Comparative Biochemical Properties of RNA-directed DNA Polymerases from Rauscher Murine Leukemia Virus and Avian Myeloblastosis Virus

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SUMMARY
Various biochemical properties of the RNA-directed DNA polymerases of avian myeloblastosis virus and Rauscher murine leukemia virus were studied. Effects of divalent cations, pH, and temperature on the ability of the two polymerases to utilize various template primers are significantly different. The template primers tested include DNase-activated calf thymus DNA, d(A-T)ₙ, (rA)ₙ-(rU)ₙ, (rA)ₙ-(dT)ₙ, (rA)ₙ-(dT)₆, (dA)ₙ-(dT)₆, (rC)ₙ-(dG)₆, and (rl)ₙ-(dC)₆. All showed individual characteristic requirements of divalent cations, pH, and temperature for optimum activity with the two polymerases. A minor change in the optimal conditions usually resulted in a marked decrease in the detected polymerase activity. In kinetic experiments, initial deoxythymidine 5'-monophosphate incorporation appeared to be the rate-limiting step in the (rA)ₙ-(rU)ₙ-dependent activity of the murine virus polymerase, whereas this was less apparent with the avian virus polymerase. The d(A-T)ₙ-dependent activity of both purified virus polymerases is competitively inhibited by (rU)ₙ or (rG)ₙ.

INTRODUCTION
RNA-directed DNA polymerase activities have been shown in all the known RNA oncogenic viruses (11). It is almost certain that this polymerase activity is required for viral infection and transformation of susceptible cells (13, 27). Because of the unique capacity to transcribe natural RNA sequences to DNA and the obligatory role in viral oncogenesis, a thorough knowledge of the properties and characteristics of these enzymes is important. Justifiably, much effort has been applied in the use of synthetic polynucleotide template primers to increase the sensitivity of detecting viral polymerases (18, 26) and also in defining assay conditions that will selectively differentiate the viral enzyme from normal cell DNA polymerases (2, 12, 22, 30). Relatively less consideration (9, 30) has been given concerning the manner in which known viral RNA-dependent DNA polymerases from different sources react under similar reaction conditions.

We previously reported (29) that the response of DNA polymerase from RLV for a particular template primer depends strongly on a variety of controllable conditions in the assay. The present study compares the RNA-directed DNA polymerases from RLV and AMV, with emphasis on the biochemical properties important for an effective polymerase assay. The results showed that these 2 enzymes are markedly different on the basis of several of the properties compared here.

MATERIALS AND METHODS

Materials
RLV from infected mouse plasma was obtained as a 10-fold concentrate from the National Cancer Institute (Lot RPV-HL-68-4, Hazleton Laboratory). In some experiments RLV prepared in our laboratory from the plasma of infected BALB/c mice was used. Plasma containing approximately 10¹² AMV particles/ml was kindly provided by Dr. Anne Deeney, Oregon State University. Tritiated dNTP's and polyribonucleotides were obtained from Schwarz/Mann, Orangeburg, N.Y. Specific activities (Ci/mmole and mCi/mmole polynucleotide phosphorus) were: dTTP, 17.4; dATP, 18.5; dCTP, 27.4; dGTP, 4.0; (rA)ₙ, 51.0 (M.W. ~ 2.4 X 10⁵); (rU)ₙ, 7.0 (M.W. > 100,000); and (rC)ₙ, 35.0 (M.W. > 100,000). Unlabeled dNTP's were obtained from P-L Biochemicals, Milwaukee, Wis. Nonionic detergent NP-40 was obtained from the Shell Chemical Co., New York, N.Y. Calf thymus DNA, DNase I, and pancreatic RNase were from Worthington Biochemical Corp., Freehold, N.J. Nonlabeled synthetic polynucleotides of sizes ranging from 4 to 100 were obtained from Miles Laboratories, Kankakee, Ill. Oligodeoxyribonucleotides were products of Collaborative Research, Wal- tham, Mass. Hydroxylapatite (Hyapatite C) was purchased from Clarkson Chemical Co., Williamsport, Pa., and Whatman phosphocellulose, P-1, was from H. Reeve Angel & Co., Inc., Clifton, N.J.

