*ENZYMATIC JOINING OF POLYNUCLEOTIDES, V. A DNA-ADENYLATE INTERMEDIATE IN THE POLYNUCLEOTIDE-JOINING REACTION**

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In the reaction catalyzed by the polynucleotide-joining enzyme from *Escherichia coli*, phosphodiester bonds are synthesized between the 3′-hydroxyl and 5′-phosphoryl termini of properly aligned DNA chains coupled to the cleavage of the pyrophosphate bond of DPN.[1–4] The first step in this over-all reaction occurs in the absence of DNA and consists of the formation of a covalently linked enzyme-adenylate (E-AMP) intermediate and simultaneous release of NMN[5, 6] (Fig. 1).

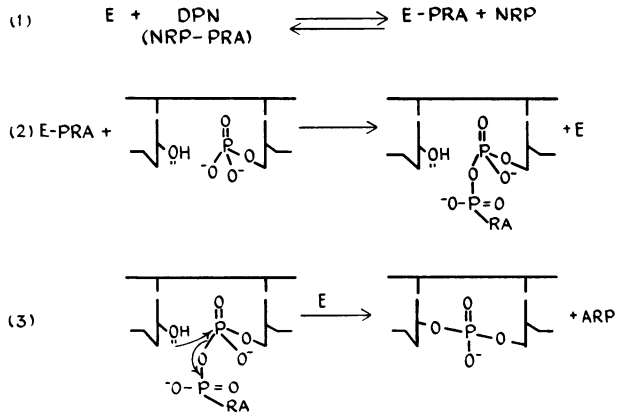

FIG. 1.—Postulated mechanism of the reaction catalyzed by the *E. coli*-joining enzyme. DPN is written as NRP-PRA to emphasize the pyrophosphate bond linking the nicotinamide mononucleotide (NRP) and adenylic acid (PRA) moieties of the DPN molecule. The designation E-PRA for enzyme-adenylate is not meant to imply that linkage of AMP to the enzyme is necessarily through the phosphate group.

This paper presents evidence for a second intermediate in the joining reaction. This intermediate is formed by reaction of E-AMP with a DNA chain to generate a new pyrophosphate bond linking the 5′-phosphoryl terminus of the DNA and the phosphoryl group of the AMP. In the final step of the joining reaction we presume that the DNA phosphate in the pyrophosphate bond of the DNA-adenylate is attacked by the 3′-hydroxyl group of the neighboring chain, displacing the activating AMP group and effecting the synthesis of the phosphodiester bond (Fig. 1).

Experimental Procedure.—Materials: Unlabeled nucleotides were purchased from Calbiochem. H^3-labeled ATP and AMP were obtained from Schwarz BioResearch. γ-P^{32}-ATP was prepared by the method of Glynn and Chappell.[7] DPN was obtained from

the Sigma Chemical Co. H^3-adenine-labeled DPN (spec. act. 1.5–2.0 × 10^3 cpm/$\mu\mu$mole) was synthesized from H^3-ATP and NMN with the hog liver DPN pyrophosphorylase[8] and purified by chromatography on DEAE-Sephadex. $d(pC)_3$ was prepared according to Khorana, Turner, and Vizsolyi.[9] Phage λ DNA was isolated from the purified phage by phenol extraction as described by Kaiser and Hogness.[10]

Single-strand scissions with 5′-phosphoryl and 3′-hydroxyl termini were introduced into λ DNA by treatment with pancreatic DNase. The reaction mixture (4.0 ml) contained 0.1 *M* Tris-HCl, pH 8.0, 10 mM $MgCl_2$, λ DNA, A_{260} = 4.0, and 0.1 μg of pancreatic DNase. After incubation at 30° for 30 min, 1 *M* EDTA, pH 8.7, was added to a final concentration of 20 mM. The solution was extracted 3 times with 2-ml aliquots of phenol (equilibrated with 0.3 *M* Tris-HCl, pH 8.6) and dialyzed (3 changes) against 1-liter portions of 10 mM Tris-HCl, pH 8.0, 10 mM EDTA, then against 1 liter of 10 mM Tris-HCl, pH 8.0. This preparation of DNA contained an average of 70 single-strand scissions per molecule as judged by the ability of the DNA to accept P^{32} from γ-P^{32}-ATP in the presence of polynucleotide kinase after treatment of the DNA with *E. coli* alkaline phosphatase at 65°.[11]

