Iida, Y., & Matsuge, A. (1974) *Mol. Gen. Genet. 129,* 27-35.
Küpper, H., Contreras, R., Khorana, H., & Landy, A. (1976) in *RNA Polymerase* (Losick, R., & Chamberlin, M., Eds.) pp 473-484, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
Losick, R., & Pero, J. (1976) in *RNA Polymerase* (Losick, R., & Chamberlin, M., Eds.) pp 227-246, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
Maitra, U., Lockwood, A., Dubnoff, J., & Guha, A. (1970) *Cold Spring Harbor Symp. Quant. Biol. 35,* 143-156.
Mangel, W., & Chamberlin, M. (1974a) *J. Biol. Chem. 249* 2995-3001.
Mangel, W., & Chamberlin, M. (1974b) *J. Biol. Chem. 249,* 3002-3006.
Mangel, W., & Chamberlin, M. (1974c) *J. Biol. Chem. 249,* 3007-3013.
Matsuge, A. (1972) *Mol. Gen. Genet. 118,* 23-31.
Miller, J. S., & Burgess, R. R. (1978a) *Biochemistry 17* (1st in a series of three papers in this issue).
Miller, J. S., & Burgess, R. R. (1978b) *Biochemistry 17* (2nd in a series of three papers in this issue).
Millette, R., Trotter, C., Herrlich, P., & Schweiger, M. (1970) *Cold Spring Harbor Symp. Quant. Biol. 35,* 135-142.
Nakanishi, S., Adhya, S., Gottesman, M., & Pastan, I. (1974) *J. Biol. Chem. 249,* 4050-4056.
Nakanishi, S., Adhya, S., Gottesman, M., & Pastan, I. (1975) *J. Biol. Chem. 250,* 8202-8208.
Pfeffer, S. R., Stahl, S., & Chamberlin, M. (1977) *J. Biol. Chem. 252,* 5403-5407.
Richardson, J. (1975) *J. Mol. Biol. 91,* 477-487.
Rosenberg, M., Weissman, S., & deCrombrugghe, B. (1975) *J. Biol. Chem. 250,* 4755-4764.
Scaife, J. (1973) *Br. Med. Bull. 29,* 214-219.
Schäfer, R., & Zillig, W. (1973) *Eur. J. Biochem. 33,* 215-226.
Seeburg, P., & Schaller, H. (1975) *J. Mol. Biol. 92,* 261-277.
Seeburg, P., Nusslein, C., & Schaller, H. (1977) *Eur. J. Biochem. 74,* 107-113.
So, A., Davie, R., Epstein, R., & Tissieres, A. (1967) *Proc. Natl. Acad. Sci. U.S.A. 58,* 1739-1746.
Stahl, S., & Chamberlin, M. (1977) *L. Mol. Biol. 112,* 577-601.
Takanami, M., Okamoto, T., & Suguira, M. (1970) *Cold Spring Harbor Symp. Quant. Biol. 35,* 179-187.
Walter, G., Zillig, W., Palm, P., & Fuchs, E. (1967) *Eur. J. Biochem. 3,* 194-201.
Wang, J. (1974) *J. Mol. Biol. 87,* 797-816.
Zillig, W., Zechel, K., Rabussay, D., Schachner, M., Sethi, V., Palm, P., Heil, A., & Seifert, W. (1970) *Cold Spring Harbor Symp. Quant. Biol. 35,* 47-58.

Enzymatic Oligoribonucleotide Synthesis with T4 RNA Ligase†

Thomas E. England and Olke C. Uhlenbeck*

ABSTRACT: The substrate specificity of T4 RNA ligase has been examined to determine whether the intermolecular reaction is sufficiently general to realize its potential in the enzymatic synthesis of oligoribonucleotides of defined sequence. Reactions between a variety of acceptor molecules with 3′- and 5′-hydroxyl groups and donor molecules with 3′- and 5′-phosphates indicate that the minimal substrates are a trinucleoside diphosphate acceptor and a nucleoside 3′,5′-bisphosphate donor. Increasing the chain length of either the acceptor or donor has little effect on the rate or extent of reaction. Although the base composition of the donor has only a small effect on the reaction rate, the presence of uridine in the acceptor greatly reduces the amount of product formed. The presence of a phosphate on the 3′ terminus of the donor molecule permits a unique intermolecular product with a 5′-hydroxyl and a 3′-phosphate. By enzymatically either adding a 5′-phosphate or removing the 3′-phosphate, a new donor or acceptor is prepared so synthesis of an oligomer chain can proceed in either direction. With the simplicity of this enzymatic pathway and the rather broad substrate specificity of T4 RNA ligase, a convenient method for the synthesis of oligoribonucleotides is established.

RNA ligase isolated from T4-infected *E. coli* catalyzes the ATP-dependent formation of a phosphodiester bond between terminal 5′-phosphates and 3′-hydroxyls of oligoribonucleotides (Silber et al., 1972; Walker et al., 1975; Kaufmann & Kallenbach, 1975). Although intramolecular cyclization can occur, intermolecular joining of an acceptor molecule with a 3′-hydroxyl and a donor molecule with a 5′-phosphate is achieved either by using a donor too short to cyclize (Walker et al., 1975) or by protecting the 3′-hydroxyl of the donor molecule (Sninsky et al., 1976; Uhlenbeck & Cameron, 1977). The intermolecular reaction proceeds through the formation of a covalent enzyme-AMP complex (Cranston et al., 1974) and the transfer of the AMP to the 5′-phosphate of the donor to form an adenylylated oligonucleotide (Ohtsuka et al., 1976; Sninsky et al., 1976). Under favorable conditions a majority of the adenylylated donor is tranferred to the acceptor to form the intermolecular product. In one case where the reaction was studied in detail, relatively high concentrations of two oligomers could be joined in excellent yield with modest amounts of enzyme (Uhlenbeck & Cameron, 1977). Thus, the intermolecular reaction of T4 RNA ligase would appear to be of great importance for the enzymatic synthesis of oligoribonucleotides of defined sequence.

In this work we will examine the substrate specificity of

† From the Department of Biochemistry, University of Illinois, Urbana, Illinois 61801. *Received December 5, 1977.* This work was supported by a grant from the National Institutes of Health (GM 19059).

0006-2960/78/0417-2069$01.00/0

RNA ligase in order to determine whether the reaction is sufficiently general to realize its synthetic potential. The approach will be to study the reaction between equimolar concentrations of an acceptor molecule with 3′- and 5′-hydroxyls and a donor molecule with 3′- and 5′-phosphates. A considerable number of donors and acceptors with different chain lengths and sequences will be tested. The presence of the 3′-phosphate on the donor prevents intramolecular cyclization or multiple additions of the donor (Sninsky et al., 1976; Uhlenbeck & Cameron, 1977) so a single intermolecular product with a 5′-hydroxyl and a 3′-phosphate is formed. If the 3′-phosphate is removed from the product with alkaline phosphatase, the molecule can be used as an acceptor in another ligase reaction. If a 5′-phosphate is added to the product with polynucleotide kinase and ATP, the oligomer can be a donor in another ligase reaction. An example of such a bidirectional synthetic pathway will be given in order to demonstrate the flexibility of oligonucleotide synthesis with RNA ligase.

