Volume 10 Number 6 1982 Nucleic Acids Research

The preparative synthesis of oligodeoxyribonucleotides using RNA ligase

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Received 18 January 1982; Accepted 17 February 1982

ABSTRACT

The synthesis of nmol quantities of defined sequences of oligodeoxyribonucleotides using T4 RNA ligase has been demonstrated. Reactions using from 18 to 200 nmol of substrates in which a single 2'-deoxyribonucleoside 3',5'-bisphosphate was added to an oligodeoxyribonucleotide resulted in yields from 13 to 95%. When two oligodeoxyribonucleotides were similarly joined using RNA ligase, the yields ranged from 10 to 50%. Although the reactions contained high concentrations of enzyme and were incubated from 5 to 21 days, there was little degradation of either substrates or products. We have also characterized an unusual product which arises when 3'-phosphate terminated oligodeoxyribonucleotides are incubated with RNA ligase and high concentrations of ATP. This product has an adenylyl group linked to the 3'-phosphate by an anhydride bond. The mechanistic and synthetic implications of forming this product are discussed.

INTRODUCTION

T4 RNA ligase has been used extensively for the synthesis of oligoribonucleotides of defined sequence (see reviews 1 and 2). The enzyme catalyzes the formation of a 3'→5' phosphodiester bond between one oligonucleotide containing a 3'-hydroxyl group (the acceptor) and another bearing a 5'-phosphate (the donor) (3). A complementary template strand is not required to align the ends of the oligonucleotides joined and a donor as short as a ribonucleoside 3',5'-bisphosphate can be added to any acceptor three or more nucleosides in length (4,5).

We have been developing RNA ligase to synthesize defined sequences of oligodeoxyribonucleotides. We have reported conditions which extend the RNA ligase reaction to DNA substrates, thereby allowing the efficient addition of 3',5'-deoxyribonucleoside bisphosphates to oligodeoxyribonucleotides (6,7) and of single-strand oligodeoxyribonucleotides to one another (8). These studies used homopolymeric molecules as models. In this paper, we describe how to synthesize preparative amounts (1 to 100 nmol) of several oligodeoxyribonucleotides of defined sequence. In addition, we report the characteri-

0305-1048/82/1006-1877$2.00/0

zation of an unanticipated product resulting from the RNA ligase catalyzed addition of an adenylyl group to the 3'-phosphate of an oligodeoxyribonucleotide. A preliminary report of some of these results has been published (9).

MATERIALS AND METHODS

Materials. The oligodeoxyribonucleotides dGpdG$(pdA)_2(pdT)_2(pdC)_2$ and pdG$(pdA)_2$ were purchased from Collaborative Research. The oligodeoxyribonucleotides dCpdCpdA and dG$(pdA)_2$ were gifts of S. A. Narang. ATP, 3'-dCMP, and adenosine 5'-phosphoromorpholidate were obtained from Sigma Chemical Co. [γ-^{32}P]ATP was purchased from New England Nuclear and [2,8-^{3}H]ATP was purchased from ICN. Unless otherwise stated, all other nucleotides and oligonucleotides were purchased from P-L Biochemicals.

T4 RNA ligase was purified as described (10) from E. coli cells infected with T4 am E4314. Wild type and PseT 1 polynucleotide kinases (11) were gifts of D. Soltis and O. C. Uhlenbeck. Other RNA ligase reaction components and enzymes used for analysis were as described (6-8).

Paper chromatography of nucleotides and oligonucleotides. The following solvents were used to develop paper chromatograms: I, 1-C_3H_7OH: conc. NH_3:water::55:10:35; II, 100 mL of 0.1 M Na_2HPO_4, pH 6.8 to which was added 60 g $(NH_4)_2SO_4$ and 2.3 mL 1-C_3H_7OH; III, 60 mL 1 M ammonium acetate plus 40 mL 95% ethanol; and IV, 70 mL 1 M ammonium acetate plus 30 mL 95% ethanol.

Preparation of nucleotides and oligonucleotides. The mononucleotide pBrdUp was prepared from pdUp and bromine by the method of Riley et al. (12). Other 2'-deoxyribonucleoside 3',5'-bisphosphates were prepared using the procedure of Barrio et al. (13) and were purified as described (6). [5'-^{32}P]pdNps were synthesized and purified as described (6).

The dinucleoside pyrophosphate dCyd-3'PP5'-Ado was synthesized by condensing the tri-n-butylammonium salt of 3'-dCMP with adenosine 5'-phosphoromorpholidate as described (14), except that the reaction was carried out in dimethylsulfoxide instead of pyridine. The product was purified by chromatography on a DEAE Sephadex A-25 column using a linear gradient of 0.1 to 1.0 M triethylammonium acetate, pH 4.5.

The acceptor 3',5'-dihydroxyl oligodeoxyribonucleotides [dN$(pdN)_n$] and the 3'- and 5'-phosphate terminated oligodeoxyribonucleotide donors [pdNp$(dNp)_n$] were prepared as described (6,8). Labeled [5'-^{32}P]oligodeoxyribonucleotides were prepared as described (8). All oligomers were purified by chromatography on prewashed Whatman 3 MM paper in solvent I. They were eluted from the paper with water and stored at -20°C.

[3'-^{32}P]dT(pdT)$_3$pdCp was prepared as follows: dT(pdT)$_3$pdC[3'→5'-^{32}P]prCp was synthesized by the addition of [5'-^{32}P]prCp to dT(pdT)$_3$pdC using RNA ligase (7). The terminal 3'-phosphate of the resulting product, dT(pdT)$_3$pdC-[3'→5'-^{32}P]prCp was removed with bacterial alkaline phosphomonoesterase. The final product [3'-^{32}P]dT(pdT)$_3$pdCp was obtained by periodate oxidation and β-elimination of the ribonucleotide terminated oligomer as described by Winter and Brownlee (15).

