A COMPARISON OF THE ENZYMATIC RESPONSES OF THE DNA POLYMERASES FROM FOUR RNA TUMOR VIRUSES

Ann A. Kiessling and Mehran Goulian
Department of Medicine, School of Medicine
University of California, San Diego
La Jolla, California 92093

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Summary: DNA polymerases from avian, feline, murine and simian RNA tumor viruses exhibit substantial differences in optimal assay conditions and vary widely in their template-primer preferences. Avian DNA polymerase utilizes both natural and synthetic template-primers efficiently in the presence of Mg^{++} as well as Mn^{++}. By contrast, the mammalian viral DNA polymerases are much more responsive to poly(A)·oligo(dT) than to other template-primers, and exhibit up to 20-fold greater activity with Mn^{++} than with Mg^{++}. In addition, simian sarcoma virus DNA polymerase shows no detectable response to poly(C)·oligo(dG) over a wide variety of conditions stimulatory to the other viral enzymes.

Synthetic template-primers [e.g., poly(A)·oligo(dT) and poly(C)·oligo(dG)] have been widely used to detect the DNA polymerases ("reverse transcriptases") of animal RNA tumor viruses (1,2,3) or of intracellular forms of candidate human RNA tumor viruses (4,5,6). However, complete information has not been available on optimal reaction parameters of a number of RNA tumor virus enzymes in the presence of synthetic or natural template-primers. Such information is necessary for specific detection of reverse transcriptases in the presence of contaminating cellular enzymes since some cellular DNA polymerases have been shown to use poly(C)·oligo(dG) and/or poly(A)·oligo(dT) with high efficiency under appropriate assay conditions (7,8,9,10). The studies reported here provide detailed comparisons of the DNA polymerases from AMV, FeLV, MuLV, and SiSV in the presence of synthetic or natural template-primers under a broad range of assay conditions. The results show large differences between these four viral enzymes in their assay optima and relative responses to each template-primer, as well as several differences from conditions commonly used to assay these enzymes.

Materials and Methods

Viruses: MuLV was produced from infected 3T3 cells (Clone I), obtained originally as a gift from Dr. Hung Fan, Salk Institute. AMV (BAI strain A) was a gift from Dr. George Beaudreau, Oregon State University. Theilin strain

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Abbreviations used: poly(mC)·oligo(dG), poly(2'-O-methyl-cytidylate)·(oligodeoxyguanylate$_{12-18}$); AMV, avian myeloblastosis virus; FeLV, feline leukemia virus; MuLV, Moloney murine leukemia virus; SiSV, simian sarcoma virus; RLV, Rauscher leukemia virus; DTT, dithiothreitol; BPA, bovine plasma albumin.

of FeLV from infected FL74 cells (Pfizer) and two lots of SiSV from infected NC-37 cells (Litton Bionetics and Pfizer) were produced for the USPHS-NCI Viral Oncology Program. All samples of virus were rebanded twice in continuous sucrose density gradients prior to use. AMV was quantified by ATPase activity (11) and MuLV by virus particle counts in electron micrographs prepared by Dr. Peter Kiessling; FeLV and SiSV were supplied with virus particle counts.

Antibodies: Goat antisera against SiSV and RLV DNA polymerases were produced for the USPHS-NCI Viral Oncology Program (Huntingdon Research Center) and were made available by the courtesy of Dr. Jack Gruber.

Synthetic Template-Primers: Poly(mC)·oligo$(dG)_{12-18}$ (1:1, w/w) and poly(dA)·oligo$(dT)_{12-18}$ (1:1, w/w) were obtained from P-L Biochemicals. Poly(C)·oligo$(dG)_{12-18}$ was either from P-L Biochemicals (1:1, w/w) or prepared by combining poly(C) with oligo$(dG)_{12-18}$ (2:1, w/w) both from Collaborative Research. Poly(A)·oligo$(dT)_{12-18}$ (2:1, w/w) was also from Collaborative Research.

