Characterization of the Deoxyribonucleic Acid Polymerase Associated with Kilham Rat Virus

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Purified preparations of the parvovirus, Kilham rat virus, have associated with them a protein with DNA polymerase activity. The enzyme has been separated from the other two or three viral proteins and purified 63-fold. The viral associated enzyme was found in a single peak of DNA polymerase activity after chromatography on DEAE-cellulose, DNA-cellulose, and phosphocellulose columns. It shares some properties in common with the host cellular DNA polymerases, described in the preceding paper (Salzman, L. A., and McKerlie, L. (1975) J. Biol. Chem. 250, 5589-5595), but also has some important distinguishing characteristics. The Kilham rat virus-associated DNA polymerase has increased enzyme activity in the presence of 0.02 M KCl and has a strong preference for a synthetic DNA polymer containing deoxyadenylate and deoxythymidylylate. The enzyme has a molecular weight of approximately 75,000 ± 3,000 and appears to contain endonuclease activity.

Kilham rat virus (KRV) is one of a ubiquitous group of small (15 to 30 nm), DNA-containing viruses called the parvoviruses (1, 2). KRV was first isolated from a rat sarcoma and can cause a variety of diseases and malformations in neonatal animals (3, 4). All members of the parvovirus group that have been studied contain one molecule of single-stranded, linear DNA with a molecular weight of 1.5 to 2.2 x 10^6 and three viral proteins (5). They are assembled in the nucleus of an infected cell and causes disintegration of the nucleolus and cytoplasmic structures before cell lysis (6, 7).

Very little information is available about the replication of a linear single-stranded viral DNA molecule in a cell. There are some reports that suggest the possibility of a double-stranded linear DNA intermediate in parvovirus replication (8-10). How this double-stranded DNA intermediate may be synthesized and how the progeny single-stranded viral DNA molecules are made is unknown. Purified KRV has been shown to have associated with it an enzyme with DNA polymerase activity (11). We decided to purify this enzyme in the hope that knowledge about the viral-associated DNA polymerase might lead to a better understanding of the process of DNA replication or its regulation in the infected cells. In the preceding report we have described the DNA polymerases from the rat nephroma cell line in which the virus is grown. Comparison of the viral-associated DNA polymerase with the cellular polymerases has shown that the viral enzyme resembles most a cytoplasmic cellular enzyme C11 but differs from this enzyme in several characteristics.

EXPERIMENTAL PROCEDURE

Materials

- H-Labeled deoxynucleotides and ribonucleotides were obtained from New England Nuclear Corp. Unlabeled nucleotides and salmon sperm DNA were obtained from Calbiochem. d(T), d(A), and d(G), d(C), were obtained from A. Weissbach, Roche Institute of Molecular Biology. Adenovirus DNA was a gift from J. Rose, National Institutes of Health. It was prepared by phenol extraction of purified virus (12). DEAE-cellulose, DNA-cellulose, and phosphocellulose were prepared as described in the preceding report (13). Pancreatic DNase I and micrococcal nuclease were purchased from Worthington. Spectrafluor was purchased from Amersham/Searle, Arlington Heights, Ill. Munktell 410 cellulose was purchased from Bio-Rad, Richmond, Calif.

Methods

Virus Purification and Cell Line—KRV strain 308 was originally obtained from L. Kilham. The virus was grown in a rat nephroma cell line as previously described (14). The infected cells were harvested 7 days after infection, frozen and thawed, and treated with receptor destroying enzyme. The virus-containing supernatant was then centrifuged in a CsCl gradient (average density 1.41 g/ml) in a Spinco model L centrifuge at 35,000 rpm in a type 40 rotor at 5°C for 24 hours. The virus was then dialyzed against 300 volumes of 0.02 M Tris, pH 8.2, and 10^-4 M EDTA. The purified virus preparation was dialyzed against 4 liters of 0.02 M Tris, pH 8.2, and 10^-4 M EDTA and was called purified virus. The purified virus preparation was found to sediment as a single peak containing both hemagglutination and DNA polymerase activity in a sucrose gradient, as previously described (11).