RNA ligase reactions. Table 1 lists the substances present in RNA ligase reactions for either single nucleotide addition reactions or deoxyoligomer joining reactions. In each case, the nucleotides, oligonucleotides, spermine, and phosphocreatine were dried together under vacuum. A solution containing the remaining ingredients was added to give a final acceptor concentration of 0.5 to 2.0 mM. Reaction mixtures were incubated at 17°C. Products were purified either by chromatography on prewashed Whatman 3 MM paper in solvents I or III or by elution from a DEAE Sephadex A-25 column (0.9 X 20.5 cm) with linear gradients of triethylammonium acetate or triethylammonium bicarbonate.

The RNA ligase catalyzed addition of 5'-AMP to the 3'-phosphate of an oligodeoxyribonucleotide was performed using reactions containing 0.25 mM [3'-^{32}P]dT(pdT)$_3$pdCp; 0.25 mM dA(pdA)$_3$; 1 mM ATP; 3 mM phosphocreatine; 70 mM Hepes[2], pH 8.0; 20 μg/ml bovine serum albumin; 10 mM $MnCl_2$; 10 mM $MgCl_2$; 20

Table 1. Reaction Conditions to Use of RNA Ligase With DNA Substrates

Single Nucleotide Addition	Deoxyoligomer Joining
8 mM pdNp[a]	1 mM donor oligomer[b]
1 mM acceptor oligomer[b]	4 mM acceptor oligomer[b]
0.4 mM ATP	0.5 mM ATP
8 mM spermine	2 mM spermine
1-40 mM phosphocreatine[c]	1 mM phosphocreatine
Buffer mix[d]	Buffer mix[d]

[a]Concentration of pdNp can range from 0.5 to 10 mM if the relative concentrations of donor, acceptor, ATP, and spermine remain as given.
[b]Molecular concentration.
[c]1-10 mM for pdAp, pdCp, pdTp, and pdUp and 40 mM for pdGp.
[d]Buffer mix: 50 mM Hepes, pH 7.9, 20 mM dithiothreitol, 10 mM $MnCl_2$, 10 ug/ml bovine serum albumin, 175 U/mL creatine kinase, 170 U/mL myokinase, and 40 μM RNA ligase.

mM dithiothreitol; 10% (v/v) dimethylsulfoxide; 170 U/mL myokinase; 120 U/mL creatine kinase; and 30 μM RNA ligase in 10 μL (8,16). Reactions were incubated at 17°C for the times indicated.

Product characterizations. Nearest neighbor analyses of the products were performed as described (17). A sequence determination of the synthesized oligodeoxyribonucleotide $dG(pdA)_2pdUpdTp$ was performed as follows: The RNA ligase product was treated with [γ-^{32}P]ATP and PseT 1 polynucleotide kinase as described (8) to yield [5'-^{32}P]$pdG(pdA)_2pdUpdTp$ (10^5 cpm). The 5'-labeled oligomer was treated with wild type polynucleotide kinase to remove the 3'-phosphate as described (11). The resulting oligomer was partially digested with snake venom phosphodiesterase to yield a series of 5'-labeled fragments using the procedures of Holley et al. (18) and Wu (19). The partial digestion products were separated by standard two-dimensional fingerprinting techniques (20).

High pressure liquid chromatography. Reverse phase high pressure liquid chromatography (HPLC) was performed using a μBondapak C_{18} column (0.4 X 30 cm) from Waters Associates. The sample was applied in 1.0 mL of 0.01 M ammonium phosphate, pH 7.0 and the column eluted with a linear gradient (100 mL) of 7 to 16% acetonitrile in the phosphate buffer.

Polyacrylamide gel electrophoresis. The denaturing polyacrylamide slab gel was prepared and run using the procedure of Maxam and Gilbert (21).

RESULTS

Single nucleotide addition reactions. The single nucleotide addition reaction of RNA ligase added 3',5'-nucleoside bisphosphates to several oligodeoxyribonucleotides (Table 2). The exact reaction conditions are given in Table 1. In general, these conditions consisted of an excess concentration of the pdNp donor over the oligomer acceptor, a low ATP concentration, and an ATP regeneration system. Previous small-scale reactions between pdAp, pdGp, pdCp, pdTp, or pdUp and the acceptor $dA(pdA)_4$ demonstrated that such conditions yielded greater than 85% product after 1-10 days of reaction (7). We employed these same conditions for the synthetic reactions between pdNp donors and other acceptors. As seen in Table 2, nmol amounts of products can be synthesized under these conditions. Yields ranged from 13% to 95%.

After the RNA ligase reactions, the substrates and products were purified either by chromatography on Whatman 3 MM paper in solvents I or III or by separation on a DEAE Sephadex A-25 column. For example, the mixture after reaction of pdUp with dGpdApdA (reaction 1) was purified by DEAE column

Table 2. Single Nucleotide Addition Reactions[a]

Reaction #	Acceptor	Acceptor Amt. (nmol)	Donor	Time (days)	Yield (%)[b]
1	dGpdApdA	100	pdUp	21	78
2	dGpdApdA	50	pdUp	9	44
3	dGpdApdA	119	pdUp	8	40
4	dGpdApdA	100	pBrdUp	21	49
5	dGpdApdA	40	pdTp	14	46
6	dGpdApdA	50	pdTp	9	31
7	dGpdApdA	135	pdTp	8	13
8	dGpdApdApdU	56	pdTp	14	80
9	dGpdApdApdU	18	pdTp	9	61
10	dGpdApdApdU	35	pdTp	13	47
11	dGpdApdApdT	25	pdUp	13	52
12	dCpdCpdA	200	pdAp	11	95
13	dCpdCpdA	189	pdAp	8	95
14	dTpdTpdTpdT	81	pdCp	5	95
15	dGpdG$(pdA)_2(pdT)_2(pdC)_2$	50	pdAp	5	31
16	dGpdG$(pdA)_2(pdT)_2(pdC)_2$	50	pdAp	11	63[c]