Materials and Methods

Enzymes. Ribonuclease A and bacterial alkaline phosphatase were purchased from Calbiochem and stored as 1 mg/mL solutions in 0.1 M NaCl, 10 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.4) at −20 °C. Spleen phosphodiesterase and venom phosphodiesterase were purchased from Worthington Biochemical Co. and the enzymatic activity was assumed to be correct without further assay. Spleen phosphodiesterase was stored at −20 °C in 10 mM EDTA, 100 mM potassium phosphate (pH 6.5) at a concentration of 17 units/mL. Venom phosphodiesterase was stored at −20 °C in 10 mM $MgCl_2$, 20 mM Tris-HCl (pH 8.5) at a concentration of 10 units/mL.

T4 RNA ligase was purified from *E. coli* infected with T4 *am* 4314 (McCoy, Lubben, & Gumport, manuscript in preparation). The enzyme used in this study was free of ribonucleases and essentially homogeneous with a specific activity of 2100 units/mg. The enzyme was stored at 2000 units/mL in 25 mM KCl, 1 mM dithiothreitol, 50 mM Hepes (pH 7.5), and 50% glycerol at −20 °C.

T4 polynucleotide kinase was purified from the same preparation of phage infected cells (Cameron & Uhlenbeck, 1977). The enzyme was stored at 1000 units/mL in 25 μM ATP, 5 mM 2-mercaptoethanol, 25 mM potassium phosphate (pH 7.0), and 50% glycerol at −20 °C.

Chromatography of Oligonucleotides. Trial synthetic reactions with polynucleotide kinase or RNA ligase and degradative identification reactions with nucleases were analyzed by descending chromatography on Whatman 3MM paper. The following (v/v) mixtures of 95% ethanol and 1 M ammonium acetate (pH 7.0) were used to develop the chromatograms: solvent A, 30/70; solvent B, 50/50; solvent C, 60/40; solvent D, 70/30. Development times varied from 12 to 48 h. Oligonucleotides were located by viewing the chromatogram under an ultraviolet lamp or, if a radiolabel was present, by cutting the chromatogram in 1-cm strips, and counting in 4% 2,5-diphenyloxazole in toluene with a liquid scintillation counter. Oligonucleotides were recovered from paper chromatograms by washing the paper with absolute ethanol to remove ammonium acetate and elution with water (Thach, 1966). Since impurities eluted from paper often inhibit enzyme reactions, it was necessary to further purify the oligomer on a Bio-Gel P2 (100–200 mesh) column equilibrated with water.

For preparative scale reactions purification was normally achieved by ion-exchange column chromatography on DEAE-Sephadex A-25. The 10-mL columns were eluted with 200–300-mL linear gradients of triethylammonium bicarbonate (pH 7.5) from 0.3 to 1.0 M. The salt was removed from pooled fractions by repeated evaporation in vacuo with methanol. A Bio-Gel P2 column was often used subsequently to ensure that all the salt had been removed.

Nucleotides and Oligonucleotides. [γ-^{32}P]ATP was a gift of R. I. Gumport. ATP and pA3′p[1] were obtained from Sigma Chemical Co. pTp, pG3′p, and pA2′p were purchased from P-L Biochemicals Inc. Cytidine, uridine, deoxyadenosine, deoxyguanosine, and deoxycytidine 3′,5′-bisphosphates were synthesized from the corresponding nucleoside 3′-monophosphates with ATP and polynucleotide kinase. In each case, the reaction mixture contained 2 mM nucleoside 3′-monophosphate, 2.2 mM ATP, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 50 mM Tris-HCl (pH 9.5), 50 μg/mL bovine serum albumin, and 47.5 units/mL polynucleotide kinase. After incubation for 90 min at 37 °C, the reaction mixture was streaked on Whatman 3 MM paper and the chromatogram was developed in solvent D (for cytidine, uridine, deoxycytidine) or solvent B (for deoxyadenosine and deoxyguanosine) to separate the 3′,5′-bisphosphate from unreacted 3′-monophosphate and ATP. The products were eluted from the paper and repurified on Bio-Gel P2 prior to use. This procedure may be used to produce modest amounts of 3′,5′-bisphosphates, but the chemical method in the following paper (Barrio et al., 1978) is more convenient for large scale preparations. [5′-^{32}P]pA3′p was produced enzymatically in the same way using [γ-^{32}P]ATP and solvent B for purification.

Acceptor oligoribonucleotides with 3′,5′-hydroxyls were obtained in a variety of means. $(Ip)_nI$ ($n = 1 \rightarrow 5$) were purchased from Miles Laboratories. $(Ap)_nA$ ($n = 1 \rightarrow 5$) and $(Up)_nU$ ($n = 1 \rightarrow 5$) were obtained by limited alkaline hydrolysis of the homopolymer, removal of the 2′(3′) terminal phosphate with alkaline phosphatase and separation of the oligomers by column chromatography (Martin et al., 1971; Uhlenbeck & Cameron, 1977). Nonradioactive and ^{3}H-labeled $(Ap)_3C$ were prepared as described by Uhlenbeck & Cameron (1977). The series of oligonucleotides $UpU(pC)_n$ and the trinucleotides ApUpG, UpApG, UpCpG, UpCpA, GpApC, GpApA, and ApApG were prepared enzymatically from the corresponding dinucleotides with polynucleotide phosphorylase (Thach, 1966). ^{3}H-labeled $(Up)_2U$ was prepared as described in Uhlenbeck et al. (1970).

Donor oligonucleotides with both 3′- and 5′-terminal phosphates were synthesized by one of two methods. In the first, T4 polynucleotide kinase was used to add a 5′-phosphate to an oligomer with a 3′-phosphate. This reaction was carried out at high pH for carefully controlled incubation times to limit the extent of removal of the 3′-phosphate by the phosphatase activity in polynucleotide kinase (Cameron & Uhlenbeck, 1977). $p(Up)_5$, $p(Cp)_2Gp$, and $p(Cp)_4Gp$ were prepared by this method from $(Up)_5$, $(Cp)_2Gp$, and $(Cp)_4Gp$, respectively, according to Uhlenbeck & Cameron (1977) and purified by column chromatography. The second method for the preparation of donor molecules involves the removal of the 3′-terminal nucleoside of an oligomer with a 5′-phosphate by periodate oxidation and β elimination (Keith & Gilham, 1974). The $(pA)_n$ series was prepared by digestion of poly(A) with *Neurospora crassa* nuclease (Walker et al., 1975) and the terminal nucleoside was removed as described by Sninsky et al. (1976) to give the $p(Ap)_n$'s ($n = 2 \rightarrow 5$).

