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Reversal of T4 RNA Ligase†

Marc Krug and Olke C. Uhlenbeck*

ABSTRACT: Unexpected products detected in oligoribonucleotide synthesis reactions catalyzed by T4 RNA ligase are shown to be a result of a partial reversal of the enzyme reaction. A transfer assay for the reversal of the third step in the RNA ligase reaction mechanism and an exchange assay for the reversal of both the second and third steps are described. Reversal is confirmed by the formation of the expected covalent intermediates, adenylylated donor and adenylyl ligase, from a reaction containing 5′-AMP, unadenylylated ligase, and the tetranucleotide $(Ap)_3Cp$. In the reverse reaction, RNA ligase shows a strong preference for hydrolysis of the 3′-terminal phosphodiester bonds of oligoribonucleotides which terminate in a 3′-phosphate. Several strategies are discussed to minimize the effects of reversal in the enzymatic synthesis of oligoribonucleotides.

T4 RNA ligase catalyzes the ATP-dependent formation of a 3′→5′ phosphodiester bond between an oligonucleotide acceptor with a 3′-hydroxyl and an oligonucleotide donor with a 5′-phosphate (Uhlenbeck & Gumport, 1981). This reaction has proven useful in the synthesis of both ribo (Ohtsuka et al., 1980) and deoxyribo (Gumport et al., 1980) oligonucleotides of defined sequence as well as the extension of the 3′ (England et al., 1980) and 5′ (Stahl et al., 1980) termini of natural RNA molecules. In analogy with the ATP-dependent DNA ligases (Kornberg, 1980), the RNA ligase reaction mechanism can be dissected into three distinct steps involving two known covalent intermediates.

$$\text{ATP} + \text{E} \rightleftarrows \text{E–pA} + \text{PP}_i \quad (1)$$

$$\text{E–pA} + \text{pX}_n \rightleftarrows \text{E[A-5′pp5′-X}_n\text{]} \quad (2)$$

$$\text{E[A-5′pp5′-X}_n\text{]} + \text{Y}_m \rightleftarrows \text{Y}_m\text{pX}_n + \text{pA} + \text{E} \quad (3)$$

The first step is the reaction of ATP with RNA ligase to form a covalent adenylylated enzyme intermediate with the release of pyrophosphate. The adenylylated protein is stable at neutral pH, and this step can be reversed by the addition of pyrophosphate (Cranston et al., 1974). The second step involves the transfer of the 5′-AMP from adenylyl ligase to the 5′-phosphate of the donor, forming an intermediate with a 5′–5′ phosphoanhydride bond (Kaufmann & Littauer, 1974). Adenylylated donors isolated from reactions or chemically synthesized are reactive in the last step of the reaction in the absence of ATP, further suggesting their role as intermediates in the mechanism (Sninsky et al., 1976). In the third step, the 5′–5′ phosphoanhydride bond is broken, and the donor forms a 3′→5′ phosphodiester bond with the acceptor, and AMP is released (Sugino et al., 1977).

In this work, we will demonstrate the reversibility of the second and third steps of this reaction. This study was prompted by the detection of anomalous products in oligonucleotide synthesis reactions. Several lines of evidence are presented indicating that the rapid reversal of RNA ligase reactions can occur under synthetic conditions. Assays for the reverse reaction are developed, and several procedures for minimizing its effect are examined.

Materials and Methods

T4 RNA ligase was purified by the procedure of Moseman-McCoy et al. (1979) and was essentially homogeneous with a specific activity of 2100 units/mg. Analysis of RNA ligase on sodium dodecyl sulfate ($NaDodSO_4$)–urea–polyacrylamide gels was carried out by the procedure of Matsudaira & Burgess (1978). Venom 5′-nucleotidase, creatine phosphinokinase, and adenylate kinase were purchased from Sigma Chemical Co.

[5′-^{32}P]AMP was prepared in two steps by first synthesizing [5′-^{32}P]pAp from 3′-AMP and [γ-^{32}P]ATP with poly-

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nucleotide kinase (England et al., 1980) and then removing the 3′-terminal phosphate of pAp by incubating it with 1000 units/mL polynucleotide kinase at pH 6.0 (Cameron & Uhlenbeck, 1977). Oligoribonucleotides were prepared either by alkaline or ribonuclease digests of ribopolymers or with primer-dependent polynucleotide phosphorylase from commercial dinucleoside monophosphates by using procedures published elsewhere (England & Uhlenbeck, 1978; Uhlenbeck & Cameron, 1977). $(Ip)_nI$ (n = 3–5) was purchased from Miles Laboratories. Oligomers labeled with tritium in the 3′-terminal nucleotide were prepared from the ^3H-labeled nucleoside diphosphate by using polynucleotide phosphorylase (Uhlenbeck et al., 1970). 5′-^{32}P-Labeled oligonucleotide donors were prepared by using [γ-^{32}P]ATP and PseT 1 polynucleotide kinase (Cameron et al., 1978). $(Ap)_3C>p$ was prepared by treating 10 mM $(Ap)_3Cp$ with 30 mM *N*-ethyl-*N*′-[3-(dimethylamino)propyl]carbodiimide hydrochloride, pH 5.5, at 25 °C for 1 h. $(Ap)_3Cp$-ribose and A_3Cp-*p*-methoxyphenol were prepared from A-5′pp-ribose and A-5′pp-4-methoxyphenyl by using the ATP-independent reaction of RNA ligase (England et al., 1977; Gumport et al., 1980). $(dTp)_4dCp$ was a gift of R. Gumport.

