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Primer Requirement and Template Specificity of the DNA Polymerase of RNA Tumor Viruses

(avian myeloblastosis virus/mouse leukemia virus/*E. coli* DNA polymerase/homopolynucleotides/oligonucleotides)

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ABSTRACT **Polyribonucleotides will act as efficient templates for the DNA polymerases found in the virions of avian myeloblastosis virus and mouse leukemia virus if a short complementary oligodeoxyribonucleotide primer is added. Synthesis of the complementary polydeoxyribonucleotide continues until an amount of polymer equal to the amount of initial template has been produced. The two viruses show slightly different specificities toward the four homoribopolymers. Polydeoxyribonucleotides are generally much poorer templates than the homologous polyribonucleotides, in most cases yielding no detectable synthesis. The DNA polymerases of RNA tumor viruses, therefore, have the same requirements for activity as do other DNA polymerases, except that they prefer polyribonucleotides over polydeoxyribonucleotides as templates.**

Virions of the RNA tumor viruses contain a DNA polymerase (1, 2) which, in the absence of added nucleic acid, synthesizes a DNA copy of the 60–70S viral RNA (3). Exogenous nucleic acids, when added to disrupted virions, can also serve as templates, often giving much higher rates of synthesis than occur on the endogenous 60–70S RNA (4, 7). Polymers containing either deoxyribonucleotides or ribonucleotides can be used as templates by the enzyme (4, 6). In order to assess the template activity of various homopolynucleotides and to learn more about the requirements of the enzyme, we have been studying the rates and extents of reaction with different templates and primers.

Known DNA polymerases are apparently unable to initiate deoxyribonucleotide polymerization *de novo* on a single-stranded template, but require a primer containing a free 3′-OH (8–11). For instance, Bollum (9) showed that a 3′-*O*-acetyl-terminated poly(dT) cannot act as a template for dAMP polymerization by the DNA polymerase of calf thymus unless an oligomer of dAMP is added [(such as $(dA)_6$]. The ability of poly(A)·poly(dT) to act as a template for the avian myeloblastosis virus (AMV) DNA polymerase, while poly(A) alone is inactive (6), suggests that the AMV enzyme may also need a free 3′-OH terminus on which to initiate synthesis. The poly(dT) in poly(A)·poly(dT) would then be necessary in order to provide a 3′-OH, but only single-stranded regions of poly(A) would function as template. A series of experiments with poly(A) and oligo(dT) has substantiated this prediction and provided a way to assay the template specificity of the DNA polymerases of AMV. The enzyme can be shown, in this way, to prefer polyribonucleotides to polydeoxyribonucleotides as templates.

Abbreviations: AMV, avian myeloblastosis virus; MLV, Moloney (mouse) leukemia virus.

MATERIALS AND METHODS

Polyribonucleotides were obtained from Miles Laboratories, Elkhart, Ind.; their concentrations were determined from the extinction coefficients provided by the manufacturer. Polydeoxyribonucleotides were a kind gift of Dr. F. Bollum; their concentrations were determined from extinction coefficients provided by Dr. Bollum. The oligodeoxyribonucleotides were a product of Collaborative Research, Waltham, Mass., and had chain lengths of 12–16 units (which are indicated as 14 in the text); their concentrations were determined from the extinction coefficients of the respective polydeoxyribonucleotides. Except where noted, concentrations are given as concentration of mononucleotide in polymer solution.

Labeled deoxynucleoside triphosphates were purchased from New England Nuclear Corp. or Amersham–Searle Corp. Unlabeled triphosphates were from P–L Biochemicals.

AMV was kindly provided by Dr. J. Beard. It was purified in our laboratory by three cycles of differential centrifugation, followed by banding in a discontinuous 0.9–1.65 M sucrose gradient. Moloney mouse leukemia virus (MLV) was purchased from Electro Nucleonics Laboratories, Bethesda, Md. and had been purified by two cycles of banding in sucrose gradients. It was dialyzed against 500 volumes of 0.01 M Tris·HCl, pH 7.5, before use.

