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Dinucleoside pyrophosphates are substrates for T4-induced RNA ligase

(RNA synthesis/RNA 3′-modification/NAD$^+$/nucleotides/enzyme mechanism)

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ABSTRACT **RNA ligase isolated from bacteriophage T4-infected *Escherichia coli* will utilize a number of different compounds with the general structure Ado-5′*PP*-X as substrates in an ATP-independent reaction. The *P*-X portions of these molecules are transferred to the 3′-hydroxyl of an oligoribonucleotide to form a phosphodiester bond, and the Ado-5′*P* (AMP) portion is released. AMP, CMP, GMP, UMP, dTMP, NMN, αNMN, reduced NMN, FMN, Rib-5*P*, phosphopantetheine, and cyanoethylphosphate all have been added to [*Cyd*-^{3}H](Ap)$_3$C from their corresponding AMP adducts. Contrary to the relative lack of specificity of RNA ligase for the *P*-X group added, the failure of NADP$^+$, deamino-NAD$^+$, εNCD$^+$, εNAD$^+$, and CoA to react indicates that the enzyme shows a high degree of selectivity for the AMP portion of the substrate. The diversity of chemical groups that can be efficiently added suggests that this reaction of RNA ligase will prove useful for the modification of the 3′ ends of RNA molecules.**

RNA ligase from bacteriophage T4-infected *Escherichia coli* catalyzes the ATP-dependent formation of a 3′ → 5′ phosphodiester bond between an oligoribonucleotide with a 3′-hydroxyl group (the acceptor molecule) and another oligoribonucleotide bearing a 5′-phosphoryl group (the donor molecule) (1–5). This reaction can be dissected into discrete steps involving covalent intermediates. RNA ligase reacts with ATP to form an adenylylated enzyme with the release of pyrophosphate (6). The adenylyl group is subsequently transferred from the enzyme to the 5′-phosphoryl group of the donor oligoribonucleotide to form an adenylylated molecule containing a 5′ → 5′ phosphoanhydride bond (4, 5, 7, 8). The formation of the phosphodiester bond between the two enzyme-bound oligomers presumably occurs *via* the nucleophilic attack of the 3′-hydroxyl group of the acceptor on the activated 5′-phosphoryl group of the donor with the elimination of AMP. Because the adenylylated donor can react with the acceptor in the absence of ATP (4, 5, 8), it is likely to be an intermediate of the reaction, as is the case with T4 DNA ligase (9).

This last step in the RNA ligase reaction between an adenylylated donor oligoribonucleotide (Ado-5′*PP*5′-RNA) and an acceptor oligoribonucleotide is the major topic of this paper. We have examined a number of analogous pyrophosphates with the general structure Ado-5′*PP*-X for their ability to serve as donors in a similar ATP-independent reaction in which the *P*-X moiety is transferred to the acceptor (Ap)$_3$C. We find that many members of this simple class of compounds exclusively donate their nonadenylyl groups to an oligoribonucleotide acceptor. AMP is the other product of the reaction. The structural variability of the groups added and the yields of products obtained suggest that this reaction of RNA ligase will be a useful method for the addition of a wide variety of compounds to the 3′ terminus of RNA molecules.

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MATERIALS AND METHODS

Materials. NAD$^+$, NADH, NADP$^+$, ADP-ribose, CoA, dephospho-CoA, AMP, ADP, CMP, GMP, dTMP, UMP, and Ado-5′*PPP*5′-Guo were purchased from P-L Biochemicals, Inc. Ado-5′*PP*5′-Ado, FAD, αNAD$^+$, deaminoNAD$^+$, and cyanoethylphosphate were obtained from Sigma Chemical Co. [*U*-^{14}C]Ado-5′*PP*5′-Nir (280 Ci/mol) and Ado-5′*PP*5′ [*carbonyl*-^{14}C]Nir (53 Ci/mol) were purchased from Amersham/Searle Corp. εNAD$^+$ was a gift of J. R. Barrio. Spleen and venom phosphodiesterases (SPD and VPD) were purchased from Worthington Biochemical Corp. and RNase A and bacterial alkaline phosphatase (BAP) were from Calbiochem. Bio-Gel P-2 was obtained from Bio-Rad Laboratories. 2-Cyanoethylphosphorylimidazolidate was a gift of B. Placek.

