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Inhibition of the DNA Polymerase of Rauscher Leukemia Virus by Single-Stranded Polyribonucleotides

[mouse/poly(U)]

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ABSTRACT **The DNA polymerase of Rauscher murine leukemia virus is strongly and specifically inhibited by nontemplate, single-stranded polyribonucleotides with either the resident viral RNA, native calf-thymus DNA, or poly[d(A-T)] as templates. These inhibitory homopolymers are apparently bound to the template site of the polymerase, since they interact competitively with the template. The strength of the inhibition depends on the particular homopolymer used: poly(U) > poly(G) ≫ poly(A) > poly(C). The K_i for poly(U) was 0.08 μg/ml, which represents an apparent affinity six times greater than that observed for viral RNA. No such inhibition was observed with a highly purified DNA polymerase from mouse embryos or the *Escherichia coli* enzyme.**

There are now many reports of RNA-dependent DNA polymerases in oncogenic RNA viruses (1–8). Although similar activities have been detected in partially purified preparations from normal (9, 10) and leukemic (11–13) cells, a satisfactory assay for the specific determination of these enzymes in cell extracts has not been described. The required specificity can only be achieved when properties that are unique to the viral enzymes are clearly understood. The work described here concerns one possible unique property.

The published findings of Spiegelman *et al.* (5) show that synthetic, single-stranded homopolyribonucleotides do not serve as templates for the DNA polymerase of the avian myeloblastosis virus. The present communication describes the effects of these homopolymers on the polymerization of deoxyribonucleotides by the enzyme of the Rauscher murine leukemia virus. The enzyme–polynucleotide interactions described here may provide a basis for a specific assay for viral polymerases.

MATERIALS AND METHODS

Virus purification

Rauscher murine leukemia virus (RLV) was purified from two different sources. One source was a 10-fold mouse (strain CFW-S) plasma concentrate (lot number RPV-HL-68-4 of Hazleton Laboratories) that contained 5.48 log units (spleen enlarging)/ml. The viruses from this source were purified according to published methods (3). Rauscher virus from JLS-V5 tissue culture fluids was also purified by this procedure after an initial sedimentation at 78,000 × *g*.

Abbreviation: RLV, Rauscher murine leukemia virus.

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DNA polymerase preparations

Crude Rauscher virus DNA polymerase was prepared by detergent lysis of purified RLV at 0–4°C for 10 min in a solution containing 0.05 M Tris·HCl (pH 7.5), 0.1 M NaCl, 1 mM EDTA, 8 mM $MgCl_2$, 4 mM dithiothreitol, 0.1% (v/v) Nonidet P-40, and 150–300 μg of viral protein per ml.

DNA polymerase was repeatedly purified from 16- to 18-day-old BALB/c mouse embryos by a method similar to that described by Alberts *et al.* (14) for the purification of DNA polymerase I from *Escherichia coli*. In the procedure, decapitated embryos were homogenized at 4°C in a pH 8.1 buffer containing 5 M NaCl–0.05 M $MgCl_2$–5 mM 2-mercaptoethanol–0.02 M Tris·HCl. A 105,000 × *g* supernatant was prepared from the homogenate after it had been subjected to sonication (Bronwill Biosonik with a 12-mm diameter probe at maximum output) and filtered through cheesecloth. A nucleic acid-free solution was then prepared by the use of a two-phase, polyethyleneglycol–dextran extraction system (15). The top phase was passed over a column of DNA-cellulose after the salt had been removed by dialysis against a pH 8.1 buffer containing 0.02 M Tris·HCl–0.1 M NaCl–5 mM 2-mercaptoethanol–1 mM EDTA. The enzyme was eluted from the column by a similar buffer containing 0.6 M NaCl. The resulting DNA polymerase preparation contained more than 95% of the activity (assayed with a poly[d-(A-T)] template) and only 1.2% of the protein that was originally present in the dialyzed top phase. This "80-fold purified" preparation was occasionally purified an additional 4-fold by dialysis against a 0.02 M Tris·HCl (pH 7.5)–5 mM 2-mercaptoethanol–1 mM EDTA–0.02 M NaCl, and then passage of the enzyme preparation over a DEAE-cellulose column equilibrated with the same buffer.

The *E. coli* DNA polymerase-I was a partially purified product of Miles Laboratories, Inc.