Enzyme Preparation—To the purified virus preparation (16 ml) was added diethiothreitol to make the solution 5 mm, and Nonidet P-40 to a final concentration of 4%. The solution was then sonicated for 15 s at 4°C with the microtip of a Sonifier cell disruptor model WL40D at a setting of 7. The solution was then incubated at 37°C for 20 min. Deoxycholate was added to a final concentration of 0.5% and the solution kept in ice for an additional 20 min. The treated disrupted virus (19 ml) was then dialyzed against 300 volumes of 0.02 M potassium phosphate, pH 7.4 and 0.001 M EDTA for 16 hours with one change of dialysis buffer.

DEAE-cellulose Chromatography—The dialyzed disrupted virus preparation (16 ml) was added to a DEAE-column (18 x 2 cm) equilibrated with 0.02 M potassium phosphate, pH 7.4, 0.001 M EDTA, and 5 x 10^-5 M diethiothreitol, and washed with the same buffer until
all unadsorbed protein was removed. The enzyme was eluted with a 300-ml linear gradient of 0.02 to 0.5 M potassium phosphate, 5 \times 10^{-4} M dithiothreitol, and 1 \times 10^{-4} M EDTA. The active fractions were pooled and concentrated in dialysis tubing with aquacide to about one-half of their volume.

**DNA-cellulose Chromatography**—DNA-cellulose was prepared using a modification of the procedure of Alberts and Herrick (15). The concentrated enzyme fractions were dialyzed against 200 volumes of 0.1% Triton X-100, 0.02 M Tris, pH 7.4, 5 \times 10^{-4} M EDTA, and 10% glycerol. The enzyme (6 ml) was added to a DNA-cellulose column (9 \times 1 cm) equilibrated with the above buffer until all of the unadsorbed protein was removed. The enzyme was then eluted with 100 ml of a linear gradient of 0 to 1 M NaCl in the above buffer. The active fractions were combined (6 ml) and dialyzed against 500 volumes of the 0.1% Triton X-100-10% glycerol buffer above.

**Phosphocellulose Column Chromatography**—An aliquot (3.5 ml) of dialyzed enzyme fractions eluted from DNA-cellulose was added to a phosphocellulose column (7 \times 1 cm). The column was washed with 0.02 M potassium phosphate, pH 7.4, 5 \times 10^{-4} M dithiothreitol, 0.1% Triton X-100, 1 \times 10^{-4} M EDTA, and 10% glycerol until unadsorbed protein was removed. The enzyme was then eluted with a 60-ml linear gradient of 0.02 to 0.9 M potassium phosphate, pH 7.4, in the above buffer. The fractions were assayed and the peak of activity was pooled. The enzyme could be stored at -17°C for several months without appreciable loss of activity.

**Assay of KRV-associated DNA Polymerase**—Incubation mixtures contained in 200 ml: 20 mM Tris-HCl, pH 8.9; 2 mM \( \beta \)-mercaptoethanol, 5 \( \mu \)g of bovine serum albumin; 10 mM MgCl\(_2\); 0.02 mM KCl; 60 \( \mu \)g of activated salmon sperm DNA; 10 mM each of dCTP, dGTP, TTP, and [\( ^3H \)]dATP, 0.5 mCi, with a specific activity 0.5 mCi/\( \mu \)mol. The DNA was precipitated in 5% trichloroacetic acid at 4°C, collected on a Whatman GF/C filter, washed with 5 \( \alpha \)-aliquots of 5 ml of 5% trichloroacetic acid, dried, and counted in 10 ml of Spectrafluor in a Beckman scintillation counter.

**Determination of Nuclease Activity in KRV-associated DNA Polymerase**—DNA exonuclease activity was measured by the release of acid-soluble radioactivity from radioactive double-stranded and single-stranded adenovirus DNA within 1 hour at 37°C. Single-stranded adenovirus DNA was prepared by heating the native DNA molecule at 100°C for 10 min and quick cooling in ice. Standard polymerizing conditions with 1.2 \( \mu \)g of enzyme were used but in the absence of deoxynucleoside triphosphates.

