The Inhibition of Avian Myeloblastosis Virus Deoxyribonucleic Acid Polymerase by Synthetic Polynucleotides†

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ABSTRACT: Nine synthetic pyrimidine homopolyribonucleotides were analyzed as regards their ability to inhibit the DNA polymerase of the avian myeloblastosis virus (AMV). Substitutions in the 4 and 5 position of the ring and the 2′ position on the sugar were found to significantly alter inhibitory activity. The most potent class of inhibitors, 2′-halogeno-2′-deoxyribopolynucleotides, were also found to be most active against the DNA polymerase activity of feline leukemia virus and Rous sarcoma virus. Studies concerning the mechanism of inhibition demonstrated a single-stranded requirement, unaltered inhibition kinetics in the presence of either Mg^{2+} or Mn^{2+}, and that the inhibition was not affected by actinomycin D. These results, in addition to the analysis of the kinetics of enzyme inhibition, suggest that inhibitory polymers compete for the template binding site on the enzyme but are inactive as templates for the polymerization reaction. The 2′-halogeno-2′-deoxyribopolymers did not inhibit the RNase H activity that copurifies with the AMV DNA polymerase nor did they interfere with the DNA polymerases of *Micrococcus luteus* and calf thymus.

The inhibition of viral polymerases by synthetic polynucleotides was first reported in a study of the RNA-dependent RNA polymerase isolated from *Escherichia coli* cells infected with the bacteriophage Qβ (Haruna and Spiegelman, 1966). This study demonstrated that both poly(U)[1] and poly(A) strongly inhibited the *in vitro* replication of the viral genome while poly(C) and poly(G) were ineffective. An analysis of the kinetics of inhibition suggested that the polyribonucleotides interfered with the initiation of replicase activity and not with the elongation phase of the polymerization reaction. In experimentally related studies it was shown that bacterial DNA-dependent RNA polymerase could not utilize poly(I) as a template for synthesis but that poly(I) could compete very effectively with normal DNA templates for the enzyme binding site (Hirschbein *et al.*, 1967).

Similar results in studies using the DNA polymerase contained within the oncornaviruses have suggested that specific synthetic polynucleotides may act as competitive inhibitors of the enzyme–template binding reaction. The ability of this viral polymerase to utilize various double-stranded synthetic polymers as templates was shown to vary markedly (Spiegelman *et al.*, 1970; Wells *et al.*, 1972). Single-stranded polyribonucleotides were demonstrated to act as efficient templates for the DNA polymerase in the presence of the complementary oligodeoxyribonucleotide primer and the avian myeloblastosis virus (AMV) enzyme was observed to utilize poly(A), poly(C) and poly(I) as template strands, whereas poly(U) was inactive (Baltimore and Smoler, 1971). It was subsequently demonstrated that poly(U) was a potent competitive inhibitor of the DNA polymerase of the Rauscher murine leukemia virus (RMLV) and exhibited some selectivity in that the polymer did not inhibit DNA-dependent DNA polymerase activity isolated from *E. coli* or mouse embryo (Tuominen and Kenney, 1971).

These results suggested that minor modification of the chemical composition of synthetic polynucleotides might significantly alter the interaction between that polymer and specific classes of DNA polymerases. An investigation concerning the relationship between the structure of synthetic polynucleotides and their ability to act as either templates for synthesis or inhibitors of enzyme activity could provide important information regarding certain aspects of the function of DNA polymerases. This report presents data on our initial studies on the relationship between polynucleotide structure and activity using the RNA dependent DNA polymerase of AMV. In addition, investigations concerning the mechanism of inhibition of a very potent inhibitor, poly(2′-fluoro-2′-deoxyuridylic acid) [poly(dUfl)], are described in detail.

Experimental Section

Materials. AMV was kindly provided by Dr. J. Beard of Duke University. Feline leukemia virus (Theilen) and Rous sarcoma virus (Schmidt-Ruppin) were purchased from Virgo Reagents. Tritiated deoxynucleoside triphosphates were obtained from Schwarz/Mann and the unlabeled substrates from Pabst Laboratories. Oligodeoxyribonucleotides of defined chain length were products of Collaborative Research. Nonidet P-40 was provided by the Shell Chemical Co.

Micrococcus luteus DNA polymerase, calf thymus DNA polymerase, poly(A), poly(dA), poly(C), poly(dU), poly(dT), poly[d(A-T)], poly(U), and [^{3}H]poly(A) were supplied by the Research Products Department of Miles Laboratories. Poly(dC) was purchased from Biopolymers, Inc. Poly(dUfl), poly(2′-chloro-2′-deoxyuridylic acid) [poly(dUcl)], poly(2′-chloro-2′-deoxycytidylic acid) [poly(dCcl)], and poly(5-bromouridylic acid) [poly(U5br)] were synthesized in this laboratory by Drs. M. P. Kotick, J. L. Colbourn, and D. P. Wilson using published procedures (Janik *et al.*, 1972; Hobbs *et al.*, 1972). Poly(1-vinyl U) was provided by Dr. J. Pitha of the Gerontology Research Center, NIH. All of the polymers used in this study possessed a sedimentation coefficient greater than 4 S. This requirement is due to the reported dependence of the K_i on the chain length of polymers with less than 200 nucleotide residues/molecule (Erickson *et al.*, 1973).

