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DNA Polymerase in Virions of a Reptilian Type C Virus

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A study was made of the DNA polymerase of reptilian type C virus isolated from Russell's viper spleen cells. Simultaneous detection experiments demonstrated the presence of 70S RNA and RNA-dependent DNA polymerase activity in reptilian type C virions. The endogenous activity was dependent on the addition of all four deoxynucleotide triphosphates and demonstrated an absolute requirement for a divalent cation. The reptilian viral DNA polymerase elutes from phosphocellulose at 0.22 M salt. In this respect, it is similar to the avian (avian myeloblastosis virus; AMV) viral enzyme but is different from the mammalian (Rauscher leukemia virus; RLV) viral enzyme which elutes at 0.4 M salt. The molecular weight of the viper DNA polymerase as estimated from glycerol gradient centrifugation is 109,000. It is a smaller enzyme than the AMV DNA polymerase (180,000 daltons) and somewhat larger than the RLV enzyme (70,000 daltons). A comparison of other properties of the type C reptilian DNA polymerase with the enzyme found in other type C oncogenic viruses is made.

RNA-dependent DNA activity has been demonstrated in type C virus isolated from mammalian (1), avian (11), and reptilian (8) tumor cells. The enzyme has been the subject of recent reviews (3, 4) and data on the characteristics of the DNA polymerase from avian and murine type C viruses have been presented. No data, however, on the properties of the enzyme from the reptilian type C virus were available to permit a comparison with other type C viral DNA polymerases. Type C virus particles have been demonstrated in a viper cell line (VSW) established from the spleen of Russell's viper (12). Virus production in these cells were shown to be inhibited by actinomycin D (5), and endogenous DNA polymerase activity has been demonstrated in ether extracts of viper type C virions (8). Immunological experiments also suggested that the viper virus possesses a group-specific antigen distinct from the avian and murine type C viruses (5). This study examines some of the biochemical and physical properties of the RNA-dependent DNA polymerase found in reptilian type C virions, and a comparison of this enzyme with the DNA polymerase found in avian and murine type C viruses is made.

MATERIALS AND METHODS

Virus. Reptilian type C virus used in these experiments was supplied by Electronucleonics, Inc., Bethesda, Md. The virus was grown in cultures of Douglas viper cells (VSW) as previously described (2). Highest viral titers were recovered from supernatants of VSW cultures maintained at 30 C. Culture medium containing released virus was centrifuged for 10 min at 4,080 × *g* to remove cells and other debris. Virus was purified by double-density gradient zonal centrifugation, and viral particles banding on the region of 1.14 to 1.17 density were pelleted and suspended in a solution containing 0.1 M NaCl, 0.01 M Tris-chloride, pH 7.0, and 0.001 M EDTA. Viral concentrations, usually 10^{11} virus particles/ml, were determined by electron microscopy of negatively stained samples.

RNA-dependent DNA polymerase assay. DNA polymerase activity was determined by measuring the conversion of [^{3}H]TTP into acid-insoluble material. The standard assay (endogenous reactions) contained, in a total volume of 0.1 ml: 50 mM Tris-chloride (pH 7.8); 5 nM $MgCl_2$; 0.5 mM dithiothreitol; 0.2 mM each of dATP, dGTP, and dCTP; 40 mM KCl; 0.08 mM [^{3}H]thymidine triphosphate; 3,000 counts per min per pmol; and the amount of viral protein as indicated in each experiment. Reaction mixtures used to assay the partially purified viral DNA polymerase also contained 2 μg of the synthetic template poly rA·dT_{10} (Miles Laboratories). Reaction mixtures were incubated at 37 C for the times indicated (Fig. 6), and the reaction was stopped by the addition of 0.5 ml of a solution containing *Escherichia coli* carrier RNA and 0.02 M sodium pyrophosphate, followed by precipitation with cold 10% trichloroacetic acid. The precipitate was then collected on Whatman glass fiber filter disk, washed with cold 5% trichloroacetic acid, and counted in a liquid scintillation counter.