DNA endonuclease activity was determined by incubation of the enzyme with the closed circular form (form I) of SV40 DNA labeled with [\( ^3H \)]thymidine. Incubation was at 37°C for 1 hour in the absence of deoxynucleoside triphosphates. The incubation mixture was then sedimented in a neutral sucrose gradient under conditions in which the conversion of form I (21 S) to form II (16 S) can be followed (16).

The conversion is effected by a single endonucleolytic scission of one DNA strand (17). The form I II was isolated after gradient centrifugation and dialyzed against 0.02 M Tris, pH 7.4, 0.01 M EDTA. An aliquot was then adjusted to a pH of 11.2 and sedimented in an alkaline sucrose gradient under conditions that separate linear (16 S) from circular (18 S) single-stranded SV40 DNA molecules. Fractions were collected from the gradient and the radioactivity in each fraction determined.

**Determination of Sedimentation Coefficient and Molecular Weight**—The method of Martin and Ames (18) was used to determine the sedimentation behavior of the KRV-associated DNA polymerase in relation to proteins of known sedimentation coefficient and molecular weight.

**RESULTS**

Separation of the KRV-associated DNA polymerase from the virion proved to be a difficult task. Treatment with various detergents and several concentrations of ammonium sulfate, and sedimentation through sucrose gradients containing detergent and salt either resulted in a failure to separate the enzyme from the rest of the virus particle or in a loss of the enzyme activity. We have found that brief sonication of the purified virions in the presence of 5 mM dithiothreitol and 4% NP-40 followed by incubation with 0.5% deoxycholate at 4°C resulted routinely in a good recovery of virus polymerase activity. After dialysis the preparation was placed on a DEAE-cellulose column and eluted with a linear gradient of 0.02 to 0.5 M potassium phosphate. As seen in Fig. 1A, the DNA polymerase was eluted from DEAE-cellulose in a large peak of activity at 0.04 M potassium phosphate. The active fractions (Fractions 28 to 45) were combined and accounted for 55 to 97% of the recovered enzymatic activity. The small peak at Fraction 55 was not routinely reproducible and was not examined further. After dialysis the combined fractions were added to a DNA-cellulose column, and the enzyme activity was eluted with a linear gradient of 0 to 1.0 M NaCl. A single very sharp peak of enzyme activity was eluted with 0.095 M NaCl (Fig. 1B). An aliquot of the enzymatically active fractions (Fractions 15 to 19) were combined, dialyzed, and added to a phosphocellulose column. The enzyme was eluted from the phosphocellulose with a linear gradient of 0.02 to 0.9 M potassium phosphate, pH 7.4, and dialyzed. The DEAE-cellulose-purified enzyme fraction (6 ml) was added to a DNA-cellulose column (9 \times 1 cm) (B). The column was washed with 0.02 M Tris buffer, pH 7.4, and the enzyme activity eluted with a gradient of 0 to 1.0 M NaCl. The gradient was started at Fraction 10. Fractions (2.0 ml) were collected and a 0.05-ml aliquot from each fraction assayed for enzyme activity. Fractions (15 to 19) eluted with 0.095 M NaCl were combined and dialyzed for further purification.
The DNA polymerase activity eluted in a single sharp peak with 0.02 M potassium phosphate. Table I summarizes the purification of the KRV-associated DNA polymerase. The enzyme was purified to a specific activity of 1125, a 63-fold purification from the disrupted virus particles which originally contained only three major proteins detectable by polyacrylamide gel electrophoresis ([H]dATP was used as a labeled substrate except when dATP was absent from the mixture. In these cases [H]TTP of approximately the same specific activity was used as the radioisotope. The activity is presented as a percentage of that obtained in the complete system in which 2.7 nmol of deoxynucleotide were incorporated by 1.2 μg of enzyme protein per hour.