DNA Polymerases: Virus lysates were prepared by adjusting purified virus to 0.25% NP-40 (Shell), 10 mM DTT, and 50% glycerol. DNA polymerase was freed from viral nucleic acids by passing through a column of DEAE cellulose in 0.01 M Tris-HCl (pH 7.4), 10% glycerol, 1 mM DTT, and 1 mM EDTA (Buffer A) containing 0.3 M KCl. Further purification was by chromatography on DNA agarose (12) with a linear gradient of 0.05-0.7 M KCl in Buffer A containing 100 µg/ml BPA. All the viral DNA polymerases eluted at 0.2-0.25 M KCl, and were concentrated by centrifugation overnight at 40,000 rpm in a Beckman SW 50.1 rotor. Final enzyme yields were >90% with at least 100-fold purification and could be stored in Buffer A with 50% glycerol and 100 µg/ml BPA at -20°C without loss of activity for 7 months.

Assays of DNA Polymerase Activity: Standard enzyme assays (20 µl) containing only endogenous viral nucleic acids as template-primer or 500 µg/ml "activated" DNA (13) are 30 mM Tris-HCl (pH 8.3), 2 mM DTT, 500 µg/ml BPA, 0.2 mM in each nonradioactive dNTP, 2 µM in ^{3}H-dTTP [62 mCi/µmole, prepared in this laboratory from ^{3}H-TdR (Schwarz/Mann)(14)], and contain $MgCl_2$ or $MnCl_2$, KCl, and enzyme as specified. Assays containing synthetic template-primers (100 µg/ml) are the same with the substitution of 0.1 mM nonradioactive complementary dNTP for the 3 dNTP's, and 5 µM ^{3}H-dGTP (28 mCi/µmole, New England Nuclear) for ^{3}H-dTTP when oligo(dC) primers are present. Reactions are carried out in wells of Microtiter (flow) plates, which are sealed and floated in a 37°C water bath for 20 min (reactions are linear for at least 40 min). Assays are terminated and processed for liquid scintillation counting (22% efficiency) by a rapid micro method (to be published) that gives lower backgrounds and higher yields of radioactive product (due to inclusion of short chain material) than the conventional filtration of acid precipitable product onto either glass fiber or nitrocellulose filters.

Results

Optimal assay conditions were determined for each of the viral enzymes in the presence of endogenous viral nucleic acids, poly(A)·oligo(dT), poly(C)·oligo(dG), or activated calf thymus DNA (Table I). For each template-primer, Mn^{++} and Mg^{++} were varied from 0.05 mM to 20 mM, and for each divalent cation concentration KCl was varied from 5 mM to 250 mM.

DNA polymerase activity on endogenous viral nucleic acids, as found by assaying DNA polymerase activity in virus lysates without adding nucleic acids, is approximately the same for equivalent numbers of each of the viruses in the presence of Mn^{++}. For this "endogenous reaction" the avian system is the only one showing a preference for Mg^{++}; in contrast, the simian system seems to

TABLE I. Optimal Reaction Conditions for Viral DNA Polymerases with Natural and Synthetic Template-Primers.