Methods
Virus Preparation. RLV obtained from the National Cancer Institute was pelleted by centrifugation at 164,000 X g for 30

1 Research jointly sponsored by the Cancer Virus Program of the National Cancer Institute and the United States Atomic Energy Commission under contract with Union Carbide Corporation.

2 The abbreviations used are: RLV, Rauscher murine leukemia virus; AMV, avian myeloblastosis virus; dNTP, deoxynucleotide triphosphate; NP-40, Nonidet P-40.
min and suspended in 50 mM NaCl and 15% glycerol to a protein concentration of 1.5 mg/ml. The viruses were isolated from plasma essentially according to the method of Duesberg and Robinson (7). They were sedimented through a 25% sucrose layer onto a 60% sucrose cushion, containing 50 mM Tris-Cl (pH 7.5), 100 mM NaCl, and 1 mM EDTA, at 25,000 rpm for 90 min in a SW27 Spinco rotor. The banded virus was then sedimented to equilibrium in a 25 to 60% sucrose gradient in the same buffer. The band that formed at a density of about 1.16 g/ml was pooled, pelleted, and suspended as described above. The final protein concentrations in 2 different AMV preparations were 3.4 and 5.2 mg/ml, and in another RLV preparation was 5 mg/ml, as determined by the method of Lowry et al. (17). Fifty-μl aliquots of the virus preparations were stored in liquid nitrogen until used.

**Determination of Polymerase Activity.** A typical reaction mixture contained 30 mM Tris-Cl (pH 8.0 at 22°):50 to 100 mM KC1:bovine serum albumin, 0.17 mg/ml:2 mM dithiothreitol:appropriate dNTPs:3H, 17.5 μCi/ml. The labeled dNTP's were diluted by the addition of the same unlabeled dNTP at 0.1 mM. When required, the other unlabeled dNTP's were present at 0.2 mM. Contents and concentrations of template primers and divalent cations, as well as temperatures, for the incubations are indicated in the figure legends. Tris buffers shift considerably in pH at different temperatures, therefore, in the study of pH optima, pH measurements were performed with 30 mM Tris-Cl buffer at the indicated temperature of the reaction. Usually, 5 to 10 μl of enzyme fraction were assayed in a total volume of 25 to 100 μl. Twenty-μl aliquots were taken at various time intervals of incubation, and the incorporated radioactive activity was measured by the paper disc method of Bollum (3) with slight modifications. The modifications involved washing the discs 4 times in an ice-cold acid solution (5% glacial acetic acid:0.7% HCl:1% sodium pyrophosphate), twice in ethanol:ether (1:1), 4 times in an ice-cold acid solution (5% glacial acetic acid:0.7% HCl:1% sodium pyrophosphate), twice in ethanol:ether (1:1), and finally once in ether. The discs were dried under a heat lamp and placed in vials with 5 ml of 0.4% 2,5-bis[2-(5-fluoro-2-butanoyl)]thiophene in toluene. The incorporation of 1 pmole of dNMP registered 45 cpm under our conditions of scintillation counting.

**Template Primer Preparations.** Calf thymus DNA was activated with DNase I by the method of Aposhian and Kornberg (1) prior to use. (rA)n-(rU)n, with a molar A:U ratio of 0.77 as obtained commercially, was adjusted to an A:U ratio of 2.47 with (rA)n. The mixture, at a total polyribonucleotide concentration of 0.4 mg/ml in 10 mM NaCl, was heated to 45° for 10 min and then slowly cooled and stored frozen at -20° until used. Preliminary experiments indicated that the same result was obtained whether the polyribonucleotide:oligodeoxyribonucleotide complexes used were formed by such preannealing or by simply mixing them together in the reaction mixture.

**Enzyme Purification.** Viral lysates were made by diluting the virus preparation with an equal volume of 50 mM Tris-Cl (pH 8.0) containing 100 mM NaCl. This was then diluted 2-fold by the addition of 40 mM MgCl2 (or 4 mM MnCl2):20 mM dithiothreitol:0.8% NP-40. This mixture, which resulted in a visible clearing of the virus suspension, was incubated in an ice bath for 15 min. The sample was then adjusted with 2.5 M KC1 to contain 500 mM KC1 and then frozen and thawed 3 times in liquid nitrogen to ensure complete disruption. (Lysates prepared in this manner can be stored for 6 to 8 weeks in liquid nitrogen without substantial loss in activity.) If the lysate was to be directly subjected to phosphocellulose chromatography, it was appropriately diluted to contain 50 mM KC1.