**Table II**

| Requirement of KRV-associated DNA polymerase | % Activity |
|---------------------------------------------|------------|
| Complete                                    | 100        |
| MgCl2                                       | 14         |
| Bovine serum albumin                       | 90         |
| β-Mercaptoethanol                           | 100        |
| DNA (activated)                             | 0          |
| Enzyme                                      | 0          |
| KCl                                         | 47         |
| dGTP                                        | 49         |
| dCTP                                        | 40         |
| TTP                                         | 27         |
| dATP                                        | 14         |
| dCTP, dGTP, TTP                             | 20         |
| dATP + dADP                                 | 71         |

**Table I**

| Purification of KRV-associated DNA polymerase |
|---------------------------------------------|
| Specific activity is units per mg of protein. A unit is defined as 1 nmol of [H]dNMP incorporated into an acid-insoluble form in 60 min at 37°. |

| Fraction          | Volume (ml) | Total protein (mg) | Activity recovered from disrupted virus (units/mg protein) |
|-------------------|-------------|--------------------|----------------------------------------------------------|
| Disrupted virus   | 16          | 78.2               | 1400                                                     |
| DEAE-cellulose peak | 17         | 24.0               | 1372                                                     |
| DNA cellulose peak | 6          | 0.672              | 524                                                      |
| Phosphocellulose peak | 2        | 0.120              | 135                                                      |

*Only one-half of the activity recovered from the DNA-cellulose column was further purified on the phosphocellulose column.*

**Fig. 2.** Kinetics of incorporation of four dNTPs by KRV-associated DNA polymerase. The reaction mixtures contained in a total volume of 0.2 ml, 20 mM Tris-Cl, pH 8.9; 5 μg of bovine serum albumin; 2 mM β-mercaptoethanol, 60 μg of activated salmon sperm DNA; 10 mM MgCl2, 0.02 M KCl, and 0.6 μg of KRV-associated DNA polymerase. One mixture (●●●●) contained 10 μM each of dGTP, dCTP, TTP, and dATP and [H]dATP (0.5 mCi/μmol). The second mixture (○○○○) contained 40 μM dATP, only, plus [H]dATP (0.5 mCi/μmol). Incubation was carried out at 37° for the indicated times.
Activated DNA that is heat-denatured serves as a template for the enzyme with about 60% of the activity of activated DNA. The enzyme also requires 3'OH termini for activity, and when template. Single-stranded DNA does not serve as a template. Only 30% as active in incorporating deoxynucleotides into DNA when native double stranded salmon sperm DNA is used as a template. Single-stranded DNA does not serve as a template. Activated DNA that is heat-denatured serves as a template for the enzyme with about 60% of the activity of activated DNA. The enzyme also requires 3'-OH termini for activity, and when

**Prime-template Studies**—The activity of the KRV-associated DNA polymerase is dependent on added primer-template (Table III). Optimal enzyme activity is achieved with 60 μg of activated salmon sperm DNA in a incubation mixture containing 1.2 μg of enzyme protein. If only 12 μg of activated DNA were used in an identical incubation mixture the enzyme activity was reduced by about 50%.

The ability of the KRV-associated DNA polymerase to use various primer-templates is shown in Table III. The enzyme is only 30% as active in incorporating deoxynucleotides into DNA when native double stranded salmon sperm DNA is used as a template. Single-stranded DNA does not serve as a template. Activated DNA that is heat-denatured serves as a template for the enzyme with about 60% of the activity of activated DNA. The enzyme also requires 3'-OH termini for activity, and when

**Table III**

| Template                    | Amount          | Substrate                  | % Activity |
|-----------------------------|-----------------|----------------------------|------------|
| DNase I-treated double-stranded DNA (activated DNA) | 60 | [3H]dNTP<sup>a</sup> | 100        |
| Double-stranded DNA         | 60 | [3H]dNTP<sup>a</sup> | 30         |
| Single-stranded DNA         | 60 | [3H]dNTP<sup>a</sup> | 5          |
| Denatured DNase I-treated double-stranded DNA | 60 | [3H]dNTP<sup>a</sup> | 60         |
| Micrococcal nuclease-treated double-stranded DNA | 60 | [3H]dNTP<sup>a</sup> | 16         |
| poly(dG-dC)                 | 60 | [3H]dGTP<sup>a</sup>, dCTP<sup>a</sup> | 90         |
| d(G)<sub>12</sub>-d(C)<sub>12</sub> | 60 | [3H]dATP<sup>a</sup>, dTTP<sup>a</sup> | 100        |
| poly[d(A-T)]                | 60 | [3H]dATP<sup>a</sup>, dTTP<sup>a</sup> | 973        |
| d(T)<sub>12</sub>-r(A)<sub>12</sub> | 60 | [3H]dATP<sup>a</sup>, dTTP<sup>a</sup> | 1800       |
| Yeast RNA                   | 60 | [3H]NTP<sup>a</sup> | 0          |
| DNase I-treated double-stranded DNA | 60 | [3H]NTP<sup>a</sup> | 0          |

