in general agreement with the apparent K_m values calculated for pdTp (0.7 mM) and dA(pdA)$_4$ (2.1 mM) in the single-addition reaction (Hinton et al., 1978). On the assumption of a single active site per 43 000-dalton polypeptide (McCoy et al., 1979), turnover numbers of 0.2 and 0.08 h^{-1} were calculated for dA(pdA)$_5$ and (pdT)$_4$ pdCp, respectively, under these nonsaturating cosubstrate conditions. These extremely low turnover numbers explain the long incubation times required for this reaction to achieve high yields.

Donor and acceptor concentrations were also varied together at equimolar ratios in reactions from 0.1 to 1.0 mM (Figure 6). Both yields and initial velocities (Figure 6, insert) increased as substrate concentration was increased. The final yields of the reactions, in terms of the substrate joined, were 38–40% for the reactions at 100–500 μM substrate and 31% for the reaction containing 1 mM reactants. (Higher yields have been obtained by using higher enzyme concentrations.) These results show that reactions with equimolar acceptor/donor ratios can be carried out throughout this range of concentrations and that by using the same amount of enzyme increased amounts of product can be obtained by using higher substrate concentrations.

Other Reaction Variables. As reported by Hinton et al. (1978), we found that incubation at low temperatures (5–22 °C) was required for the intermolecular DNA joining reaction. The optimal temperature was ~17 °C. The initial velocity observed when both $MnCl_2$ and $MgCl_2$ are present is slightly higher than when $MnCl_2$ alone is used and about sixfold higher than when $MgCl_2$ is used alone. In order to provide an energy source for the reaction without inhibiting product formation with high levels of ATP (Sugino et al., 1977; Hinton et al., 1978; Hinton & Gumport, 1979), we performed the reaction in the presence of an ATP regeneration system. Phosphocreatine was the energy source, and creatine kinase and myokinase catalyzed the regeneration of AMP to ATP. Phosphocreatine concentrations of 10 mM or higher were found to inhibit the RNA ligase reaction, but levels of 2–5 mM could be used with satisfactory results.

In the presence of the regeneration system, ATP concentration in the 250 μM equimolar joining reaction was varied from 25 μM to 1 mM (Figure 7). At ATP concentrations of 25, 100, and 200 μM, increasingly higher levels of intermediate (I) and I′ were formed as the ATP concentration

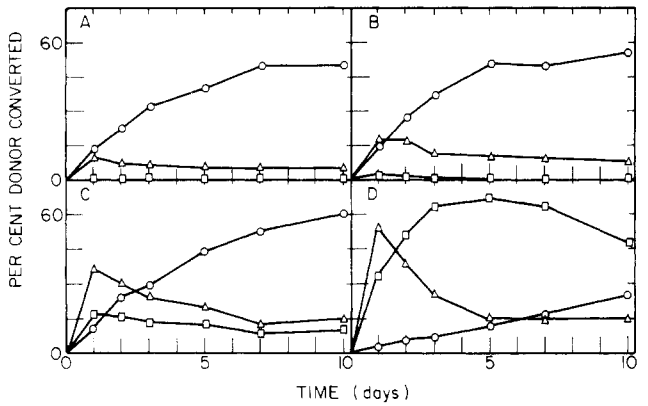

FIGURE 7: Product, intermediate, and I′ formation as a function of ATP concentrations. Each reaction contained 250 μM dA(pdA)$_5$, 250 μM [5′-^{32}P](pdT)$_4$pdCp, the ATP regeneration system, and 30 μM RNA ligase. ATP concentrations were (A) 25 μM, (B) 100 μM, (C) 200 μM, and (D) 1 mM. (○) Product; (△) intermediate; (□) I′. Reactions were analyzed as described in Figure 1.

increased but very similar rates of product formation were observed in each case. At the highest ATP concentration, little product was obtained but I′ accumulated in high yield. The intermediate (I) appeared to be the precursor of I′. These results along with those characterizing I′ indicate that the difference between I and I′ is probably at the 3′ end of the molecule. When acceptor dA_6 concentration was increased in a reaction mixture containing high ATP levels, the production of I′ was greatly diminished (data not shown), suggesting that the formation of I′ may involve binding of the 3′ end of the donor molecule to the acceptor site and that increasing acceptor concentration can prevent this binding of donor.

Other experiments with equimolar acceptor and donor concentrations at 0.5 and 1.0 mM (data not shown) confirmed that although the amount of intermediate in the reaction increased as the ATP concentration was increased, the rate of product formation did not vary significantly as long as the ATP was not in excess of donor. When the ATP concentration was greater than that of the donor, the reaction was inhibited. These results indicate that synthesis reactions can be carried out at ATP concentrations less than those required for stoichiometric joining in order to minimize accumulation of intermediate and I′ while still attaining maximum yields of product.