Initial purification was by glycerol gradient centrifugation on 10 to 30% glycerol (4 ml) containing 50 mM Tris-Cl (pH 8.0):5 mM MgCl2:5 mM dithiothreitol:0.1% NP-40:500 mM KC1. The gradients were centrifuged at 55,000 rpm at 4° for 12 hr in a SW56 titanium Spinco rotor. Fractions were collected from the bottom of the tube and assayed for polymerase activity. Both the RLV and AMV polymerases sedimented at positions consistent with their reported molecular weights (15, 22). Characteristically, the RLV enzyme sedimented as a sharp band whereas the AMV enzyme, at equivalent activity, sedimented as a broader band. Thus, the AMV lysate may contain a polymerase:nucleic acid complex, similar to that demonstrated in the Rous sarcoma virus preparations (6, 8).

Further purification was attained by applying the active fractions from glycerol gradient centrifugation, adjusted to contain 0.1 M KC1, to a hydroxylapatite column that had been equilibrated with 10 mM potassium phosphate buffer (pH 7.2) containing 5 mM MgCl2:5 mM dithiothreitol:0.2% NP-40:0.1 M KC1:15% glycerol. The enzyme was eluted with a linear gradient from 10 to 150 mM phosphate (pH 7.2). Either of the 2 viral polymerases eluted from hydroxylapatite as a single peak.

The pooled enzyme fractions from hydroxylapatite were diluted 4 times with a medium [50 mM Tris-Cl (pH 8.0):3 mM MgCl2 or 0.4 mM MgCl2:5 mM dithiothreitol:0.2% NP-40:15% glycerol] containing 50 mM KC1 and applied to a phosphocellulose column preequilibrated with the same dilution medium. The enzyme was eluted with a linear KC1 gradient (50 to 500 mM) in the same medium. The RLV enzyme eluted as a single peak whereas AMV was resolved into 2 peaks, as shown in "Results." The 2nd AMV enzyme peak was that used in most of these studies. Active fractions from the phosphocellulose columns were pooled, adjusted to contain 0.5 M KC1, and used immediately for the experiments.

Based on enzyme activity with activated calf thymus DNA and protein concentration determined by the method of Bramhall et al. (4), purification of the polymerase preparations was usually in the range of 50 to 100 times for AMV and 100 to 150 times for RLV (Table 1). Recovery of the polymerase activity usually ranged from 200 to 400% (AMV) and 100 to 200% (RLV) of that of the virus lysate, presumably due to removal of inhibitors (14).

**RNase Determination.** The purified polymerase preparation (final concentration of 1.0 μg/ml) or the crude virus lysate (10.0 μg/ml) was incubated at 37° up to 12 hr in a solution containing labeled polyribonucleotide, 2.5 μg/ml:50 mM Tris-Cl (pH 8.0):2.0 mM MnCl2:5 mM dithiothreitol:50 mM KC1. RNase H activity was determined using a similar reaction mixture with 5.0 μg/ml (rA3H)-(dT)n (1:1) (19). The extent of hydrolysis was measured by the paper disc method (3).
RESULTS

Hydroxylapatite and Phosphocellulose Chromatography. RLV polymerase readily adsorbs to hydroxylapatite in a solution of 0.5 M KCl and 10 mM potassium phosphate buffer at pH 7.2 and, with a linear gradient of phosphate solution, elutes at about 25 mM phosphate concentration (31). In contrast, the AMV polymerase does not bind to hydroxylapatite in 10 mM phosphate unless the KCl concentration of the medium is lowered to 0.25 M, and then it elutes as a single peak at about 40 mM phosphate concentration.

Phosphocellulose column chromatography of RLV polymerase showed a single peak (data not shown). In contrast, AMV polymerase gave 2 distinct peaks, 1 of which bound only slightly, if at all, to phosphocellulose in the initial 50 mM KCl buffer solution (Chart 1). This was observed with crude AMV lysates as well as with preparations from glycerol gradient centrifugation. This observation of 2 polymerase activity peaks from crude lysates, which gave a broad and more diffuse peak. These results, with the observation on polyribonucleotide inhibition experiments described later, would suggest that the 2 chromatographic peaks of AMV represent 2 forms of the same polymerase and that Peak 1 may be a complex of Peak 2 with RNA (6).