Virus DNA Polymerase and Template-Primers*	Reactions with Mg^{++}			Reactions with Mn^{++}		
	$MgCl_2$(mM)**	KCl(mM)**	Product*** (pmoles/20 min)	$MnCl_2$(mM)**	KCl(mM)**	Product*** (pmoles/20 min)
Endogenous						
AMV	8 (4-12)	20 (0-30)	.67	1 (0.4-1.5)	10 (0-20)	0.16
FeLV	4 (2-8)	40 (0-50)	.02	1 (0.4-2)	20 (0-30)	0.08
MuLV	2 (1-5)	0 (0-15)	.06	1 (0.4-1.5)	0 (0-10)	0.17
SiSV	--	--	<.01	1 (0.4-3)	30 (0-50)	0.10
Poly(A)·oligo(dT)						
AMV	4 (2-6)	20 (0-40)	11.6	0.3 (0.1-0.8)	100 (60-175)	28.3
FeLV	1 (0.8-3)	0 (0-5)	4.2	0.2 (0.1-0.4)	90 (40-130)	26.6
MuLV	1 (0.5-4)	0 (0-5)	1.2	0.4 (0.1-1)	80 (30-130)	20
SiSV	2 (0.5-6)	0 (0-10)	1.1	0.3 (0.2-0.5)	75 (50-100)	16.8
Poly(C)·oligo(dG)						
AMV	4 (0.8-12)	0 (0-5)	53.2	0.5 (0.1-1)	0 (0-5)	21.5
FeLV	6 (0.5-8)	0 (0-5)	0.7	0.4 (0.2-1)	0 (0-5)	2.8
MuLV	1 (0.4-1.5)	0 (0-5)	1.1	0.3 (0.1-0.8)	0 (0-5)	3.5
SiSV	--	--	<.01	--	--	<0.04
Activated DNA						
AMV	8 (4-12)	40 (20-60)	2.4	0.4 (0.2-0.5)	60 (20-80)	0.5
FeLV	8 (4-12)	10 (0-40)	1.8	2 (1-3)	20 (0-40)	0.6
MuLV	6 (4-12)	40 (20-60)	.6	2 (1-3)	40 (10-50)	0.2
SiSV	8 (2-12)	40 (30-60)	.1	1.5 (1-2.5)	40 (10-50)	0.06

* Each assay contained 5 x 10^9 lysed virus particles or equivalent DNA polymerase activity ($\leq$0.1 µg enzyme protein), which was saturated at 25 µg/ml poly(A)·oligo(dT) or poly(C)·oligo(dG) and 500 µg/ml activated DNA. Poly(A)·oligo(dT) and poly(C)·oligo(dG) were present at 100 µg/ml and activated DNA at 500 µg/ml. Values were determined for both DNA polymerases in detergent disrupted particles and purified DNA polymerases (except FeLV which was used only as a viral lysate); no differences in assay optima were noted with purification.

** Concentrations for maximum activity; values in parentheses are the concentration ranges that yield greater than 65% of maximum activity.

*** Amount of labeled dNMP incorporated at optimal conditions at 37°C. Backgrounds (0.005-0.01 pmole) have been subtracted.

have an absolute requirement for Mn^{++}, whereas, the feline and murine can use Mg^{++} at 25-35% of their activity with Mn^{++}. With either divalent cation, KCl stimulates AMV 2-fold, FeLV and SiSV only 1.2- to 1.6-fold, and it inhibits MuLV at all concentrations.

In poly(A)·oligo(dT) directed reactions under optimal conditions for each divalent cation, Mn^{++} is more stimulatory to the DNA polymerases than Mg^{++} by a factor of 2 for AMV, 6 for FeLV, 17 for MuLV, and 15 for SiSV. KCl stimulates the Mn^{++}-poly(A)·oligo(dT) reaction 8-fold for AMV, 5-fold for FeLV, 4-fold for MuLV, and 7-fold for SiSV, but does not stimulate the Mg^{++}-poly(A)·oligo(dT) reaction.

AMV DNA polymerase is the only enzyme showing a preference for Mg^{++} in the poly(C)·oligo(dG) directed reactions, which is also the case for the endogenous reactions. With poly(C)·oligo(dG), under optimal conditions, the ratio of enzyme activity with Mn^{++} to activity with Mg^{++} is 0.4 for AMV, 4 for FeLV, and 3 for MuLV. KCl inhibits this reaction at all concentrations with either Mn^{++} or Mg^{++}, and alters the Mn^{++} response curve by shifting the peak of the Mn^{++} curve to higher concentrations; e.g. with FeLV, from 0.4 mM at 0 KCl to 5.0 mM at 80 mM KCl, accompanied by loss of 50% of the activity at 0 KCl. KCl does not affect the Mg^{++} optima with poly(C)·oligo(dG), or either Mg^{++} or Mn^{++} optima in the endogenous, poly(A)·oligo(dT), or activated DNA systems.