Single-stranded DNA chains with H^3-riboadenylate at their 3′ termini were synthesized as described by Richardson and Kornberg.[12]

The DNA-adenylate intermediate isolated by CsCl density gradient centrifugation was purified by filtration through Sephadex G-25. H^3-containing fractions banding at the density of λ DNA (Fig. 2) were pooled and applied to a column (20 × 1 cm) of Sephadex G-25 equilibrated with 1 mM Tris-HCl, pH 8.0–1 mM EDTA–10 mM KCl. The DNA-bound AMP appeared in the void volume and was well separated from the bulk of the H^3, presumably unreacted DPN; it was concentrated approximately tenfold by evaporation under reduced pressure.

Poly dT-adenylate was prepared by condensing H^3-AMP with $p^{32}TpTpT$ by the morpholidate method of Moffatt and Khorana;[13] deoxythymidylate residues were then added to the 3′-hydroxyl end of the $d(pT)_3$ moiety by using the H^3-App^{32}TpTpT as an initiator in a reaction catalyzed by calf thymus deoxynucleotidyl transferase.[14] The average chain length was 100 deoxythymidylate residues. The P^{32} in the product was acid-precipitable and was insusceptible to alkaline phosphatase except after being heated at 100° in 1 *N* HCl for 15 min, or after treatment with *E. coli* exonuclease I and snake venom phosphodiesterase. Details of the preparation and characterization of the poly dT-adenylate will be published elsewhere.

Pancreatic DNase, *E. coli* alkaline phosphatase, micrococcal nuclease, spleen, and snake venom phosphodiesterases were purchased from the Worthington Biochemical Co. *E. coli* exonuclease I (DEAE-cellulose fraction) was purified by the method of Lehman and Nussbaum.[15] *E. coli* polynucleotide-joining enzyme (fraction V) was prepared and assayed as described previously;[1] it was further purified by gradient chromatography on DEAE-Sephadex.[16]

Methods: Density gradient sedimentation was carried out at 25° in the International B60 centrifuge with the SB 405 rotor. To 2.7 gm of solution to be analyzed (containing 30 μmoles of 1 *M* Tris-HCl, pH 8.6, and 30 μmoles of EDTA, pH 8.7) were added 3.5 gm CsCl, and the solution was centrifuged for 30 hr at 53,000 rpm. The bottom of the tube was then punctured and 15-drop fractions (usually a total of 16) were collected. Acid-insoluble radioactivity was determined as described elsewhere.[17]

Paper electrophoresis of nucleotides was performed at 20° in 0.015 *M* sodium citrate buffer, pH 5.5, at a potential of 5000 volts for 30–75 min. Descending paper chromatography on Whatman 3 MM paper was carried out with the 1-propanol–ammonia–water system of Hanes and Isherwood.[18] After electrophoresis or chromatography, the paper was cut into strips and the radioactivity determined. Measurements of radioactivity were made using a Nuclear-Chicago model 724 liquid scintillation counter.

Results.—Isolation of DNA-adenylate: When joining enzyme, H^3-labeled DPN, and phage λ DNA containing multiple single-strand scissions were briefly

incubated (5 min) at 0°, a small peak of acid-precipitable H^3 with the buoyant density of λ DNA was identified by density gradient centrifugation (Fig. 2). This DNA-adenylate peak amounted to approximately 1 per cent of the H^3 found at the top of the gradient where E-AMP would be expected to band. Assay of the fractions from the CsCl gradient showed that joining-enzyme activity was exclusively at the top of the gradient; less than 0.01 per cent could be detected at the density of λ DNA. DNA-adenylate did not appear when denatured DNA was used in place of native λ DNA. Similarly, termination of the reaction after mixing H^3-DPN and enzyme, but before the addition of DNA, prevented the accumulation of H^3 in the position of the gradient occupied by λ DNA (Table 1). In both of these cases, despite the absence of DNA-adenylate, E-AMP was formed, as judged by the appearance of high levels in acid-insoluble radioactivity at the top of the density gradient. When joining enzyme which had been denatured by heating for two minutes at 100° was used, acid-insoluble H^3 could not be detected at any point in the gradient.