Both Peak 2 polymerase of AMV and the single polymerase peak of RLV were similarly eluted from phosphocellulose at 0.25 M KCl. However, the RLV enzyme appeared to be more stable to storage at 4°C and ~20°C, respectively, similar to the previous reports (15, 22).

Nuclease Activities. Table 1 shows the polymerase and nuclease activities of the 2 viral enzyme preparations. Low levels of RNase activity were detected in the crude virus lysate, particularly that of AMV. After purification, both AMV and RLV polymerase preparations showed no nuclease activity with radioactive polyribonucleotides as substrates, which gave no sign of decreased sedimentation rate by glycerol gradient centrifugation following 8 hr of incubation. Most of the RNase H activity present in the crude RLV lysate appeared to be removed after purification of this polymerase; in contrast, the RNase H activity remained associated with the polymerase activity after application of the purification procedures to AMV. The RNase H activities of the 2 viruses functioned optimally at 2 mM Mn²⁺ using (rA)n-(dT)n as substrate. Although there was evidence of inhibitors in our viral lysate preparations, polymerase inhibition of the potency described by other workers (14) was not observed in our study; such inhibitors were completely removed from our purified polymerase preparations.

Metal Requirements for Various Template Primers. Scohnick et al. (25) have shown that the preference of viral polymerase for Mn²⁺ or Mg²⁺ depends on the template primer that is being used. We have shown further (29) that certain combinations of Mn²⁺ and Mg²⁺ are better than either alone for certain

Table 1

Polymerase and nuclease activities of RLV and AMV enzyme preparations

<table>
<thead>
<tr>
<th>Activity</th>
<th>RLV enzyme</th>
<th>AMV enzyme</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Lysate</td>
<td>Purified</td>
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<tr>
<td>Polymerase with</td>
<td>Lysate</td>
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</tr>
<tr>
<td>Activated DNA</td>
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<td>59,260</td>
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<tr>
<td>Nuclease with</td>
<td>Lysate</td>
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</tr>
<tr>
<td>(rA)n-(dT)n</td>
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<td>0</td>
</tr>
<tr>
<td>(rC)n</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>(rU)n</td>
<td>5</td>
<td>0</td>
</tr>
</tbody>
</table>

a Prepared by the sequential steps of glycerol gradient centrifugation, hydroxylapatite chromatography, and phosphocellulose chromatography (Peak 2 of AMV).

b Expressed as pmoles dTMP incorporated per µg protein during 30-min incubation at 37°C (activated DNA), at 30°C (rA)n-(dT)n, (AMV), and at 20°C [(rA)n-(dT)n, RLV].

c Expressed as pmoles nucleotide monophosphate hydrolyzed per µg protein in a 2-hr incubation at 37°C.

Metal concentrations were: for DNA, 1.7 mM Mg²⁺; and for (rA)n-(rU)n, 0.5 mM Mn²⁺; 6 mM Mg²⁺. *, (rA)n-(rU)n; , activated DNA.
higher concentrations, is stimulated maximally at 1 to 2 mM. Mn$^{2+}$ alone at the optimal concentration (0.5 mM) is less than 20% as effective as Mg$^{2+}$ with either enzyme. Furthermore, the addition of Mn$^{2+}$ at optimal Mg$^{2+}$ concentration causes inhibition with both enzymes. The template primer d(A-T)$_n$ has metal requirements very similar to those shown for activated calf thymus DNA (Chart 2).

In contrast to the situation with DNA, Mn$^{2+}$ rather than Mg$^{2+}$ is the preferred cation when (rA)$_n$-(rU)$_n$ is the template primer for dTMP incorporation. RLV polymerase shows no activity when Mn$^{2+}$ is absent from the reaction. Requirement for Mn$^{2+}$ is less absolute by the AMV polymerase, which gives some activity with (rA)$_n$-(rU)$_n$ by using 6 to 10 mM Mg$^{2+}$ only. Although both polymerases show high activity with this RNA template primer in the presence of Mn$^{2+}$ alone, combined use of Mn$^{2+}$ and Mg$^{2+}$ may give higher enzyme activity than does the use of Mn$^{2+}$ only. The optimal divalent cation combinations are 0.5 to 1.0 mM Mn$^{2+}$ plus 1 to 2 mM Mg$^{2+}$ for RLV polymerase and 0.5 mM Mn$^{2+}$ plus 2 to 6 mM Mg$^{2+}$ for AMV polymerase. A significant difference is that, at the optimal Mn$^{2+}$ concentration, RLV polymerase-catalyzed dTMP incorporation with (rA)$_n$-(rU)$_n$ is markedly inhibited by Mg$^{2+}$ concentrations greater than 2 mM, whereas Mg$^{2+}$ begins to inhibit the AMV activity only at concentrations greater than 6 mM.