Reaction conditions for the AMV polymerase reaction were 0.05 M Tris (pH 8.3), 6 mM $Mg(acetate)_2$, 0.02 M dithiothreitol, 0.06 M NaCl, 0.2% Nonidet P-40, and the indicated concentrations of deoxyribonucleotides and polymers. For MLV, the conditions were identical except that the $Mg(acetate)_2$ was replaced by 1 mM $MnCl_2$, and 0.05% Nonidet P-40 was used. All reactions in which yields of product were determined were done in sealed vials and a nitrogen atmosphere to prevent air oxidation of thiols. All reactions, including single points in time courses, were performed in a 0.1-ml volume, and amounts of added substrates are given per 0.1 ml. Reaction mixtures contained about 0.5 μg of AMV protein or 2.5 μg of MLV protein.

RESULTS

Poly(A) as a template

Incubation of virions of AMV with [^{3}H]TTP in the absence of other deoxyribonucleoside triphosphates led to little or no incorporation. The addition of poly(A) alone stimulated in-

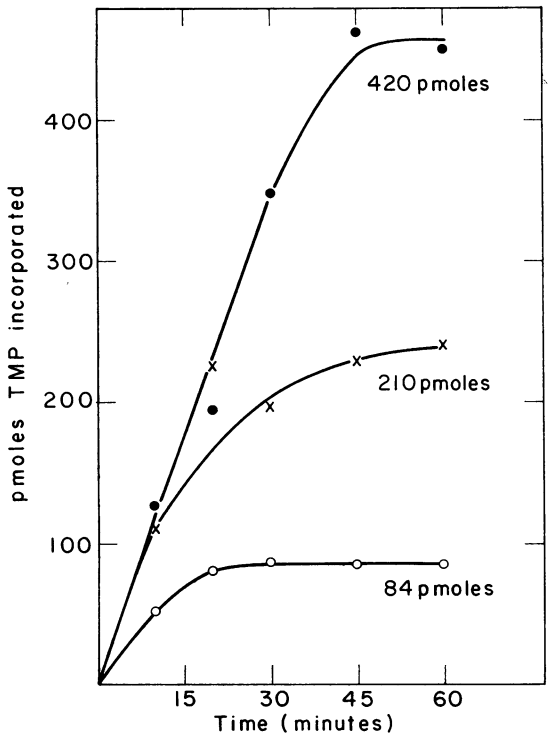

FIG. 1. Poly(A) as a template for the DNA polymerase of avian myeloblastosis virus. Data represent the incorporation of [^{3}H]TMP in standard reaction mixtures containing 18 nmol of [^{3}H]TTP (44 cpm/pmol), 170 pmol of $(dT)_{14}$, and 420, 210, or 84 pmol of poly(A).

corporation only slightly, but if either poly(dT) or $(dT)_{14}$ was also present, a marked stimulation was observed (12, 13; Table 1). Stimulation by the primer oligomer was specific to oligo(dT); neither oligo(dA), oligo(dC), nor oligo(dG) would stimulate incorporation (Table 1).

Measurements of rates of synthesis with a single concentration of poly(A) and different concentrations of primers showed that $(dT)_{14}$ could support a higher rate of synthesis than poly(dT), but that poly(U) was inactive (Table 2). Furthermore, the amount of primer that yielded a maximal rate of synthesis with 2100 pmol of poly(A) was about 20 pmol of $(dT)_{14}$ and about 500 pmol of poly(dT). Since the average chain length of the poly(dT) was about 800 units, maximal rates of incorporation occurred at about equimolar concentrations of

TABLE 1. *Various oligomers as primers for the AMV DNA polymerase with poly(A) as a template*

Additions	cpm [^{3}H]TMP incorporated
Poly(A)	234
Poly(A) + $(dT)_{14}$	15,800
Poly(A) + $(dC)_{14}$	505
Poly(A) + $(dG)_{14}$	315
Poly(A) + $(dA)_{14}$	140

Samples were incubated for 60 min at 37°C. Standard reaction mixtures contained 18 nmol of [^{3}H]TTP (44 cpm/pmol), 210 pmol of poly(A), and either 300 pmol of $(dA)_{14}$, 290 pmol of $(dC)_{14}$, 205 pmol of $(dG)_{14}$, or 170 pmol of $(dT)_{14}$.