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[1] Abbreviations of synthetic polynucleotides are in accordance with IUPAC–IUB Recommendations (*J. Biol. Chem. 245*, 5171 (1970)). Abbreviations used are: AMV, avian myeloblastosis virus; FeLV, feline leukemia virus; RMLV, Rauscher murine leukemia virus; and RSV, Rous sarcoma virus.

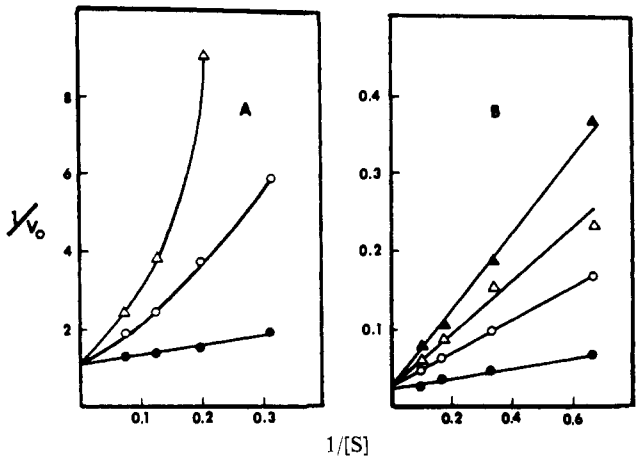

FIGURE 1: Kinetic analyses of poly(U) inhibition of DNA polymerase activity in detergent disrupted AMV preparations using synthetic primer-templates. Ordinates expressed in pmoles of deoxynucleoside monophosphate incorporated min^{-1}/ml^{-1} and abscissas in μg of poly(U)/ml. In experiment A the primer-template was $d(pT)_{10}\cdot$poly(A) and inhibition concentrations were: (○) 8 μg/ml; (△) 40 μg/ml; and (●) the uninhibited reaction. In experiment B $d(pG)_{10}\cdot$poly(C) was the primer-template and poly(U) concentrations were: (○) 0.5 μg/ml; (△) 0.8 μg/ml; (▲) 1.6 μg/ml; and (●) the uninhibited reaction.

Virus and Enzyme Purification. Virus particles were concentrated by centrifugation at 60,000*g* for 60 min onto 100% glycerol cushions. The virus was collected, dialyzed against buffer (0.15 M NaCl–3 mM EDTA–0.01 M Tris-HCl (pH 8.5)), layered on a 25–60% sucrose gradient and centrifuged at 40,000*g* at 4° for 90 min. The visible band of virus was removed, dialyzed against 0.15 M NaCl in 0.01 M Tris-HCl (pH 8.5), and stored at −70° in 10% glycerol.

The AMV DNA polymerase was isolated from purified virus essentially by the method previously described (Kacian *et al.*, 1971). The virus was disrupted by exposure to 1.0% sodium deoxycholate and 1.0% Nonidet P-40 (Shell Chemical Co.), and centrifuged at 16,000*g*, and the supernatant was applied to a Whatman DEAE-cellulose DE52 column. The active fractions were pooled, diluted, and applied to a Reeve Angel phosphocellulose P-11 column. The enzyme samples were stored in 2 mM dithiothreitol and 50% glycerol at −20°.

Assay for RNA Directed DNA Polymerase Activity. The polymerization of either dGMP or dTMP using the complementary primer-templates, $d(pG)_{10}\cdot$poly(C) or $d(pT)_{10}\cdot$poly(A), respectively, was carried out at 37° in the following standard reaction mixture: 10 mM Tris-HCl buffer (pH 8.5); 7 mM KCl; 2 mM dithiothreitol; 10 mM $MgCl_2$; 0.6 mM dCTP *or* dATP; and 0.15 mM dGTP *or* dTTP. Tritiated dGTP *or* dTTP was added at a level of 0.01 Ci/l. assay mixture or approximately 20 cpm/pmol. Only the two complementary deoxynucleoside triphosphates were added to each reaction. The primer-templates were prepared in a weight ratio of 1:6 unless stated otherwise.

When the polymerase activity in purified virions was assayed, the particles were first disrupted in 0.2% Nonidet P-40 at 4° for 20 min and then added to the assay mixture at a level of 10 ng of protein/μl. Due to a lack of material, the protein concentration of the purified enzyme could not be determined; however, using our standard assay conditions and saturating levels of primer-template, 1.0 μl of enzyme solution polymerized 3.0 pmol of dGMP/min. Trichloroacetic acid insoluble product was collected and washed on nitrocellulose filters. The filters were dried, placed in a toluene-based scintillation fluid, and assayed in a liquid scintillation spectrometer.

Assay for M. luteus and Calf Thymus DNA Polymerase Activity. The *M. luteus* DNA polymerase was assayed by procedures described by Zimmerman (1966). Under these conditions and using $d(pT)_{10}\cdot$poly(dA) as the primer-template at a concentration of 4.0 μg/ml, our preparation incorporated 0.77 μmol of dTMP min^{-1} μg of $protein^{-1}$. Calf thymus DNA polymerase was purified by published procedures (Yoneda and Bollum, 1965) and had a specific activity of 120 pmol of dTMP incorporated min^{-1} μg of $protein^{-1}$ using poly[d(A-T)] as primer-template (4.0 μg/ml). Calf thymus DNA polymerase assay mixture was prepared as follows: 50 mM potassium phosphate buffer (pH 7.2), 1 mM mercaptoethanol, 1 mM $MgCl_2$, 0.20 μM deoxynucleotide triphosphates, and 0.01 μCi of the labeled substrate per μl of reaction mixture.