[1] Abbreviations used: one letter abbreviations for oligonucleotides and nucleoside 3′(2′),5′-bisphosphates; pA3′p, adenosine 3′,5′-bisphosphate; pA2′p, adenosine 2′,5′-bisphosphate; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid. BAP, SPD, and RNase A are bacterial alkaline phosphatase, spleen phosphodiesterase, and ribonuclease A, respectively.

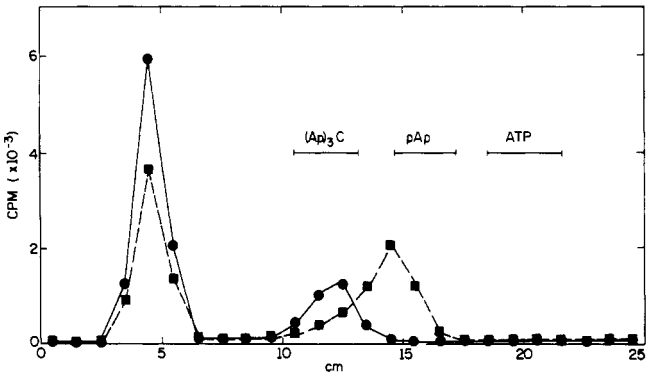

FIGURE 1: Intermolecular reaction of $(Ap)_3C$ and pA3′p. [*Cyd*-^{3}H]-$(Ap)_3C$ (0.1 mM, 460 Ci/mol) and [5′-^{32}P]pA3′p (0.13 mM, 15 Ci/mol) were incubated in the usual Hepes buffer (see Materials and Methods) with ATP (0.5 mM) and RNA ligase (210 units/mL) for 1 h at 37 °C. The chromatogram was developed with solvent A for 16 h. Circles represent ^{3}H label and squares are ^{32}P label.

RNA Ligase Reactions. The standard assay to measure the reactivity of a donor utilized a common acceptor, $(Ap)_3C$. Reactions (30 μL) were carried out in 200-μL polyethylene tubes (Bel-Art) under the following conditions: 0.1 mM [*Cyd*-^{3}H]$(Ap)_3C$ (330 Ci/mol), 0.1 to 0.3 mM donor, 0.5 mM ATP, 20 mM $MgCl_2$, 3.3 mM dithiothreitol, 50 mM Hepes (pH 8.3), 10 μg/mL bovine serum albumin, and 7–350 units/mL T4 RNA ligase. Fixed-time assays were incubated at 37 °C for 60 min and the entire reaction mixture was spotted at the origin of a paper chromatogram. For kinetic studies, $(Ap)_3C$ of higher specific activity was used and aliquots were withdrawn and spotted at the appropriate times. Paper chromatograms were developed in solvent A and the yield was determined as the percentage of the total radioactivity present in the product.

Assays to compare the reactivity of different acceptors employed a common donor, pA3′p. The reactions were carried out in the same manner as above with 0.1 mM acceptor and 0.2 mM pA3′p. The yield was calculated as the fraction of acceptor converted into product either from ^{3}H label in acceptor or from ^{32}P label in donor.

The enzymatic synthesis of $(Ap)_3CpApUp$ was carried out in two steps. In a total volume of 30 μL, 1 mM [*Cyd*-^{3}H]-$(Ap)_3C$ (330 Ci/mol), 2.8 mM pA3′p, 3.3 mM ATP was incubated for 1 h at 37 °C with 175 units/mL T4 RNA ligase in the same buffer used above. The 3′-terminal phosphate was removed from the product of the reaction, $(Ap)_3CpAp$, by first incubating the reaction at 100 °C for 5 min to inactivate the RNA ligase and then adding 5 μg of alkaline phosphatase and incubating for 1 h at 37 °C. The $(Ap)_3CpA$ product (yield > 90%) was purified on DEAE-Sephadex as described above. The $(Ap)_3CpApUp$ was prepared by incubating in a 30-μL reaction of 0.25 mM [*Cyd*-^{3}H]$(Ap)_3CpA$ (330 Ci/mol), 0.47 mM pUp, 1 mM ATP, and 175 units/mL RNA ligase in the usual buffer for 1 h at 37 °C.

$(Ap)_3Cp(Ap)_3CpAp$ was also synthesized in two steps. First, $(Ap)_3CpAp$ was made as outlined above and purified by column chromatography. The donor oligomer, $p(Ap)_3CpAp$, was obtained by incubating 0.25 mM [*Cyd*-^{3}H]$(Ap)_3CpAp$ (330 Ci/mol) and 0.83 mM [γ-^{32}P]ATP (80 Ci/mol), with 19 units/mL polynucleotide kinase in 10 mM $MgCl_2$, 50 μg/mL bovine serum albumin, and 0.1 M cyclohexylaminoethanesulfonic acid (pH 9.5). After incubation for 2 h at 37 °C, the reaction mixture was purified on DEAE-Sephadex. The product, [5′-^{32}P,*Cyd*-^{3}H]$p(Ap)_3pCpAp$ (yield 31%), was identified as the peak containing both radiolabels. The ligation reaction to form $(Ap)_3Cp(Ap)_3CpAp$ contained 0.25 mM $(Ap)_3C$, 0.05 mM [5′-^{32}P,*Cyd*-^{3}H]$p(Ap)_3CpAp$, 0.25 mM ATP, and 330 units/mL T4 RNA ligase in the usual buffer. After incubating at 37 °C for 1 h, the reaction was analyzed by descending paper chromatography as shown in the results.

Product Characterizations. Oligonucleotides were eluted from paper chromatograms as described above and subjected to degradation in one or more of the following reaction mixtures: (a) 20 mM Tris-HCl (pH 8.5), 20 mM $MgCl_2$, 1 unit/mL venom phosphodiesterase; (b) 100 mM potassium phosphate (pH 6.5), 1 mM EDTA, and 1.7 units/mL spleen phosphodiesterase; (c) 0.2 M Tris-HCl (pH 8.2), 10 mM $MgCl_2$, 0.2 M NaCl, and 0.1 mg/mL ribonuclease A; and (d) same as c plus 0.1 mg/mL alkaline phosphatase. In all cases, the 30-μL identification reactions contained 1 to 4 nmol of oligomer and were incubated at 37 °C for 1 to 4 h. The reaction mixtures were analyzed by descending paper chromatography with appropriate markers.