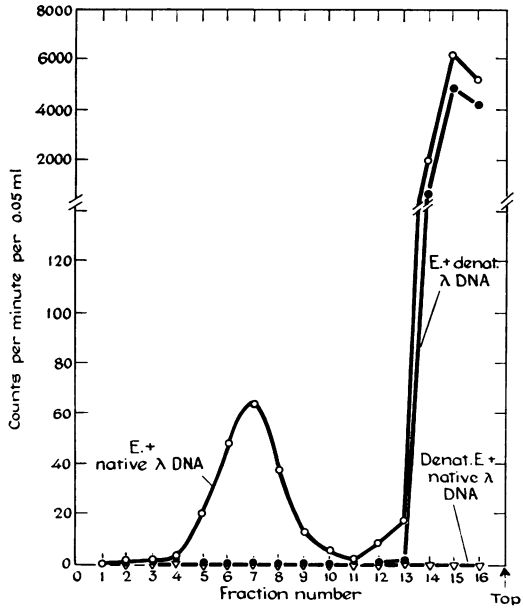

FIG. 2.—Demonstration of DNA-adenylate by CsCl density gradient centrifugation. A reaction mixture (0.1 ml) containing 25 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 2.5 mM EDTA, 12.5 μg of bovine plasma albumin, 70 units of joining enzyme (*E*), and 0.68 μM H^3-DPN (1.8×10^3 cpm/μμmole) was incubated for 5 min at 30°. Pancreatic DNase-treated λ DNA (0.1 ml) was added, and the mixture incubated for 5 min at 0°. More DNA (0.1 ml) and H^3-DPN (34 μμmoles) was added, the solution mixed rapidly at 0°, and the reaction immediately terminated by the addition of 0.02 ml of 0.66 *M* glycine-NaOH, pH 10.2, containing 0.33 *M* EDTA. Two control reaction mixtures were prepared and treated as above, except that in one the enzyme had been heated to 100° for 2 min and in the second the λ DNA had been heated to 100° for 2 min. CsCl density gradient centrifugation and determination of acid-insoluble H^3 were then performed as described in *Methods*.

Radioactivity found in the position of λ DNA appeared within 0.5 minute of incubation at 0° and then fell precipitously with further incubation. On the other hand, the amount of radioactivity in the E-AMP fraction remained relatively constant and even increased slightly with longer periods of incubation (Table 1). After incubation for five minutes at 30°, there was no detectable DNA-adenylate peak.

When the number of single-strand breaks in the DNA was raised by about tenfold, the yield of DNA-adenylate was increased. Under these conditions, the DPN concentration became limiting and the levels of both DNA-adenylate and E-AMP fell upon incubation; however, the ratio of the two remained relatively constant (14.7% at 0.5 min and 6.7% at 5 min).

TABLE 1. *Effect of incubation conditions on yield of DNA-adenylate.*

Time of incubation at 0° (min)	Units of enzyme	H^3 in E-AMP (cpm)	H^3 in DNA-adenylate (cpm)	Yield (%)
0	250	30,300	<10	<0.03
0.5	290	12,200	970	7.9
5.0	145	25,300	406	1.6
5.0	250	38,000	250	0.7

The reaction mixtures (0.1 ml) for the samples incubated for 5 min contained 25 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 2.5 mM EDTA, 1 μM H^3-DPN (1.8×10^3 cpm/μμmole), 12.5 μg of bovine plasma albumin, and the indicated amounts of enzyme. After 5 min at 30° the mixtures were chilled to 0° and 0.2 ml of pancreatic DNase-treated λ DNA (prepared as described in *Methods*) was added. After 5 min the reactions were terminated by adding glycine-EDTA as described in the legend to Fig. 2. The "0-min" reaction mixture was prepared in the same way except that the glycine-EDTA was added before the λ DNA. The composition of the reaction mixture for the sample incubated for 0.5 min was the same as that described above, except that it was scaled up to 0.3 ml. After the 5-min preincubation period at 30°, the mixture was chilled to 0° and 0.5 ml of λ DNA was added. After 0.5 min the reaction was terminated. All of the samples were then subjected to CsCl density gradient centrifugation as described in *Methods*. An aliquot (0.1 ml) of each fraction was used to determine acid-insoluble H^3.