RNA ligase reactions were carried out in 30 mM $MgCl_2$, 3 mM dithiothreitol, and 50 mM *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid buffer at the pH indicated. Incubations were at 25 °C unless otherwise noted. Reaction mixtures were analyzed by descending paper chromatography on Whatman 3MM paper by using one of the following mixtures (v/v): solvent A (50:50 1 M ammonium acetate: 95% ethanol), solvent B (35:65:5 1.0 M ammonium acetate:95% ethanol:glacial acetic acid), or solvent C (25:70:5 1.0 M ammonium acetate:95% ethanol:1.0 M ammonium hydroxide). Oligonucleotides were located on the chromatogram by viewing with ultraviolet light and by cutting the paper in 1-cm strips and counting in 4% diphenyloxazole in toluene in a liquid scintillation counter. Oligonucleotides were recovered from the chromatogram and subjected to nuclease digestion for identification as described by England & Uhlenbeck (1978).

Results

Detection of Unexpected Products in an RNA Ligase Reaction. The only product expected from the reaction catalyzed by RNA ligase joining equal concentrations of [*Guo*-^3H]-ApCpG and [5′-^{32}P]pGpApUp is the hexamer [^3H,^{32}P]Ap-CpGpGpApUp. This is because the 3′-phosphate on the donor molecule is a successful blocking group and prevents multiple additions of the donor to the acceptor (Uhlenbeck & Cameron, 1977). However, when this reaction was carried out under standard conditions and analyzed by descending paper chromatography (Figure 1A), three oligomer products (peaks I, II, and III) were observed in addition to the two starting materials. Calculations based on the specific activities indicated that while peak II contained an equimolar ratio of [^3H]guanosine to [^{32}P]phosphate, peak I had twice as much ^{32}P as ^3H, and peak III contained only ^3H label. The fact that peak II had the expected ratio of radioactivity and migrated similarly to a $(Ap)_5Up$ standard on the paper chromatogram suggested that it was the expected product ApCpGpGpApUp. This identification was confirmed by several experiments. First, the presence of a 3′-terminal phosphate could be inferred by the resistance of this oligomer to snake venom phosphodiesterase and the small increase in its chromatographic mobility upon treatment with bacterial alkaline phosphatase. Second, treatment of peak II with ribonuclease T_1 produces an oligomer with ^3H and ^{32}P label which comigrates with an

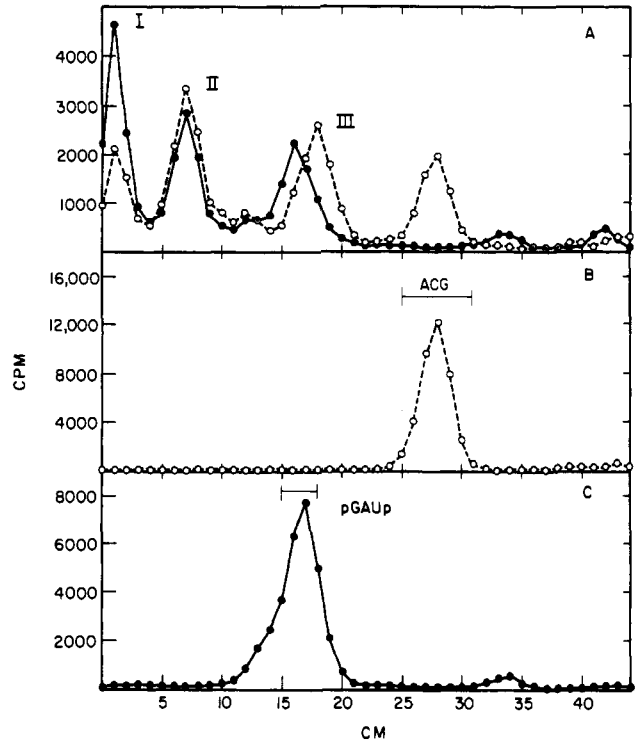

FIGURE 1: Equimolar joining of ApCpG and pGpApUp. [*Guo*-^3H]ApCpG (0.1 mM, 22 Ci/mol) and [5′-^{32}P]pGpApUp (0.1 mM, 9 Ci/mol) were incubated in pH 8.3 buffer with 0.5 mM ATP and 220 units/mL RNA ligase for 8 h. The chromatogram was developed with solvent A for 36 h. Open circles and dashed lines represent ^3H label; closed circles and solid lines are ^{32}P label. (A) Complete reaction mixture; (B) pGpApUp omitted; (C) ApCpG omitted.

ApCpGp marker. Third, treatment of peak II with ribonuclease T_1 and alkaline phosphatase produces two radiolabeled peaks which comigrate with the expected products [*Guo*-^3H]ApCpG and $^{32}P_i$. Finally, peak II that had been treated with alkaline phosphatase was digested with snake venom phosphodiesterase, and the products were analyzed by high-voltage electrophoresis. Both the ^3H and ^{32}P labels comigrated with a pG marker, consistent with the G(3′→5′)pG linkage between the donor and acceptor. Similar analysis revealed the identities of the two other new peaks in Figure 1. Peak I was found to be ApCpGpGpApGpApUp with ^3H label in the guanosine that is the third nucleotide from the 5′ terminus and ^{32}P label in the third and fifth phosphates from the 5′ terminus. Peak III was identified as [*Guo*-^3H]-ApCpGpUp.