Assay for RNase H Activity. The substrate for the detection of nuclease H activity was $d(pT)_{10}\cdot[^3H]$poly(A) prepared in a weight ratio of 1:6, respectively. Other reaction constituents were: Tris-HCl (pH 8.5), 10 mM; KCl, 7 mM; dithiothreitol, 2 mM; $MgCl_2$, 10 mM; dATP and dTTP, 0.6 mM. The two deoxynucleoside triphosphates were required for the demonstration of nuclease activity and, therefore, both synthesis of the DNA–RNA hybrid and degradation of the labeled template strand occurred simultaneously. The purified enzyme preparation was added to the assay mixture and incubated at 37°. Acid insoluble material was precipitated with a mixture of trichloroacetic acid (100%)–saturated sodium pyrophosphate–saturated sodium phosphate (monobasic) in a volume ratio of 1:1:1 at 4°. The precipitate was collected and quantitated as described for the DNA polymerase assay. The decrease in the isotopically labeled material retained on the filter was used as a measure of RNase H activity.

Results

Standardization of the Inhibition Reaction. For comparative studies on polynucleotide inhibitors of the oncornavirus DNA polymerase, it was essential to employ a standardized methodology that would be unbiased as regards the pyrimidine base moiety of the inhibitor. The reasoning underlying this statement is illustrated in Figure 1. When the primer–template was $d(pT)_{10}\cdot$poly(A) and the kinetics of poly(U) inhibition were studied, complex, nonlinear plots of $1/V_0$ *vs.* 1/primer–template were obtained (Figure 1A). It was reasoned that this may have been due to base pairing between nonprimed regions of the template strand, poly(A), and the inhibitor. Hence, the poly(A)·poly(U) could have been either an inactive template or an inactive inhibitor. To circumvent this type of problem, the primer–template was changed to $d(pG)_{10}\cdot$poly(C) and the Lineweaver–Burk plot was consistent with that expected for a competitive inhibitor of enzyme activity (Figure 1B) and corroborated previously published data (Tuominen and Kenney, 1971). For the comparative analyses we therefore used $d(pG)_{10}\cdot$poly(C) as primer–template for poly(U) analogs and $d(pT)_{10}\cdot$poly(A) for poly(C) related compounds.

If poly(U) competes with the template strand for the enzyme binding site, then the order of addition of reactants should also significantly affect inhibition kinetics. This was observed previously in studies on the inhibition of the $Q\beta$ replicase by poly(U) (Haruna and Spiegelman, 1966) and we have found that the K_i for a specific polynucleotide inhibitor can be reduced 3–4-fold by preincubation of the enzyme with inhibitor before template addition. This is true for both poly(U) and poly(dUfl) and is strong support for the argument that the

polymers interact directly with the enzyme. In the experiments to be described in this report, primer–template and inhibitor were added to the reaction mixture which lacked enzyme, the tube mixed thoroughly and enzyme added last.

The reaction constants for the enzyme activity in detergent-disrupted virions and purified enzyme preparations using either $d(pG)_{10}\cdot poly(C)$ or $d(pT)_{10}\cdot poly(A)$ as primer–template are summarized in Table I. These values were determined by Lineweaver–Burk plots of $1/V_0$ *vs.* 1/[S] in which the primer–template was treated as a true substrate. The avian viruses appear to prefer poly(C) as a template whereas FeLV uses poly(A) more efficiently.

Comparative Analyses of the Inhibitory Activity of Selected Polynucleotides. Using the values in Table I we computed the K_i for various synthetic polynucleotides from the expression, $V_i/V_{max} = [S]/[K_m(1 + [i]/K_i + [S]]$, where V_i is the initial velocity of the inhibited reaction, [S] is the primer–template concentration, and [i] is the concentration of the inhibitory polynucleotide.

The results of an initial screen of available polynucleotides are shown in Table II. All of the calculations were made using our standard reaction conditions at [S] values of 0.8 and 4.0 μg/ml in duplicate samples. The results indicate that both pyrimidine base and sugar are of great importance as regards inhibitory activity of the polymer. The significant enhancement of activity due to the substitution of a 2′-halogen in both poly(U) and poly(C) was of major interest and studies into the mechanism and characteristics of this reaction are the subject of the following studies.

Kinetics of the Inhibition of AMV DNA Polymerase by Poly(dUfl). Previous data have shown that poly(U) is a competitive inhibitor of both the RMLV enzyme (Tuominen and Kenney, 1971) and the AMV enzyme (see Figure 1B) and our first experiments were designed to characterize the type of inhibition of poly(dUfl). Unexpectedly, the kinetics for poly(dUfl) inhibition suggested noncompetitive or partially noncompetitive inhibition. This observation was true for both the detergent-disrupted virions (Figure 2A) and the partially purified enzyme preparation (Figure 2B).