Evidence that DNA-adenylate is an intermediate in the reaction: The isolated DNA-adenylate displayed the properties expected of an intermediate in the joining reaction. Thus, the H^3-AMP was quantitatively released into an acid-soluble form upon treatment of the DNA-adenylate with joining enzyme in the absence of added DPN (Table 2). The liberated H^3 was identified chromatographically as 5′-AMP; after treatment with phosphatase it cochromatographed

TABLE 2. *Release of AMP from DNA-adenylate in the absence of DPN.*

Units of enzyme	"Native" DNA-adenylate (% of H^3 made acid-soluble)	Heat-denatured DNA-adenylate (% of H^3 made acid-soluble)
6.0	100	35*
0.2	60	—
0.06	32	2

Reaction mixtures (0.1 ml) contained 10 mM Tris-HCl, pH 8.0, 2 mM $MgCl_2$, 1 mM EDTA, 5.0 μg of bovine plasma albumin, the indicated amounts of joining enzyme and either native DNA-adenylate (2 μg of DNA, 20 cpm) or DNA-adenylate denatured by heating at 100° for 2 min. After incubation at 37° for 30 min, the reaction mixtures were heated at 100° for 2 min and acid-insoluble H^3 was determined. A minimum of 400 counts over background was recorded for the sample in which all the AMP was retained in the DNA-adenylate.

* The acid-soluble H^3 formed in this sample was not free AMP (see text).

with adenosine. When the DNA-adenylate was heat-denatured before incubation with joining enzyme, approximately one third of the AMP was converted to an acid-soluble form (Table 2). However, after treatment with phosphatase and chromatography, essentially all (>90%) of the H^3 remained at the origin, well separated from the 5′-AMP and adenosine markers and at a position where oligonucleotide material would be anticipated in the solvent system used. The release of AMP linked to oligonucleotide from heat-denatured DNA-adenylate is therefore most probably due to nuclease contamination of the joining enzyme preparation, which became significant at the relatively high levels of enzyme used in this experiment.

Evidence that AMP is linked to the 5′-terminus of DNA: The two most probable sites in DNA to which AMP may be linked are at the 5′-phosphoryl and the 3′-hydroxyl termini. *E. coli* exonuclease I should be capable of distinguishing between these two possibilities. This enzyme degrades single-stranded DNA sequentially from the 3′-hydroxyl end, and produces 5′-mononucleotides but leaves the 5′-terminal dinucleotide intact.[15] Thus, if the AMP were linked to the 5′-phosphoryl terminus of the DNA, digestion by exonuclease I should yield a trinucleotide in which the AMP is linked to the terminal dinucleotide through a pyrophosphate bond. On the other hand, if the AMP were in phosphodiester linkage at the 3′ end of the DNA, it should be released as free AMP.[15]

The isolated DNA-adenylate intermediate and a control DNA preparation bearing AMP at its 3′ terminus were denatured, treated with exonuclease I and alkaline phosphatase, and then chromatographed. In the case of the DNA with AMP at its 3′ terminus, the only product formed was adenosine (Fig. 3); AMP must therefore have been released by the exonuclease I treatment. On the other hand, treatment of the isolated intermediate with exonuclease I and alkaline phosphatase yielded products which formed a rather broad radioactive peak on the chromatogram at the position expected of a mixture of trinucleotides (Fig. 3). Digestion of synthetic poly dT-adenylate (see below) with these enzymes produced a similar peak of radioactivity in the trinucleotide region of the chromatogram. These data indicate that the AMP is linked at the 5′ end of the isolated DNA-adenylate. Moreover, the finding that the product of exonuclease I digestion migrated to the position occupied by a trinucleoside triphosphate even after phosphatase treatment is consistent with the presence of an internal pyrophosphate group linking the AMP to the 5′-phosphoryl terminus of the DNA.

Activity of synthetic poly dT-adenylate as a substrate for the joining enzyme: To determine directly whether the polynucleotide-joining reaction involves formation of a pyrophosphate linkage between AMP and the 5′-phosphoryl terminus of the polynucleotide chain, the presumptive intermediate was synthesized and tested as a substrate for the joining enzyme.

Poly dT-adenylate was prepared in which the 5′-terminal phosphate of the poly dT was labeled with P^{32} and the adenylate labeled with H^3. The double label permitted simultaneous measurement of the release of AMP from the polynucleotide and the incorporation of the terminal phosphate of poly dT into phosphodiester linkage.