Synthesis of Nucleotides. Adenosine 5′-phosphoromorpholidate was prepared by the method of Moffatt and Khorana (10). The dinucleoside 5′-pyrophosphates containing Ado and Cyd, Guo, dThd, or Urd were synthesized from the AMP-morpholidate and the corresponding nucleotide by procedures described by Moffatt and Khorana for the synthesis of UDP-Glc (10), except that the reactions with CMP and GMP were carried out in dimethyl sulfoxide rather than pyridine. The products were purified by elution from DEAE-Sephadex A-25 (acetate) columns with gradients of triethylammonium acetate (pH 5.0) and precipitation from methanol with acetone and diethyl ether. ADP-cyanoethanol was synthesized in dimethyl formamide from the pyridinium salt of AMP and 2-cyanoethylphosphorylimidazolidate as described by Symons (11). The product was purified by treatment with BAP, chromatography on Whatman 3MM paper in 50% (vol/vol) 1 M ammonium acetate/95% ethanol (solvent A), and desalting by gel filtration chromatography on Bio-Gel P-2. εNCD$^+$ was synthesized as described by Greenfield *et al.* (12).

Preparation of Oligoribonucleotides. (Ap)$_3$C was obtained by RNase A hydrolysis of poly(A,C), separation of the (Ap)$_n$Cp series by RPC-5 column chromatography, and treatment of (Ap)$_3$Cp with BAP (3). Tritium-labeled (Ap)$_3$C (12 Ci/mmol) was obtained by adding a single tritiated cytidine residue to an excess of (pA)$_3$ primer with primer-dependent polynucleotide phosphorylase in the presence of RNase A and treating the reaction mixture with BAP (13).

Purification of RNA Ligase. T4-induced ligase was purified from *E. coli* cells infected with T4 *am* E4314 (gene 43 amber mutant) by a modification of the method described by Walker

Abbreviations: Abbreviations following the IUPAC-IUB Commission on Biochemical Nomenclature recommendations [*J. Mol. Biol.* **55**, 299 (1971)] are used throughout. SPD, VPD, and BAP, spleen phosphodiesterase, venom phosphodiesterase, and bacterial alkaline phosphatase, respectively; εNCD$^+$, nicotinamide 3,N^4-ethenocytosine dinucleotide; εNAD$^+$, nicotinamide 1-N^6-ethenoadenine dinucleotide; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

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et al. (3) with Sephadex G-100 in place of Sephadex G-75. In addition, the enzyme was further purified by gradient elutions from columns of hydroxylapatite and DEAE-cellulose. One unit of RNA ligase activity is defined as 1 nmol of $(pA)_{12}$ circularized in 30 min at 37° in the following reaction mixture (2): 50 mM *N*-2-hydroxypiperazine-*N'*-2-ethanesulfonic acid (Hepes) (pH 7.5), 10 mM $MgCl_2$, 0.25 mM dithiothreitol, bovine serum albumin (50 μg/ml), 0.1 mM ATP, 1 μM $(pA)_{12}$, and 2.5–20 milliunits of enzyme. The enzyme used in this study had a specific activity of 2100 units/mg.

Assay of Joining Reaction. Reactions between $(Ap)_3C$ and the compounds with structure Ado-5′*PP*-X were performed in 200-μl polyethylene microcentrifuge tubes in reaction mixtures (30 μl) containing 50 mM Hepes (pH 8.3), 20 mM $MgCl_2$, 3.3 mM dithiothreitol, bovine serum albumin at 10 μg/ml, 8.3% (vol/vol) glycerol, 0.5 mM dinucleoside pyrophosphate, 0.1 mM [*Cyd*-^{3}H]$(Ap)_3C$ (120 Ci/mol), and RNA ligase at 330 units/ml. Each reaction was incubated at 37° for 1 hr. The entire mixture was spotted on Whatman 3MM paper and developed by descending paper chromatography for 48–72 hr with solvent A.