Results

Joining of $(Ap)_3C$ and pAp. Nucleoside 3′,5′-bisphosphates can be shown to be donors in the ATP-dependent T4 RNA ligase reaction by analysis of the reaction between [*Cyd*-^{3}H]-$(Ap)_3C$ and [5′-^{32}P]pA3′p by descending paper chromatography (Figure 1). After reaction, the majority of the ^{3}H label originally present in $(Ap)_3C$ migrates as a single, less mobile peak suggesting that a longer oligomer has been made. Coincident with the ^{3}H label in the product was ^{32}P label originally present in pA3′p. From the specific activities of $(Ap)_3C$ and pA3′p it can be calculated that equal amounts of donor and acceptor are present in the product. The product could be confirmed to have the structure $(Ap)_3CpAp$ by a variety of criteria. In Figure 2A, rechromatography of the isolated product again shows comigration of the two radiolabels. An identical profile is obtained when the product is treated with venom phosphodiesterase. The resistance of the product to this exonuclease confirms the presence of a 3′-phosphate. Treatment of the product with alkaline phosphatase (Figure 2B) results in a slight increase in mobility indicating removal of the 3′-phosphate, but continued comigration of the two labels indicating that the labeled 5′-phosphate of pA3′p is now in an internal position. When the product is treated with spleen phosphodiesterase (Figure 2C), the ^{3}H and ^{32}P labels comigrate with a 2′(3′)-CMP marker, confirming that ligation has occurred in the expected position and the 5′ terminus of the product is a free hydroxyl. When the product is reacted with ribonuclease A (Figure 2D), a single peak of ^{3}H and ^{32}P radioactivity comigrating with $(Ap)_3Cp$ marker is obtained. Confirmation that the ^{32}P label originally present on the 5′-phosphate of pA3′p is now on the 3′-terminus of $(Ap)_3Cp$ is obtained by treatment of the product with ribonuclease A and alkaline phosphatase (Figure 2E). In this case, the ^{3}H label migrates with $(Ap)_3C$ and the ^{32}P label migrates as inorganic phosphate. Thus, the analysis in Figure 2 confirms the product peak in Figure 1 to be $(Ap)_3CpAp$.

It may be noted in Figure 1 that a majority of the ^{32}P radioactivity not in product no longer migrates as pA3′p but rather in a slightly slower position. This slower moving material can be identified as the intermediate in the reaction A5′pp5′Ap by the fact that the ^{32}P label is resistant to alkaline phosphatase. Adenylylated donors of the same sort have been reported by other workers (Sninsky et al., 1976; Ohtsuka et al., 1976; Sugino et al., 1977). RNA ligase reactions run at a high pH (Uhlenbeck & Cameron, 1977) and with a slight molar excess of donor over acceptor minimize the reduction of yield caused

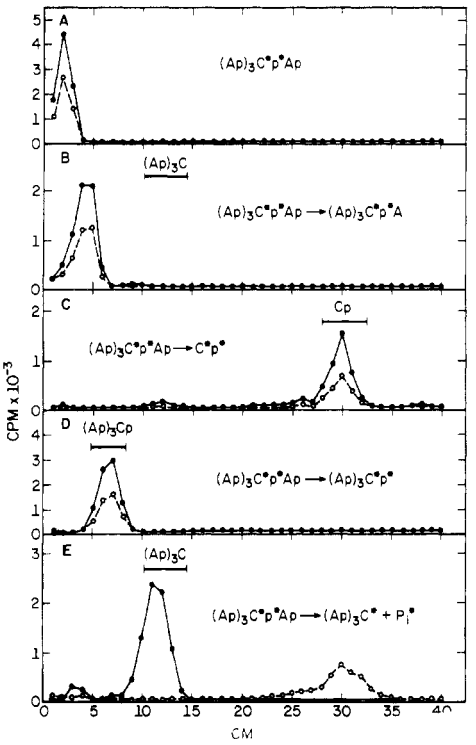

FIGURE 2: Nuclease degradation of [^{3}H, ^{32}P](Ap)$_3$CpAp. After hydrolysis with nucleases (see Materials and Methods), reaction mixtures were analyzed by descending paper chromatography in solvent B (36 h): (A) untreated (Ap)$_3$CpAp; (B) treatment with BAP to produce [^{3}H, ^{32}P](Ap)$_3$CpA; (C) treatment with SPD to produce [^{3}H, ^{32}P]Cp; (D) treatment with RNase A to produce [^{3}H, ^{32}P](Ap)$_3$Cp; and (E) treatment with RNase A plus BAP to produce [^{3}H](Ap)$_3$C and [^{32}P]phosphate. Solid circles are ^{3}H label and open circles are ^{32}P label.

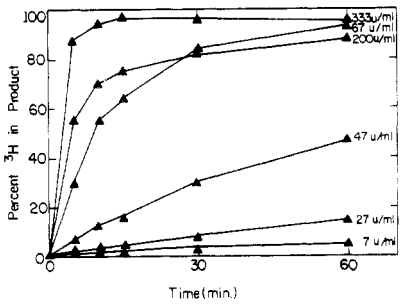

FIGURE 3: Kinetics of the reaction of (Ap)$_3$C and pA3′p at varying concentrations of RNA ligase (see Materials and Methods).

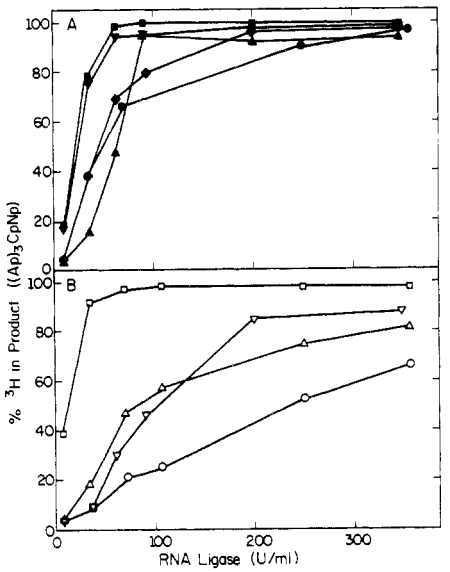

FIGURE 4: Ligation of [*Cyd*-^{3}H](Ap)$_3$C and the ribonucleoside 3′,5′-bisphosphates (A) and deoxyribonucleoside 3′,5′-bisphosphates (B) as a function of enzyme concentration. The reactions were performed under the conditions given in Materials and Methods with: (A) (■) pC3′p (0.25 mM); (▼) pU3′p (0.28 mM); (▲) pA3′p (0.23 mM); (●) pG3′p (0.28 mM); (♦) p(Up)$_5$ (0.10 mM); and (B) (□) pdCp (0.24 mM); (▽) pTp (0.25 mM); (△) pdAp (0.20 mM); (○) pdGp (0.23 mM).

by the presence of this second product.

The kinetics of the intermolecular reaction between (Ap)$_3$C and pA3′p at varying enzyme concentrations are presented in Figure 3. At high enzyme concentrations very rapid and nearly quantitative conversion of acceptor into product is observed. However, at lower enzyme concentrations the initial rate is unexpectedly low and the reaction yield at long incubation times is also less than expected. Similar low reaction yields have been seen in the intermolecular reaction between (Ap)$_3$C and p(Up)$_5$ (Uhlenbeck & Cameron, 1977). It is likely that this phenomenon is due to enzyme inactivation during the course of the reaction.