Assay of DNA polymerase

DNA polymerase activity was measured at 37°C in 110–225 μl of an assay mixture that contained, as constant constituents, 0.02 M Tris·HCl, 0.05 M KCl, 10 mM $MgCl_2$, and 2.5 mM dithiothreitol at pH 7.5, unless otherwise stated. The concentrations of radioactively labeled and unlabeled deoxyribonucleoside triphosphates, templates, inhibitors, and enzymes used are given in the figure legends. Aliquots (25–100 μl) of the assay mixtures were removed at various times, spotted onto 3 MM filter-paper discs, and prepared for esti-

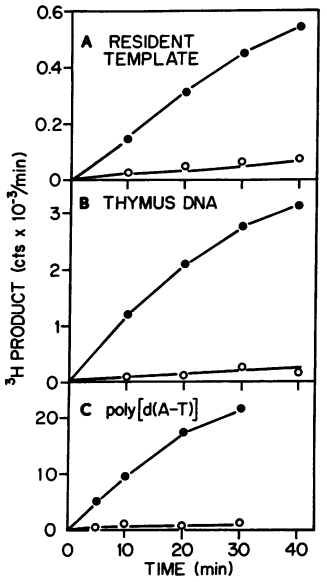

FIG. 1. Inhibition of Rauscher leukemia virus DNA polymerase by polyuridylate. In *A* and *B*, 50-μl aliquots were harvested from 225-μl assay mixtures that contained (per ml) 62 μg of RLV protein; 0.61 μmol each of dATP, dCTP, and dGTP; and 4.8 nmol of [^{3}H]dTTP at 660 cpm/mol. Native calf-thymus DNA was present in *B* at 8.0 μg/ml. In *C*, 25-μl aliquots were removed from 110-μl assay mixtures that contained (per ml) 30 μg of RLV protein, 0.32 μmol of dATP, 0.011 μmol of [^{3}H]dTTP at 160 cpm/pmol, and 28 μg of poly[d(A-T)]. Assays containing 32 μg/ml of poly(U) are represented by *open circles*, while *closed circles* represent controls.

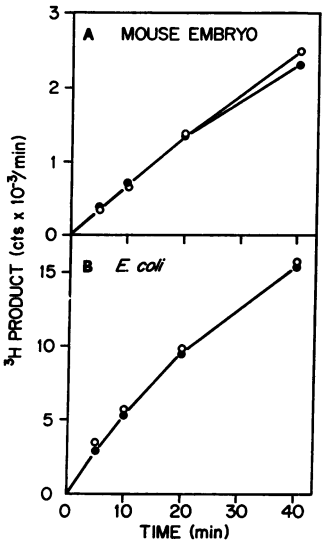

FIG. 2. Effect of polyuridylate on the rate of DNA polymerization by enzymes from mouse embryos and *E. coli*. The 210-μl assays contained (per ml) 0.28 μmol of dATP, 0.022 μmol of [^{3}H]-dTTP at 390 cpm/pmol, 14 μg of poly[d(A-T)], and either no poly(U) (*closed circles*) or 65 μg of poly(U) (*open circles*). The reactions in *A* were initiated by the addition of 2.7 μg of "300-fold purified" mouse-embryo enzyme (see *Methods*), while those in *B* were initiated with 0.07 unit of *E. coli* DNA polymerase. Aliquots of 50 μl and 25 μl were harvested in *A* and *B*, respectively.

mation of acid-insoluble radioactivity according to the method of Bollum (16). All initial velocity determinations were made under conditions that yielded a linear increase in product with time.

Polynucleotides

All the synthetic polynucleotides used were products of Miles Laboratories, Inc. The concentrations of stock solutions were determined by dilution of the material in 0.1 M phosphate buffer at pH 7.0 and determination of the absorbance at 260 nm. The extinction coefficients used were (expressed as reciprocal millimolar concentrations of polynucleotide phosphorus): polyriboadenylate, 9.75; polyribocytidylate, 5.65; polyriboguanylate, 10.51; polyribouridylate, 9.61. Conversions to mg/ml concentrations were made with the following factors for average μmol P/mg polymer: poly(A), 2.29; poly(C), 2.27; poly(G), 1.99; poly(U), 2.50 (data of Miles Laboratories, Inc.). A 1 mg/ml solution of poly[d(A-T)] was assumed to contain 20 A_{260} units/ml.

RESULTS

The inhibition of the DNA polymerase of Rauscher leukemia virus by polyuridylate is illustrated in Fig. 1. Although greatly different rates of deoxyribonucleotide polymerization were observed with poly[d(A-T)], native calf-thymus DNA, and the resident RNA template, all reactions were similarly inhibited by polyuridylate. More than 90% inhibition was observed with each of the reaction systems.