RESULTS

Detection and characterization of endogenous DNA polymerase activity. Type C viper virions were disrupted with detergent and incubated in the presence of all the components of

the polymerization reaction in the absence of added template. The products of the reaction were isolated by phenol extraction and layered on linear sucrose gradients (7). The DNA synthesized sediments in the 70S region as indicated by the AMV 70S RNA marker. When reaction mixtures were preincubated with RNase before sedimentation, no labeled material was seen in the 70S region. This experiment demonstrates the presence of an RNA-dependent DNA polymerase activity in viper virions which utilizes 70S RNA as template. The endogenous polymerase activity requires all four deoxynucleotide triphosphates for maximum activity, and the omission of a single deoxynucleotide triphosphate from the reaction mixture resulted in a 90% reduction in the incorporation of [^{3}H]TMP into acid-insoluble material. The reaction demonstrates an absolute requirement for divalent cations and is stimulated by the addition of dithiothreitol, an effect which has been observed for other viral DNA polymerases. Reaction mixtures preincubated with RNase demonstrated minimal incorporation (usually less than 5%); the labeled product of the viper polymerase reaction is DNase sensitive. The endogenous activity was inhibited by increasing concentrations of oligo (dT_{10}), but since disrupted virions contain many degradative enzymes (10) the significance of this observation is difficult to assess.

Partial purification of viper DNA polymerase: phosphocellulose chromagraphy. Supernatants from detergent-disrupted virions were placed on phosphocellulose columns (2) and eluted with a linear phosphate gradient (Fig. 2). Fractions were assayed by using the synthetic template poly rA·dT_{10}. The enzyme eluted as a single peak of activity at approximately 0.20 M KPO_4 and peak fractions of activity were used in subsequent experiments.

Glycerol gradient centrifugation. Analysis of the phosphocellulose enzyme on linear 10 to 30% glycerol gradients in the presence of high salt (0.50 M KCl) demonstrated a single peak of DNA polymerase activity sedimenting between the marker proteins aldolase and bovine serum albumin (Fig. 3). The estimated molecular weight was determined to be approximately 109,000. Sedimentation of the same enzyme preparation on glycerol gradients in the presence of low salt (0.05 M KCl) demonstrated two peaks of DNA polymerase activity and possibly reflects a subunit or conformational change in the enzyme.

Kinetics of partially purified viper DNA polymerase. The rates of polymerization were proportional to the amount of enzyme added,