The metal requirements for the 2 enzymes using (rA)$_n$-(dT)$_6$ are shown in Chart 3. RLV polymerase has a metal optimum of 0.5 mM Mn$^{2+}$ plus 1 to 2 mM Mg$^{2+}$, and Mg$^{2+}$ at concentrations higher than 4 mM becomes inhibitory (Chart 3A). In contrast, AMV polymerase prefers 8 to 10 mM Mg$^{2+}$ alone over any Mn$^{2+}$:Mg$^{2+}$ combinations or Mn$^{2+}$ alone (Chart 3B).

Other template primers for which we have determined optimal metal conditions (Table 2) include the following. (a) dGMP incorporation with (rC)$_n$-(dG)$_6$: RLV, 8 mM Mg$^{2+}$; AMV, 12 mM Mg$^{2+}$; Mn$^{2+}$ substitutes but is less effective; there is no stimulation by combined Mg$^{2+}$ and Mn$^{2+}$. (b) dAMP incorporation with (rU)$_n$-(dA)$_6$: RLV, 1 to 2 mM Mn$^{2+}$; AMV, 2 mM Mn$^{2+}$; Mg$^{2+}$ is ineffective alone but does not seem to be inhibitory in combination with Mn$^{2+}$. (c) dCMP incorporation with (rI)$_n$-(dC)$_6$: RLV, 2 to 8 mM Mg$^{2+}$; AMV, 1 to 2 mM Mg$^{2+}$; Mn$^{2+}$ is effective alone and inhibitory in combination with Mg$^{2+}$. (d) dTMP incorporation with (rA)$_n$-(dT)$_6$: RLV and AMV, both 5 to 12 mM Mg$^{2+}$ concentration; the inclusion of Mn$^{2+}$ (0.5 to 1 mM) strongly inhibits the AMV activity but only slightly inhibits the RLV activity. Although the relative activities of the 2 enzymes are approximately equal when (rC)$_n$-(dG)$_6$ or (rU)$_n$-(dA)$_6$ are used as template primers, there is a striking difference in the ability of the 2 enzymes to utilize (rI)$_n$-(dC)$_6$. The (rI)$_n$-(dC)$_6$-dependent activity with RLV polymerase is barely detectable, whereas with AMV polymerase it is comparable to the activity seen with (rA)$_n$-(rU)$_n$ or activated calf thymus DNA. Nuclease activity against (rI)$_n$ was not specifically tested (Table 1). However, since AMV preparations generally had greater nuclease content than did RLV preparations, and since purified RLV preparations also fail to utilize (rI)$_n$-(dC)$_6$ efficiently, we consider it unlikely that this difference is due to nuclease.

We have used various metal conditions and temperatures in an effort to get (rA)$_n$-(rU)$_n$-dependent dAMP incorporation. We have observed none with either enzyme, even in the presence of (dA)$_6$. This observation probably relates to the greater stability of (rA)$_n$-(rU)$_n$ product (dA)$_n$.(rU)$_n$ (21).

Effect of Temperature. We reported previously (29) that the optimum temperature for the RLV polymerase reaction varies considerably, depending on the template primer used. The observed optimum temperature values appeared to correlate well with the thermal stability of the template primer. For example, (rI)$_n$-(rC)$_n$ had a higher temperature optimum (47°) than did (rA)$_n$-(rU)$_n$ (40°), and (rC)$_n$-(dG)$_6$ was more effective at a higher temperature (40°) than was (rA)$_n$-(dT)$_6$ (20°).