[a]Reaction mixtures were incubated as described in Table 1 and Materials and Methods.
[b]Yields are based on the amount of the limiting substrate, the acceptor, converted to product.
[c]Reaction mixture included 50 nmol RNase A.

chromatography. The profile (Figure 1) shows materials absorbing 260 nm light eluting at concentrations of 0.5, 0.75, and 1.1 M triethylammonium acetate. The yield, calculated with respect to the amount of acceptor converted to product, was 78%. This elution profile also demonstrates the absence of either significant contaminating products arising from nucleases or of the reaction intermediate, A-5'pp5'-dUp. The absence of such substances is critical if this general methodology is to be useful for synthesis.

After the single nucleotide addition reactions, the products containing 3'-phosphates could be converted to acceptors by treatment with phosphomonoesterase to allow a second single nucleotide addition. For example,

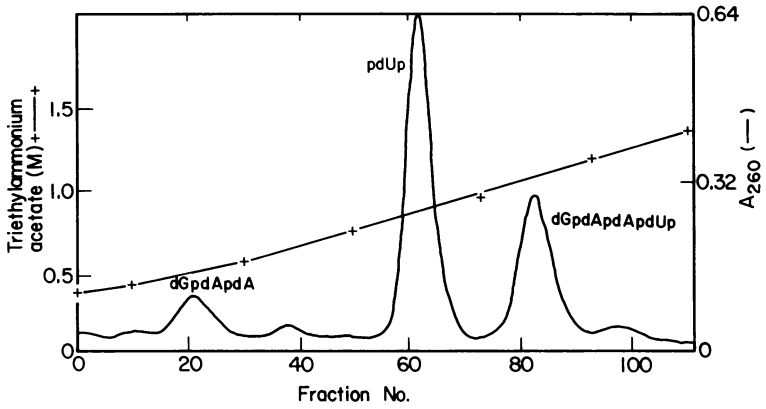

Figure 1. Ion exchange chromatography of the reaction mixture of pdUp and dGpdApdA (reaction 1) on a DEAE Sephadex A-25 (acetate) column. The column was eluted with a linear gradient of 0.4 to 1.5 M triethylammonium acetate, pH 5.8.

dGpdApdApdUp obtained from the reaction of dGpdApdA with pdUp (reactions 1-3) was dephosphorylated and converted to dGpdApdApdUpdTp by the addition of pdTp (reactions 8-10). Thus, the single nucleotide addition reaction of RNA ligase may be used for the stepwise synthesis of deoxyoligomers.

The product from each of the single nucleotide addition reactions listed in Table 2 was characterized by its UV absorption spectrum. The observed spectral ratios were consistent with reported or calculated values (data not shown). In particular, higher 270/260 and 280/260 ratios were obtained for dGpdApdApBrdUp than for dGpdApdApdUp, consistent with the ratios for the mononucleotides UMP and BrdUMP (12).

All the products were also characterized by their mobilities on Whatman 3 MM paper in solvents I and III. Paper chromatography in these solvents separates nucleotides and oligonucleotides primarily on the basis of charge although purines tend to migrate more slowly than pyrimidines. As an example of the effects seen, the product dGpdApdApdUp [R_m = 0.5 (relative to pdTp)] migrated more slowly than either the donor pdUp (R_m = 0.86) or the acceptor dGpdApdA (R_m = 1.2). Removal of the 3'-phosphate by treatment with phosphomonoesterase yielded more rapidly migrating material, consistent with the conversion of the product dGpdApdApdUp to dGpdApdApdU (R_m = 1.1).

In addition to these analyses, the dGpdApdApdUpdTp, obtained after the stepwise addition of pdUp and pdTp to dGpdApdA, was partially digested with snake venom phosphodiesterase and the products separated by standard two-

dimensional fingerprinting techniques (20). The observed fingerprint pattern (Figure 2) was consistent with the expected sequence pdGpdApdApdUpdT.

Deoxyoligomer joining reactions. The deoxyoligomer joining reaction of RNA ligase was used to synthesize two oligodeoxyribonucleotides: $dT(pdT)_7$-$(pdG)_2(pdA)_2pdUpdTp$ (reactions 17, 18, and 19) and $dT(pdT)_7(pdG)_2(pdA)_2$-$(pdT)_2(pdC)_2pdAp$ (reaction 20) (Table 3). The reaction conditions, which were developed from previous small-scale reactions (8,22), involve a four-fold excess of acceptor over donor, a low ATP concentration, and an ATP regeneration system. As seen in Table 3, these conditions resulted in yields