After drying, the product oligomers were located by observation under ultraviolet light. The products were always less mobile than the acceptor, $(Ap)_3C$. Radioactivity was determined by cutting the chromatograms into 1-cm strips and assaying in a scintillation counter. Yields were calculated from the ratio of radioactivity in the product region to the total present on the chromatogram.

Product Characterization. The isolated product oligomers (*ca.* 0.6 nmol, 120 Ci/mol) were degraded in the following reaction mixtures (30 μl): (*i*) 20 mM Tris·HCl, pH 8.5/10 mM $MgCl_2$/VPD, 1 unit/ml; (*ii*) 100 mM potassium phosphate, pH 6.5/1 mM EDTA/SPD, 1.7 units/ml; and (*iii*) 200 mM Tris·HCl, pH 8.2/200 mM NaCl/RNase A, 0.1 mg/ml or, in addition, BAP, 0.2 mg/ml, at 37° for 1–4 hr. The reaction mixtures, along with appropriate markers, were analyzed by paper chromatography in solvent A. In reactions using NADH and FAD the product oligomers were fluorescent, and all products containing quarternary nicotinamide residues became fluorescent after development of the chromatograms in a 2-butanone/NH_3 atmosphere (14).

RESULTS AND DISCUSSION

Reaction of $(Ap)_3C$ and NAD$^+$. RNA ligase catalyzed the formation of a phosphodiester bond between the 3′-hydroxyl group of $(Ap)_3C$ and the 5′-phosphoryl group of NMN when the oligoribonucleotide was incubated with NAD$^+$ (Ado-5′-*PP*5′-Nir). When a reaction mixture containing [*Cyd*-^{3}H]-$(Ap)_3C$ (0.1 mM) and [*Nir*-^{14}C]NAD$^+$ (0.2 mM) was incubated with RNA ligase and subsequently analyzed by descending paper chromatography, the results shown in Fig. 1*A* were obtained. The majority of the ^{3}H label originally present in $(Ap)_3C$ migrated more slowly, suggesting that a longer oligomer had been made. A small amount of ^{3}H label migrated with $(Ap)_3C$ and represents unreacted acceptor. Coincident with the ^{3}H label in the product was ^{14}C label originally present in the nicotinamide portion of NAD$^+$. The remaining ^{14}C label comigrated with unreacted NAD$^+$. It can be calculated from the specific activities of the $(Ap)_3C$ and NAD$^+$ that one NMN was transferred to each acceptor molecule converted to product. These results suggest that RNA ligase can transfer a single NMN from NAD$^+$ to $(Ap)_3C$ to form $(Ap)_3$CpNir.

In an identical reaction performed with NAD$^+$ labeled in the Ado portion ([*Ado*-^{14}C]NAD$^+$), the results shown in Fig. 1*B*

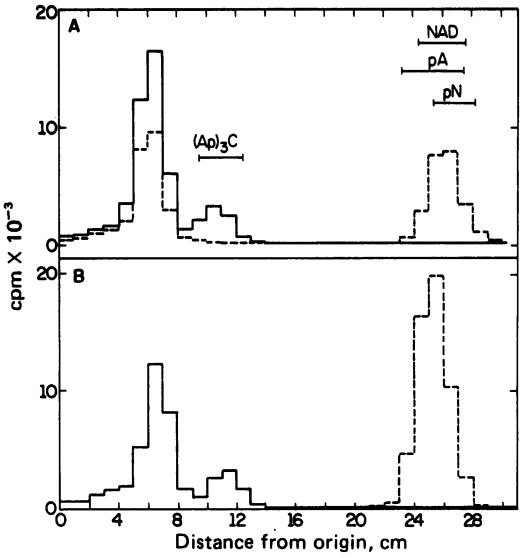

FIG. 1. Intermolecular ligation of $(Ap)_3C$ and NAD$^+$. (*A*) [*Cyd*-^{3}H]$(Ap)_3C$ (0.1 mM, 190 Ci/mol) and [*Nir*-^{14}C]NAD$^+$ (0.2 mM, 7.5 Ci/mol). (*B*) [*Cyd*-^{3}H]$(Ap)_3C$ (0.1 mM, 190 Ci/mol) and [*Ado*-^{14}C]-NAD$^+$ (0.2 mM, 8 Ci/mol). Incubation was at 37° for 60 min with RNA ligase at 500 units/ml. Analysis was by descending paper chromatography in solvent A (16 hr). The solid line represents ^{3}H label; the dashed line is ^{14}C label.

were obtained. Again the majority of the ^{3}H-labeled $(Ap)_3C$ was converted to a single, more slowly migrating product. However, none of the ^{14}C label from the Ado portion of NAD$^+$ was incorporated into the product; rather, it all migrated with NAD$^+$ and AMP. This experiment suggests that only the NMN and not the AMP portion of NAD$^+$ is transferred by RNA ligase to the acceptor oligoribonucleotide.