Nucleoside 3′,5′-bisphosphates are the smallest effective donors in the ATP-dependent RNA ligase reaction. At high enzyme concentrations where nearly quantitative yield is obtained with pA3′p, no detectable product is observed with 5′ AMP or with pA2′p. Thus, the 3′ phosphate must be present for activity. In addition, a twofold excess of pA2′p present in a reaction with pA3′p does not alter the rate or extent of the reaction (data not shown). Nucleoside 2′,5′-bisphosphates are therefore neither substrates nor effective inhibitors in the RNA ligase reaction.

Comparison of Donors. In order to compare the relative reactivity of various donors with a common acceptor, 1-h reactions were performed at varying enzyme concentrations. In Figure 4A the four ribonucleoside 3′,5′-bisphosphates were compared as donors using (Ap)$_3$C as acceptor. Although the reaction shows some preference for the pyrimidine substrates, nearly quantitative yields can be obtained with all four substrates above 250 units/mL RNA ligase. In Figure 4B, the four deoxyribonucleotide 3′,5′-bisphosphates are shown also to be donors in a similar series of fixed-time assays. Except for pdCp, the deoxyribonucleoside 3′,5′-bisphosphates were significantly poorer substrates than the corresponding ribonucleosides and even at the higher enzyme levels less than quantitative yields were obtained.

The reactivities of oligoribonucleotide donors are similar to mononucleotide donors when compared with the same acceptor. As seen in Figure 4A, the donor p(Up)$_5$ reacts with (Ap)$_3$C only slightly less well than pU3′p at comparable enzyme concentrations. Two other oligonucleotide donors, p(Cp)$_2$Gp and p(Cp)$_4$Gp, were also observed to react approximately as well as pCp (Table I). Finally, in Table I, the reactivities of the homologous series of donors, p(Ap)$_n$ (n = 1 → 5), with (Ap)$_3$C as acceptor are compared. It is apparent that the chain length of the donor does not greatly affect the extent of reaction. We can therefore conclude that, although the base composition of a donor can affect the extent of a reaction to a limited degree, the chain length of a donor does not contribute significantly to its reactivity in the intermolecular RNA ligase reaction.

Comparison of Acceptors. The relative reactivities of different acceptors were compared by using a 1.5 molar excess of a common donor, pA3′p, and measuring the amount of product formed after 1-h incubation at three (or four) enzyme concentrations (Table II). From these data it is apparent that

TABLE I: A Comparison of Donors.

Donor	12.5 U/mL	37.5 U/mL	62.5 U/mL	125 U/mL	250 U/mL
pAp	8[a]	34	58	88	94
$p(Ap)_2$	6	30	63	88	93
$p(Ap)_3$	7	21	40	67	92
$p(Ap)_4$	5	33	70	92	93
$p(Ap)_5$	5	16	53	80	95
$p(Cp)_2Gp$	51	93	94	98	
$p(Cp)_4Gp$	28	64	74	81	

[a] Yield is expressed as fraction of $(Ap)_3C$ acceptor converted into product.

TABLE II: A Comparison of Acceptors.

Acceptor	14 U/mL	35 U/mL	170 U/mL	350 U/mL
ApA	0[a]	0	0	0
$(Ap)_2A$	58	84	>95	>95
$(Ap)_3A$	66	92	>95	>95
$(Ap)_4A$	78	93	>95	>95
$(Ap)_5A$	86	95	>95	>95
IpI		0	0	0
$(Ip)_2I$		2	12	64
$(Ip)_3I$		14	59	81
$(Ip)_4I$		16	53	53
$(Ip)_5I$		21	66	87
UpU		0	0	0
$(Up)_2U$		0	6	15
$(Up)_3U$		0	5	13
$(Up)_4U$		0	5	19
$(Up)_5U$		4	7	14
UpUpC		0	6	11
$UpU(pC)_2$		13	46	78
$UpU(pC)_3$		16	42	72
$UpU(pC)_4$		17	35	57
$UpU(pC)_5$		30	81	100
ApUpG		2	23	45
UpApG		4	14	80
UpCpG		8	46	79
UpCpA		8	18	32
GpApC		30	62	95
GpApA		60	77	88
ApApG		31	69	100

[a] Yield is expressed as fraction of acceptor converted into product.

none of the dinucleoside monophosphates take part in the intermolecular reaction. Further attempts to utilize a number of different dimers at higher concentrations and under different reaction conditions were not successful. All 11 trinucleoside diphosphates tested were acceptors in the intermolecular reaction, establishing the minimal chain length required for an active acceptor. After this minimal length requirement has been met, very little chain length dependence upon yield is observed for the three homologous series tested. At a given enzyme concentration, yields with tetramer acceptors are only slightly higher than those with the corresponding homologous trimer.

On the other hand, for a given chain length, the base composition of the acceptor has a large effect on yield. With the three homologous series tested, the oligo(A)s react more readily than the oligo(I)s and the oligo(U)s react the least well. While nearly quantitative intermolecular yields could be obtained with the oligo(A)s and the oligo(I)s, even the highest enzyme concentrations used did not achieve high yields with oligo(U) acceptors. The yield of the intermolecular reaction does not simply depend upon the identity of the 3′-terminal nucleotide of the acceptor. A comparison of the relative reactivity of 11 trimers (Table II) reveals that, if the trimer contains a combination of only A, G, and C residues, relatively high yields are obtained, but if the trimer contains a uridine residue at any position, much lower yields are found. For example, ApUpG and UpApG are poorer acceptors than ApApG. This strong effect of composition upon the efficiency of an acceptor can also be seen with the $UpU(pC)_n$ series (Table I). Since good yields are obtained for the longer oligomers, the poor reactivity of UpUpC is not due to the 3′-terminal C residue but to the internal U residues. A more complete survey of acceptors will be required before the efficiency of an acceptor in an intermolecular reaction can be accurately predicted.

Since the buffer conditions chosen for the reactions in Table II were those optimized for the addition of $(Ap)_3C$ to $p(Up)_5$ (Uhlenbeck & Cameron, 1977), it was of interest to determine whether higher yields could be obtained with the "poor" uridine containing acceptors when different conditions were used. For this reason, the reaction between tritium-labeled $(Up)_2U$ and pAp was studied under a variety of substrate concentrations, pH, and buffer conditions. Although it was found that the low reaction yields for a given enzyme concentration could not be substantially improved by increasing the $(Up)_2U$ concentration, the yield of $(Up)_3Ap$ could be nearly doubled by reducing the $MgCl_2$ concentration in the reaction from 20 to 7.5 mM. Attempts to improve the yield by the addition of sodium or potassium salts or various stabilizers (glycerol, formamide, urea, Carbowax) were generally unsuccessful except in the case of dimethyl sulfoxide. In the presence of 15% Me_2SO, the yield of $(Up)_3Ap$ was more than doubled (to 37% with 350 units/mL RNA ligase). Unfortunately, the increased reaction yield with Me_2SO does not occur at 7.5 mM $MgCl_2$ nor does it occur when $(Ap)_3C$ was used as an acceptor. From these preliminary experiments it appears that the optimal conditions for intermolecular reactions will depend upon the identity of the acceptor. Nevertheless, satisfactory yields may be often obtained under a variety of conditions, including those which optimized the $(Ap)_3C$ plus $p(Up)_5$ addition.