Requirement for Single-Strandedness for Enzyme Inhibition. Poly(dUfl) forms a 1:1 complex with poly(A) and the thermal stability of this duplex is greater than that observed for poly(U)·poly(A) (Janik *et al.*, 1972). To assess the inhibitory activity of poly(A)·poly(dUfl) we mixed the polymers in equal weight ratios in 0.01 M Tris-HCl (pH 8.0) and 10 mM $MgCl_2$ at 4° for 18 hr. The degree of hypochromicity was 0.20. The complex, poly(dUfl), poly(A) or the buffer was then added to standard reactions using the purified enzyme in the presence of 4 μg/ml of $d(pG)_{10}\cdot poly(C)$. After 10-min incubation the control reaction polymerized 53.4 pmol of dGMP, in the presence of 10 ng/ml of poly(A) 52.9 pmol of dGMP were incorporated, only 18.0 pmol of dGMP were incorporated when incubated with 10 ng/ml of poly(dUfl), and 52.8 pmol of dGMP were incorporated in the presence of 20 ng of the duplex, poly(A)·poly(dUfl). Hence, the inhibitory polynucleotide must be single stranded.

The observation that double-stranded polymers do not interfere with polymerase activity may account for the kinetics described previously (Figure 1). When excess template can base pair with the inhibitory polymer another variable is introduced into the system and the K_i would increase (Figure

TABLE I: Summary of the Polymerization Reaction Constants for RNA Directed DNA Polymerase Activity of Selected Oncornaviruses.

Enzyme Preparation	Constant	Primer–Template $d(pG)_{10}\cdot poly(C)$	Primer–Template $d(pT)_{10}\cdot poly(A)$
Purified AMV enzyme	K_m[a]	0.88	6.6
	V_{max}[b]	76	38
Disrupted AMV	K_m	1.3	6.4
	V_{max}	34	7.8
Disrupted FeLV	K_m	14	7.6
	V_{max}	8.8	22
Disrupted RSV	K_m	3.3	10
	V_{max}	16	7.8

[a] Expressed as μg/ml of primer–template. [b] Expressed as pmol min^{-1} ml^{-1} of deoxynucleoside triphosphate.

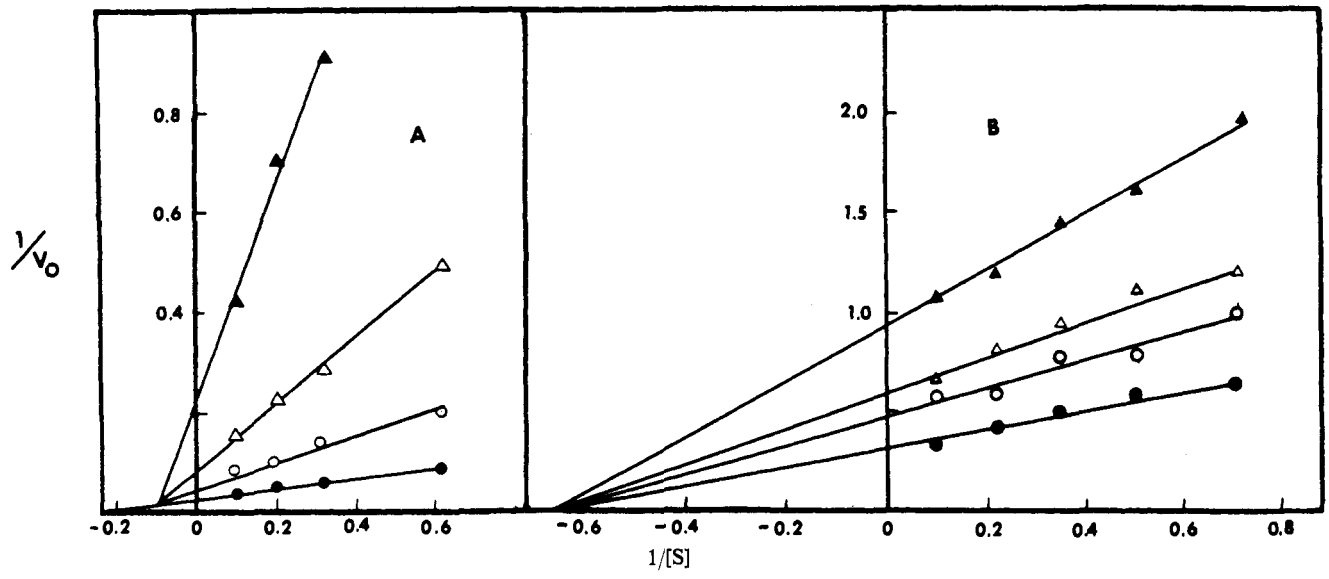

FIGURE 2: Kinetic analyses of poly(dUfl) inhibition of the AMV DNA polymerase activity. In experiment A the DNA polymerase in detergent-disrupted virions was assayed using poly(dUfl) concentrations of: (○) 0.1 μg/ml; (△) 0.25 μg/ml; (▲) 0.5 μg/ml; and (●) the uninhibited reaction. In experiment B purified AMV DNA polymerase was employed using poly(dUfl) concentrations of: (○) 4.0 ng/ml; (△) 10 ng/ml; (▲) 16 ng/ml; and (●) the uninhibited reaction.

TABLE II: The Inhibition of Oncornavirus RNA Directed DNA Polymerase Activity by Synthetic Homopolynucleotides.