Product Characterization. When the chromatograms presented in Fig. 1 were treated with an NH_3/2-butanone atmosphere (14), fluorescent spots characteristic of quarternary nicotinamide compounds developed in locations coincident with the product and NAD$^+$. Thus, the presence of an Nir moiety in the product oligoribonucleotide is indicated.

The product of the RNA ligase catalyzed reaction of [*Cyd*-^{3}H]$(Ap)_3C$ and [*Nir*-^{14}C]NAD$^+$ shown in Fig. 1*A* was isolated by elution from a paper chromatogram and was characterized by degradation with specific enzymes. Rechromatography of the isolated material is illustrated in Fig. 2*A* and shows the coincidence of migration of the two radiolabels. When the isolated product was treated with VPD, an exonuclease requiring a 3′-hydroxyl group and yielding 5′-nucleoside monophosphates, the ^{3}H label migrated with 5′-CMP and the ^{14}C label with NMN (Fig. 2*B*). Treatment of the product with SPD, an exonuclease requiring a 5′-hydroxyl group and yielding 3′-nucleoside monophosphates, resulted in the formation of a ^{3}H-labeled product migrating with 2′(3′)-CMP and a ^{14}C-labeled product chromatographing with nicotinamide (Fig. 2*C*). Digestion of the product with RNase A yielded a ^{3}H-labeled oligoribonucleotide that comigrated with authentic $(Ap)_3$Cp and ^{14}C-labeled material migrating with nicotinamide (Fig. 2*D*). These results confirm that a 3′ → 5′ phosphodiester bond links the Cyd and Nir residues in the product oligoribonucleotide. Because only one NMN residue is added, the structure of the product is $(Ap)_3$CpNir.

Reaction of $(Ap)_3C$ and Other Pyrophosphates. A number of substrates of the general structure Ado-5′*PP*-X were tested as substrates for RNA ligase. The enzyme catalyzed the transfer of the nonadenylyl portion of a number of these compounds to