In order to determine which step in the intermolecular reaction determines the low reaction rate with poor acceptors, the time courses for the formation of both the adenylylated intermediate and the product are examined in the $(Up)_2U$ plus pA3′p reaction. Since the available paper chromatographic systems are not able to separate all the starting materials and products in the reaction, two identical reactions were carried out, one with [^{3}H]$(Up)_2U$ and the other with [2,8-^{3}H]ATP. The product, $(Up)_3Ap$, in the first reaction is detected as the ^{3}H-labeled peak migrating slower than $(Up)_2U$ upon paper chromatography with solvent D. Treatment of the second reaction with alkaline phosphatase and separation by paper chromatography in solvent D yield the intermediate as ^{3}H-labeled peak comigrating with A5′pp5′A. Unreacted ATP and the AMP released during the ligation reaction are degraded to adenosine. In Figure 5, the rapid formation of adenylylated intermediate and slow formation of product are observed. Thus, the rate-limiting step in $(Up)_3Ap$ synthesis appears to be the transfer of the adenylylated donor to the acceptor. This view is confirmed in Figure 5 where, in a reaction performed under similar conditions, the transfer of an adenylylated monomer A5′pp5′A to $(Up)_2U$ is also extremely inefficient. However, when a "good" acceptor such as $(Ap)_3C$ is used, the transfer of A5′pp5′A is very rapid (England et al., 1977).

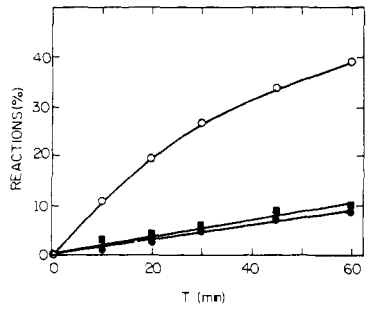

FIGURE 5: Time course of $(Up)_2U$ + pA3'p reaction and ATP-independent reaction of $(Up)_2U$ + A5'pp5'A. Reactions were performed in the usual buffer with $(Up)_2U$ (0.1 mM) and either pAp (0.23 mM) and ATP (0.5 mM) or A5'pp5'A (0.25 mM) at 37 °C with 175 units/mL RNA ligase: (○) formation of adenylylated intermediate A5'pp5'Ap, 100% reaction of pA3'p = 7 nmol; (●) formation of product $(Up)_3Ap$ in ATP-dependent reaction, 100% reaction of $(Up)_2U$ = 3 nmol; (■) formation of product $(Up)_3A$ in ATP-independent reaction, 100% reaction of $(Up)_2U$ = 3 nmol.

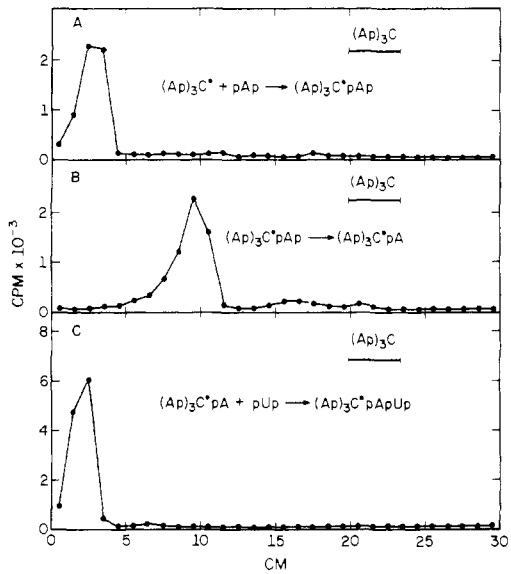

FIGURE 6: Stepwise synthesis of $(Ap)_3CpApUp$. Aliquots of the following reactions were analyzed by descending paper chromatography in solvent B (100 h): (A) RNA ligase joining of [*Cyd*-^{3}H]$(Ap)_3C$ and pA3'p; (B) BAP treatment of [*Cyd*-^{3}H]$(Ap)_3CpAp$; and (C) RNA ligase joining of [*Cyd*-^{3}H]$(Ap)_3CpA$ and pUp.

Multistep Synthesis. In order to illustrate the flexibility of synthesis with RNA ligase, two oligomers were made which both used as a first step, the addition of $(Ap)_3C$ to pA3'p to form $(Ap)_3CpAp$. In the first example, synthesis was continued in the 3' direction to make $(Ap)_3CpApUp$ and in the second example synthesis was continued in the 5' direction to make $(Ap)_3Cp(Ap)_3CpAp$. The stepwise synthesis of $(Ap)_3CpApUp$ as analyzed by descending paper chromatography is summarized in Figure 6. In Figure 6A an aliquot of the preparative scale reaction of [*Cyd*-^{3}H]$(Ap)_3CpAp$ is analyzed. Subsequent treatment of the reaction mixture with alkaline phosphatase resulted in the faster migrating oligonucleotide [*Cyd*-^{3}H]-$(Ap)_3CpA$ (Figure 6B). After purification of the pentamer, the addition of pUp to give the less mobile product [*Cyd*-^{3}H]$(Ap)_3CpApUp$ is shown in Figure 6C. As is evident from the three paper chromatography profiles, both RNA ligase reactions and the removal of the 3'-phosphate were essentially quantitative, suggesting that sequential additions of donors to the 3' end of an oligomer may be performed efficiently.

The synthesis and identification of $(Ap)_3Cp(Ap)_3CpAp$ are

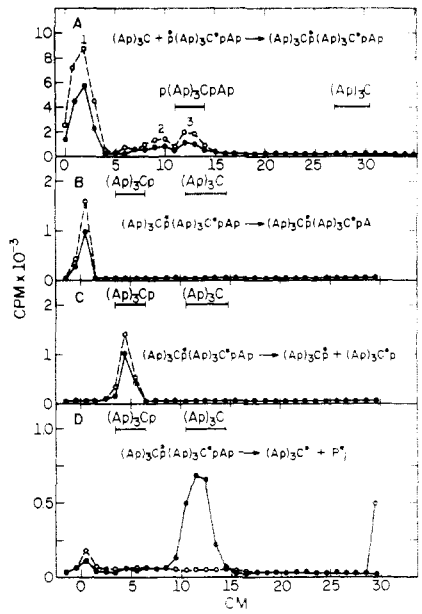

FIGURE 7: Ligation of $(Ap)_3C$ and [5'-^{32}P, *Cyd*-^{3}H]p$(Ap)_3CpAp$ and characterization of product [^{32}P, ^{3}H]$(Ap)_3Cp(Ap)_3CpAp$. The ligation reaction (A) and the enzymic degradations (B–D) were analyzed by descending paper chromatography with solvent A (96 h) and solvent B (96 h), respectively. (B) Treatment with BAP to yield [^{32}P, ^{3}H]$(Ap)_3Cp(Ap)_3CpA$; (C) treatment with RNase A to yield [*Cyd*-^{3}H]$(Ap)_3Cp$ and [3'-^{32}P]$(Ap)_3Cp$; and (D) treatment with RNase A plus BAP to yield [*Cyd*-^{3}H]$(Ap)_3C$ and [^{32}P]phosphate. Solid circles are ^{3}H label and open circles are ^{32}P label.