The DNA polymerases isolated from both samples of SiSV do not respond to poly(C)·oligo(dG) throughout a wide range of assay conditions. With purified SiSV DNA polymerase, the presence of poly(C)·oligo(dG) results in a slight increase in acid-precipitable ^3H over assays containing no template-primer, but this small amount of acid-precipitable radioactivity does not increase with incubation time nor is it affected by the wide range of ionic conditions tested in the assay. A 5-fold increase in concentration of either purified enzyme or viral lysate [resulting in 5-fold increases in response to poly(A)·oligo(dT)] and/or an increase in poly(C)·oligo(dG) to 1 mg/ml still results in no convincing evidence of SiSV DNA polymerase activity in this assay system. The presence of SiSV DNA polymerase in a poly(C)·oligo(dG) reaction either before, during, or after the addition of AMV DNA polymerase has no effect on the amount of product synthesized relative to assays containing the avian enzyme alone, indicating the absence of an inhibitor.

All four viral DNA polymerases prefer Mg^{++} to Mn^{++} for the reactions with activated DNA, and KCl stimulates the reaction 2- to 3-fold at optimal concentrations of Mg^{++}. $(NH_4)_2SO_4$ can partially substitute for KCl in the DNA reactions (as well as the endogenous and poly(A)·oligo(dT) reactions; NaCl is less effective than either $(NH_4)_2SO_4$ or KCl in stimulating the AMV, FeLV, and SiSV enzymes, and inhibits the murine DNA polymerase.

Doubling the concentration of dCTP or enzyme in the standard assay with poly(C)·oligo(dG) has no detectable effect on the optimal concentrations of Mn^{++} or Mg^{++}. Doubling the concentration of ^{3}H-dGTP or poly(C)·oligo(dG) increases the Mn^{++} optimum slightly (e.g., for AMV, by 0.5 mM), but has no appreciable effect on the Mg^{++} optimum.

Under assay conditions optimal for poly(C)·oligo(dG) stimulation, the methylated derivative poly(mC)·oligo(dG) stimulates AMV, FeLV, and MuLV DNA polymerases to 5-20% of the level of stimulation with poly(C)·oligo(dG), but gives no detectable stimulation of SiSV DNA polymerase. In accord with previous reports (4), poly(dA)·oligo(dT) (with either Mg^{++} or Mn^{++}) is not an effective template-primer for the viral DNA polymerases, stimulating to only 5-10% of the level of synthesis with activated DNA and to less than 1% of the level of activity with Mn^{++}-poly(A)·oligo(dT).

Yeast RNA (DNA-free) stimulates the viral DNA polymerases to 5-20% of the level of activity observed with activated DNA. This low level of template activity of natural RNA has been observed before using 70S viral RNA as template-primer for viral DNA polymerases (15,16,17).

All the viral enzymes have a pH optimum in the range of 8.0-8.6 (Tris-HCl) with peak activity at pH 8.3 under optimal ionic conditions for both poly(A)·oligo(dT) and poly(C)·oligo(dG) when assayed at 37°C (all pH measurements at 25°C). Nonreactive protein in the assay, i.e. BPA or rabbit immunoglobulin, stimulates the viral DNA polymerases approximately 5-fold above the level of template-primer stimulation alone, with 1-4 mg/ml providing maximum stimulation. In the absence of added protein, NP-40 at concentrations of 0.05-2% stimulates approximately 2-fold above the level of template-primer stimulation but is not additive with the stimulation by protein. The wide range of permissible NP-40 concentrations with either disrupted virus or purified DNA polymerase is in contrast to reported narrow permissible range of Triton X-100 (18).

Product formation by the viral DNA polymerases in the presence of poly(A)·oligo(dT) has a temperature optimum of 25°-30°C; in contrast, the incorporation of ^{3}H-dGMP into product with poly(C)·oligo(dG) is higher at 50°C, particularly for the Mg^{++}-poly(C)·oligo(dG) system with AMV DNA polymerase (Figure 1). Optimal concentrations for Mg^{++} or Mn^{++} are essentially the same at 30° and 37°C with poly(A)·oligo(dT), or at 50° and 37°C with poly(C)·oligo(dG) (data not shown). Similar temperature optima with these synthetic template-primers have been observed previously (19). Although largely accounted for by differences between the template-primers, the results may also be related in part to differences between the enzymes in stability at elevated temperatures (20,21). Even at the higher temperatures the SiSV DNA polymerase does not respond detectably to poly(C)·oligo(dG).