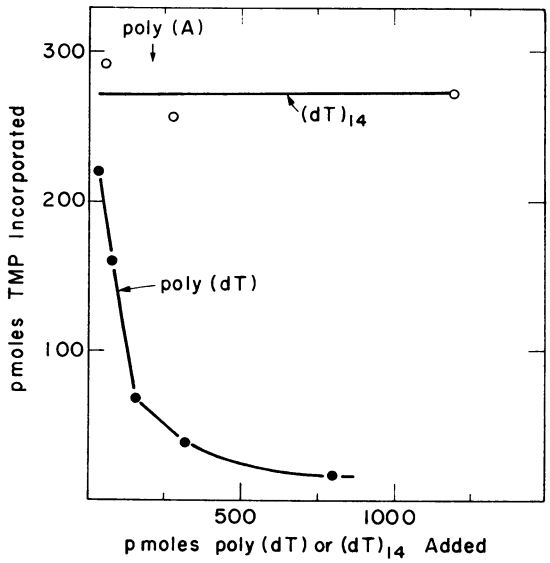

FIG. 2. Poly(dT) and $(dT)_{14}$ as primers for poly(A) with the DNA polymerase of avian myeloblastosis virus. Standard reaction mixtures were used with 210 pmol of poly(A) (marked by *arrow*), 18 nmol of [^{3}H]TTP (50 cpm/pmol), and either poly(dT) or $(dT)_{14}$ as indicated.

$(dT)_{14}$ and poly(dT) *molecules*, indicating that the number of free 3'-OH ends is the critical factor.

The previous experiment measured rates of synthesis. Measurements of *yields* of synthesis with a fixed concentration of $(dT)_{14}$ and three different concentrations of poly(A) showed that the yield of [^{3}H]poly(dT) was 100 ± 10% of the added poly(A) (Fig. 1), which indicates that a poly(A)·poly(dT) duplex is the final product.

When yields were measured with different concentrations of poly(dT) or $(dT)_{14}$ at a fixed poly(A) concentration, the two primers acted very differently. The yield was about 100% of

TABLE 2. *Poly(dT) and $(dT)_{14}$ as primers for the DNA polymerase of AMV with poly(A) as a template*

poly(dT) (pmol)	$(dT)_{14}$ (pmol)	poly(U) (pmol)	pmol [^{3}H]TMP incorporated
—	—	—	9
—	3.4	—	188
—	6.8	—	351
—	17	—	418
—	34	—	560
—	68	—	642
—	136	—	521
36	—	—	186
90	—	—	243
180	—	—	247
360	—	—	311
720	—	—	358
1800	—	—	360
—	—	11 to 1100	10

Samples were incubated for 60 min at 37°C. Standard reaction mixtures contained 19 nmol of [^{3}H]TTP (42 cpm/pmol) and 2100 pmol of poly(A).