Effect of Acceptor Chain Length and Composition. The effect of the chain length of the acceptor on the RNA ligase reaction was investigated with a series of dA(pdA)$_n$ acceptors (Table I). The dinucleoside monophosphate did not react, but acceptors from 3 to 12 residues long joined to the (pdT)$_4$pdCp donor. There is a sharp contrast between the rates obtained with the deoxyoligomer acceptors and that of the oligoribonucleotide acceptor rA(prA)$_3$, with the ribooligomer reacting over 200 times faster and giving an 81% yield in 1 h. The best velocities and yields with deoxy substrates were obtained with acceptors 4–8 nucleotides long, while the trimer and dodecamer gave both lower rates and yields.

Oligo(deoxyribonucleotides) containing dT, dG, and dC residues were also compared to the dA(pdA)$_n$ acceptors to investigate the effect of base composition upon the reaction (Table I). The highest rates and yields were obtained with the dA-containing acceptors and the lowest with dC(pdC)$_5$. This result is in contrast to data obtained with the single nucleoside bisphosphate addition reaction which indicated dC-terminated acceptors to be the best substrates (Hinton et al., 1978). The last reaction listed in the table illustrates that

Table I: Effect of Acceptor Chain Length and Composition on Initial Velocity and Yield of the Single-Strand Joining Reaction of RNA Ligase[a]

acceptor	V_i (pmol/h)	yield (%)[b]
rA(prA)$_3$	>2100	84[c]
dApdA	0	0
dA(pdA)$_2$	3	12
dA(pdA)$_3$	10	27
dA(pdA)$_5$	9	27
dA(pdA)$_7$	9	28
dA(pdA)$_{11}$	4	15
dT(pdT)$_7$	2	13
dT(pdT)$_7$pdG	3	12
dC(pdC)$_5$	1	7
dC(pdC)$_5$[d]	13	41[d]

[a] Each reaction contained 250 μM [5′-^{32}P](pdT)$_4$pdCp, 25 μM ATP, 250 μM acceptor, 32 μM RNA ligase, and the ATP regenerating system described under Materials and Methods. Product was assayed by paper chromatography as described under Materials and Methods. Points from the linear portion of the product vs. time curve were used for initial velocity calculations. [b] Yield after 7 days. [c] Yield after 1 h. [d] This reaction contained the components listed above except 47 μM ligase, 500 μM dC(pdC)$_5$ acceptor, 10% Me$_2$SO, and 2 mM spermine; the yield was determined after 8 days.

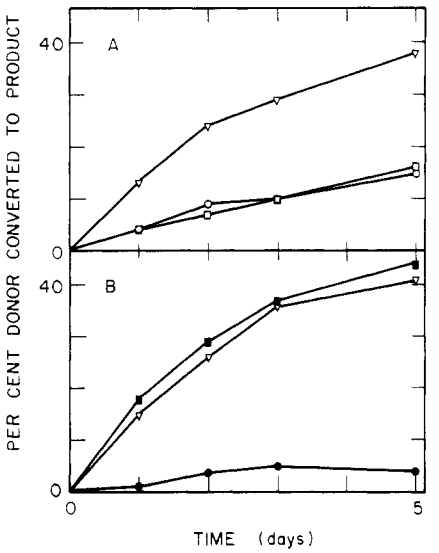

FIGURE 8: Effect of complementary and noncomplementary oligonucleotides on the RNA ligase catalyzed joining of dA(pdA)$_7$ to (pdT)$_4$pdCp. Each reaction contained 250 μM [5′-^{32}P](pdT)$_4$pdCp, 25 μM ATP, and the ATP regenerating system. (A) Enzyme was 19 μM: (▽) 500 μM dA(pdA)$_7$ acceptor; (○) 500 μM dT(pdT)$_7$ acceptor; (□) 500 μM dA(pdA)$_7$ + 500 μM dT(pdT)$_7$. (B) Enzyme was 28 μM: (▽) 500 μM dA(pdA)$_7$; (●) 250 μM dC(pdC)$_5$; (■) 500 μM dA(pdA)$_7$ + 500 μM dC(pdC)$_5$. Reactions were analyzed as described in Figure 1.

the poor yield obtained with dC(pdC)$_5$ can be significantly improved by changing the reaction conditions. Increasing the acceptor and enzyme concentrations and adding Me$_2$SO and spermine (Hinton & Gumport, 1979) to the reaction mixture made possible a 40% yield (with respect to the donor). These results indicate that DNA acceptors 3 or more nucleotides long and terminated with any common deoxynucleotide can participate in an RNA ligase intermolecular reaction. Since the single nucleoside bisphosphate addition reaction has also indicated that any common 2′-deoxynucleoside 3′,5′-bisphosphate can act as donor in the reaction (Hinton et al., 1978), it is likely that the joining of any two single-strand oligo(deoxyribonucleotides) is possible with RNA ligase. We are continuing