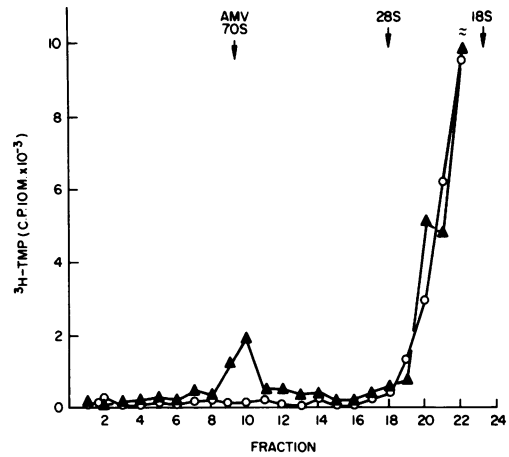

FIG. 1. *Simultaneous detection of reverse transcriptase activity and 70S RNA in detergent-disrupted viper virions by sucrose gradient centrifugation. In this experiment, a 1-ml viral suspension (10-inch viral particles/ml) in 0.01 M Tris-chloride (pH 8.3), 0.001 M EDTA, and 0.15 M NaCl (TNE buffer) was centrifuged at 105,000 × g for 1 h at 4 C, and the viral pellet was resuspended in 0.1 ml of TNE buffer containing 0.5 M KCl. Virions were disrupted with 0.05% Nonidet detergent (final concentration), and 0.05-ml samples of this suspension were incubated for 15 min at 37 C in 0.5-ml reaction mixtures as described in Materials and Methods. Higher concentrations of detergent inhibited viral DNA polymerase activity. The reactions were stopped by the addition of NaCl and sodium dodecyl sulfate to final concentrations of 0.4 M and 1%, resepectively. Approximately 15,500 counts/min were trichloroacetic acid-precipitable after the reaction was terminated. An equal volume of TNE-saturated phenol was added, and the mixture was shaken for 10 min at 22 C and centrifuged at 6,000 × g for 15 min at 4 C. The aqueous phase (5,500 trichloroacetic acid-precipitable counts/min was removed and layered over a linear glycerol gradient (10 to 30%) prepared in TNE buffer and centrifuged at 4 C at 40,000 rpm for 4 h in a Spinco SW41 swinging bucket rotor. RNA markers were 70S RNA from AMV and 18S and 23S RNA. The gradient was monitored at 254 nm, collected fractions were precipitated with 10% cold trichloroacetic acid, and radioactivity in the precipitates was determined. In the control experiment, detergent-disrupted virions were pretreated with RNase A (50 μg/ml) and incubated for 30 min at 22 C before being used in reaction mixtures. Symbols: ▲, [^{3}H]TMP-labeled product of the endogenous reaction (no added template); ○, [^{3}H]TMP-labeled product of the endogenous reaction from detergent-disrupted virions pretreated with RNase.*

and reactions were linear for the time indicated when the double-stranded synthetic homopolymer poly rA·dT_{10} was used as template (Fig. 4). The endogenous activity, however, was linear for only 10 min, after which a decrease in

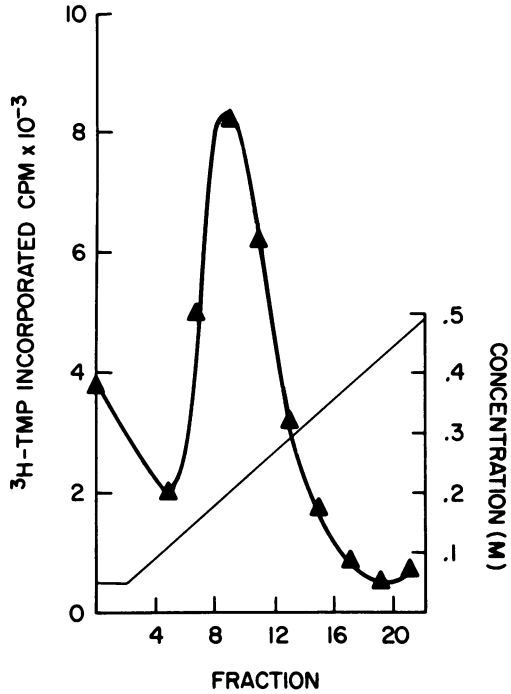

FIG. 2. *Phosphocellulose chromatography of viper RNA-dependent DNA polymerase. Protein (0.2 mg) from detergent-disrupted virions was applied to a phosphocellulose column (0.5 by 5 cm) previously equilibrated with a solution containing 0.02 M K_2PO_4, 20% glycerol, and 0.001 M dithiothreitol, washed with 2 ml of equilibration buffer, and eluted with a 10-ml linear gradient of 0.02 M to 0.5 M KPO_4. Fractions were collected and assayed for [^{3}H]TMP incorporation by using the synthetic template rA·dT_{10}. Reaction mixtures (0.1 ml), as described in Table 1, were modified and contained 2.5 μg of rA·dT_{10} and 20 μliters of enzyme and were incubated for 15 min at 37 C. Symbol: ▲, [^{3}H]TMP incorporated into acid-insoluble product.*

acid-precipitable [^{3}H]TMP-labeled product was observed. The presence of phosphatases and nucleases in preparations of disrupted virions has been reported (10) and probably is related to loss of acid-precipitable product seen in the endogenous viper DNA polymerase reaction.

Divalent cation requirement. The viper virus DNA polymerase requires either manganese or magnesium for the polymerization reactions when the synthetic template poly rA·dT_{10} is used (Fig. 5). The enzyme demonstrates optimal activity at 10 mM magnesium; manganese can replace magnesium only about 50% as efficiently at an optimal concentration of 2 mM manganese.

Temperature optimum of the reptilian viral DNA polymerase. The temperature dependence of the endogenous polymerization reaction is shown in Fig. 6. The incorporation of [^{3}H]TMP into acid-insoluble product is a function of the temperature at which reaction mixtures are incubated. Maximal incorporation is seen in the range of 35 to 40 C, after which heat inactivation of the enzyme probably occurs.