Because of the unexpected results with the SiSV enzyme, antigenic properties

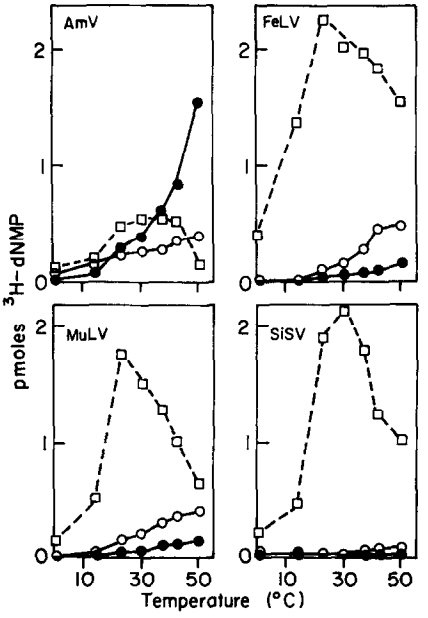

Figure 1

Temperature responses of virus DNA polymerases. Standard assay mixtures of poly(C)·oligo(dG) with 1.25 mM Mg^{++} (●—●), poly(C)·oligo(dG) with 0.4 mM Mn^{++} (0—0), or poly(A)·oligo(dT) with 0.25 mM $MnCl_2$, 80 mM KCl (□–□) were portioned into 10 µl aliquots and incubated for 20 min at each temperature.

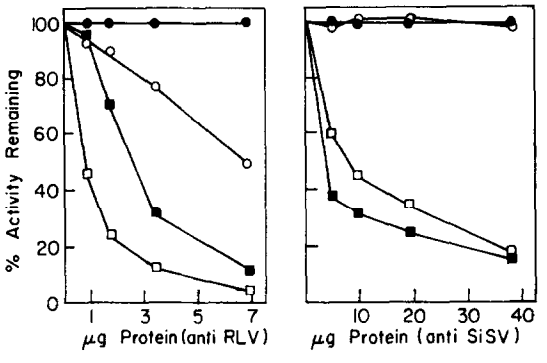

Figure 2

Antibody neutralization of virus DNA polymerases. Enzyme protein was mixed with the indicated amount of goat antisera at 0°C for 1 h in the presence of all assay components for the Mn^{++}-poly(A)·oligo(dT) system except the poly(A)·oligo(dT) and ^{3}H-dTTP. Controls (100% activity) were with normal goat serum. Non-neutralized enzyme activity was measured by addition of poly(A)·oligo(dT) and ^{3}H-dTTP followed by incubation at 30°C for 20 min. 100% ^{3}H-dTMP incorporations were: AMV (●—●), 8 pmoles; FeLV (0—0), 9 pmoles; MuLV (□—□), 22 pmoles; SiSV (■—■), 15 pmoles.

were also examined. Antibody against SiSV DNA polymerase inhibits the SiSV DNA polymerase used in these studies and to a lesser extent MuLV DNA polymerase but not AMV or FeLV DNA polymerases (Figure 2), a result different from a previous study by Scolnick, et al. (3), which indicated that the simian virus DNA polymerase does not share antigenic determinants with murine (or feline) DNA polymerases. The possibility that the two antibodies used here are recognizing two different classes of antigenic determinants in the viral DNA polymerases is indicated by the fact that FeLV, SiSV, and MuLV are all recognized by anti-RLV DNA polymerase, but only MuLV and SiSV are recognized by anti-SiSV DNA polymerase.