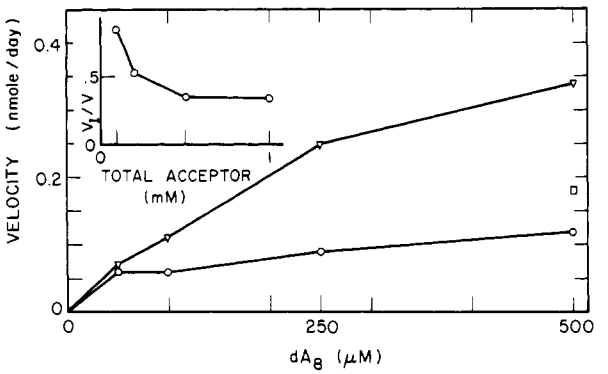

FIGURE 9: Inhibition of the RNA ligase catalyzed joining of dA(pdA)$_7$ to (pdT)$_4$pdCp by dT(pdT)$_7$ as a function of total acceptor concentration. Each reaction contained 100 μM [5′-^{32}P](pdT)$_4$pdCp, 25 μM ATP, the ATP regeneration system, and 28 μM RNA ligase: (▽) dA(pdA)$_7$ alone; (○) dA(pdA)$_7$ + an equal concentration of dT(pdT)$_7$; (□) 500 μM each of dA(pdA)$_7$ and dT(pdT)$_7$ + 3.7 mg/mL RNase A. The insert shows the ratio of the inhibited velocity (V_I) with dT(pdT)$_7$ present to the velocity (V) observed with dA(pdA)$_7$ alone as a function of acceptor concentration. Reactions were analyzed as described in Figure 1.

to optimize reaction conditions and to apply the reaction to a variety of substrates.

Effect of Secondary Structure. The acceptors dA(pdA)$_7$ and dT(pdT)$_7$ were reacted with [5′-^{32}P](pdT)$_4$pdCp separately and in equimolar combination in order to determine the effect of duplex structure on the RNA ligase reaction (Figure 8A). The reaction with dT(pdT)$_7$ alone produced much less product than with dA(pdA)$_7$ alone (cf. Table I); when both acceptors were present the predominant product was dA(pdA)$_7$(pdT)$_4$pdCp, but less of this product was obtained than when dA(pdA)$_7$ was used alone. The joining of each of the acceptors to (pdT)$_4$pdCp was inhibited by the presence of the complementary acceptor. The poor acceptor dC(pdC)$_5$ did not inhibit the joining of dA(pdA)$_7$ to (pdT)$_4$pdCp (Figure 8B), suggesting that the inhibition by dT(pdT)$_7$ was due to its complementarity to dA(pdA)$_7$ rather than to some other factor relating to its poor reactivity as an acceptor.

Similar reactions with dA(pdA)$_7$ alone or in equimolar combination with dT(pdT)$_7$ were carried out over a range of concentrations (Figure 9). The presence of the complementary oligonucleotide had little effect on the reaction at 50 μM concentrations of each acceptor, but the inhibition was more pronounced at higher concentrations, as shown by the ratio of the velocities of dA(pdA)$_7$ joining in the presence and in the absence of dT(pdT)$_7$ (Figure 9, insert). One point on the graph also indicates that the addition of RNase A (a single-strand DNA binding protein; Jenson & von Hippel, 1976) facilitated the joining of the complementary acceptors, even though the amount present (270 μM) was considerably less than the total concentration of oligo(deoxyribonucleotides) (1.0 mM) present in the reaction. These results suggest that duplex oligonucleotides are poorer acceptors in the RNA ligase reaction that single-strand substrates. The inhibition of a reaction by the addition of an oligonucleotide complementary to the acceptor is concentration dependent, as would be expected for the formation of duplex structures between separate oligonucleotide molecules, and can be at least partially alleviated by the addition of the DNA "melting" protein, RNase A. We have found that RNase A also stimulates the addition of single deoxynucleoside 3′,5′-bisphosphates to oligo(deoxyribonucleotides), suggesting that its activity may not be solely related to disruption of duplex structure (Hinton & Gumport, 1979).