The results shown in Chart 4 indicate that not only the
Table 2

Comparison of divalent metal requirements and pH and reaction temperature optima for various template primers with the DNA polymerases from RLV and AMV

DNase-activated calf thymus DNA was used at a concentration of 100 µg/ml; (A-T)₆ was used at 10 µg/ml. The other RNA templates were used at 10 µg/ml. Oligomers at a concentration of 10 µM were used in all cases except for the optimum temperature determinations with (rA)₆-(rU)₆ and (rA)₆ as templates, where 1 µM oligomer was used.

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<th>Polymer</th>
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<th>dNTP-3H incorporated</th>
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<th>Optimum pH</th>
<th>Optimum temperature</th>
<th>[Mn²⁺] (mM)</th>
<th>[Mg²⁺] (mM)</th>
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² Data were obtained using purified polymerase preparations also.

nature of the template primer but also the source of the enzyme is undoubtedly very important in determining the optimum reaction temperature. The RLV enzyme shows optimal activity at 25° when (rA)₆-(dT)₆ is the template primer, whereas the AMV enzyme is maximally active at 35° with the same template primer (Chart 4). The optimum temperature for both enzymes increases slightly when the concentration of (dT)₆ is increased from 1 to 10 µM. The higher temperature optima of the reaction of the AMV enzyme with (rA)₆-(dT)₆ is not due to the higher levels of Mg²⁺ in the assays. With all of the template primers studied, higher optimum reaction temperatures have been observed for the AMV enzyme than for the RLV enzyme (Table 2). Since the same Tris-Cl buffer (pH 8.0 at 22°) was used throughout these experiments for optimum temperature determination, the question should be asked whether the observed differences in the optimum temperatures for the 2 polymerases reflect effects of pH shift caused by temperature change on the polymerase activity (see "Effect of pH"). A careful examination showed that the effect of temperature and the effect of pH on the polymerase activity are independent.

Effect of pH. Using the respective optimal metal conditions at various temperatures, we investigated the effects of pH on the activities of the 2 viral polymerases in 30 mM Tris-Cl buffers. These results can be summarized as follows: with (rA)₆-(dT)₆ at 25°, pH 7.8 to 8.0 for RLV and pH 7.7 to 7.9 for AMV; with (A-T)₆ at 40°, pH 7.2 for RLV and pH 8.0 to
8.4 for AMV; with \((rA)_n\cdot(rU)_n\) at 40°C, pH 7.9 for RLV and pH 7.5 to 7.8 for AMV; with activated calf thymus DNA at 40°C, pH 7.7 to 7.9 for RLV and pH 8.0 to 8.3 for AMV; and with \((rA)_n\cdot(dT)_n\) at 40°C, pH 7.8 to 8.0 for RLV and pH 8.1 to 8.3 for AMV. The pH measurement varies considerably with temperature for a single Tris-Cl buffer, so that a buffer measuring pH 8.0 at 22°C gives pH 7.6 at 40°C. A control experiment indicated that the pH optimum for the polymerase with a given template primer remains constant at different reaction temperatures, although it takes different Tris-Cl buffers to maintain this constant pH. When the 2 enzymes were assayed under identical instead of their respective optimal metal conditions, the same results were obtained, indicating that the pH optimum properties of the 2 enzymes are independent of the metal contents in the reaction.

**Kinetic Observations.** Characteristically, DNA synthesis, with activated calf thymus DNA as template primer, is linear with time for either enzyme (Chart 5), and the same is true when \((A-T)_n\) is the template primer. Linear kinetics with the RLV enzyme is most obvious at enzyme concentrations lower than that shown in this chart. However, the kinetics of the 2 enzymes with \((rA)_n\cdot(rU)_n\) as template primer are quite different. The lag observed with the RLV polymerase is not seen with the AMV enzyme (Chart 5). The lag seen with the RLV enzyme appears to reflect a difficulty in initial dTMP incorporation, presumably onto the \((rU)_n\) primer, and it is eliminated when oligomers of deoxythymidine are added. We must emphasize that the \((rA)_n\cdot(rU)_n\) used in all these studies was adjusted to an A:U ratio of 2.47, the ratio empirically determined to give maximal template primer activity. A linear rate of synthesis is observed with the AMV enzyme. It would appear that initiation is rate limiting to a much greater degree with the RLV polymerase than with the AMV enzyme. In this regard, when tested under optimal conditions for \((rA)_n\cdot(rU)_n\)-dependent DNA synthesis, 10 \(\mu M\) \((dT)_n\) stimulates the rate by approximately 20-fold with the RLV enzyme. However, under conditions optimized for \((rA)_n\cdot(rU)_n\)-dependent synthesis, the AMV enzyme is stimulated only about 2-fold by the addition of 10 \(\mu M\) \((dT)_n\).