Although several different pathways could be proposed, the formation of the two unexpected products ApCpGpUp and ApCpGpGpApGpApUp seems to involve the removal of the 3′-terminal pUp from pGpApUp. For example, one possible mechanism involves first forming the expected product Ap-CpGpGpApUp and then cleaving the 3′-terminal internucleotide bond to give ApCpGpGpA and pUp. The pUp could then be added to the acceptor ApCpG to produce ApCpGpUp (peak III), and ApCpGpGpA could be added to the donor pGpApUp to produce ApCpGpGpApGpApUp (peak I). Thus, the appearance of the unexpected products in Figure 1A can be explained as if there is a hydrolytic activity which removes the 3′-terminal pUp.

Several lines of evidence indicated that this apparent hydrolytic activity was closely related to the action of RNA ligase itself and not an independent nuclease activity contaminating the enzyme preparation. Incubation of complete reactions missing only the acceptor (Figure 1B) or the donor (Figure 1C) showed no indication of degradation of either oligomer.

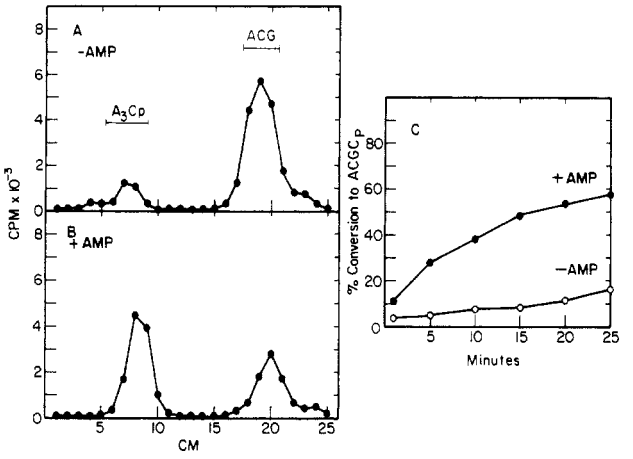

FIGURE 2: Transfer reaction with $(Ap)_3Cp$ and ApCpG. [*Guo*-^{3}H]ApCpG (0.1 mM, 20 Ci/mol) and 0.5 mM $(Ap)_3Cp$ were incubated in pH 7.0 buffer without (panel A) or with (panel B) 0.2 mM 5′-AMP and 20 units/mL RNA ligase for 25 min (panels A and B) or the indicated times (panel C). The chromatogram was developed with solvent A for 18 h.

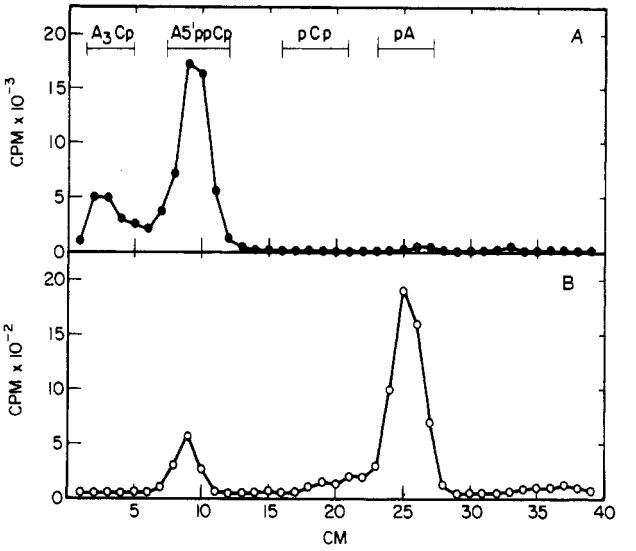

FIGURE 3: Detection of adenylylated intermediate. ApApA[3′→5′-^{32}P]pCp (3 nM, 1.0 Ci/μmol) and 40 μM AMP (panel A) or [^{3}H]AMP (10 μM, 100 Ci/mol) and 5 mM $(Ap)_3Cp$ (panel B) were incubated in pH 7.0 buffer with 40 units/mL RNA ligase for 60 min. The chromatogram was run in solvent B for 18 h.

A similar control incubating the isolated product ApCpGpGpApUp with RNA ligase without ATP also produced no breakdown. Furthermore, a variety of different ligase preparations isolated by different procedures all produced similar ratios of peaks I, II, and III. Any contaminating nuclease activity might be expected to be present in different amounts in different enzyme preparations. Finally, the relative amounts of peaks I, II, and III remained approximately constant at several concentrations of reactants and several different incubation temperatures.

We will demonstrate that the apparent hydrolysis and generation of pUp are actually the result of reversal of the last step in the RNA ligase reaction and are catalyzed by 5′-AMP generated in the forward reaction. ApCpGpGpApUp reacts with 5′-AMP to form the intermediates A-5′pp5′-Up and ApCpGpGpA. The A-5′pp5′-Up subsequently reacts with ApCpG to produce peak III, and ApCpGpGpA is an acceptor with pGpApUp donor to form peak I. Thus, the unexpected products accumulate in RNA ligase reactions after sufficient AMP and product are produced in the normal forward reaction.