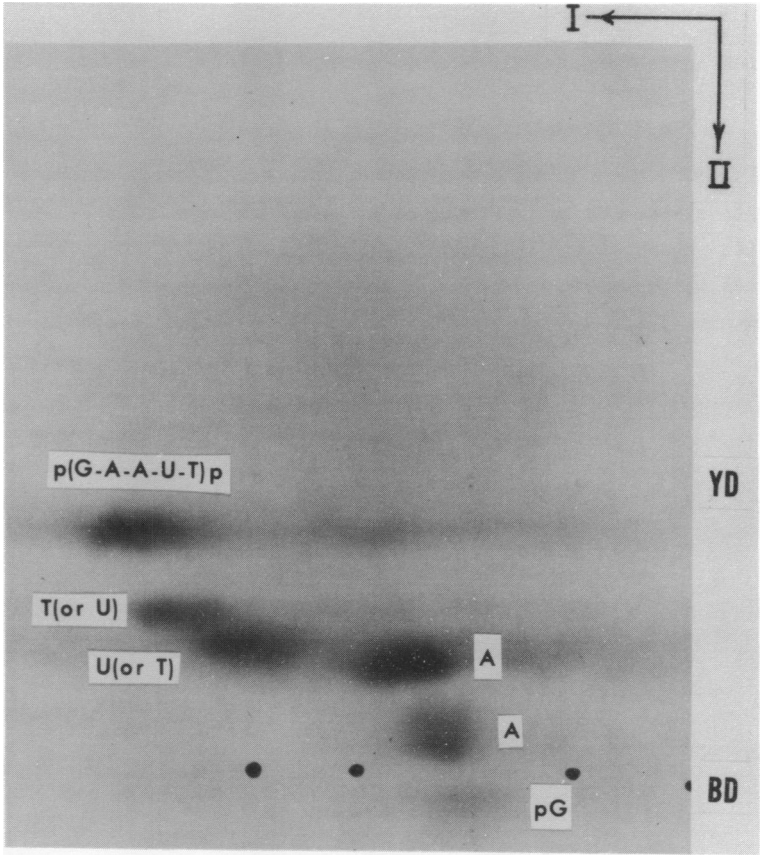

Figure 2. Autoradiogram of a two-dimensional fingerprint of a partial snake venom phosphodiesterase digestion of [5'-^{32}P]pdGpdApdApdUpdT (reaction 8). BD is the dye xylene cyanol FF and YD is orange G. I represents the direction of electrophoresis and II that of homochromotography.

Table 3. Deoxyoligomer Joining Reactions[a]

Reaction #	Acceptor	Amt.[b] (nmol)	Donor	Time (days)	Yield (%)[c]
17	$dT(pdT)_7pdG$	32	pdGpdApdApdUpdTp	11	50
18	$dT(pdT)_7pdG$	35	pdGpdApdApdUpdTp	10	10
19	$dT(pdT)_7pdG$	11	pdGpdApdApdUpdTp	10	28
20	$dT(pdT)_7$	58	$pdGpdG(pdA)_2(pdT)_2(pdC)_2pdAp$	12	19

[a]Reaction mixtures were incubated as described in Table 1 and Materials and Methods.
[b]Amount of acceptor.
[c]% yields are based on the amount of the limiting substrate, the donor, converted to product.

ranging from 10 to 50% and demonstrated that this reaction of RNA ligase can be used to synthesize nmol amounts of oligodeoxyribonucleotides.

After incubation with RNA ligase, the products and the unreacted substrates were separated by chromatography on Whatman 3 MM paper in solvent III. Figure 3 shows the results of this separation for the reaction of

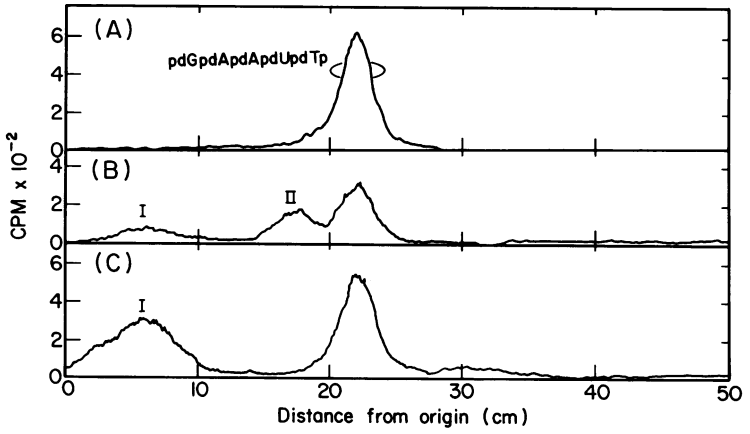

Figure 3. Paper chromatography of the reaction mixture of $dT(pdT)_7pdG$ with [5'-^{32}P]pdGpdApdApdUpdTp. The chromatograms were developed in solvent III. Panel A: control of [5'-^{32}P]pdGpdApdApdUpdTp before incubation; panel B: complete reaction mixture at 24 hr; panel C: complete reaction mixture at 170 hr.

$dT(pdT)_7pdG$ with [5'-^{32}P]pdGpdApdApdUpdTp. Chromatography of the donor alone demonstrated that greater than 95% of the radioactivity comigrated with pdGpdApdApdUpdTpA. After incubation with $dT(pdT)_7pdG$ in an RNA ligase reaction mixture, two new peaks of radioactivity were observed, equivalent to 25% (peak I) and 36% (peak II) transfer of the radioactivity to product and intermediate, respectively (B). Continued incubation for 170 hr resulted in the elimination of the reaction intermediate and a transfer of 50% radioactivity to product (C).

The products isolated from the deoxyoligomer joining reactions were characterized by nearest neighbor analyses as described in Materials and Methods. Analyses of the product of the reaction of $dT(pdT)_7pdG$ with [5'-^{32}P]pdGpdApdApdUpdTp demonstrated that 91% of the radioactivity was located between two dGuo residues (Figure 4, A and B). This result is consistent with the formation of the expected RNA ligase product, $dT(pdT)_7$-pdG[3'→5'-^{32}P]pdG$(pdA)_2$pdUpdTp. The remainder of the radioactivity (9%) was located between a 5'-dG residue and a 3'-dT. This result suggested that a slight amount of the acceptor was degraded during the course of the reaction, producing a dT-terminated acceptor.