A linear relationship is observed with increasing AMV polymerase concentration when DNA is the template primer (Chart 6). A slightly sigmoidal curve is seen when \((rA)_n\cdot(rU)_n\) is the template primer. DNA-dependent synthesis with the RLV polymerase is linear with increasing protein concentration. In contrast, the increase in the rate of \((rA)_n\cdot(rU)_n\)-dependent DNA synthesis is enhanced with increasing protein concentration. A highly purified preparation of RNA-directed DNA polymerase from RLV-infected mouse spleens (31) exhibits the same properties with DNA or \((rA)_n\cdot(rU)_n\) as template primers, as is shown in Chart 6 for the RLV purified polymerase.

**Polyribonucleotide Inhibition.** DNA-directed polymerase activity of RLV lysates was shown to be extremely sensitive to inhibition by single-stranded polyribonucleotides (28). Chart 7 illustrates this observation with \((rG)_n\). It also shows that the AMV polymerase, prepared by glycerol gradient centrifugation, appears less sensitive to this type of inhibition. This is particularly true of AMV lysates, which sometimes showed almost complete resistance to the inhibition as reported by Gallo (10). When the AMV polymerase was isolated in a highly purified state, such as Peak 2, from phosphocellulose chromatography, it became susceptible to \((rG)_n\) or \((rU)_n\)
inhibition to the same extent as did the RLV polymerase (Chart 7). Although pretreatment of an AMV lysate significantly increased the proportion of the polymerase that eluted as Peak 2, such pretreatment did not increase the sensitivity of an AMV lysate to (rG)ₙ inhibition. Thus, this marked difference of the 2 viruses could have been due to factors other than the polymerase molecules per se in the virus lysate. Therefore, we performed subsequent comparative studies with polymerase preparations of highest purity in our stock. The data of inhibition experiments were analyzed by Cleland’s kinetic method (5). With both RLV and AMV polymerases, plots of 1/v versus 1/A (reciprocal plots) indicate competitive inhibition of the d(A-T)ₙ-directed activity, namely that polyribonucleotides alter the slope but not the intercept of the reciprocal plots. Replots of the slope versus the inhibitor (rU)ₙ concentration, however, indicate that the 2 enzymes are inhibited in a different manner, i.e., a linear curve with Kᵢ of 3 to 4 μg/ml for the RLV polymerase and a hyperbolic curve for the AMV polymerase (Chart 8).

**DISCUSSION**

Reported molecular weights of the 2 enzymes (14, 15, 22) indicate that proteins with the same function but from different oncogenic RNA viruses can be markedly different in molecular weight. Similar findings with other structural proteins components of the oncogenic RNA viruses have been observed. Completely different protein patterns have been demonstrated for AMV and RLV lysates by sodium dodecyl sulfate polyacrylamide gel electrophoresis (23). Even with phylogenetically more closely related viruses, such as feline leukemia virus and RLV, a careful comparison by the same electrophoretic technique showed that some protein components migrate differently, possibly suggesting different molecular weights (24).

Two facts are obvious from our data on the divalent metal requirements for the DNA polymerase assays: (a) for a single DNA polymerase from an oncogenic RNA virus the metal requirements for optimal activity differ greatly, depending on the template primers used; (b) for 2 different polymerases the optimum metal requirements for the same template primer may also differ greatly. Similar conclusions can be drawn from studies on pH effects, namely, that the same viral polymerase may require different pH conditions with different template primers and that different viral polymerases may require different pH conditions with the same template primer. The effect of divalent cations and the effect of pH seem to be independent events, since no correlation can be established. However, both metal and pH conditions exert profound influences on the outcome of polymerase assays. It is not uncommon to obtain less than 50% of the maximal polymerase activity as a result of a minor change of these conditions, such as assaying (rA)ₙ-(rU)ₙ-dependent activity of the RLV polymerase at pH 7.6 (optimal for AMV) instead of pH 7.9 (optimal for RLV), or using a combination of 0.5 mM Mn²⁺ and 6.0 mM Mg²⁺ instead of 0.5 mM Mn²⁺ and 2.0 mM Mg²⁺.