Transfer Reaction. For demonstration of the reversal of the third step of the RNA ligase reaction more directly, an assay was developed which allows this reversal to occur in the absence of the ATP-dependent forward reaction. This was done by omitting both ATP and a 5′-phosphorylated oligomer from the reaction mixture. Such a transfer reaction contains 5′-AMP, a nonradioactive 3′-phosphorylated oligomer, $(Ap)_3Cp$, and a ^{3}H-labeled acceptor oligomer, [*Guo*-^{3}H]-ApCpG. Upon incubation with RNA ligase, reversal of the third step of the reaction converts $(Ap)_3Cp$ and AMP to ApApA and A-5′pp5′-Cp. The [^{3}H]ApCpG can subsequently react with A-5′pp5′-Cp to form [^{3}H]ApCpGpCp in the ATP-independent forward reaction. Thus, transfer of the 3′-terminal pCp from $(Ap)_3Cp$ to ApCpG can be accomplished by the third step of the RNA ligase reaction acting first in the reverse direction and then in the forward direction. In Figure 2, analysis of this reaction at a 5:1 molar input ratio of $(Ap)_3Cp$ and [^{3}H]ApCpG is shown. Figure 2A,B shows the appearance of a slower moving radioactive product upon incubation with RNA ligase in the absence and presence of added AMP. This new product migrates closely to $(Ap)_3Cp$ and can be identified as ApCpGpCp by several criteria. These include resistance to snake venom phosphodiesterase and hydrolysis with ribonuclease T_1 to produce [^{3}H]ApCpGp and Cp. Figure 2C shows the time course for the appearance of ApCpGpCp. In the presence of added AMP, the reaction continues until it approaches the theoretical 83% value expected for equilibrium between a 5:1 molar ratio of $(Ap)_3Cp$ and ApCpG. Repeating this experiment at a 1 to 1 input molar ratio of $(Ap)_3Cp$ to ApCpG gave the expected equilibrium value of 50% ApCpGpCp. Although added 5′-AMP strongly stimulates the rate of the reaction, significant transfer activity occurs even in the absence of added 5′-AMP, presumably due to trace amounts of 5′-AMP present in the RNA ligase preparation.

Direct demonstration of reversal of the third step in the RNA ligase reaction requires the isolation of the intermediate adenylylated donor which is predicted to form. Since the equilibrium constant for formation of A-5′pp5′-Cp from 5′-AMP and $(Ap)_3Cp$ may not be great, it could be difficult to detect this intermediate by using an equimolar input of 5′-AMP and $(Ap)_3Cp$. Thus, in order that the proportion of intermediate formed could be increased, two parallel incubations were carried out by using high concentrations of either one or the other of the reactants. In Figure 3A, an excess of unlabeled AMP was utilized to convert a significant proportion of [^{32}P]$(Ap)_3Cp$ into the ^{32}P-labeled intermediate. In Figure 3B, an excess of unlabeled $(Ap)_3Cp$ was used to convert [^{3}H]AMP into the ^{3}H-labeled intermediate. The two putative intermediate peaks formed in Figure 3 were eluted from the chromatogram and mixed. These ^{3}H- and ^{32}P-labeled oligomers were then shown to comigrate precisely on descending paper chromatography in three different solvent systems (A, B, and C). The mixture was confirmed to be [*Ado*-^{3}H,^{32}P]-A-5′pp5′-Cp by several criteria. First, treatment of the mixture with calf alkaline phosphatase produced a faster moving peak in solvent C that contained both labels and comigrated with a chemically synthesized A-5′pp5′-C marker (England et al., 1977). Second, treatment of the mixture with snake venom phosphodiesterase yielded two peaks upon paper chromatography. As expected, the faster moving peak contained only ^{3}H label and comigrated with 5′-AMP while the slower peak contained only ^{32}P label and comigrated with pCp. Finally, when the mixture was combined with ApApA and T4 RNA ligase, the products of the reaction were [^{32}P]$(Ap)_3Cp$ and

Table I: 5′-Monophosphate Requirement of the Transfer Reaction

nucleotide	% ApCpGpCp at 60 min	
	30 units/mL	300 units/mL
pA	44	72
pdA	2	32
pI	3	44
pG	3	21
pC	2	28
pU	2	24
Ap	1	22
none	2	22

Table II: Different Donors in the Transfer Reaction

oligomer	% transfer	oligomer	% transfer
$(Ap)_3$	0	$(pA)_4$	0
$(Ap)_4$	52	$(pA)_5$	0
$(Ap)_3Gp$	24	$(pU)_4$	0
$(Ap)_3Cp$	66	$(pU)_5$	0
$(Ap)_3Up$	12	$(Up)_5U$	2
$(Ap)_4Cp$	45	$(Ap)_3C$	0
$(Cp)_3Gp$	60	$(Ap)_5C$	0
$(Up)_4$	11	$(Ip)_3I$	0
$(Cp)_{11}Gp$	46	$(Ip)_4I$	3
$(dTp)_4dCp$	4	$(Ip)_5I$	12[a]

[a] Mixture of products; see text.

[^{3}H]AMP. Thus, the putative intermediate made by reversal was shown to be competent to react in the ATP-independent forward reaction.

The assay in Figure 2 was used to demonstrate a pH optimum of 7.0 and a requirement for magnesium ion for the transfer reaction. Under the conditions used in Figure 2, the initial transfer reaction rate was measured as a function of 5′-AMP concentration. The half-maximal catalytic rate was obtained at 4 μM 5′-AMP.

Several other nucleotides were tested for their ability to substitute for 5′-AMP in the transfer reaction under the same conditions as those of Figure 2 (Table I). Only 5′-IMP and 5′-dAMP have any significant activity above that of no added AMP. This is consistent with the specificity for ATP in the forward RNA ligase reaction, where dATP and ITP were the only additional active triphosphates (Cranston et al., 1974). This also supports the contention that the same binding site on RNA ligase is used to bind 5′-AMP in the reverse reaction as is used to bind ATP in the forward reaction.