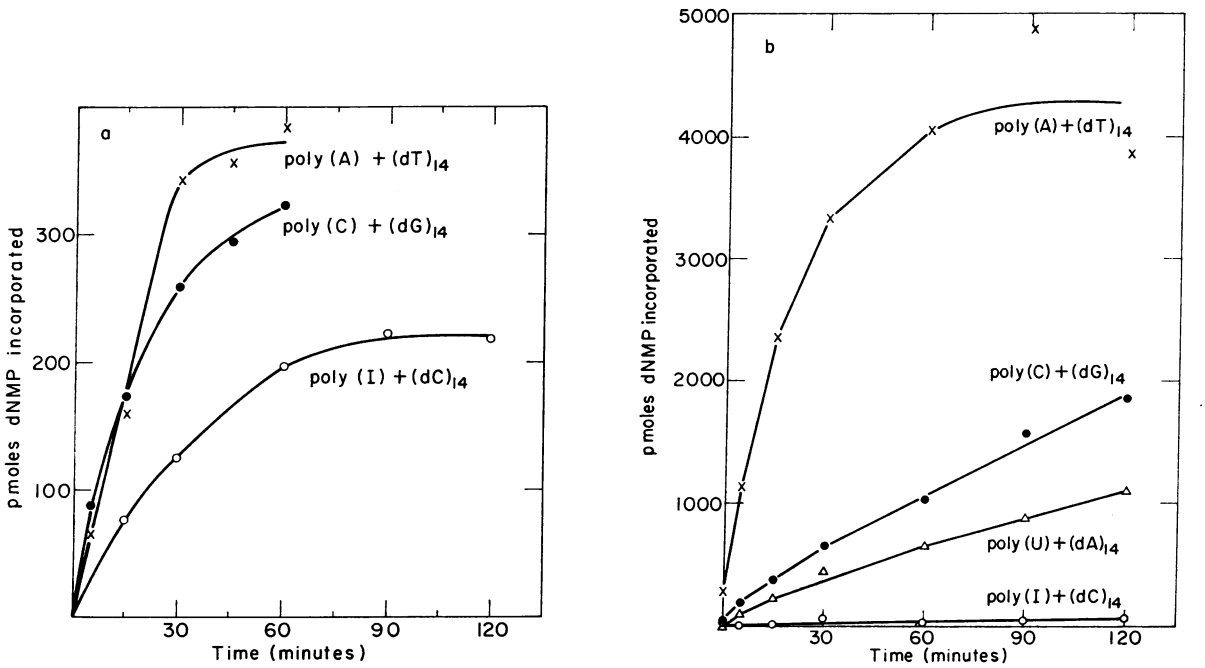

FIG. 3. Relative efficiency of different homopolymers as templates for tumor virus DNA polymerases.

(*a*) DNA polymerase of avian myeloblastosis virus. Standard reaction mixtures were used containing 330 pmol of a given homopolymer, 250 pmol of the complementary oligodeoxyribonucleotide, and 20 nmol of the complementary [^{3}H]deoxyribonucleoside triphosphate (40 cpm/pmol). No activity was demonstrable with poly(U) plus $(dA)_{14}$ and [^{3}H]dATP.

(*b*) Moloney mouse leukemia virus. Standard reaction mixtures were used containing 3300 pmol of a given homopolymer, 2500 pmol of the complementary oligodeoxyribonucleotide, and 20 nmol of the complementary [^{3}H]deoxyribonucleoside triphosphate (40 cpm/pmol).

the input poly(A) at all concentrations of $(dT)_{14}$, from 0.5 to 5 times the poly(A) concentration (Fig. 2). However, as the concentration of poly(dT) was increased, the yield was reduced (Fig. 2). At a 1:1 input of poly(A) to poly(dT) the yield was about 20% of the yield with $(dT)_{14}$. The poly(dT) presumably covers the single-stranded regions of poly(A) that act as template, while $(dT)_{14}$ does not fill these sites, probably because at 37°C it is only weakly bound to the poly(A).

Poly(I), poly(C), and poly(U) as templates

When poly(C) was added to the AMV polymerase, the presence of $(dG)_{14}$ was necessary to stimulate synthesis (Table 3). Similarly, poly(I) required $(dC)_{14}$ to stimulate dCMP polymerization. Neither $(dC)_{14}$ nor $(dG)_{14}$ could stimulate incorporation by itself. Poly(U) would not act as template for dAMP polymerization by the AMV DNA polymerase, even in the presence of $(dA)_{14}$.

The relative rates of polymerization of dCMP on poly(I), dGMP on poly(C), and dTMP on poly(A) are shown in Fig. 3*a*. Poly(A) and poly(C) supported about equal rates of synthesis, but poly(I) was less efficient. The yield of poly(dG) was about 100% of the added poly(C), but the yield of poly(dC) was only about 50% of the added poly(I).

The activity of MLV with different templates is shown in Fig. 3*b*. Poly(A) was the most efficient template in this case. Poly(C) was much less effective, poly(U) even less so, and poly(I) only barely stimulated incorporation.

Poly(dA), poly(dC), poly(dI), and poly(dT) as templates

Of the four polydeoxyribonucleotides, only poly(dC) would act as template, even in the presence of the complementary oligomer (Table 4). The rate of synthesis on poly(dC) was less than the rate on poly(C) (Fig. 4). When an excess of enzyme was used, however, the yield of poly(dG) on poly(dC) ultimately was about 100% of the input poly(dC) (unpublished results).

TABLE 3. *Poly(I), poly(C), and poly(U) as templates for the DNA polymerase of AMV*

Additions	cpm incorporated [^{3}H]dCMP	[^{3}H]dGMP	[^{3}H]dAMP
Expt. 1			
None	<200		
Poly(I)	<200		
Poly(I) + $(dC)_{14}$	23,648		
$(dC)_{14}$	<200		
Expt. 2			
None		<200	
Poly(C)		<200	
Poly(C) + $(dG)_{14}$		420,466	
$(dG)_{14}$		<200	
Expt. 3			
None			<200
Poly(U)			<200
Poly(U) + $(dA)_{14}$			<200

Samples were incubated for 60 min at 37°C. Standard reaction mixtures contained 94 pmol [^{3}H]dCTP (8450 cpm/pmol), 1650 pmol of poly(I), and 2900 pmol of $(dC)_{14}$ in Expt. 1; 274 pmol of [^{3}H]dGTP (2910 cpm/pmol), 1300 pmol of poly(C), and 2050 pmol of $(dG)_{14}$ in Expt. 2; and 228 pmol of [^{3}H]dATP (3520 cpm/pmol), 1100 pmol of poly(U), and 300–3000 pmol of $(dA)_{14}$ in Expt. 3.