In view of these results for metal and pH requirements, the experimental data commonly found in the current literature concerning oncogenic RNA virus polymerase, in which the relative efficiencies of various template primers are compared...
under a single set of assay conditions, probably do not give a true indication of the transcribing potential of the polymerase tested. Similarly, the responses of different oncogenic RNA virus polymerases to a given template primer under a single set of uniform reaction conditions should not be taken as indicative of the maximal potential response.

In a study of RNA synthesis by Escherichia coli RNA polymerase, using polyribonucleotides as templates and complementary oligoribonucleotides as primers, Niyogi and Wilton (20) have demonstrated a definite correlation between the chain length of the oligomer and the optimum temperature of the reaction and have shown that this is a reflection of the melting temperature of the oligomer:polymer complex. Studying the ability of RLV polymerase to utilize various poly(rA)-oligo(dT) complexes for dTMP incorporation, we have observed the same phenomenon, namely, the optimum temperature for the reaction increases with increasing chain length of the oligo(dT) used (W.-K. Yang and L. C. Waters, unpublished results). If these results suggest a particular preferred degree of oligomer:polymer association for optimal activity of the polymerase, then the present results indicate that the nature of the polymerase is also very important in determining the utilization of the complex. This is illustrated by the marked differences in temperature optima for the 2 polymerases with regard to the priming activity of (dT)n (Chart 4). The AMV enzyme apparently uses (rA)n-(dT)n most effectively near its melting temperature, whereas the RLV polymerase may require a more strongly associated form of this complex at a lower temperature. For all the template primers tested, the AMV polymerase has higher temperature optima than the RLV polymerase (Table 2). These results emphasize that it is important to consider the reaction temperature when potential template primers are being selected for the polymerase of a particular oncogenic RNA virus.

Several observations with the RLV polymerase suggest that initiation of synthesis is the rate-limiting step in RNA-primed DNA synthesis but not in DNA-primed DNA synthesis (L. C. Waters and W.-K. Yang, unpublished results). However, there is little, if any, lag in the rate of DNA synthesis with the AMV polymerase using either DNA or (rA)n-(rU)n as template primers (Chart 5). Related to this is the comparison of the 2 polymerases with regard to the kinetic curve correlating the rate of DNA synthesis and the enzyme concentration in the reaction mixture. For RLV polymerase, a sigmoidal curve is obtained with (rA)n-(rU)n as template primer but not with DNA template primers. Similar curves are found for AMV polymerase, although the sigmoidal relation with (rA)n-(rU)n is less pronounced (Chart 6). In this regard, Leis and Hurwitz (16) have also reported a sigmoidal relationship between the rate of DNA synthesis and AMV enzyme concentration, using RNA isolated from AMV or calf thymus DNA as template primer. All these kinetic observations present interesting problems regarding a possible difference in subunit structure for the RLV and AMV polymerase and the relationship of enzyme structure to template primer preference.

Tuominen and Kenney (28) showed that the DNA-directed polymerase activity of RLV lysates was very sensitive to inhibition by single-stranded polyribonucleotides. Gallo (10) maintained that some RNA tumor virus preparations could be relatively insensitive to such inhibition. Our results with the lysate polymerase preparations of RLV and AMV confirm both reports. However, the fact that an extensive purification of the AMV polymerase rendered it highly sensitive to the inhibition indicates that the observed difference may be due to factors other than polymerase per se in the virion. For this reason, we have used highly purified preparations and demonstrated a hyperbolic competitive inhibition with AMV polymerase and a linear competitive inhibition with RLV polymerase. It remains, however, to be determined whether such differences in enzyme kinetic behavior represent different complex structures of the 2 virus polymerases, which are obvious from analyzing molecular weight and the associated RNase H activity, or still insufficient purity of the AMV polymerase preparation.

The experimental results presented above demonstrated that the RNA-directed DNA polymerases from RLV and AMV are definitely very different by biochemical parameters. Many essentially identical results, like those described, were obtained using crude viral lysates. We should emphasize, however, that biologically highly active plasma viruses were used in our study. Perhaps studies like those reported will be useful in the definitive diagnosis of a presumptive human tumor virus.

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