In Table II, a variety of different oligomers were tested as donors in the transfer reaction by using [^{3}H]ApCpG or [^{3}H]CpApG as acceptors and the reaction conditions in Figure 2. The shortest oligomer active in the transfer reaction is a tetranucleotide with a 3′-terminal phosphate. $(Ap)_3$ gave no detectable product even when 10-fold higher enzyme concentration was used. Although the yields varied somewhat, all tetranucleotides with a 3′-terminal phosphate were active in the transfer reaction and resulted in similar products. Hydrolysis always occurred at the 3′-terminal internucleotide linkage, yielding [^{3}H]ApCpGpNp as the only product. Even when longer oligomers were used, little if any hydrolysis occurred at internal positions to give longer tritiated products. This remarkable preference for hydrolysis at the 3′-terminal internucleotide linkage appears to be related to the presence of the 3′-terminal phosphate. $(Ap)_3C$ and $(Ip)_3I$ gave no detectable product even when 10-fold higher enzyme concentrations were used. However, some of the longer oligomers with a 3′-terminal hydroxyl gave transfer products at considerably lower yields. For example, when $(Ip)_4I$ was incubated with AMP and ApCpG, the only product was ApCpG-

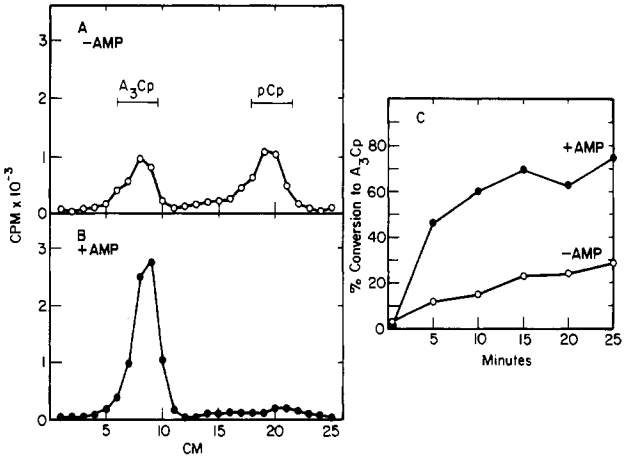

FIGURE 4: Exchange reaction between pCp and $(Ap)_3Cp$. [5′-^{32}P]pCp (0.1 mM, 7.2 Ci/mol) and 0.5 mM $(Ap)_3Cp$ were incubated in pH 8.3 buffer without (panel A) or with (panel B) 0.2 mM 5′-AMP and 200 units/mL RNA ligase for 25 min (panels A and B) or the indicated times (panel C). The chromatogram was developed in solvent A for 18 h.

$(pI)_2$. When $(Ip)_5I$ was used, the products were $ApCpG(pI)_2$ in 8% yield and $ApCpG(pI)_3$ in 6% yield. In both cases, no ApCpGpI was obtained. Thus, when the transfer reaction is examined with oligomers with a 3′-terminal hydroxyl, hydrolysis of the phosphodiester bond can occur at several positions in the molecule but not at the 3′-terminal position.

The substrate specificity and reaction products of the reverse transfer reaction can be understood in terms of the known oligomer binding sites on RNA ligase deduced from the forward reaction. It is generally believed that the acceptor site encompasses a trinucleoside diphosphate and the donor site encompasses a 5′,3′-bisphosphate (Uhlenbeck & Gumport, 1981). For reversal, an oligomer must bind both the donor and acceptor sites and place an internucleotide linkage at the catalytic site. Thus, the smallest oligomer which can fill both the donor and acceptor sites is a tetramer with a 3′-terminal phosphate. In this case, hydrolysis will occur at the 3′-terminal internucleotide linkage. If the oligomer ends with a 3′-terminal hydroxyl, both sites will not be filled unless a pentamer is used. Hydrolysis will then occur at the second internucleotide linkage from the 3′ terminus so that the donor site can be filled.

Exchange Reaction. Facile reversal of the third step of the RNA ligase reaction suggests that the second step might be reversible as well. Reversal of both the second and third steps of the ligase mechanism can be assayed by an exchange reaction between [5′-^{32}P]pCp and $(Ap)_3Cp$. If the second step can indeed reverse, then the adenylylated donor intermediate A-5′pp5′-Cp formed by reversal of the third step will react with the RNA ligase to form adenylyl ligase and pCp. The radioactive pCp will then exchange with the nonradioactive pCp, react with the adenylyl ligase to form [^{32}P]A-5′pp5′-Cp, and finally react with ApApA to form [^{32}P]$(Ap)_3Cp$. Thus, the reversal of the second and third steps in the ligase mechanism followed by the same two steps in the forward direction will result in the exchange of [5′-^{32}P]pCp into the terminal position of $(Ap)_3Cp$. Figure 4 shows the result of this reaction with a 5 to 1 molar input ratio of $(Ap)_3Cp$ to [^{32}P]pCp with (Figure 4B) and without (Figure 4A) added 5′-AMP. The ^{32}P label is converted from pCp to a form comigrating with $(Ap)_3Cp$. This product was confirmed to be [^{32}P]$(Ap)_3Cp$ by its resistance to snake venom phosphodiesterase and its sensitivity to spleen phosphodiesterase. The only radioactive product of spleen phosphodiesterase digestion was the expected [3′-^{32}P]Ap. Figure 4C shows the time course of the exchange