Polynucleotide	K_i (μg/ml)			
	Enzyme Preparation			
	AMV Enzyme[a]	Disrupted AMV[b]	Disrupted RSV[b]	Disrupted FeLV[b]
Poly(U)	4.9×10^{-2}	6.3×10^{-1}	3.1×10^{-1}	1.2×10^{-1}
Poly(dU)	1.3×10^{-1}	8.4×10^{-1}	ni[c]	2.0
Poly(dT)	5.4×10^{-2}	1.8	nd[d]	5.1×10^{-2}
Poly(C)	3.8	ni	ni	ni
Poly(5Ubr)	1.3×10^{-2}	5.0×10^{-1}	1.7×10^{-1}	1.8×10^{-1}
Poly(dUfl)	5.0×10^{-3}	4.4×10^{-2}	4.5×10^{-3}	1.9×10^{-2}
Poly(dUcl)	3.6×10^{-3}	4.0×10^{-2}	1.4×10^{-3}	2.8×10^{-2}
Poly(dCcl)	8.8×10^{-3}	nd	2.2×10^{-3}	nd
Poly(I-vinyl U)	4.1×10^{-1}	nd	nd	nd

[a] Enzyme that was partially purified as described in Methods. [b] Enzyme activity in detergent-disrupted purified virus. [c] Not inhibitory at 5 μg/ml. [d] Experiment has not been done.

1A). On the other hand, when base pairing cannot occur the kinetic analysis is more simple and less inhibitor is required (Figure 1B).

The Effect of the Divalent Cation on Polymerase Inhibition. One of the variables affecting template preference has been shown to be the divalent cation required in the reaction mixture. Mouse leukemia virus has been shown to accept poly(U) as a template in the presence of Mn^{2+} but not Mg^{2+} (Baltimore and Smoler, 1971). Although the AMV enzyme cannot utilize poly(U) in the presence of either Mg^{2+} or Mn^{2+}, the inhibition of enzyme activity could have been dependent on the cation in our standard reaction, Mg^{2+}. To assess this possibility we first determined the optimal concentration of Mn^{2+} (0.5 mM), calculated the reaction constants, and then compared the K_i of both poly(U) and poly(dUfl) using the two experimental conditions. As demonstrated in Table III, the nature of the two cations used in this test had no significant influence on the K_i.

Influence of Actinomycin D on Inhibition by Poly(dUfl). Actinomycin D has been shown to have little effect on the synthesis of DNA from a single-stranded RNA template but blocks the utilization of the RNA–DNA hybrid as a template (McDonnell *et al.*, 1970). The data presented to this point, as will be subsequently discussed, suggest that poly(dUfl) inhibits the initial binding of polymerase to single-stranded regions of the RNA template. If this is the case, then we can predict that these two polymerase inhibitors act at different sites in the reaction and that the presence of actinomycin D will not change the K_i of poly(dUfl).

To aid in the assessment of these possibilities a minor modification was made in our standard reaction in that the ratio of primer:template was changed from 1:6 to 1:1.5. It was reasoned that this alteration would increase the sensitivity of our assay by providing more primed template regions on the single-stranded polyribonucleotide and, hence, more enzyme binding sites. These conditions did not significantly alter the K_m of the reaction but did reduce the V_{max} from 78 to 44 pmol of dGMP min^{-1} ml^{-1} (Tables III and IV). The addition of 30 μg/ml of actinomycin D caused a further decrease in the V_{max} to 13 pmol of dGMP min^{-1} ml^{-1}. The K_i for poly(dUfl) was then calculated in the presence and absence of actinomycin D and no significant change was observed (Table IV). These results suggest that the antibiotic and poly(dUfl) act on different stages of the polymerization reaction, the polymer presumably inhibiting the binding of enzyme to template.

Resistance of Inhibition by Poly(dUfl) to Pancreatic RNase. Substitution by fluorine in the 2′ position of poly(U) results

TABLE III: Comparison of the Inhibition of Purified AMV DNA Polymerase by Poly(dUfl) and Poly(U) in the Presence of Mg^{2+} or Mn^{2+}.

	Divalent Cation[a]	
	Mg^{2+}	Mn^{2+}
V_{max} (pmol of dGMP/min)[b]	78	48
K_m (μg/ml)[b]	0.88	1.52
K_i [μg of poly(U)/ml][c]	4.9×10^{-2}	5.9×10^{-2}
K_i [μg of poly(dUfl)/ml][c]	5.0×10^{-3}	5.6×10^{-3}

[a] Optimal concentrations of cations determined to be 10.0 mM for Mg^{2+} and 0.5 mM for Mn^{2+}. [b] Value obtained from graphical plots of $1/V_0$ *vs.* 1/[S]. [c] K_i values were determined at poly(U) concentrations of 0.2, 0.5, and 1.0 μg/ml and poly(dUfl) concentrations of 0.1, 0.25, and 0.5 μg/ml as described in text. The concentration of the primer–template, $d(pG)_{10}$·poly(C), was 2.5 μg/ml.

TABLE IV: Comparison of the Inhibition of Purified AMV Polymerase by Poly(dUfl) in the Presence and Absence of Actinomycin D.