summarized in Figure 7. The purified oligomer [*Cyd*-^{3}H]-$(Ap)_3CpAp$, from a preparative scale reaction (Figure 6A), was reacted with [γ-^{32}P]ATP and polynucleotide kinase to produce [5'-^{32}P, *Cyd*-^{3}H]p$(Ap)_3CpAp$ as described in Materials and Methods. This 3',5'-phosphorylated oligomer was then used as a donor in a ligase reaction with unlabeled $(Ap)_3C$. The analysis of this reaction by paper chromatography is shown in Figure 7A. Peak 3 is the unreacted donor oligomer, peak 2 is probably the adenylylated intermediate, [^{32}P, ^{3}H]-A5'pp5'$(Ap)_3CpAp$, on the basis of its resistance to alkaline phosphatase (data not shown), and peak 1 was identified as the ligation product [^{32}P, ^{3}H]$(Ap)_3Cp(Ap)_3CpAp$ (yield 73%). The characterization of the product was based on the following identification reactions: (a) the resistance of ^{32}P label in the product to alkaline phosphatase (Figure 7B) indicates that the 5'-terminal phosphate originally in p$(Ap)_3CpAp$ is now in an internal position; (b) hydrolysis of the product with ribonuclease A gives both the ^{3}H and ^{32}P label comigrating with $(Ap)_3Cp$ (Figure 7C); and (c) including alkaline phosphatase in the ribonuclease digestion results in the ^{3}H label migrating with $(Ap)_3C$ and the ^{32}P label as inorganic phosphate (Figure 7D). The last two experiments confirm that the 5'-phosphate on the p$(Ap)_3CpAp$ donor takes part in a ribonuclease sensitive internucleotide linkage and, after hydrolysis, the phosphate is on the 3' end of $(Ap)_3Cp$.

Discussion

An important feature of the strategy of oligoribonucleotide synthesis with T4 RNA ligase is the presence of a removable blocking group on the 3' terminus of the donor molecule. This group prevents three major side reactions and the synthesis of a single product by the intermolecular ligation of acceptor and donor can be achieved. If the 3'-terminal blocking group were absent, the donor molecule could react with itself or multiple additions of the donor to the acceptor could occur. These side

reactions will lead to substantially less of the desired product if the unblocked donor is a better acceptor than the intended acceptor (Ohtsuka et al., 1976). In addition, if the 3′-terminal blocking group is absent and the donor is longer than eight nucleotides (Kaufmann et al., 1974), the intramolecular cyclization reaction will compete effectively with the intermolecular reaction (Kaufmann & Kallenbach, 1975).

A 3′-terminal phosphate is a particularly useful blocking group since it is frequently obtained in the course of oligoribonucleotide synthesis and it can be easily removed with alkaline phosphatase. Thus, oligomers with 3′-terminal phosphates are obtained as a result of alkaline hydrolysis of homopolymers or nuclease digestion of random copolymers. In addition, oligomers with 3′-phosphates are the products of synthetic reactions combining polynucleotide phosphorylase and a nuclease (Thach et al., 1966). Finally, 3′-phosphates can be introduced by periodate oxidation of the terminal 2′,3′-*cis*-diol and subsequent β elimination (Keith & Gilham, 1974).

In this paper we have studied the RNA ligase reaction between combinations of donors having terminal 5′- and 3′-phosphates with acceptors having terminal 5′- and 3′-hydroxyls. In general, intermolecular products are formed when the donor is at least a nucleoside 3′,5′-bisphosphate and the acceptor a trinucleoside diphosphate.

The minimal donor requirement of the reaction is complicated. While pNp's are donors, 5′-AMP is not. However, when 5′-AMP is adenylylated to form A5′pp5′A, it is an effective substrate in the ATP-independent RNA ligase reaction (England et al., 1977). Thus, the minimal donor requirement seen for the intermolecular reactions is determined by the first step in the reaction mechanism where the donor is adenylylated. The subsequent transfer of the adenylylated intermediate to the product does not require the 3′-phosphate. The lack of activity of nucleoside 2′,5′-diphosphates implies that the 3′-phosphate group must be in the same position as the correct 3′ → 5′ internucleotide linkage of an oligomer in order for the adenylylation step to occur. Presumably oligonucleotides with 2′ → 5′ internucleotide linkage between the first and second residues would also be inactive donors. There is relatively little difference in the rate of ligation for a variety of mononucleotide and oligomer donors with $(Ap)_3C$ as an acceptor. This is consistent with the observation of similar rates of ligation of various adenylylated donors (England et al., 1977). Clearly, the main determinant of the success of the ligation reaction is the identity of the acceptor.

A comparison of a variety of acceptors with a given donor reveals large differences in the enzyme concentration required to obtain adequate yields. All attempts by us and others (Kaufman & Kallenbach, 1975; Sugino et al., 1977) to use dinucleoside monophosphates as acceptors have failed. It would appear that the acceptor binding site requires at least three nucleosides for activity. However, as little additional chain length dependence on rate is observed, it is unlikely that the acceptor binding site will accommodate more than three nucleosides. The composition of the acceptor has a large effect upon the extent of the ligation reaction. If a uridine residue is present in any of the three positions adjacent to the ligation site, the yield is considerably lower. This observation has been noted for a variety of donor–acceptor pairs (Ohtsuka et al., 1976; Sninsky et al., 1976). Higher yields with U containing acceptors can be achieved by increasing the enzyme concentration and, to a certain degree, by changing the reaction conditions. The low reaction yield is apparently the result of inefficient transfer of the adenylylated intermediate to the acceptor. Since the acceptor must apparently be present for the adenylylated intermediate to form (Sugino et al., 1977), it is possible that both oligomers dissociate from the enzyme before ligation occurs and that rebinding of "poor" acceptors and adenylylated donors is relatively inefficient. This view is consistent with the low rate of reaction with $(Up)_2U$ and A5′pp5′A.