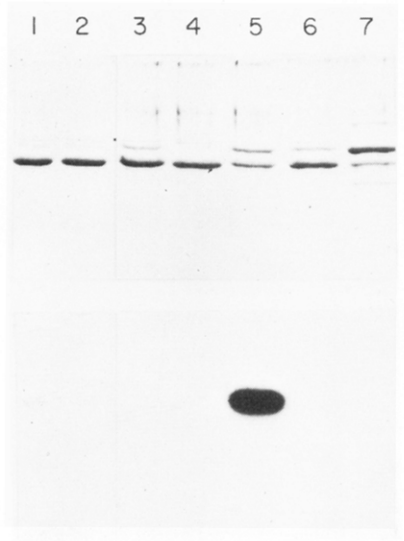

FIGURE 5: Detection of adenylyl ligase in the exchange reaction on a $NaDodSO_4$–urea gel. In lane 5, ^{32}P-labeled 5′-AMP (20 μM, 500 Ci/mol) and 1 mM $(Ap)_3Cp$ were incubated in pH 8.3 buffer with 280 units/mL RNA ligase for 30 min. Control incubations omitted both substrates (lane 1), omitted 5′-AMP (lane 2), replaced $(Ap)_3Cp$ with 1 mM pCp (lane 3), replaced $(Ap)_3Cp$ with 1 mM ApApA (lane 4), or replaced both substrates with 11 mM ATP (lane 6) or with 11 mM ATP and 6 units/mL pyrophosphatase (lane 7). The upper panel is the stained gel, and the lower panel is the autoradiogram of that gel.

reaction with and without added AMP. As in the transfer reaction, the exchange reaction is stimulated by the addition of 5′-AMP, but some reaction occurs without it due to small amounts of 5′-AMP in the enzyme preparation. The reaction approaches the equilibrium value expected for the 5:1 molar ratio of $(Ap)_3Cp$ and pCp. Other input ratios of the two components confirmed that free exchange between [^{32}P]pCp with the terminal pCp of $(Ap)_3Cp$ appears to occur.

In order to prove that the exchange reaction involves the formation of adenylyl ligase, it is necessary to demonstrate the formation of this intermediate in the reaction. This is simplified by the observation that adenylyl ligase separates from the nonadenylylated form on a $NaDodSO_4$–urea–polyacrylamide gel (Higgens et al., 1977). As shown in Figure 5, the unadenylylated enzyme (lane 1) moves slightly faster than the adenylylated enzyme (lane 7). Incubation of enzyme with A_3Cp (lane 2) or [5′-^{32}P]AMP plus ApApA (lane 4) does not lead to adenylylated enzyme. However, incubation with $(Ap)_3Cp$ and [5′-^{32}P]AMP (lane 5) produces a significant amount of adenylylated enzyme, and examination of the autoradiogram indicates that it is ^{32}P labeled. This shows that adenylylated RNA ligase can form in the reaction and that the adenylyl group is derived from AMP added to the reaction mixture.

The pH optimum of the exchange reaction in Figure 4 is found to be about 8.3, which is considerably higher than the optimum of 7.0 for the transfer reaction. Since the exchange reaction includes the identical reverse and forward steps contained in the transfer reaction, a different pH optimum suggests that either the forward or the reverse direction of the second step in the RNA ligase mechanism is rate limiting. This is consistent with the fact that a 10-fold higher enzyme concentration was needed in the exchange reaction in Figure 4 than in the transfer reaction in Figure 2. The experiment in Figure 6 compares the relative rates of the exchange and transfer reactions under conditions where both can occur simultaneously. In this experiment, equimolar amounts of $(Ap)_3Cp$, [5′-^{32}P]pCp, and [^3H]ApApA are incubated with

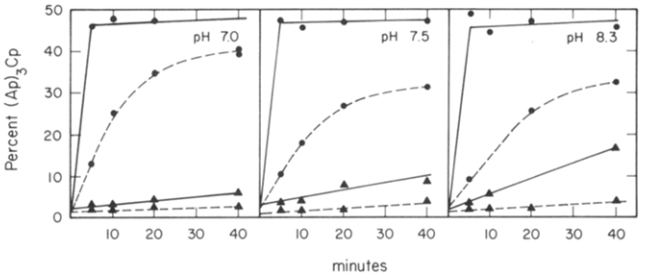

FIGURE 6: Simultaneous transfer and exchange reactions. [^3H]ApApA (0.1 mM, 18 Ci/mol), [5′-^{32}P]pCp (0.1 mM, 4.5 Ci/mol), 0.1 mM $(Ap)_3Cp$, and 0.1 mM AMP were incubated at the indicated pH with 30 units/mL (dashed lines) or 300 units/mL (solid lines) RNA ligase. After paper chromatography in solvent A, the amount of ^3H (circles) or ^{32}P (triangles) in $(Ap)_3Cp$ was determined.

5′-AMP and RNA ligase. At all three pH values, the rate of [^3H]$(Ap)_3Cp$ formation is at least 10 times faster than [^{32}P]$(Ap)_3Cp$ formation. Thus, the transfer reaction is much more rapid than the exchange reaction and indicates that the second step in the RNA ligase mechanism does not reverse as readily as the third step.