When poly dT-adenylate was incubated with joining enzyme in the absence of

DPN, the H^3 was released as an acid-soluble product, identified chromatographically as AMP, and a nearly equivalent amount of the P^{32} was converted to a form which was insensitive to alkaline phosphatase after heating in 1 *N* HCl at 100° for 15 minutes (Table 3). Upon degradation of the product to 3′-mononucleotides by the combined action of micrococcal nuclease and spleen phosphodiesterase, all of the P^{32} was found to be associated with 3′-dTMP, a result which is consistent with its incorporation into a phosphodiester bond. Thus, there is a stoichiometric correspondence between cleavage of the pyrophosphate bond link-

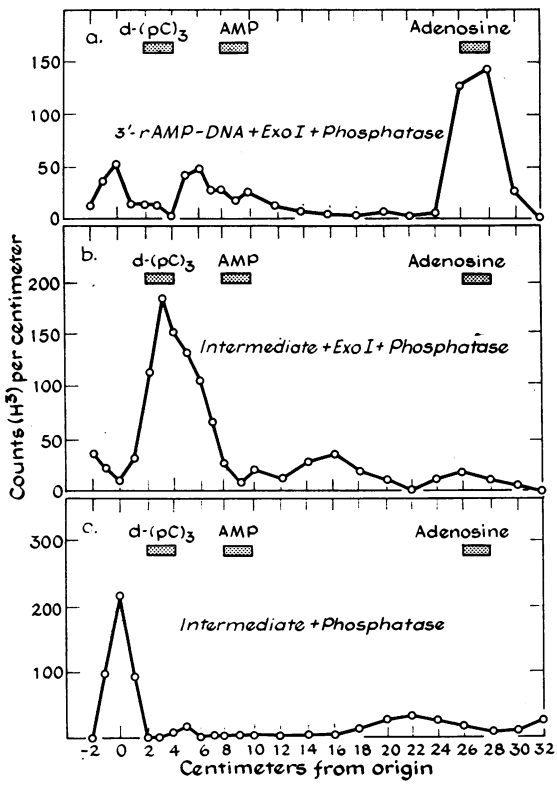

FIG. 3.—Paper electrophoresis of DNA-adenylate intermediate and DNA chains with riboadenylate at their 3′-termini (*3′-rAMP-DNA*) after treatment with exonuclease I (*Exo I*) and phosphatase. Three reaction mixtures (0.2 ml each) were prepared containing 80 mM glycine-NaOH, pH 9.5, 8 mM $MgCl_2$, and 2.5 mM β-mercaptoethanol. To one (reaction mixture *a*) were added single-stranded DNA chains with H^3-AMP at their 3′-termini (60 μg DNA, 800 cpm), 17 units of exonuclease I,[15] and 5 units of phosphatase.[23] To the other two were added heat-denatured DNA-adenylate (19 μg DNA, 250 cpm) and either exonuclease I and phosphatase (*b*) or phosphatase alone (*c*). The reaction mixtures were incubated at 37° for 30 min, then chromatographed for 18 hr in the 1-propanol–ammonia–water system. The paper was dried, the nucleotides were identified, and the radioactivity was determined as described in *Methods*. The values shown correspond to the total number of counts, corrected for background recorded for each strip.

ing poly dT and AMP on the one hand and phosphodiester bond formation on the other. Both the release of AMP and the incorporation of the P^{32} into phosphodiester linkage required that poly dA be present.

The reaction mechanism for the polynucleotide-joining enzyme as proposed in Figure 1 predicts that adenylylation of the enzyme (to form E-AMP) would render it inactive in the reaction with poly dT-adenylate. Consistent with this prediction was the observation that preincubation of the enzyme with 0.3 mM DPN before the addition of poly dT-adenylate resulted in 95 per cent inhibition of phosphodiester bond formation.

Discussion.–The data presented here show that an intermediate in which AMP is bound in pyrophosphate linkage to the 5′-phosphoryl termini of DNA is formed

TABLE 3. *Reactivity of synthetic poly dT-adenylate in the polynucleotide-joining reaction.*

Enzyme	Poly dA	P^{32} in phosphodiester linkage ($\mu\mu$moles)	H^3-AMP released ($\mu\mu$moles)
−	+	5	7
+	+	38	46
+	−	2	3