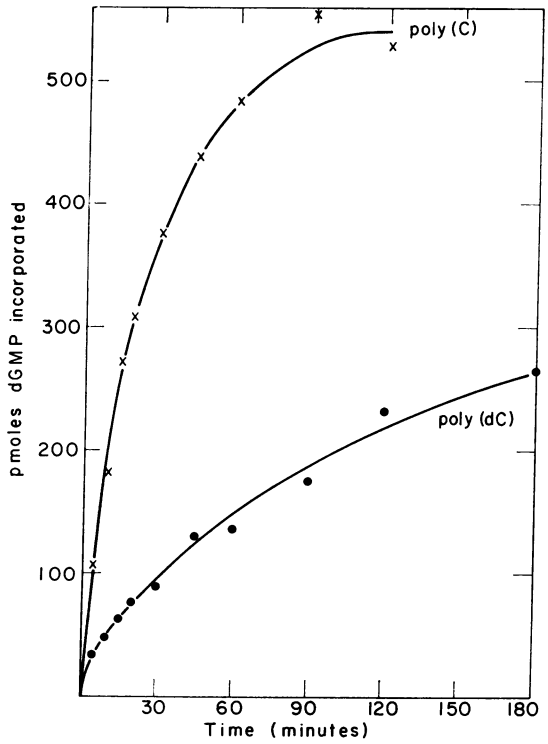

FIG. 4. Poly(C) and poly(dC) as templates for the avian myeloblastosis virus DNA polymerase. Data represent the incorporation of [^{3}H]dGMP in standard reaction mixtures containing 16 nmol of [^{3}H]dGTP (40 cpm/pmol), 370 pmol of $(dG)_{14}$, and 440 pmol of poly(C) or 440 pmol of poly(dC).

Virions of MLV were tested with deoxyribohomopolymers. They were inactive with poly(dA), poly(dI), and poly(dT), but were active with poly(dC). In contrast to AMV, the rate of synthesis with poly(dC) was about 3-fold higher than the rate with poly(C).

DISCUSSION

These experiments demonstrate that both a template and a primer are necessary for stimulation of the AMV DNA polymerase. Only a primer that can pair with the template to form standard base-pairs is active (Table 1 and unpublished results of I. Verma). For poly(A), poly(U) is not an effective primer, which suggest that the primer must be a poly*deoxyribo*nucleotide.

The polymerase reaction with poly(A) plus $(dT)_{14}$ proceeds until the template is covered and then stops (or falls to a very low rate). In different experiments, with poly(A) and poly(C), yields of product of 70–130% of the added template have been recorded; however, since doubling the template concentration always doubles the yield, it would appear that the deviation from 100% is due to technical errors and inaccuracies in specific activities and extinction coefficients. The ability of

TABLE 4. *Comparison of polydeoxyribonucleotides and polyribonucleotides as templates for the DNA polymerase of AMV*

Polymer added	pmol incorporated
Expt. 1	
Poly(A)	530
Poly(dA)	7
Expt. 2	
Poly(C)	240
Poly(dC)	240
Expt. 3	
Poly(I)	155
Poly(dI)	4
Expt. 4	
Poly(U)	3
Poly(dT)	2

Samples were incubated for 60 min at 37°C. In Expt. 1, 420 pmol of poly(A) or 270 pmol of poly(dA) was incubated with 170 pmol of $(dT)_{14}$ and 18 nmol of [^{3}H]TTP (50 cpm/pmol). In Expt. 2, either 260 pmol of poly(C) or 370 pmol of poly(dC) was incubated with 205 pmol of $poly(dG)_{14}$ and 16 nmol of [^{3}H]dGTP (50 cpm/pmol). In Expt. 3, either 330 pmol of poly(I) or 170 pmol of poly(dI) was incubated with 290 pmol of $(dC)_{14}$ and 17 nmol of [^{3}H]dCTP (47 cpm/pmol). In Expt. 4, 220 pmol of poly(U) or 180 pmol of poly(dT) was incubated with 300 pmol of $(dA)_{14}$ and 15 nmol of [^{3}H]dATP (53 cpm/pmol).