The product of the reaction of $dT(pdT)_7$ with [5'-^{32}P]pdGpdG$(pdA)_2(pdT)_2$-$(pdC)_2$pdAp was also characterized by nearest neighbor analysis (Figure 4, C and D). A transfer of 83% of the ^{32}P label from a 5'-dG to a 3'-dT was observed. This transfer is consistent with the formation of the product $dT(pdT)_6pdT$[3'→5'-^{32}P]pdGpdG$(pdA)_2(pdT)_2(pdC)_2$pdAp. However, any degradation of the acceptor $dT(pdT)_7$ during the reaction could not be monitored by this analysis since the reaction of the donor with degraded acceptor molecules would also result in a 5'-dG to 3'-dT transfer of ^{32}P. The remainder of the radioactivity (17%) migrated with a mobility similar to that of the intermediate derivative Ado-5'PP5'-dGuo suggesting that the isolated product contained intermediate as a contaminate.

The product $dT(pdT)_7pdG$[3'→5'-^{32}P]pdGpdApdApdUpdTp was further characterized by reverse phase HPLC. The product was eluted from the column with a linear gradient of acetonitrile and the profile is shown in Figure 5. Two peaks of radioactivity were observed. Comparison of this profile with one obtained with the oligodeoxyribonucleotide $dT(pdT)_7pdG$ (Panel B) demonstrated that under these conditions peak II eluted almost simultaneously with this marker. Samples from peaks I and II were treated with high specific activity [γ-^{32}P]ATP and polynucleotide kinase and applied to a denaturing polyacrylamide gel as described in Materials and Methods. An autoradiogram of the gel

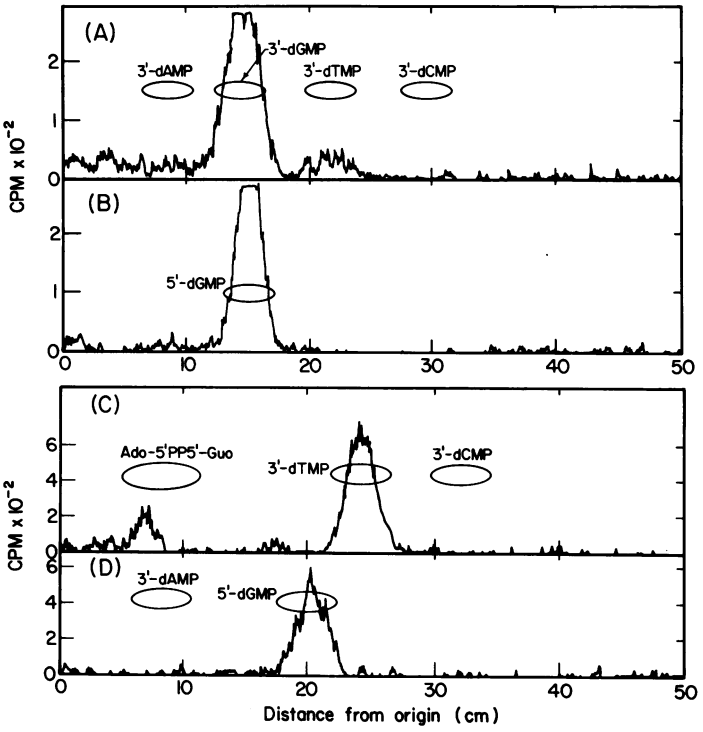

Figure 4. Nearest neighbor analyses of the products isolated after the deoxyoligomer joining reactions. Panels A and B: product from the reaction of $dT(pdT)_7pdG$ with [5'-^{32}P]pdGpdApdApdUpdT, after incubation with spleen phosphodiesterase and micrococcal nuclease (A) or with snake venom phosphodiesterase and pancreatic DNase I (B); panels C and D: product of the reaction of $dT(pdT)_7$ with [5'-^{32}P]pdGpdG$(pdA)_2(pdT)_2(pdC)_2$pdAp as in A (C) or as in B (D).

(Figure 6) demonstrated that the material from peak II migrated as a single component that moved more slowly than authentic [5'-^{32}P]$pdT(pdT)_{11}$. These results are consistent with the formation of the expected product $dT(pdT)_7$-$(pdG)_2(pdA)_2$pdUpdTp. (The material in peak I was a mixture of the 5'-labeled donor and the expected product.)

3'-phosphate modification reaction of RNA ligase. In earlier studies of the deoxyoligomer joining reaction, when there was an excess of ATP over the acceptor or donor concentrations, we observed an unusual product (8,16). This product, designated I', arose from a modification of the terminal 3'-phosphate of the donor molecule. In order to determine the structure of

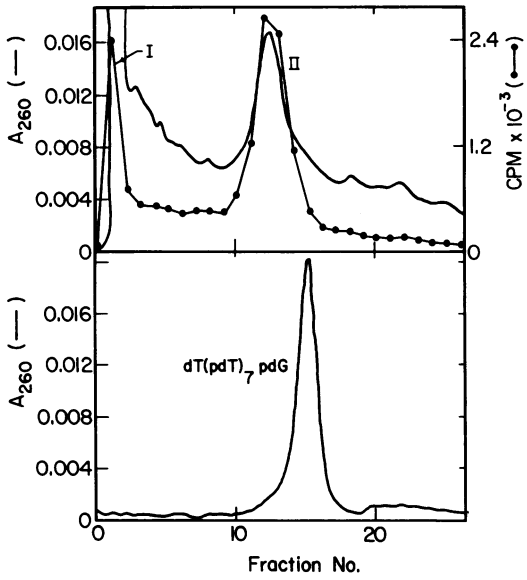

Figure 5. HPLC chromatography of the product dT(pdT)$_7$[3'→5'-^{32}P]pdGpdGpdApdApdUpdT (reactions 18 and 19). Panel A: elution profile of the product of the reaction of dT(pdT)$_7$pdG with [5'-^{32}PpdGpdApdApdUpdTp after treatment with phosphomonoesterase. Panel B: elution profile of dT(pdT)$_7$pdG. A_{260} (——), radioactivity (●——●).