<sup>a</sup> [3H]dNTP refers to a mixture of [3H]dATP and unlabeled dGTP, dCTP, and dTTP (see "Methods").

<sup>b</sup> The radioactive and nonradioactive deoxynucleoside triphosphates were added to each incubation at a final total concentration of 40 nM per incubation.

<sup>c</sup> [3H]NTP refers to a mixture of [3H]ATP (0.5 mCi, specific activity 62 mCi/μmol) and unlabeled GTP, CTP, ATP, and UTP (50 nM each per incubation).

maximum incorporation of one deoxynucleotide is less than 30% of that found when 4 deoxynucleotides are present. Addition of fresh enzyme to the incubation mixture containing four dTNP's after a 180-min incubation reproduces the initial reaction kinetics without any lag period. The biphasic kinetics suggests that a rapid rate of initiation is followed by a slower rate of polymerization, the extent of which may be limited by some enzyme instability.

**Effect of Inhibitors on KRV-associated DNA Polymerase**—The effect of several chemical inhibitors on the activity of the KRV-associated DNA polymerase is presented in Table IV. As is generally true for DNA polymerases, pyrophosphate inhibited the enzyme. Sodium pyrophosphate at 5 mM inhibited enzyme activity by 59%; 10 mM concentration inhibited the enzyme activity by 82%. Ethidium bromide (90 μM) and p-chloromercuribenzoate (0.025 mM) also inhibited the DNA polymerase by 50 to 60%. The enzyme was strongly stimulated by the presence of the monovalent cation KC1. In the presence of the optimal concentration of KC1 (0.02 M), the enzyme stimulation was 2- to 3-fold over that observed in the absence of the KC1. High concentrations of salt have also been shown to be required for maximal activity of the Herpes simplex virus-induced DNA polymerase (26).

**Molecular Weight of Enzyme**—The molecular weight of the enzyme is unknown and is not clear. The oligomer homopolymer d(G)12,d(C)12, is as good a template for the enzyme as activated DNA and the enzyme activity is independent of which radioisotope [3H]dTTP or [3H]dCTP is used. Pol d(A-T) is an excellent template for the enzyme whether dATP or TTP is supplied as labeled substrate. The enzyme is 9.7 to 18 times as active with pol (d(A-T)) as with activated DNA.

Oligomer-homopolymers which have a polyribostrand (T)4-(A), in place of the polydeoxyribostrand do not serve as a template in the absence of yeast RNA or activated DNA as a template under the conditions described here or that described for RNA-dependent RNA polymerase (22). The inability of the enzyme to copy a polyribostrand differentiates the KRV-associated DNA polymerase from the R-DNA polymerase in eukaryotic cells (21, 23) and the RNA-dependent DNA polymerase found in RNA tumor viruses (24, 25).

**Effect of p-Chloromercuribenzoate, 0.025 mM**

**Effect of Sodium Pyrophosphate, 5 mM**

**Effect of Ethidium Bromide, 0.025 mM**

**Effect of KCl, 0.1 M**

**Table IV**

| Addition | % Activity |
|----------|------------|
| Standard assay | 100        |
| Sodium pyrophosphate, 5 mM | 41        |
| Ethidium bromide, 20 mM | 42        |
| p-Chloromercuribenzoate, 0.025 mM | 44        |
| NaCl, 0.125 M | 100        |
| KCl, 0.1 M | 155        |
phosphocellulose-purified enzyme was determined by sedimentation in a sucrose gradient with markers of known sedimentation coefficients ($s_{20,w}$) and molecular weight. When the KRV-associated DNA polymerase was sedimented in a 5 to 20% sucrose gradient (Fig. 3), the enzyme was found in a sharp peak of activity between Escherichia coli alkaline phosphatase (A) ($s_{20,w} = 6.3$, MW = 80,000) and bovine serum albumin (B) ($s_{20,w} = 4.4$, MW = 68,000). Using the bovine serum albumin as a marker, the KRV-associated DNA polymerase has an approximate calculated $s_{20,w} = 5$ and a MW = 72 to 78,000.