Discussion

The reaction of DNA substrates with RNA ligase is most clearly understood in light of the reaction mechanism of the enzyme. RNA ligase reacts with ATP to form a covalent enzyme–AMP complex with the release of PP$_i$ (Cranston et al., 1974). The adenylyl group is subsequently transferred to the 5′-phosphate of the donor oligonucleotide to form a 5′→5′-phosphoanhydride linkage with the resulting compound referred to as the intermediate I in this paper (Kaufmann & Littauer, 1974; Sninsky et al., 1976; Ohtsuka et al., 1976; Sugino et al., 1977). In the final step of the reaction a phosphodiester bond is formed between the 3′-hydroxyl of the acceptor and the activated 5′-phosphate of the donor, with the release of AMP. This third step of the reaction is inhibited by ATP (Sugino et al., 1977) and by analogy to DNA ligase (Harvey et al., 1971) probably requires free enzyme rather than the adenylylated from (Sugino et al., 1977; Hinton et al., 1978; Hinton & Gumport, 1979).

RNA ligase efficiently catalyzes the joining of RNA oligonucleotide substrates to each other (Uhlenbeck & Cameron, 1977; Kikuchi & Sakaguchi, 1978). DNA oligonucleotides (Snopek et al., 1976) or deoxyribonucleoside 3′,5′-bisphosphates (England & Uhlenbeck, 1978) have been joined as donors to RNA acceptors at 37 °C. Although Sugino et al. (1977) reported intermolecular joining involving a DNA acceptor using conditions similar to those used for RNA acceptors, we have observed that only the first two steps of the reaction occur when DNA substrates are used at 37 °C, even at very high enzyme concentrations. In our hands, low temperatures are required for reactions involving DNA acceptors as well as very high levels of enzyme and long reaction times for both the single nucleoside bisphosphate addition reaction (Hinton et al., 1978; Hinton & Gumport, 1979) and the oligo(deoxynucleotide) joining reaction.

The concentration of ATP has a complex effect on the reaction with DNA substrates. As reported by Hinton et al. (1978) for the single nucleotide addition reaction, increasing concentrations of ATP cause a decrease in the reaction velocity, presumably by sequestering the enzyme in the adenylylated form. Higher ATP concentrations, however, did result in higher final yields of products, presumably by producing and maintaining higher levels of adenylylated intermediate (I). These two effects were most favorably balanced by the use of an acceptor/ATP/pdNp donor ratio of 1:2:8 (Hinton et al., 1978). In order to perform an RNA ligase reaction between equimolar concentrations of oligo(deoxyribonucleotides), we have found it useful to use low levels of ATP and an ATP regenerating system. The use of an ATP regeneration system also significantly improves the rates and yields of the single nucleotide addition reaction (Hinton & Gumport, 1979). In the presence of such a system, a wide range of ATP concentrations gives very similar results, as long as donor concentration is in excess of ATP. Excess donor concentration, with respect to ATP, probably promotes the availability of free enzyme through the reaction of adenylylated enzyme with donor to form I. In contrast, when ATP is in excess of donor, there is sufficient ATP to convert most of the donor to intermediate and in addition to maintain most of the enzyme in the adenylylated form. With little free enzyme available and little donor to regenerate it, product formation is inhibited.

A new compound (I′) is formed from donor in yields greater than 90% in reactions containing high ATP concentrations. This compound has the same 5′-adenylylated phosphate terminus as the intermediate as judged by enzymatic degradations but appears to be larger than I or donor upon paper and RPC-5

chromatography. These results indicate that I′ differs from I at its 3′ terminus. It appears in a reproducible kinetic pattern which suggests that it is formed from the intermediate (I). We are currently investigating the possibility that I′ may contain an adenylyl group in a phosphoanhydride linkage to its 3′-phosphate as well as its 5′-phosphate. This product may arise through the binding of I in the acceptor site with its 3′-phosphate in position to accept the adenylyl group from the adenylylated enzyme. Modification of the 3′ end of the donor, I, or product may account for some of the minor peaks observed upon RPC-5 chromatography of the reaction mixtures (Figure 3), and interaction of the donor with the acceptor site may explain the inhibition of the joining reaction by high concentrations of donor (Figure 5).

The possibility of joining single-strand DNA oligonucleotides with RNA ligase simplifies the synthesis of large DNA oligonucleotides of defined sequence. There is no need to synthesize complementary oligonucleotides capable of forming a stable duplex structure as requisite substrates for DNA ligase. A single-strand polymer could be converted to a duplex with the use of a single oligonucleotide primer and DNA polymerase I (Richardson et al., 1964). Although very high levels of enzyme are required for the RNA ligase reaction, yields of up to 60% have been achieved at equimolar concentrations of model substrates, with a relatively low level of nuclease degradation. Higher yields have been obtained when excess acceptor has been used to drive the reaction. Quantities of DNase-free RNA ligase sufficient for practical syntheses can be readily obtained (McCoy et al., 1979). We, therefore, expect the combination of the single deoxyribonucleoside bisphosphate addition and single-strand deoxyoligomer joining reactions of RNA ligase to be useful in the synthesis of defined sequence deoxyoligomers.

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