	Experimental Conditions	
	a	*b*
V_{max} (pmol of dGMP min^{-1} ml^{-1})[c]	44	13
K_m (μg)[c]	0.88	0.96
K_i (μg)[c]	5.0×10^{-3}	5.7×10^{-3}

[a] Standard reaction conditions employed with the exception that $d(pG)_{10}$·poly(C) was prepared in a ratio of 1:1.5, respectively, and used at a concentration of 4.0 μg/ml. [b] Conditions were the same as above with 30 μg/ml of actinomycin D added. [c] Reaction constants calculated as described in Table III.

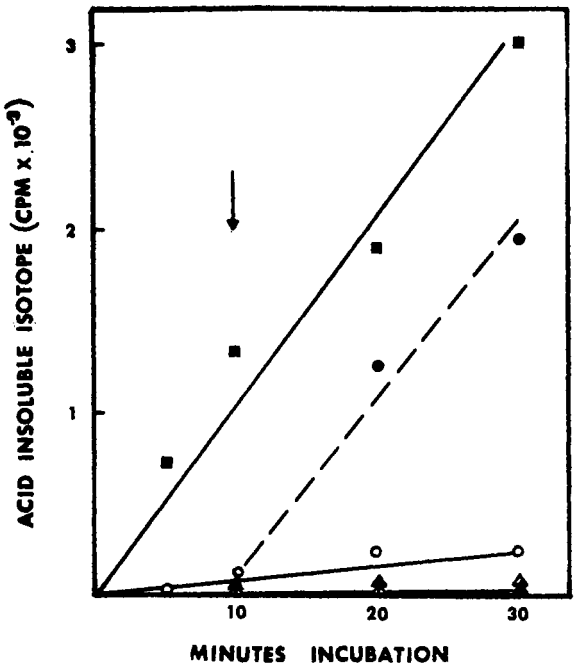

FIGURE 3: Effect of RNase on the inhibition of AMV DNA polymerase activity by poly(U) and poly(dUfl). The primer–template was poly[d(A-T)] used at a concentration of 20 μg/ml and pancreatic RNase (10 μg/ml) was added at time indicated by arrow. Poly(U) (0.4 μg/ml) and poly(dUfl) (0.1 μg/ml) were added to the reaction before the purified enzyme: (■) control; (○) poly(U); (●) poly(U) and RNase; (△) poly(dUfl); and (▲) poly(dUfl) and RNase.

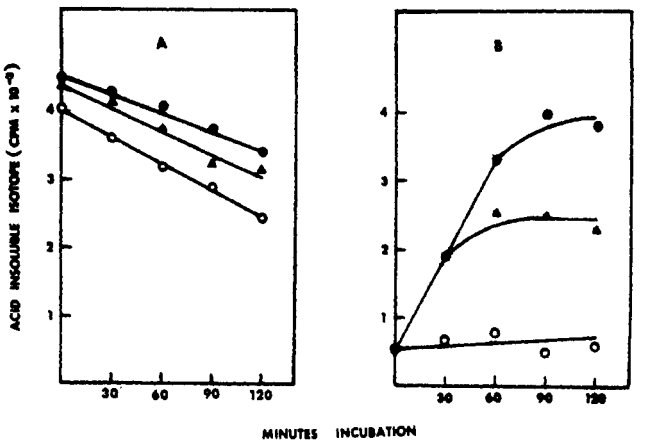

FIGURE 4: Effect of poly(dCcl) on the RNase H activity in purified preparations of AMV DNA polymerase. Experiment A represents the degradation of [³H]poly(A) (5×10^{-4} Ci/g): (○) 0.1 μg/ml of poly(dCcl) added at 0 min; (▲) poly(dCcl) added at 30 min; and (●) control. Experiment B represents the polymerization of [³H]-TMP: (○) poly(dCcl) added at 0 min; (▲) poly(dCcl) added at 30 min; and (●) control.

in a polymer that is resistant to hydrolysis by deoxyribonuclease I and pancreatic ribonuclease (Janik *et al.*, 1972). This property of poly(dUfl) should render its inhibitory activity insensitive to the presence of these degradative enzymes in our assay system. The fact that the AMV enzyme can utilize certain DNA polymers for DNA synthesis allows the assessment of the above prediction by following this DNA-directed DNA synthesis in the presence of pancreatic RNase. The DNA template, poly[d(A-T)], was used and the incorporation of [³H]dTTP into acid insoluble material was measured. As illustrated in Figure 3, the addition of 10 μg/ml of pancreatic RNase rapidly restored the polymerase activity to the reaction inhibited by poly(U) but had no effect on the reaction blocked by poly(dUfl).

Effect of poly(dCcl) on Viral RNase H activity. Purified preparations of the AMV DNA polymerase have been shown to contain RNase H activity (Grandgenett *et al.*, 1973), a ribonuclease that specifically degrades the RNA moiety of a RNA–DNA hybrid. In view of the resistance of 2′-halogenated polynucleotides to nucleases, it was possible that poly(dUfl) and related polymers inhibited a required nuclease function rather than directly inhibiting the polymerization of DNA. Using standard reaction conditions, $d(pT)_{10}$·[³H]poly(A) was found to be a much more efficient substrate for the AMV RNase H activity than $d(pG)_{10}$·[³H]poly(C). Thus, for these experiments, poly(dCcl) was used in place of poly(dUfl) to eliminate the possible base pairing between inhibitor and substrate. All other conditions were identical with those used in the polymerization reaction except that in measuring RNase H activity the tritium-labeled compound was poly(A) and in the assay for polymerization the tritium label was dTTP.