the compound, we synthesized and reacted the pseudodonor [3'-^{32}P]dT(pdT)$_3$pdCp with RNA ligase and a four-fold excess of [^{3}H]ATP. This substrate is not a normal donor because it lacks a 5'-phosphate, however, it allows for the unambiguous characterization of the reaction at the 3'-phosphate group. As seen in Figure 7, paper chromatography of the reaction mixture at zero time yielded two peaks of radioactivity: the ^{32}P comigrated with dT(pdT)$_3$pdCp and the ^{3}H with ATP (A). Treatment of this reaction mixture with phosphomonoesterase converted all the ^{32}P to Pi; the mobility of the ^{3}H was not altered since in this system ATP and Ado comigrate (B). After incubation of the reaction mixture with RNA ligase for 75 hr, followed by treatment with phosphomonoesterase, a new peak of radioactivity (I'), containing both the ^{3}H and ^{32}P and migrating more slowly than dT(pdT)$_3$pdCp or ATP, was observed (C). The appearance of this peak indicated that the terminal 3'-phosphate of the donor molecule had been rendered resistent to phosphomonoesterase by the addition of an Ade containing residue.

Previously, we had speculated that this modification occurred through

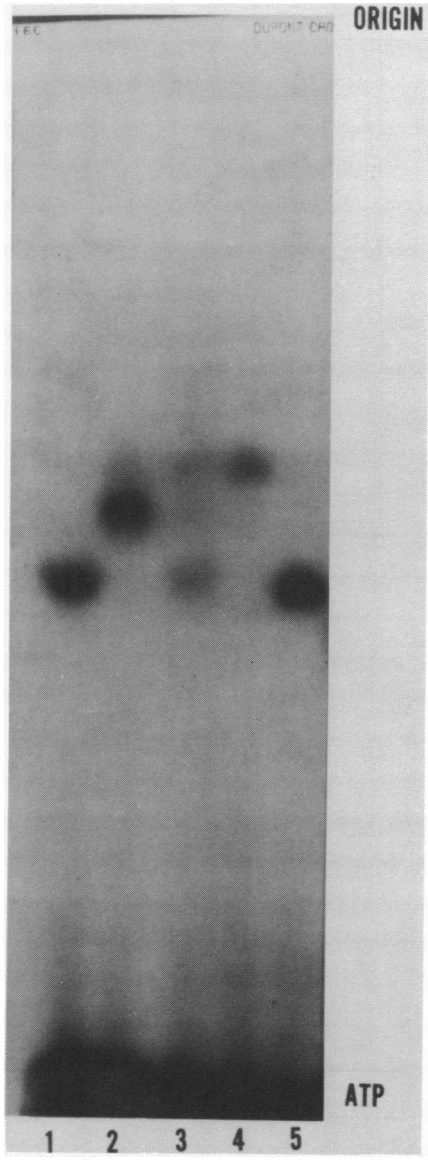

Figure 6. Polyacrylamide gel electrophoresis of [5'-^{32}P](pdT)$_8$(pdG)$_2$(pdA)$_2$-pdUpdT. Lane 1: [5'-^{32}P]pdT(pdT)$_7$pdG; Lane 2: [5'-^{32}P]pdT(pdT)$_{11}$; Lanes 3 and 4: peaks I and II, respectively, isolated from the HPLC column shown in Figure 5A; Lane 5: [5'-^{32}P]dT(pdT)$_7$pdG isolated from the μBondapak C_{18} column shown in Figure 5B.

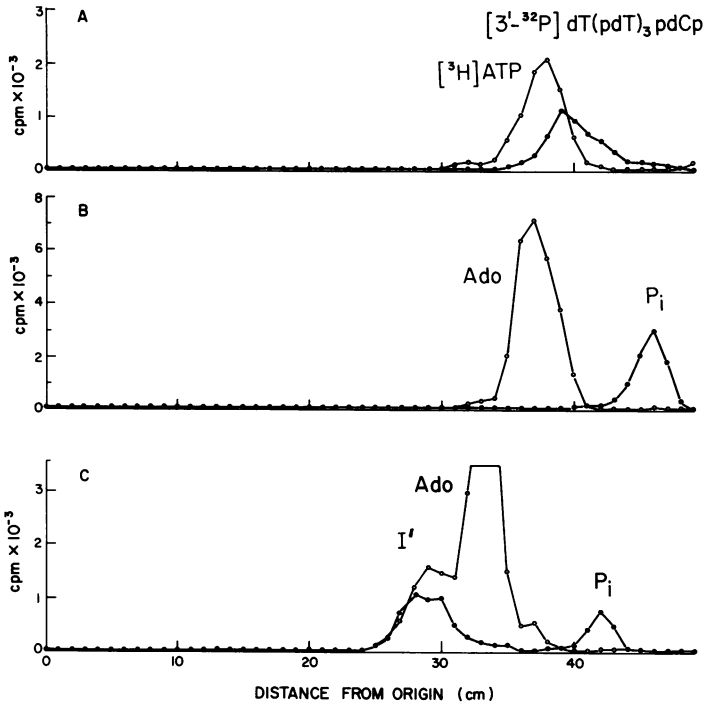

Figure 7. Paper chromatography of the reaction mixture of [3'-^{32}P]dT(pdT)$_3$-pdCp and [^{3}H]ATP. Panel A: complete reaction mixture, zero time; panel B: as in A, but after treatment with phosphomonoesterase; panel C: complete reaction mixture incubated for 75 hr followed by treatment with phosphomonoesterase. ^{32}P, (● —— ●); ^{3}H, (○ —— ○).