Other Enzyme Activities—We examined the phosphocellulose-purified KRV-associated DNA polymerase for endonuclease activity by determining its ability to “nick” the double stranded circular form of SV40 (Component I) and for exonuclease activity by the release of acid soluble radioactivity from radioactive native and denatured adenovirus DNA (see “Methods”). As seen in Fig. 4, after incubation with the enzyme (1.2 µg), 49% of SV40 Component I is converted to a form which migrates in the area of Component II (one linear strand, one circular strand in double-stranded DNA) and Component III (two linear DNA strands in double-stranded DNA). Further analysis of the enzyme-treated SV40 DNA in an alkaline sucrose gradient to denature the DNA and separate the strands into 18 S (single-stranded circular DNA) and 16 S (single stranded circular DNA) species showed that the KRV-associated DNA polymerase-treated DNA sedimented in fragments of 6 S and smaller. The DNA polymerase endonuclease activity appears to have cleaved both strands of SV40 Component I in several places.

We were unable to demonstrate an exonuclease activity against either native or denatured adenovirus DNA (<1% of polymerizing activity). An exonuclease activity preferring single-stranded DNA as substrate has been found with purified preparations of a vaccinia virus-directed DNA polymerase (27). A very low exonuclease activity was recently found with the Herpes simplex virus-induced DNA polymerase (26) that could not be detected with a natural DNA such as we used here, but was detected only by solubilization of poly[d(A·T)] or poly(dG·dC). We have not carried out this assay.

DISCUSSION

KRV was purified from possible contaminating cellular components by two or three successive isopycnic centrifugations in CsCl gradients. The purified virion sediments as a single peak during velocity centrifugation in sucrose. The peak has associated with it the virion hemagglutinin and an enzyme with DNA polymerase activity (11). We have separated the enzyme from disrupted virions and disassociated it from the other known virion proteins. From results obtained by sedimentation in sucrose gradients with known protein markers it appears to have a molecular weight of 72,000 to 78,000, and a sedimentation coefficient $s_{20.w}$ of 5. KRV has previously been reported (19) to contain a main capsid protein (with a molecular weight of about 62,000) which comprises approximately 75% of the virion protein. The purified virus also contains two minor protein components with molecular weights of 72,000 and 55,000 that represent approximately 15 and 11.4% of the virion protein, respectively. The DNA polymerase may account for the largest molecular weight protein associated with the virions. This protein is present in approximately 8 to 10 copies per virion (19).

In a preceding paper (13), we have characterized the DNA polymerases isolated from the rat nephroma cell. The stimulation of the KRV-associated DNA polymerase by 0.02 M NaCl is a property shared by only one cellular enzyme, the cytoplasmic DNA polymerase C11. The viral-associated polymerase and C11...
have a common pH optimum of 8.0 and similar $K_m$ of dNTP, $K_m$ of activated DNA, and $Mg^{2+}$ optimum. They are both inhibited by pyrophosphate, ethidium bromide, and $p$-chloromercuribenzoate. Like all of the cellular enzymes with the exception of $C_1$, the KRV-associated enzyme utilizes the synthetic polymer [d(A-T)] best of all the primer-templates tested. The viral-associated enzyme uses this primer-template with much greater efficiency than the cellular enzymes. For the KRV-associated enzyme the molecular weight is 72,000 to 78,000 and the $s_{20, w}$ is 5. The cellular $C_{II}$ enzyme has not been purified to the same high degree as the viral-associated enzyme, but its molecular weight is believed to be about 70,000 and the $s_{20, w}$ between 4 and 5.5.