The potent inhibition of the RLV polymerase was sharply contrasted by the absence of any effect of poly(U) on catalysis by the polymerases from BALB/c mouse embryos or *E. coli* (Fig. 2). It is possible that the apparent lack of inhibition was due to the presence of ribonuclease or some other anti-poly(U) factors in the mouse and *E. coli* polymerase preparations. However, this explanation was ruled out by experiments which showed that reaction mixtures containing poly (U) completely inhibited the RLV polymerase even after 20 min in the presence of the mouse embryo or *E. coli* enzymes. Thus, it was concluded that the apparent specific inhibition of the RLV polymerase was due to an intrinsic property not present in the *E. coli* or mouse enzymes, rather than to a more trivial factor such as ribonuclease contamination.

The potent and apparently specific inhibition of the enzyme from leukemia virus by polyuridylate encouraged a more comprehensive characterization of this phenomenon. The data presented in Table 1 demonstrate that polymeriza-

TABLE 1. *Effect of pH and buffer composition on poly(U) inhibition*

Assay conditions	% Inhibition
pH 8.3	97.8
pH 8.3 + K-PO$_4$	91.5
pH 7.5	98.7
pH 7.5 + K-PO$_4$	93.4

Aliquots of 25 μl were harvested after 5, 10, 20, and 40 min incubation of 110-μl reaction mixtures that contained (per ml) 0.28 μmol of dATP, 0.011 μmol of [^{3}H]dTTP at 390 cpm/pmol, 28 μg of poly[d(A-T)], and either no poly(U) or 32 μg of poly(U), in both the presence and absence of 42 μmol of potassium phosphate at pH 7.5 or 8.3. All other conditions were as described in *Methods*.

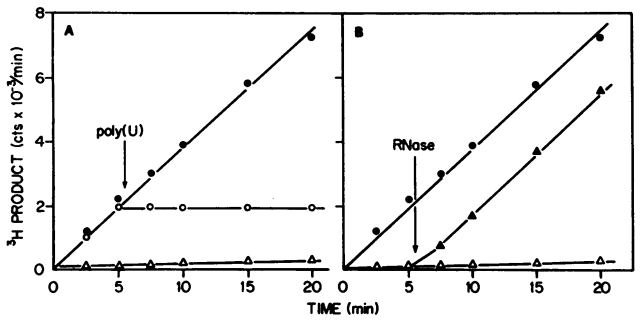

FIG. 3. Reversibility of polyuridylate inhibition of RLV polymerase. Aliquots of 25 μl were removed from 210-μl reactions that contained (per ml) 0.31 μmol of dATP, 0.018 μmol of [^{3}H]dTTP at 200 cpm/pmol, 28 μg of poly[d(A-T)], and 23 μg of RLV protein. Reactions represented by *circles* contained no poly(U) at zero time, whereas those represented by *triangles* contained 52 μg/ml of poly(U) at zero time. Poly(U) (52 μg/ml) was added to one reaction, in *A*, at 5.2 min. In *B*, 100 μg of pancreatic ribonuclease A was added to a poly(U)-containing reaction (▲) at 5.2 min.

tion by the RLV enzyme was inhibited similarly at both pH 7.5 and pH 8.3. In addition, the presence of inorganic phosphate did not significantly relieve the inhibition, suggesting that it was not the result of a nonspecific phosphate effect.

The extent of inhibition of the RLV polymerase was observed to be the same whether poly(U) was present at zero time or added after polymerization had commenced (Fig. 3*A*). This implies that poly(U) may interfere with the elongation phase of polymerization in addition to its effect on the initial binding of enzyme to the poly[d(A-T)] template. The experiment illustrated in Fig. 3*B* demonstrates that polyuridylate inhibition of the RLV polymerase is readily reversible. The addition of ribonuclease 5 min after the initiation of polymerization rapidly removed the inhibitory effect and allowed catalysis to proceed normally.

The data in Fig. 3 suggested that poly(U) may have been competing with a component of the reaction mixture for polymerase. An experiment to determine whether the competition was between poly(U) and the template, poly[d(A-T)], was therefore performed. The data (presented in Fig. 4) are clearly interpretable in terms of a classical competitive inhibition. No such competition has been observed with any other reaction component.