The kinetics for both the polymerization of dTMP and the degradation of poly(A) are shown in Figure 4. Incorporation of [³H]TMP into acid-insoluble material is linear for approximately 60 min whereas the [³H]poly(A) is degraded at a constant rate during the 120 min time interval studied. When poly(dCcl) is added at the beginning of the experiment before enzyme addition, polymerization is blocked completely while the degradation of poly(A) occurs at a rate similar to that of the control reaction. If the inhibitory polymer is added after the initiation of synthesis, polymerization of [³H]dTMP stops after approximately a 40 percentage increase and, again, there is no change in the rate of degradation of poly(A). Hence, it appears that it is possible to block the template binding site on the AMV DNA polymerase without altering the RNase H activity and that 2′-halogenated derivatives of poly(U) specifically block the polymerization reaction.

Specificity of Inhibition by Poly(dUfl). Poly(U) has been shown to be an inactive template for the AMV enzyme when primed with $d(pA)_{10}$ (Baltimore and Smoler, 1971) and not to interfere with DNA synthesis catalyzed by *E. coli* DNA polymerase I or polymerase activity extracted from mouse embryo (Tuominen and Kenney, 1971); however, poly(U) does inhibit DNA polymerase I of normal human lymphocytes (Abrell *et al.*, 1972). In order to determine if the specificity of inhibition by poly(dUfl) was similar to that described for poly(U) we obtained preparations of *M. luteus* and calf thymus DNA-dependent DNA polymerases. The primer–template for these reactions was $d(pG)_{10}$·poly(dC) at a concentration of 0.8 μg/ml and the enzyme preparations were employed at levels which incorporated approximately 25–50 pmol of dGMP/min. Neither poly(U) nor poly(dUfl) inhibited these enzymes at inhibitor concentrations up to 40 μg/ml. In addition, poly(dUfl) has been found to inhibit DNA polymerase I of normal human lymphocytes but not DNA polymerase II from the same cells (J. W. Abrell and R. C. Gallo, personal communication). Hence, poly(dUfl) appears to retain the same degree of inhibitory specificity as poly(U).

The 2′-halogenated derivatives were also found similar to poly(U) in that they were not active templates for the AMV when primed with the complementary oligonucleotide. The primer $d(pA)_{10}$ was annealed to poly(dUfl) at a ratio of 1:6, as described previously, and the complex was used as a primer–template for the *M. luteus* or AMV DNA polymerase. The results showed that poly(dUfl) could serve as a template for the *M. luteus* DNA polymerase but was completely inactive as a template for the AMV enzyme (Figure 5). The individual

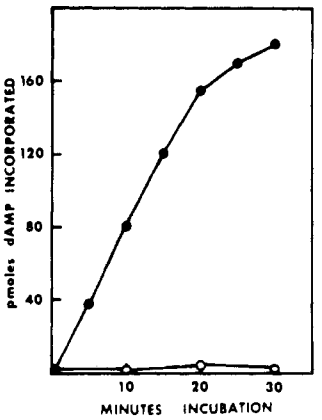

FIGURE 5: Template activity of poly(dUfl) for the DNA polymerases of *M. luteus* and AMV. The two DNA polymerase preparations were diluted in their respective buffers to approximately equal activities (*i.e.*, 200–250 pmol min^{-1} ml^{-1} using $d(pG)_{10}$·poly(C) for the AMV polymerase and d(pG)·poly(dC) for the *M. luteus* DNA polymerase). The primer–template $d(pA)_{10}$·poly(dUfl) was prepared in a ratio of 1:6 as described and used at a concentration of 5 μg/ml: (●) *M. luteus* DNA polymerase; (○) AMV polymerase.

components, poly(dUfl) and $d(pA)_{10}$, were not active in either system.

Discussion

The observation that polymerase enzymes bind to single-stranded template strands in the absence of primer is known to occur in the case of *E. coli* polymerase I. φX174 DNA was found to bind as many as 20 enzyme molecules per single-stranded circle (Englund *et al.*, 1968). Zone centrifugation studies on the Qβ replicase–Qβ RNA complex indicated that only one enzyme molecule was bound per linear RNA genome and that this binding was reversible in the absence of chain elongation (August *et al.*, 1968). In view of the fact that all DNA polymerases examined thus far do require a primer–template like secondary structure for the initiation of synthesis, it is no surprise that single-stranded synthetic polynucleotides (*i.e.*, unprimed templates) can act as inhibitors of the polymerization reaction. On the other hand, the observations that the degree of inhibition is strongly dependent upon the chemical composition of the polynucleotide and that different classes of DNA polymerases respond to these polymers in markedly different manners (*i.e.*, template utilization or enzyme inhibition) are of interest. In the case of poly(dUfl), the data suggest that the *M. luteus* DNA polymerase can bind to a "primed" template strand, initiate, and polymerize the complementary strand of poly(dA). The AMV DNA polymerase, however, can apparently bind to poly(dUfl) but cannot initiate and/or sustain polymerization.