There are, however, several important differences between the viral-associated enzyme and the cellular enzyme, $C_{II}$. The elution pattern of the two enzymes from DEAE-cellulose and DNA-cellulose is different. $C_{II}$ enzyme elutes from DEAE-cellulose with 0.1 M KPO$_4$ and from DNA-cellulose at 0.55 M NaCl. The viral-associated enzyme eluted from DEAE-cellulose with 0.04 M KPO$_4$ and from DNA-cellulose with 0.09 M NaCl. The viral-associated enzyme activity is stimulated 2.5-fold by Mn$^{2+}$ in comparison to Mg$^{2+}$ as the divalent cation. Mn$^{2+}$ in the presence or absence of KCl is as good a source of divalent cation as Mg$^{2+}$ plus KCl for this enzyme. The cellular enzyme, $C_{II}$, in the presence of Mn$^{2+}$ with or without KCl, has only 32% of the activity found in the presence of Mg$^{2+}$. A difference in DNA template specificity appears to exist between these two enzymes. The rat nephroma enzyme $C_{II}$ can utilize the ribostrand of d(G)$_{12}$ d(C)$_n$ to polymerize thymidylic acid, thus exhibiting R-DNA polymerase activity. The KRV-associated enzyme does not have R-DNA polymerase activity. The cytoplasmic cellular enzyme $C_{II}$ can utilize native DNA as a primer-template just as efficiently as activated DNA. It cannot, however, effectively copy the synthetic heteropolymer d(G)$_{12}$ d(C)$_n$ and exhibits only 10 to 20% of the optimal activity with this template. The viral-associated enzyme does not utilize native DNA as an optimal DNA template and the enzyme activity falls to 30 to 40% of that found with an equal weight of activated DNA. This enzyme can copy effectively the synthetic heteropolymer d(G)$_{12}$ d(C)$_n$. The KRV enzyme can also utilize denatured, activated DNA as a template, whereas the $C_{II}$ enzyme has virtually no activity with this template. We do not know the conformation of this denatured, activated DNA. Since salmon sperm has A-T-rich regions, there may be regions of hairpin turns or folding back of the ends of the DNA. This finding is especially interesting if the viral-associated enzyme is involved in copying the single-stranded KRV DNA which contains 56% A-T.

The viral-associated enzyme shares some properties and has some characteristic differences from the other rat nephroma cell polymerases. The enzymes all have similar $K_m$ values for dNTP and activated DNA. The pH optimum is similar for the KRV-DNA polymerase, the cytoplasmic enzyme $C_{II}$, and nuclear enzyme $N_2$ and $N_1$. The nuclear enzymes have a similar $Mg^{2+}$ optimum, although none of the cellular enzymes can substitute Mn$^{2+}$ for Mg$^{2+}$. All of the enzymes are inhibited to varying degrees by pyrophosphate, ethidium bromide, and $p$-chloromercuribenzoate. As reported in the preceding report none of the other cellular enzymes except $C_{II}$ appear to be stimulated by salt. In fact, the cytoplasmic enzyme $C_1$ and the major nuclear enzyme $N_2$ are strongly inhibited by KCl (90 to 70% inhibition in 0.1 M KCl). The cytoplasmic enzyme $C_1$ does not utilize the synthetic template poly[d(A-T)] and poly[d(G-C)]. The elution properties of the cellular enzymes from the three cellulose columns used in purification differ from the elution properties of the viral-associated enzyme. The nuclear enzyme $N_2$ and the cytoplasmic enzyme $C_1$ are the only two cellular enzymes with calculated values for $s_{20, w}$ and molecular weights close to the values found for the viral-associated enzyme.

The KRV-associated enzyme appears to be a single DNA polymerase activity made in an infected cell containing multiple DNA polymerases. The viral-associated enzyme does not appear to be identical in properties to any of the cellular enzymes. These facts tend to support the idea that the viral-associated enzyme may play a role in viral DNA synthesis and may be a specific enzyme for viral replication. However, these experiments do not rule out the possibility that the KRV-associated enzyme may represent a modification of a cellular enzyme such as $C_{II}$ or be synthesized following derepression of a host specified DNA polymerase not normally detected in the cell.

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