Discussion

The results in this study tend to set the avian RNA tumor viral DNA polymerase apart from the mammalian viral enzymes in several respects. In reactions with either synthetic template-primer or endogenous viral nucleic acid, the mammalian enzymes are much more active with Mn^{++} than with Mg^{++} (as high as 20:1), whereas the AMV enzyme is less specific in its divalent metal requirement, even showing a preference for Mg^{++} in its reactions with poly(C)·oligo(dG) and with endogenous viral nucleic acid. In addition, comparison of the four viral enzymes emphasizes the striking differences between their relative activities with poly(A)·oligo(dT), poly(C)·oligo(dG), and DNA at their respective optimal assay conditions (Table II). AMV shows 2-fold greater activity with poly(C)·oligo(dG) over poly(A)·oligo(dT), in contrast to the average 6:1 preference of the enzymes from FeLV and MuLV for poly(A)·oligo(dT) over poly(C)·oligo(dG). Differences between the mammalian enzymes themselves are illustrated by the striking preference

TABLE II. Comparison of relative activities of viral DNA polymerases with different template-primers.

	poly(A)·oligo(dT)	Activated DNA	poly(C)·oligo(dG)
AMV	12	1	22
FeLV	15	1	2
MuLV	33	1	6
SiSV	168	1	<0.6

Values are calculated from the data in Table I and are the ratios of enzyme activities at optimal conditions with synthetic template-primers to activities at optimal conditions with activated DNA.

for poly(A)·oligo(dT) exhibited by the simian enzyme, its inability to utilize Mg^{++} ion for the endogenous reaction, and its further inability to respond to poly(C)·oligo(dG) with either Mn^{++} or Mg^{++}.

Previous reports have described SiSV DNA polymerase as showing a response to poly(C)·oligo(dG) (2, 22, 23, 24). The relative responses to each of the template-primers (as well as assay conditions) vary widely between the reports, e.g. in one case a ratio for poly(A)·oligo(dT) to poly(C)·oligo(dG) stimulation of 10:1 (22), as compared to a ratio of 3:1 for enzyme from virus grown in a different cell type (23). Variable results, including no response, of SiSV DNA polymerase to poly(C)·oligo(dG) have also been observed by others (M. Robert-Guroff and R. C. Gallo, personal communication).

Possible explanations for the lack of response of the simian virus DNA polymerase are (1) a defect in the poly(C)·oligo(dG) template-primers; (2) an alteration of the viral DNA polymerase possibly secondary to purification and storage of the virus; and (3) an alteration of the virus population itself with tissue culture passage. The first possibility cannot be ruled out, but would imply a requirement for a very specific poly(C)·oligo(dG) structure since the preparations used here gave the expected levels of activity for avian and murine DNA polymerases (20). The second possibility implies that the simian DNA polymerase has undergone a structural alteration rendering it unable to use poly(C)·oligo(dG) but still allowing it to use poly(A)·oligo(dT). Such selective loss of template-primer utilization, although conjectural at this time, could be of great importance if true. The third possibility implies either selection of a specific virus population, or an alteration of the DNA polymerase in the entire virus population.

The number of virus particles that can be detected by an assay for viral DNA polymerase has been estimated to be from 2×10^4 (25) to 2×10^8 (26). The former value is from a viral particle estimate based on quantification by laser beat frequency spectroscopy, which is not yet correlated with more standard methods of virus titration. The results in the present study are consistent with most reports and indicate a sensitivity of $1\text{-}10 \times 10^5$, depending on specific activity of radioactive precursors.

It is evident from the studies presented here that the differences between the viral enzymes in their assay requirements and responses are greater than has been ordinarily appreciated. Further, with the synthetic template-primer systems, the mammalian viral DNA polymerases have more features in common with normal cellular DNA polymerases (7,8,9,10) than with AMV DNA polymerase. To date, normal cellular DNA polymerases have not been shown to utilize natural RNA or poly(mC)·oligo(dG) (27) and therefore these systems appear specific for RNA tumor virus DNA polymerases. However, as illustrated here, natural RNA and

poly(mC)·oligo(dG) are at least 2 orders of magnitude less sensitive than the Mn^{++}-poly(A)·oligo(dT) assay for all 4 viral enzymes, and for SiSV poly(mC)·oligo(dG) is at least 4 orders of magnitude less sensitive than Mn^{++}-poly(A)·oligo(dT)(3). It therefore seems difficult or impossible to specify at this time one set of assay conditions that will detect small amounts of all RNA tumor viral DNA polymerases, particularly in the presence of contaminating cellular enzymes.

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