TABLE 5. *Template specificity of DNA polymerases*

Enzyme	Homopolymers							
	A	C	I	U	dA	dC	dI	dT
AMV DNA polymerase	+++	+++	++	0	0	++ [<poly(C)]	0	0
MLV DNA polymerase	+++	++	+	++	0	+++ [>poly(C)]	0	0
E. coli DNA polymerase I	+ [<poly(dA)]	0	0	0	+++	++	+	++

Relative activities of different templates for the viral enzymes were estimated from the data in Figs. 3 and 4 and from other experiments in which rates were determined with precursors of high specific activity. Where active polymers are designated +++ or ++, inactive polymers ("0" in the table) had less than 1% of the activity of their active homologues.

The data on *E. coli* DNA polymerase I were obtained with a highly purified preparation (fraction 7; ref. 15) kindly provided by Dr. Arthur Kornberg and assayed with 0.05 M potassium phosphate buffer, pH 7.4 and 6 mM Mg $(acetate)_2$ (13). Templates, primers, and substrates were varied as in the assays of the viral DNA polymerases. Poly(dA) was 10-fold more active than poly(A) in an experiment analogous to that in Fig. 4.

poly(T) to depress the yield of [^{3}H]poly(T) synthesis on poly(A) supports the idea that only single-stranded regions of homopolymers can be copied.

All of the experiments described here were performed with the DNA polymerase activity of whole virions of AMV or MLV disrupted with Nonidet P-40. All of the basic experiments, however, have been repeated with an enzyme fraction purified by either DEAE-Sephadex or phosphocellulose chromatography of disrupted AMV virions and have yielded essentially identical results, except for a lowered level of activity with poly(I) (I. Verma, unpublished data).

The template specificities of AMV and MLV are summarized in Table 5 and are compared with the template specificity of *Escherichia coli* DNA polymerase I ("Kornberg enzyme"), determined in the same way. The preference of the tumor virus enzymes for poly*ribo*nucleotides as opposed to the preference of *E. coli* DNA polymerase I for poly*deoxyribo*nucleotides is evident. The only exception is the higher rate of MLV DNA polymerase with poly(dC) than with poly(C). Spiegelman *et al.* (6) have previously noted that homopolymers of either cytidylate or deoxycytidylate are very good templates for a number of different types of nucleic acid polymerases. The reason for this preference is not known.

The exact order of preference of the tumor virus enzymes for polyribonucleotides depends upon a number of variables, so the results in Fig. 3 are true only under the specified assay conditions. For instance, replacement of manganese ion by magnesium ion in the MLV polymerase assays markedly decreases the activity of poly(A) and increases the activity of poly(C). However, using various manganese and magnesium ion concentrations, we were unable to make any of the polydeoxyribonucleotides except poly(dC) into effective templates. Also, manganese ion did not allow the AMV DNA polymerase to utilize poly(rU) · $(dA)_{14}$. Differential effects of magnesium and manganese ions have been noted previously (16).

The results with homopolymers demonstrate that DNA-dependent DNA polymerases and RNA-dependent DNA polymerases are distinguishable. The use of homopolymer templates with polydeoxyribonucleotide primers provides a methodology for testing unknown enzymes for their template preference. A battery of such tests must be used for screening, because each enzyme has its own properties.

The requirement for a single-stranded template and a hydrogen-bonded primer for synthesis by the DNA polymerase found in the virions of RNA tumor viruses is similar to the requirements of other DNA polymerases (8, 11). These properties suggest that the known DNA polymerases are "repair enzymes", that is, they will cover any single-stranded region of a template with the appropriate polydeoxyribonucleotides if a 3′-OH is in position to initiate the synthesis. This raises the question of the physiological role of the virion DNA polymerase. If, as is believed, it is an enzyme designed to copy viral RNA into a DNA copy, how is synthesis on the viral RNA initiated? The small amount of DNA in the virion (14) could serve this function, although there is no evidence for such a role for this DNA. On the other hand, the RNA itself could initiate synthesis, in which case a covalently linked RNA–DNA molecule would be formed.

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