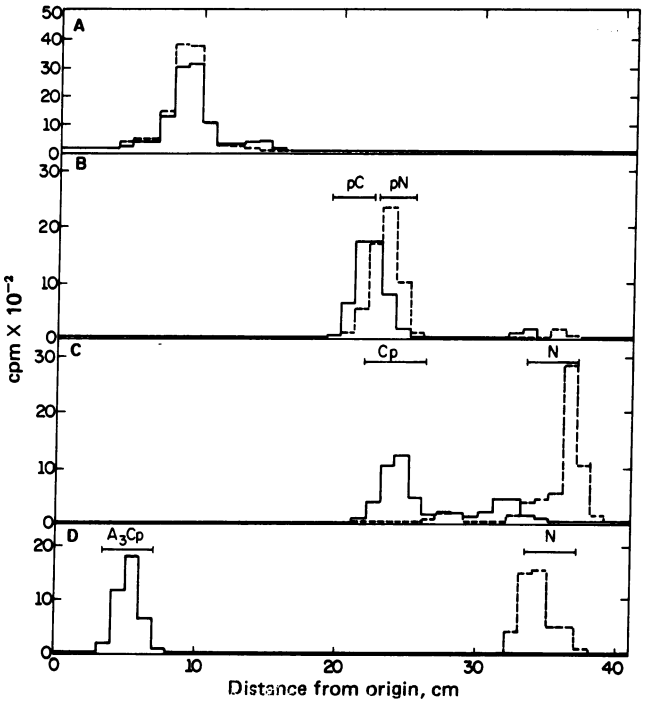

FIG. 2. Nuclease degradation of [*Cyd*-^{3}H, *Nir*-^{14}C](Ap)$_3$CpNir. Reaction mixtures were analyzed by descending paper chromatography in solvent A (36 hr) after hydrolysis with nucleases as described in *Materials and Methods*. (*A*) Untreated (Ap)$_3$CpNir; (*B*) treatment with VPD to produce [^{3}H]pC and [^{14}C]NMN; (*C*) treatment with SPD to produce [^{3}H]Cp and [^{14}C]Nir; and (*D*) treatment with RNase A to produce [^{3}H](Ap)$_3$Cp and [^{14}C]Nir. The solid line represents ^{3}H label; the dashed line is ^{14}C label.

[*Cyd*-^{3}H](Ap)$_3$C under the conditions described in *Materials and Methods* (Table 1). The yield was determined by separating the components of the reaction mixture by paper chromatography and calculating the fraction of ^{3}H label converted into product. When the pyrophosphate substrates were composed of AMP and the four common ribonucleotides, the product was itself susceptible to further addition. Good yields of a short distribution of the (Ap)$_3$C(pN)$_n$ series were obtained. The relatively low yield of (Ap)$_3$CdT was probably due to a DNA exonuclease activity that contaminates the enzyme preparation.

NADH and αNAD$^+$ were also excellent substrates, indicating that the charge on the Nir moiety and the conformation about the *N*-glycosyl bond in the group transferred is not crucial for enzyme recognition. Because ADP-ribose is also a good substrate, the presence of a base in the donor portion of the pyrophosphate is not required. However, even ribose cannot be considered to be the minimum substrate required for the donor portion of the pyrophosphate because dephospho-CoA, FAD, and ADP-cyanoethanol also are substrates for RNA ligase.

Each of the products of the successful reactions listed in Table 1 were detected as a slower migrating peak of ^{3}H label on paper chromatography. Transfer of *P*-X to the oligoribonucleotide was confirmed with RNase A and RNase A plus BAP treatments. The degradations produced radiolabel comigrating with authentic (Ap)$_3$Cp and (Ap)$_3$C, respectively. For example, when the isolated product of the RNA ligase-catalyzed reaction of FAD and [*Cyd*-^{3}H](Ap)$_3$C was treated with RNase A, the radioactivity migrated faster than the product and was coincident with (Ap)$_3$Cp (Fig. 3*A*, solid line). Treatment of the product with RNase A plus BAP regenerated the acceptor

Table 1. Substrates tested with RNA ligase:
(Ap)$_3$C + Ado-5′*PP*-X → (Ap)$_3$CpX + Ado-5′*P*

Substrate	Yield*
Ado-5′*PP*5′-Ado	61 (22)
Ado-5′*PP*5′-Cyd	51 (10)
Ado-5′*PP*5′-Guo	48 (52)
Ado-5′*PP*5′-Urd	56 (27)
Ado-5′*PP*5′-dThd	19 (7)
Ado-5′*PP*5′-Rib	91
Ado-5′*PP*5′-Nir	94
Ado-5′*PP*5′-Nir (reduced)	100
Ado-5′*PP*5′-αNir	70
Ado-5′*PP*4-pantetheine	55
Ado-5′*PP*5′-riboflavin	32
Ado-5′*PP*1-cyanoethanol	80
Ado-5′*PP*1-Glc	6
Ado-5′*PP*	1
Ado-5′*PPP*5′-Guo	5
P-2′-Ado-5′*PP*5′-Nir	1
P-3′-Ado-5′*PP*4-pantetheine	2
ϵCyd-5′*PP*5′-Nir	3
ϵAdo-5′*PP*5′-Nir	4
Ino-5′*PP*5′-Nir	2

* The numbers in parentheses represent products containing more than one added residue.

(Ap)$_3$C (Fig. 3*A*, dashed line). In two further examples, the products of the reactions of ADP-ribose and dephospho-CoA with acceptor were similarly isolated and degraded (Fig. 3 *B* and *C*, respectively). These analyses indicate that the phosphodiester bond formed with the *P*-X portion of the pyrophosphate used the 3′-hydroxyl of the cytidine of the acceptor. If the RNA ligase had added to the 2′-hydroxyl, the product would not have been sensitive to RNase A.