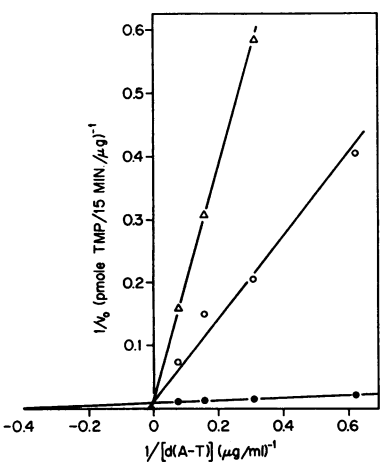

FIG. 4. Competitive interaction of template and polyuridylate. Aliquots of 100 μl were harvested at 15 min from 110-μl reaction mixtures that contained a variable concentration of poly[d(A-T)], and either no poly(U) (●), 4.5 μg/ml of poly(U) (○), or 9.0 μg/ml of poly(U) (△). Other assay conditions were as described for Fig. 3.

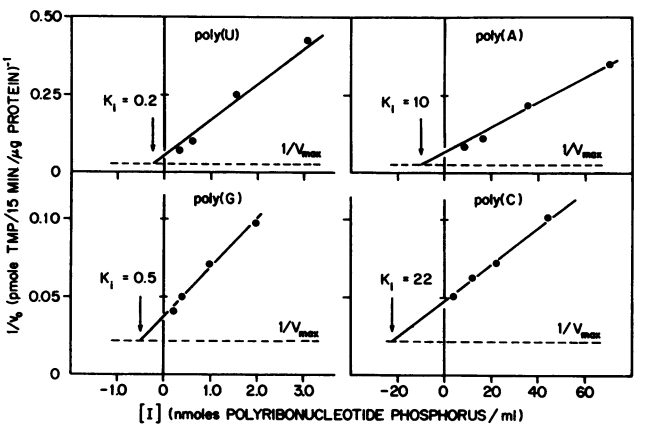

FIG. 5. Quantitation of polyribonucleotide inhibition of RLV polymerase. Aliquots of 100 μl were harvested at 15 min from 110-μl reaction mixtures that contained (per ml) 0.28 μmol of dATP, 0.025 μmol of [^{3}H]dTTP at 215 cpm/pmol, 1.52 μg of poly[d(A-T)], 10.6 μg of RLV protein, and a variable concentration of either poly(U), poly(A), poly(G), or poly(C). The $1/V_{max}$ value shown was obtained from a double-reciprocal plot similar to that shown in Fig. 4.

It became of interest to determine what the effects of single-stranded polyribonucleotides other than poly(U) were on the RLV-catalyzed polymerization. The effects of other polyribonucleotides were pertinent, since the observed poly(U) inhibition may have simply been due to the sugar–phosphate backbone of the polymer. The results presented in Fig. 5 show clearly that the base composition of the polyribonucleotide does play an important part in determining the strength of the inhibition. The apparent dissociation constants (K_i's) for the four polyribonucleotides tested ranged from 0.2 (micromolar concentration of polynucleotide phosphorus) for poly(U) to 22 for poly(C). Expressed in terms of μg/ml, the K_i's were 0.08 for poly(U), 0.25 for poly(G), 4.4 for poly(A), and 9.7 for poly(C).

Single-stranded polyribonucleotides do not serve as templates for polymerization by the DNA polymerases of RNA tumor viruses under the usual reaction conditions (5). The data presented here demonstrate that these polymers do, however, bind to the enzyme. It was of interest, therefore, to determine whether the viral RNA (17, 18) present in the RLV polymerase preparations used here is also bound tightly by the enzyme under the assay conditions. This RNA has already been shown to be an inferior template for polymerization (Fig. 1; ref. 19). The results presented in Fig. 6 show that some viral RNA is bound during poly[d(A-T)] synthesis and acts as a competitive inhibitor of poly[d(A-T)] binding. The saturation data for the template, poly[d(A-T)], were obtained before and after ribonuclease treatment of the RLV polymerase. The apparent dissociation constant for poly[d(A-T)] was decreased about 2-fold by the ribonuclease treatment (1.5 μg/ml after versus 2.6 μg/ml before). The presence of a constant amount of viral RNA does not, however, influence the K_i's that were obtained in Fig. 5 (20).