the formation of phosphoanhydride bond between the 3'-phosphate of a donor and the α-phosphate of ATP. Such a reaction would yield the product dT(pdT)$_3$dC-3'pp5'-A (8,16). We tested this hypothesis by isolating I' (Figure 8A) and digesting it with various enzymes. In addition, we synthesized the dinucleoside pyrophosphate dC-3'pp5'-A and compared the digestion products derived from it with those from I'. As seen in Figure 8B, treatment of I' with snake venom phosphodiesterase, an enzyme which requires a 3'-hydroxyl group and liberates 5'-nucleoside monophosphates, yielded [^{3}H]AMP and the pseudodonor [3'-^{32}P]dT(pdT)$_3$pdCp. Incubation of dC-3'pp5'-A with snake venom phosphodiesterase cleaved the 3'→5' phosphoanhydride bond, yielding the products dCp and pA (data not shown). Treatment of I' with pancreatic DNase I in addition to snake venom phosphodiesterase yielded

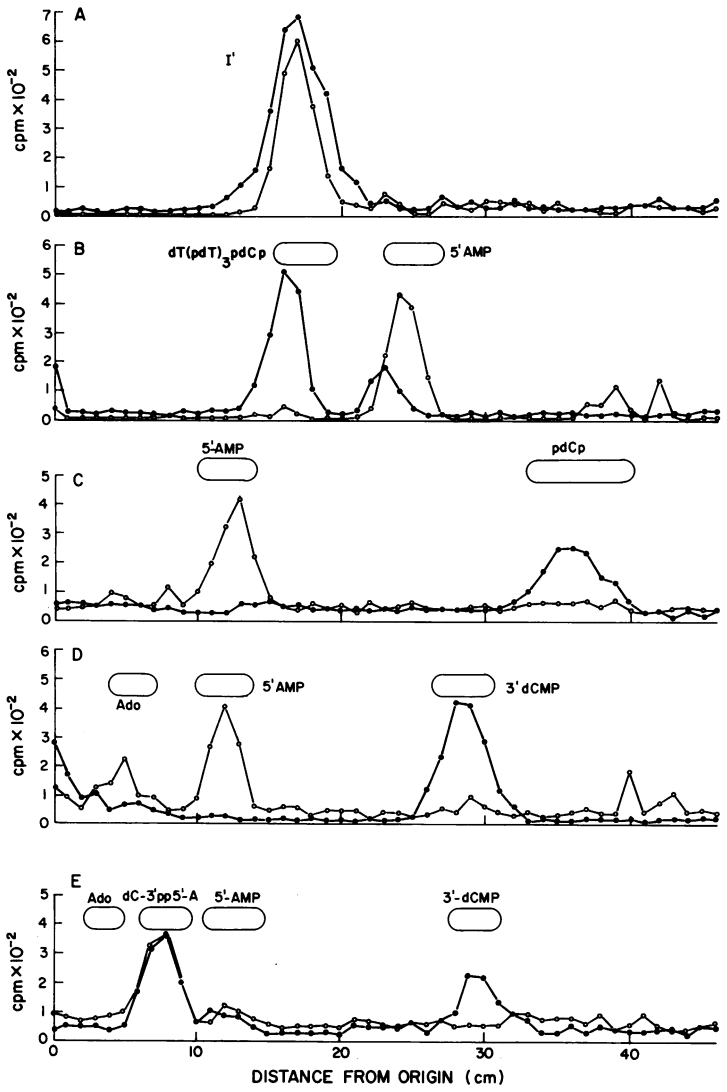

Figure 8. Characterization of the RNA ligase product, I'. Isolated I' was digested and the products separated by chromatography on Whatman No. 1 paper in solvents I (panels A and B) or II (panels C, D, and E). Isolated I' before digestion (A); treated with snake venom phosphodiesterase (B); treated as in panel A plus pancreatic DNase I (C); treated with spleen phosphodiesterase (D); and treated with micrococcal nuclease (panel E). ^{32}P, (●——●); ^{3}H, (○——○).

the radioactive products [3'-^{32}P]pdCp and [^{3}H]AMP (C). Treatment of I' with spleen phosphodiesterase, an exonuclease which requires a 5'-hydroxyl group and yields 3'-nucleoside monophosphates, generated [3'-^{32}P]dCMP and [^{3}H]AMP (D). Similarly, incubation of dC-3'pp5'-A with spleen phosphodiesterase yielded dCp and pA. Finally, digestion of I' with micrococcal nuclease, an endonuclease that yields 3'-nucleoside monophosphates, resulted in a product containing both ^{32}P and ^{3}H and comigrating with authentic dC-3'pp5'-A (Panel E). Previous analyses had shown that the chemically synthesized dC-3'pp5'-A was not cleaved by this enzyme. The production of some labeled dCMP and AMP after cleavage of I' by micrococcal nuclease may reflect the ability of the enzyme to hydrolyze longer oligonucleotides much more efficiently than dinucleoside monophosphates (23). Taken together, these results are consistent with the addition of AMP to the 3'-phosphate of the oligomer, dT(pdT)$_3$pdCp to give dT(pdT)$_3$pdC-3'pp5'-A in which the linkage is *via* a phosphoanhydride bond.

DISCUSSION

Synthetic reactions. We have demonstrated that RNA ligase can be used to synthesize nmol quantities of oligodeoxyribonucleotides having defined sequences. The sequential addition of single 2'-deoxyribonucleoside 3',5'-bisphosphates to an acceptor allows stepwise syntheses (Table 2). Furthermore, the reaction between two oligodeoxyribonucleotides was used to synthesize larger oligodeoxyribonucleotides to form products fourteen and seventeen nucleotides long (Table 3). No template strand was required to align the ends of the two joined oligomers in either case. RNA ligase can extend deoxyoligomers in either the 5'→3' or the 3'→5' direction. For example, dGpdG(pdA)$_2$(pdT)$_2$(pdC)$_2$ was extended at the 3'-end to form dGpdG-(pdA)$_2$(pdT)$_2$(pdC)$_2$pdAp and this product, in turn, was lengthened at its 5'-terminus by reaction with dT(pdT)$_7$ to form the heptadecamer dT(pdT)$_7$-(pdG)$_2$(pdA)$_2$(pdT)$_2$(pdC)$_2$pdAp.