The only common feature of the successful substrates in Table 1 is the structure Ado-5′*PP*-CH_2-. ADP by itself was unable to transfer a phosphate to (Ap)$_3$C under various condi-

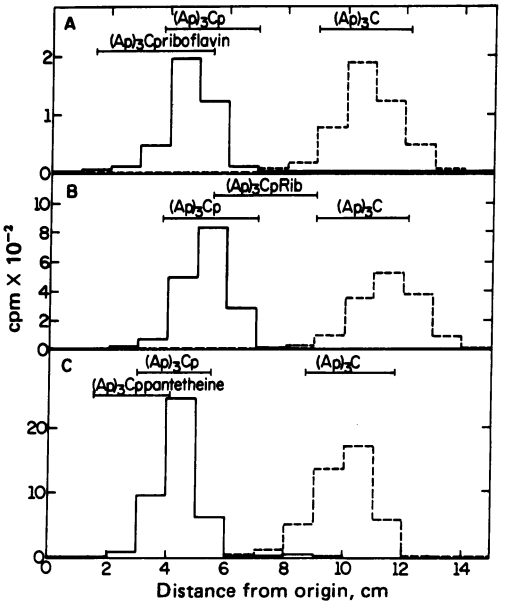

FIG. 3. Enzymatic degradation of products. The ^{3}H-labeled oligonucleotides were treated with RNase A (solid line) and RNase A plus BAP (dashed line) and analyzed by descending paper chromatography in solvent A (24 hr). (*A*) (Ap)$_3$Cp-riboflavin; (*B*) (Ap)$_3$CpRib; and (*C*) (Ap)$_3$Cp-pantetheine.

tions of divalent metal concentration, pH, and nucleotide concentration. Thus, the X moiety in Ado-5′*PP*-X cannot be hydrogen. The pyrophosphate must be substituted on both phosphates to be a substrate in the RNA ligase reaction. Because Ado-5′*PPP*5′-Guo was not reactive, X cannot be phosphate. Finally, the failure of ADP-glucose to serve as a substrate may be explained either by the electronic character of the phosphoacetal linkage or by steric factors.

Unlike the results with the common nucleosides, only one residue was added to $(Ap)_3C$ in all the other successful reactions. In some cases, no 3′-hydroxyl residue was available to transfer a second *P*-X moiety onto the product oligonucleotide. In cases in which a 3′-hydroxyl is available, it may be concluded that the substrate requirement for the acceptor oligonucleotide is more stringent than the donor. Relatively large differences in the rate of the intermolecular reaction have been observed for different acceptor sequences (4, 5).

The general lack of specificity observed for the *P*-X moiety is to be contrasted with the high degree of specificity that RNA ligase has for the Ado portion of the pyrophosphate—the group that is eliminated as AMP in the successful reaction. Modification of either the adenine (ϵNCD^+, deamino-NAD^+, or ϵNAD^+) or the ribose (CoA, $NADP^+$) moiety renders the substrates unreactive. This finding is consistent with the fact that only ATP and, to a lesser extent, dATP are cofactors in the circularization reaction catalyzed by RNA ligase (1).

RNA ligase will catalyze the ATP-dependent addition of a single 3′,5′-ribonucleoside bisphosphate to oligoribonucleotide acceptors (unpublished data). This reaction also shows little specificity for the base of the nucleotide added but does require that there be a phosphate on the 3′-hydroxyl in order to form an adenylylated intermediate and transfer to the 3′ end of the acceptor oligonucleotide. Nucleoside 5′-monophosphates were not found to be active donors in the ATP-dependent RNA ligase reaction. Because we find here that dinucleoside pyrophosphates are active ATP-independent donors, it is clear that, once the pyrophosphate is formed, the specificity for the group transferred is greatly diminished.

The remarkable lack of specificity of adenylylated compounds with RNA ligase extends the kinds of residues that can be added to oligoribonucleotides and represents a facile and versatile method for the modification of the 3′ ends of RNA molecules. The introduction of fluorescent groups, of antigenic determinants, of radiolabels, or of specific protecting groups are examples of possible applications of this reaction.

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