Due to the complexity of chemical techniques, enzymatic methods have been developed to prepare oligonucleotides of defined sequence. An important advantage of using enzymes for oligoribonucleotide synthesis is that the formation of the correct 3′ → 5′ internucleotide linkage is ensured. The most extensively used enzymatic procedure thus far has been the controlled addition of nucleoside diphosphates to the 3′ end of an oligoribonucleotide primer using primer dependent polynucleotide phosphorylase (Thach, 1966). Although the yields of this reaction can be improved substantially by using 3′ blocked diphosphates (Bennett et al., 1973; Walker & Uhlenbeck, 1975), the stepwise nature of the procedure limits the complexity of the sequences which can be made. In addition, as the primer molecule becomes longer, the reverse, phosphorolysis reaction rate increases which leads to scrambling of the sequence (Chou & Singer, 1970; Sninsky et al., 1974). Finally, the preference of polynucleotide phosphorylase for purine rich primers limits the sequences which can be made.

The RNA ligase reaction seems to solve many of the difficulties encountered with polynucleotide phosphorylase. As RNA ligase does not appear to saturate in its binding of donor or acceptor at concentrations as high as 1 mM (Uhlenbeck & Cameron, 1977), reactions can be performed at relatively high oligomer concentrations. Coupled with the nearly quantitative yields obtained in most cases, large scale preparations of oligomers with reasonable amounts of enzyme are possible. While more than 20 000 units (~10 mg) of RNA ligase can be obtained from 100 g of phage infected cells (McCoy, Lubben, & Gumport, manuscript in preparation), we calculate that the quantitative addition of a pA3′p residue to 1 mg of $(Ap)_3C$ would require less than 75 units (36 μg) of RNA ligase.

Another advantage of using RNA ligase is that the pathway for the synthesis of a complex oligonucleotide can involve the joining of oligomer blocks and is not limited to the stepwise addition of single residues to the 3′ end of an oligomer as with polynucleotide phosphorylase. Aside from the obvious improvement in overall reaction yield and ease of purification, this procedure allows considerable flexibility in introducing modifications at specific points in the sequence. The fact that 3′,5′-nucleoside bisphosphates are donors in the ligase reaction should allow insertion of an arbitrary residue at a specific locus in a synthetic scheme. In the following paper (Barrio et al., 1978) it is shown that a variety of modified nucleotides can be introduced into a oligomer sequence in a similar manner.

In contrast to polynucleotide phosphorylase, RNA ligase does not seem to catalyze any degradative reaction. Although many preparations of the enzyme contain a deoxyribonuclease activity (Sugino et al., 1977), no evidence for any ribonuclease activity has been found. Both oligomers and high molecular weight RNA remain undegraded. Thus, unlike polynucleotide phosphorylase, relatively long oligomers can be made with RNA ligase.

Three difficulties of using RNA ligase for the synthesis of oligomers can be presently identified. The first is that a certain percentage of the donor is converted to the adenylylated intermediate but not transferred onto acceptor. The extent of this side reaction seems to vary with the acceptor that is used (Ohtsuka et al., 1976). This difficulty can to a great extent be overcome by increasing the pH of the reaction buffer (Uhlenbeck & Cameron, 1977). The use of a slight excess of donor to acceptor also optimizes the amount of intermolecular

product formed. The second difficulty of RNA ligase synthesis is the evident lack of reactivity of uridine-containing acceptors. Although the reaction yields can be improved by altering conditions to some degree, the best procedure to avoid this difficulty is to design a synthetic strategy which avoids ligations to sequences with uridine. Finally, although RNA ligase is stable on storage, its apparent inactivation during incubation at 37 °C causes a rapid decrease in rate during the course of reaction. Thus, much larger amounts of enzyme are required for quantitative yield than would be predicted from the initial rate. Attempts to find reaction conditions to stabilize RNA ligase have thus far been unsuccessful. Recent experiments suggest that greater yields can be obtained by running the reaction for longer times at reduced temperatures. Investigation of this possibility is continuing.

In summary, we feel that the high yields, broad substrate specificity and simplicity of the RNA ligase reaction make it the most convenient method for the synthesis of oligoribonucleotides of defined sequence.

Acknowledgments

We wish to thank R. I. Gumport, V. Cameron, G. Knapp, and A. Studencki for gifts of nucleotides and B. Parker for technical assistance. We also appreciate the helpful discussions with R. I. Gumport.

References

Barrio, J. R., Barrio, M. G., Leonard, N. J., England, T. E., & Uhlenbeck, O. C. (1978) *Biochemistry 17* (following paper in this issue).

Bennett, G. N., Mackey, J. K., Wiebers, J. L., & Gilham, P. T. (1973) *Biochemistry 12,* 3956–3962.

Cameron, V., & Uhlenbeck, O. C. (1977) *Biochemistry 16,* 5120–5126.

Chou, J. Y., & Singer, M. F. (1970) *J. Biol. Chem. 245,* 1005–1011.

Cranston, J. W., Silber, R., Malathi, V. G., & Hurwitz (1974) *J. Biol. Chem. 249,* 7447–7456.

England, T. E., Gumport, R. I., & Uhlenbeck, O. C. (1978) *Proc. Natl. Acad. Sci. U.S.A. 74,* 4389–4842.

Kaufmann, G., & Kallenbach, N. R. (1975) *Nature (London) 254,* 452–454.

Kaufmann, G., Klein, T., & Littauer, U. Z. (1974) *FEBS Lett. 46,* 271–275.

Keith, G., & Gilham, P. T. (1974) *Biochemistry 13,* 3601–3606.

Martin, R. H., Uhlenbeck, O. C., & Doty, P. (1971) *J. Mol. Biol. 57,* 201–215.

Ohtsuka, E., Nishikawa, S., Sugiura, M., & Ikehara, M. (1976) *Nucleic Acids Res. 3,* 1613–1623.

Silber, R., Malathi, V. G., & Hurwitz, J. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69,* 3009–3013.

Sninsky, J. J., Bennett, G. N., & Gilham, P. T. (1974) *Nucleic Acids Res. 1,* 1665–1674.

Sninsky, J. J., Last, J. A., & Gilham, P. T. (1976) *Nucleic Acids Res. 3,* 3157–3166.

Sugino, A., Snopek, T. J., & Cozzareli, N. R. (1977) *J. Biol. Chem. 252,* 1732–1738.

Thach, R. E. (1966) *Proced. Nucleic Acid Res. 1,* 520–534.

Thach, R. E., Sundararajan, T. A., Dewey, K. F., Brown, J. C., & Doty, P. (1966) *Cold Spring Harbor Symp. Quant. Biol. 31,* 85–97.

Uhlenbeck, O. C., & Cameron, V. (1977) *Nucleic Acids Res. 4,* 85–98.

Uhlenbeck, O. C., Baller, J., & Doty, P. (1970) *Nature (London) 225,* 508–510.

Walker, G. C., & Uhlenbeck, O. C. (1975) *Biochemistry 14,* 817–824.

Walker, G. C., Uhlenbeck, O. C., Bedows, E., & Gumport, R. I. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72,* 122–126.