Reversal and Oligoribonucleotide Synthesis. The formation of undesirable side products in RNA ligase joining reactions due to reversal occurs frequently. For example, the equimolar joining reactions between ApUpG and pUpCpGp, ApCpG and $p(Ap)_3Cp$, $(Ap)_2CpG$ and $p(Ap)_2Gp$, and ApUpG and pApUpGp all gave large amounts of side products. However, in the additions of $p(Up)_5$ and pApUpGp to $(Ap)_3C$, no side products were detected. The relative rates of the forward and reverse reactions for different donor–acceptor pairs therefore dictate the distribution of products. When a donor such as pApUpGp is used with a good acceptor such as $(Ap)_3C$, the forward reaction is completed and the acceptor used up before the reverse reaction can compete. However, when the same donor is used with a poor acceptor such as ApUpG, the slow forward reaction permits reversal to occur. Since only certain acceptor–donor pairs have a sufficiently fast forward reaction rate, the reverse reaction will affect many joining reactions. The problem must therefore be overcome if RNA ligase is to be an effective tool for oligomer synthesis.

One possibility of controlling the reverse reaction would be to alter the reaction conditions to favor the ATP-dependent forward reaction over the reverse reaction. However, changing the time, temperature, or pH of incubation over a wide range did not greatly improve the ratio of side products to desired product in the ApCpG plus pGpApUp reaction. The use of Mn^{2+} or a mixture of Mn^{2+} and Mg^{2+} to fulfill the divalent cation requirement was also ineffective in altering the ratio of side products to desired product. The only change which improved the situation was to increase the ATP concentration to 5 mM. Unfortunately, the improvement was only moderate, and above 5 mM ATP, the forward reaction is inhibited as much as the reverse reaction.

Since the reverse reaction is catalyzed by the 5′-AMP produced by the forward reaction, one logical method to reduce reversal would be to enzymatically remove AMP from the reaction as it is formed. Such a coupled enzyme system would have to be active under optimal RNA ligase reaction conditions and also be effective at removing very low concentrations of AMP from reaction mixtures since appreciable reversal occurs at 1–2 μM AMP. The pH optimum of adenylate deaminase was too low to permit effective use. However both venom 5′-nucleotidase and an ATP-regenerating system composed of creatine phosphokinase and adenylate kinase showed some improvement in the suppression of reversal in the ApCpG plus

Table III: Different 3′-Terminal Blocking Groups

oligomer	% transfer
$(Ap)_3Cp$	64
$(Ap)_3C>p$	32
$(Ap)_3Cp$-ribose	2
$(Ap)_3Cp$-4-methoxyphenyl	1

pGpApUp reaction. Unfortunately, in both cases, at levels of enzyme which were effective, commercial enzyme preparations contained sufficient ribonuclease activity to cause unacceptable degradation of starting materials. If uncontaminated enzyme preparations could be obtained, enzymatic removal of AMP remains a possibility for controlling the reverse reaction.

One final possibility for suppressing the reverse reaction is suggested by the much greater reactivity of 3′-phosphorylated oligomers compared to 3′-hydroxylated oligomers. Since the only function of the 3′-phosphate group on the donor is to serve as a blocking group to prevent multiple additions, some other group may be equally effective as a blocking group but not be as reactive in the reverse reaction. Thus, several different blocking groups on the 3′ terminus of $(Ap)_3C$ were prepared and tested for their ability to be substrates in the transfer reaction (Table III). The reaction conditions were the same as those in Figure 2 except that 100 units/mL RNA ligase was used in a 30-min incubation. While the 2′,3′ cyclic terminal phosphate only shows some improvement over the 3′-phosphate, both phosphoribose and *p*-methoxyphenyl phosphate show undetectable levels of transfer. These data suggest that a convenient way to avoid reversal in an RNA ligase synthetic reaction is to avoid the use of a 3′-terminal phosphate as a donor blocking group.

Discussion

All three steps of the T4 RNA ligase reaction are readily reversible. The first step of the reaction was demonstrated to be reversible by showing that RNA ligase catalyzes ATP–pyrophosphate exchange in the absence of oligonucleotides (Cranston et al., 1974). Here we demonstrate that the last two steps of the reaction are reversible as well. The formation of A-5′pp5′-Cp in reactions initially containing $(Ap)_3Cp$ and AMP demonstrates that RNA ligase has the ability to cleave a 3′→5′ phosphodiester bond and to form the pyrophosphate linkage between AMP and a 5′-phosphate. Reversal of the second step was demonstrated under similar reaction conditions by isolation of ligase–adenylate from reactions containing only $(Ap)_3Cp$, AMP, and deadenylylated ligase. This reaction presumably proceeds by formation of A-5′pp5′-Cp followed by hydrolysis of the pyrophosphate linkage and formation of the protein–AMP bond.

This reversibility of the ligase reaction formed the basis for two assays designated the transfer reaction and the exchange reaction. The transfer reaction involves reversal of the last step in the ligase reaction mechanism to form the adenylylated intermediate, followed by the same step in the forward direction with the resultant transfer of the 3′-terminal nucleoside bisphosphate from one oligomer to another. The exchange reaction requires reversal of the third and then the second step of the ligase mechanism, followed by both steps in the forward direction. Here the net result is the exchange of the 3′-terminal bisphosphate of an oligomer with free nucleoside 5′,3′-bisphosphate. The two assays overcome the difficulty of the unfavorable equilibrium constant of the reverse steps by measuring combined reverse and forward reactions. Comparison of the rates of transfer and exchange reactions indicate that the former is more than 10 times as rapid as the latter. Thus, it appears that the rate-limiting step of the exchange reaction involves either the formation or the hydrolysis of the adenylylated enzyme.