Comparative analyses of nine pyrimidine-containing homopolynucleotides as competitive inhibitors of the DNA polymerase of the oncornaviruses indicate the importance of the chemical composition for this activity. The preliminary data from this study of structure–activity relationships can be briefly summarized as follows: (1) substitution by an amino group in the 4 position of the pyrimidine ring greatly reduces inhibitory activity and allows the polymer to act as a template; (2) a bromine atom or methyl group in the 5 position on the ring increases inhibitory activity; (3) complete replacement of the ribose sugar by a vinyl polymer did not destroy the inhibitory capacity, indicating the importance of pyrimidine ring; (4) substitution by a hydrogen atom for the 2′-hydroxyl group decreased inhibitory activity; and (5) replacement of the 2′-hydroxyl group by a halogen greatly increased the inhibitory activity of the polymer. This last point is illustrated most dramatically by the observation that the K_i for poly(C) is 400 times greater than that calculated for poly(dCcl). Thus, both the pyrimidine and sugar moieties of the polymer contribute significantly to the interaction between the enzyme and polynucleotide, especially as regards the 4, 5, and 2′ positions.

The observation that poly(U) and poly(dUfl) produce different inhibition kinetics appears to indicate that the polymers belong to different classes of inhibitors. As reported previously, poly(U) is a competitive inhibitor of the viral polymerase, presumably competing for the template binding site. We feel that poly(dUfl) also competes competitively for the template binding site. The difference in kinetics may result from the fact that our assay measures polymerization, not binding, and that the deoxynucleoside triphosphates are the true substrates for this reaction rather than the primer–template. Hence, a nontemplate polymer with a very low dissociation constant for the template binding site of the enzyme (*i.e.*, the reaction may approach irreversibility) may inhibit the polymerization reaction in a noncompetitive manner. A definitive statement of the mechanism of inhibition by poly(dUfl) must be based on the future development of an enzyme–template binding assay similar to that described for the *E. coli* DNA dependent RNA polymerase (Hinkle and Chamberlin, 1972).

The conclusion that poly(dUfl) does interact with the template binding site on the viral enzyme is based upon five observations. First, the inhibition kinetics indicate that the synthetic polymers interact with the enzyme and not with the primer–template. Template–inhibitor interactions can occur, of course (see Figure 1), and since the viral genome does contain large segments of poly(A) (Gillespie *et al.*, 1972) these interactions might be quite significant. The higher K_i observed when disrupted AMV was used as the source of enzyme in contrast to similar analyses using purified preparations (Table II) might be indicative of such an interaction. Second, the K_i of a given polymer can be reduced several fold by preincubation of enzyme and inhibitor prior to the addition of the primer–template. Third, the K_i of the polymer is inversely proportional to the chain length up to a critical size of 200 nucleotide residues/molecule (Erickson *et al.*, 1973). Fourth, the inhibitory polymer must be single stranded. And fifth, the degree of inhibition appears to be independent of the divalent cation required for polymerization. These last three properties have been described for the enzyme binding site of microbial nucleic acid polymerases (Englund *et al.*, 1968; August *et al.*, 1968).

The observation that poly(dCcl) does not inhibit the degradation of poly(A) by the viral RNase H activity is of interest since both polymerase and nuclease activity have been found in one electrophoretically pure protein (Grandgenett *et al.*, 1973). If this is the case, then it appears that a synthetic polynucleotide can occupy the template binding site and, in the absence of polymerization, the enzyme can still demonstrate uninhibited nuclease activity.

Studies on the relationship between the structure of polynucleotides and their ability to interact with the viral enzymes may reveal significant information regarding such protein–nucleic acid interactions. A similar approach has been used in other microbial systems. The binding of the *lac* repressor to DNAs of defined sequence has revealed the importance of structure in this system and it is of interest to note that the bromine atom in the 5 position of the uracil ring greatly in-

creases binding of the polymers to the repressor molecule (Lin and Riggs, 1971, 1972). In addition, the polymerase binding sites of two bacteriophage genomes, Qβ (Weber *et al.*, 1972) and fd (Heyden *et al.*, 1972), have been isolated and have been found to be rich in uridylate residues or AT base pairs, respectively. The role of these pyrimidine residues in the binding of enzyme to nucleic acid may be important.

The possibility that synthetic polynucleotides may exert a direct antiviral activity, in addition to interferon induction, is suggested by recent data. Poly(A) and poly(2′-*O*-methyl A) have been shown to inhibit the replication of Moloney leukemia virus in secondary Swiss mouse embryo cells (Tennant *et al.*, 1972) and, although not proven in a definitive manner, this inhibition is presumably the result of the inhibition of the viral RNA directed DNA polymerase activity. Poly(dUfl), with a K_i several logs lower than that determined for poly(A) and being relatively nuclease resistant, should prove to be a more potent antiviral agent. Although the inhibition by poly(U) analogs may not be specific for the viral enzymes (Abrell *et al.*, 1972), such polymers may be able to interfere with the polymerases and allow host mechanisms to inactivate the viral genetic information. *In vitro* and *in vivo* studies on the antiviral properties of poly(dUfl) and related compound are now in progress. In addition, these studies on the structure–activity relationships and the mechanism of inhibition are being used as an aid in the design of other synthetic polymers of interest.

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