The reaction mixtures contained, in a final volume of 0.1 ml, 10 mM Tris-HCl, pH 8.1, 3 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 10 μg bovine plasma albumin, 0.42 μM (in dT termini) P^{32}-poly dT-H^3 adenylate (200 cpm of P^{32} and 13 cpm of H^3 per $\mu\mu$mole of termini), 48 μM (in dAMP residues) poly dA, and 6 units of joining enzyme as indicated. After 1 hr at 30°, 0.015-ml aliquots were removed from each reaction and heated with 0.05 ml 1 *N* HCl at 100° for 15 min. 2 *M* Tris-HCl, pH 8.1 (0.1 ml), and 0.12 unit of phosphatase[23] were then added and the reaction mixtures incubated at 37° for 30 min. The fraction of P^{32} adsorbable to Norit after treatment with phosphatase was then measured as described previously.[1] To the remainder of the incubation mixtures were added 0.01 ml 0.95 mM poly dA, 0.1 ml 0.1 *M* pyrophosphate, 0.1 ml calf thymus DNA (2.5 mg/ml), and 0.5 ml 3.5% perchloric acid–0.35% uranyl acetate. After 15 min at 0°, the mixtures were centrifuged and the radioactivity of the supernatant fluid was determined.

in the polynucleotide-joining reaction. The steady-state concentration of this intermediate is extremely low and it may be accumulated in detectable amounts only under rather restricted conditions (i.e., brief incubation at 0° in the presence of high concentrations of enzyme and single-strand breaks in DNA). This is presumably the result of the extreme rapidity of the final step in the reaction sequence (reaction (3), Fig. 1), in which attack of the activated DNA phosphate by the 3′-hydroxyl group of the neighboring DNA chain occurs.

The mechanism of formation of phosphodiester bonds by the DNA polymerase and polynucleotide-joining enzyme are basically similar. Thus, chain growth from the 3′-hydroxyl end of DNA by the polymerase involves an attack by the 3′-hydroxyl group of the DNA on the activated α-phosphate of the incoming deoxynucleoside triphosphate.[19] Analogously, synthesis of a phosphodiester bond in the joining reaction occurs by attack of the 3′-hydroxyl group of one DNA chain on the activated 5′-phosphoryl group of another (apposing) chain.

A closer analogy to the joining reaction is to be found in phosphodiester bond formation in the phospholipids. For example, phosphoryl choline is first linked to CMP *via* a pyrophosphate bond to form CDP-choline. An attack by the α-hydroxyl group of the diglyceride on the activated choline phosphate follows, resulting in the synthesis of the phosphodiester bond of phosphatidyl choline and the liberation of CMP.[20]

In the first step of the T4-ligase-catalyzed reaction, E-AMP is formed by reaction of the enzyme with ATP.[21, 22] It would seem likely that once E-AMP has been generated, the phage-induced ligase reaction would proceed through a similar DNA-adenylate intermediate.

Summary.—DNA with AMP in pyrophosphate linkage at the 5′-phosphoryl termini was identified as a component of the polynucleotide-joining reaction. The isolated DNA-adenylate was active as a substrate for the joining enzyme in the absence of DPN. Synthetic poly dT-adenylate was also active in the joining reaction in the absence of DPN, provided that poly dA were added. The liberation of AMP and phosphodiester bond synthesis was stoichiometric.

These findings are consistent with a mechanism for the joining reaction in which (1) E-AMP is formed by a reaction of enzyme with DPN, (2) AMP is transferred to the 5′-phosphoryl terminus of a DNA chain to form a new pyrophosphate bond, and (3) the activated 5′-phosphate group is attacked by the 3′-hydroxyl group of the apposing DNA chain, displacing the AMP and producing a phosphodiester bond linking the two chains.

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The abbreviations used are: AMP and ATP, adenosine 5′-mono- and triphosphate; CMP and CDP, cytidine 5′-mono- and diphosphate; DPN, diphosphopyridine nucleotide; NMN, nicotinamide mononucleotide; $d(pC)_3$, a trinucleotide composed of deoxycytidylate residues terminated by a 5′-phosphate group; $d(pT)_3$ or pTpTpT, a trinucleotide of deoxythymidylate residues terminated by 5′-phosphate; poly dT, a homopolymer of deoxythymidylate residues; poly dA, a homopolymer of deoxyadenylate; AppTpTpT, AMP bound in pyrophosphate linkage to the 5′-phosphate of pTpTpT; poly dT-adenylate, AMP in pyrophosphate linkage to the 5′-phosphate of poly dT; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetate; and Tris, tris(hydroxymethyl)aminomethane.

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