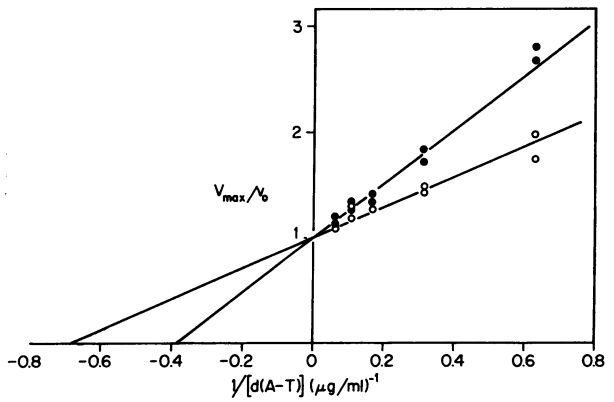

FIG. 6. Effect of ribonuclease treatment of RLV polymerase on the apparent affinity of the polymerase for poly[d(A-T)]. Aliquots of 100 μl were harvested after 20 min from reactions that contained (per ml) 0.28 μmol of dATP, 0.041 μmol of [^{3}H]dTTP at 100 cpm/pmol, a variable amount of poly[d(A-T)], and either 12.5 μg of untreated RLV polymerase (●) or 11.1 μg of ribonuclease-treated RLV polymerase (○). Ribonuclease treatment was accomplished by incubating detergent-lysed RLV (see *Methods*) with 250 μg/ml of pancreatic ribonuclease A for 15 min at 23°C. The values of V_{max} (obtained from double reciprocal plots) were 82.3 pmol of dTMP/20 min/100-μl aliquot for the untreated RLV system, and 71.5 pmol dTMP/20 min/100-μl aliquot for the ribonuclease-treated enzyme.

An apparent K_i for the resident viral template may also be estimated† from the data presented in Fig. 6. A value of K_i = 0.5 μg/ml was calculated from these data, which represents a 3-fold tighter binding than observed for poly[d((A-T)] but only one-sixth that observed for poly(U).

DISCUSSION

Although there are now many reports (1–8) of RNA-dependent DNA polymerases in oncogenic RNA viruses, the published work of Spiegelman *et al.* (5) has shown that synthetic single-stranded polyribonucleotides do not serve as templates for polymerization by these enzymes under the reaction conditions usually used. The results presented here show that these polyribonucleotides reversibly inhibit the DNA polymerase of Rauscher murine leukemia virus with either virus RNA, DNA, or poly[d(A-T)] as a template. Hence, the single-stranded polyribonucleotides are bound by the enzyme. Furthermore, the reversible competitive interaction between the template, poly[d(A-T)], and these single-stranded polymers suggests that the same template binding site is involved.

The strength of the inhibition by single-stranded polyribonucleotides is clearly dependent on the base composition of the homopolymer. Poly(U) and poly(G) were apparently bound with much greater affinity than were poly(A) or poly (C). Thus, no purine or pyrimidine preference is obvious. Furthermore, the apparent dissociation constants for poly-(U) (K_i = 0.08 μg/ml) and poly(G) (K_i = 0.25 μg/ml) are smaller than that estimated for the resident viral RNA (K_i = 0.50 μg/ml), suggesting that the viral polymerase might be inhibited *in vivo* by one of these polymers. Preliminary experiments to test this possibility are now underway. These data also suggest that viral polymerase molecules, free of bound polynucleotides, must be rare inside host cells.

The findings presented here indicate that the inhibition of DNA polymerization by synthetic homopolyribonucleotides may be a property unique to the enzymes of oncogenic RNA viruses. DNA polymerase-I of *E. coli* and the predominant enzyme of mouse embryos were clearly not inhibited by high concentrations of poly(U). These enzymes do, however, possess some properties similar to the leukemia virus polymerase studied here. First, poly[d(A-T)] is a "preferred" template for each. In addition, the *E. coli* enzyme has been shown to catalyze an RNA-dependent reaction with either natural RNA (22) or certain synthetic RNAs (23) as templates. Thus, the findings reported here suggest that poly(U) or poly(G) might serve as a rapid and simple means of differentiating cellular polymerases from the viral enzyme in crude enzyme preparations.

Both the specificity of poly(U) inhibition and the competitive interaction of poly(U) and template suggest a simple approach to simultaneous separation and purification of leukemia virus-like DNA polymerases from crude cellular homogenates. Thus, the viral enzymes might be specifically eluted from DNA-cellulose columns (14) with buffers containing poly(U).

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† When $V_{max}/v = 0$ in Fig. 6, $1/d(A - T) = -1/K_m(1 + i/K_i)$, where i, the concentration of RNA, was calculated from the known RNA/protein composition ratio of RNA tumor viruses (see ref. 21) and K_m = 1.5 μg/ml for poly[d(A − T)].

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