Although a direct comparison was not made, it is evident from oligomer synthesis reactions that reversal of the last step can proceed at a rate quite comparable to that of the forward reaction. This is in contrast to T4 DNA ligase where the reverse reaction is much slower than the forward reaction (Modrich et al., 1972). However, the rapid reversal of RNA ligase is apparent only when a 3′-terminal phosphate is present in the reaction. Reversal at internal phosphates is considerably slower, a situation more comparable to the DNA ligase reaction. It is not clear why the 3′-terminal phosphate enhances the rate of reversal at the adjacent phosphodiester bond so much. Perhaps the additional negative charge is responsible for the effect since 3′-terminal phosphodiesters are not as active.

Since oligomers with 3′-terminal phosphates are such good substrates for the RNA ligase reverse reaction, phosphate is a very poor choice for a blocking group on the 3′ terminus of the donor in oligomer synthesis reactions. Although not all donor–acceptor pairs will lead to unacceptable levels of reversal, it is presently difficult to predict the extent of correct and incorrect products. While enzymatic methods for removal of AMP from the ligase reaction may be developed, it is probably better to avoid the difficulty entirely and discontinue the use of phosphates as 3′ blocking groups. Some preliminary experiments reported here indicate that other 3′ blocking groups will be satisfactory alternatives. Several other removable 3′ blocking groups have already been successfully employed in RNA ligase reactions, including the acid-labile methoxyethylidene (Ohtsuka et al., 1978) and *O*-(α-methoxyethyl) (Sninsky et al., 1976) groups and the photolabile *o*-nitrophenyl group (Ohtsuka et al., 1979). In addition, the phosphoribose group discussed in this paper may be useful as a 3′ blocking group. It can be introduced onto the 3′ terminus of an oligomer by incubating with ADP-ribose and RNA ligase (England et al., 1977). It can be subsequently removed by periodate oxidation, β elimination, and treatment with alkaline phosphatase. Finally, in cases where the donor molecule is a poor acceptor, no 3′ blocking group is necessary to obtain reasonable yields of product.

It is possible that the reverse reaction of RNA ligase will be of use in oligomer synthesis reactions. Neilson et al. (1980) have used the reversal of the third step to remove the terminal nucleoside bisphosphate from a synthetic oligomer instead of the more traditional method of phosphatase, periodate oxidation, and β elimination. The exchange reaction could be used to conveniently alter the 3′-terminal nucleotide of a 3′-phosphorylated oligomer by exchanging a 3′,5′-bisphosphate.

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Noncovalent Binding of 7β,8α-Dihydroxy-9α,10α-epoxytetrahydrobenzo[*a*]pyrene to Deoxyribonucleic Acid and Its Catalytic Effect on the Hydrolysis of the Diol Epoxide to Tetrol†

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ABSTRACT: In the presence of native DNA the hydrolysis of benzo[*a*]pyrene-7,8-diol 9,10-epoxide (BPDE) to tetrols (BPT) is markedly accelerated (by a factor of up to ~80 at 25 °C, pH 7.0, in 5 mM sodium cacodylate buffer solution). When stopped-flow kinetic techniques are utilized, it is shown that the pseudo-first-order hydrolysis rate constant k_H is smaller by a factor of ~3 in the presence of equivalent concentrations of denatured DNA, by a factor of 8–25 in the presence of nucleotides, and by a factor of 35–45 in the presence of nucleosides (depending on the nucleotide or nucleoside). In the presence of native DNA, k_H increases with increasing DNA concentration and reaches a limiting value of $k_H = 0.684 \pm 0.04\ s^{-1}$ at DNA concentrations in excess of $\sim 5 \times 10^{-4}$ M (expressed in concentration of nucleotides). A kinetic model based on (1) rapid formation of a noncovalent BPDE–DNA complex followed by (2) slower hydrolysis of BPDE to BPT at these binding sites is consistent with the experimental data. It is shown furthermore that the DNA concentration dependence of k_H and of noncovalent intercalative binding of BPDE to DNA is similar and that addition of magnesium ions (which is known to reduce intercalative binding of planar aromatic molecules to DNA) also reduces k_H. These results suggest, but do not necessarily prove, that the DNA binding sites at which the hydrolysis of BPDE (to BPT) is catalyzed are intercalative in nature.

Benzo[*a*]pyrene is a well-known polycyclic aromatic hydrocarbon carcinogen and environmental pollutant. The mechanisms of action of BaP[1] and of other polycyclic aromatic hydrocarbons in vivo have been extensively studied. These compounds are metabolically activated to reactive epoxide derivatives principally on the microsomes of the endoplasmic reticulum catalyzed by the mixed-function oxygenase enzymes. These epoxide derivatives subsequently react with cellular macromolecules, and it is widely believed that DNA is a critical target molecule in the initiation of chemically induced carcinogenesis. Among the more than 30 known metabolites of BaP, the major reactive derivative has been identified as BPDE (Gelboin, 1980; Harvey, 1981; Selkirk et al., 1976). This diol epoxide can react with DNA either in vivo or in vitro. A covalent adduct involving a chemical bond between the 10 position of the hydrocarbon and the exocyclic amino group of guanine is formed predominantly (Jeffrey et al., 1977;

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[1] Abbreviations: BPDE, (±)-7β,8α-dihydroxy-9α,10α-epoxytetrahydrobenzo[*a*]pyrene; BPT, 7,8,9,10-tetrahydroxytetrahydrobenzo[*a*]pyrene; BaP, benzo[*a*]pyrene; THF, tetrahydrofuran; dA, deoxyadenosine; dC, deoxycytidine; dG, deoxyguanosine; dT, thymidine; pdA, deoxyadenosine 5′-phosphate; pdC, deoxycytidine 5′-phosphate; pdG, deoxyguanosine 5′-phosphate; dpT, thymidine 5′-phosphate.

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