All these reactions taken together (Tables 1 and 3) formed approximately 0.5 μmol of phosphodiester bonds and used less than 1.7 mg of enzyme, an amount equivalent to one-fifth that routinely isolated from 150 g (wet) of T4-infected *E. coli*. (10). Although the reaction mixtures were incubated from five to 21 days with high concentrations of enzyme, we observed little degradation of either products or substrates.

A difficulty encountered in these studies was the low reactivity of an acceptor that was capable of forming a stable duplex structure under our

reaction conditions. The octamer dGpdG$(pdA)_2(pdT)_2(pdC)_2$ was a poor acceptor for pdAp addition (reaction 15) even though reactions with other acceptors terminated with dCyd had resulted in good yields in other reactions (6). We showed previously that converting a single-strand homopolymer acceptor to a duplex by adding a complementary oligodeoxyribonucleotide inhibits the joining reaction (8). These results indicate that duplex structure in the acceptor interfers with productive interaction with the enzyme. We have partially circumvented this problem by disrupting the duplex with the single-strand oligonucleotide binding protein, RNase A (24). The addition of equimolar RNase A and octamer doubled the product yield (compare reactions 15 and 16).

An occasional problem due to the reversal of the RNA ligase reaction during syntheses with oligoribonucleotides has been described by Krug and Uhlenbeck (25). They have characterized an AMP-dependent removal of a pNp residue from 3'-phosphate terminated oligoribonucleotide donors to generate 3'-hydroxyl terminated oligoribonucleotide derivatives. These products can act as acceptors and can thereby compete with the intended acceptor in joining to the donor. In addition, the other product of the reversal, A-5'pp5'-Np, can add to the original acceptor. These reactions result in sequence scrambling and in reduced yields of the correct product. We have preliminary evidence that similar reverse reactions may sometimes occur with oligodeoxyribonucleotide substrates. We do not yet know whether this phenomenon will present a serious problem in deoxyoligoribonucleotide syntheses with RNA ligase.

The 3'-phosphate modification reaction. We have been pursuing enzymatic methods for the synthesis of DNA in order to produce oligodeoxyribonucleotides of very high purity. Generally, the specificity of an enzyme for its substrates and in the reaction it catalyzes should ensure the absence of undesired reaction products which can sometimes arise during chemical syntheses. However, we have observed that when we use the inefficient substrate DNA with RNA ligase, an unanticipated reaction of the 3'-phosphate of the donor molecule may be observed (8,16). We have characterized the product of this modification and find that it arises through the addition of a 5'-AMP to the 3'-phosphate of the donor to form an anhydride linkage. One can explain this reaction by considering the reaction mechanism of RNA ligase (see reviews 1 and 2). Studies of the substrate specificities of the enzyme have revealed the likely existence of three substrate binding sites. One binds ATP; another, the acceptor; and a third, the donor. The acceptor site

requires an oligonucleotide containing three nucleosides and two internucleotide phosphates for efficient binding while the donor site requires only a single nucleotide, provided it contains both 5' and 3' phosphate groups. We speculate that the 3'-phosphate modified product arises from the occasional binding of the donor oligomer in the acceptor site. This binding would occur in such a way that the 3'-phosphate of the donor was positioned so that it would accept the adenylyl group from the activated enzyme. In the usual product forming reaction, the 5'-phosphate of the donor would accept the adenylyl group and the reaction intermediate A-5'pp5'-dN_np would be formed. This scheme for the modification of the 3'-phosphate is supported by two observations. First, the addition of increasing amounts of a 3',5'-dihydroxyl acceptor molecule decreases the formation of the 3'-phosphate modified donor (8,16). Presumably, this inhibition arises because the acceptor competes with the donor for the acceptor site. Secondly, we have been unable to modify the 3'-phosphate of 3',5'-deoxyribonucleoside bisphosphate donors (data not shown). This result would be expected because the acceptor site requires a trinucleoside diphosphate for efficient binding.

Fortunately, the appearance of the 3'-modified product occurs in the presence of high ATP concentrations. The reaction conditions developed for oligodeoxyribonucleotide syntheses use a low ATP concentration and as a result we do not observe significant I' formation during synthesis reactions. This side reaction may, however, sometimes be responsible for minor products.

When a 3'-phosphate terminated oligoribonucleotide is similarly reacted with RNA ligase and ATP a low yield of the 2',3'-cyclic phosphate terminated oligomer is obtained (V. Pyle and R. Gumport, unpublished observation). This product probably arises through the adenylylation of the terminal 3'-phosphate just as it occurs with the deoxyoligomer. Because AMP is a good leaving group, it would be rapidly eliminated by the attack of the adjacent 2'-hydroxyl to form the cyclic phosphate. This latter reaction is consistent with our finding that a number of other leaving groups are eliminated from the 3'-phosphate of ribo-oligomers to form the 2',3'-cyclic phosphate terminated product (9).

Conclusion. The reaction conditions presented here represent our current view of the best methods for using RNA ligase with DNA substrates. These reactions can be a useful adjunct to currently used chemical and enzymatic techniques for DNA synthesis. In particular, they offer methods to synthesize oligodeoxyribonucleotides of high purity.

ACKNOWLEDGEMENTS

We are indebted to D. Soltis and O. C. Uhlenbeck for their gift of wild type and PseT 1 polynucleotide kinases and to P. Modrich and S. A. Narang for gifts of oligodeoxyribonucleotides. We acknowledge the technical assistance of A. Manthey and K. Browning and helpful discussions with M. Moseman McCoy. This work was supported by USPHS NIH GM 25621 and GM 07283.

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[2]Hepes is N-2-hydroxyethylpiperazine-n'-2-ethane sulfonic acid.

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