DISCUSSION

Before this study, no data on the properties of the RNA-dependent DNA polymerases from a reptilian type C virus were available to permit a comparison of this enzyme with the viral DNA polymerases from mammalian and avian type C viruses. Although the reptilian RNA-dependent DNA polymerase is basically very similar in the polymerization reaction to the enzyme found in other type C particles, it does differ in several biochemical and physical parameters.

Simultaneous detection experiments have demonstrated a DNA polymerase activity in viper virions and indicate that the enzyme

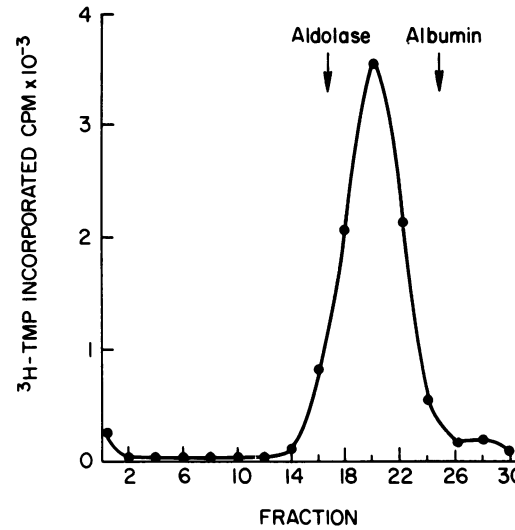

FIG. 3. *Glycerol gradient centrifugation of viper viral DNA polymerase. The peak of enzyme activity eluting from phosphocellulose at 0.22 M KPO_4 was dialyzed against a solution containing 0.01 M Tris-chloride, pH 7.8, 0.002 M dithiothreitol, and 0.5 M KCl (buffer A) and layered on a linear glycerol gradient (10 to 30%) prepared with buffer A. Samples were centrifuged at 4 C in a Spinco SW41 rotor for 5 h at 40,000 × g. Adolase and bovine serum albumin were run in parallel buckets. Absorbance at 254 nm was monitored continuously as the gradient was fractionated. Fractions were assayed with the synthetic template rA·dT_{10} as described in Fig. 2. The position of the markers are indicated by the arrows. Symbol: ●, [^{3}H]TMP incorporated into acid-insoluble product.*

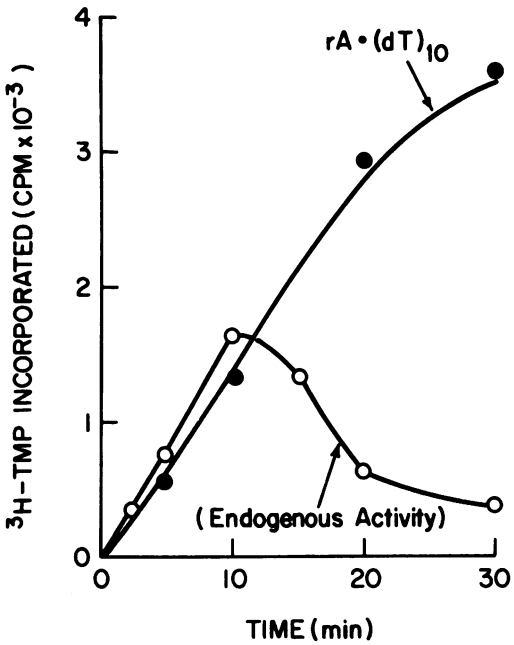

FIG. 4. *Kinetics of the viral DNA polymerase in disrupted virions and of the partially purified enzyme. Reaction mixtures (endogenous reaction), as described in Table 1, were incubated for the times indicated, and [^3H]TMP incorporation into acid-insoluble product was determined. The partially purified enzymes from glycerol gradients were assayed by using the synthetic template rA · dT_{10}, as described in Fig. 2. Symbols: O, endogenous reaction; ●, partially purified viral DNA polymerase assayed with rA · dT_{10}.*

utilizes 70S RNA as a template. The activity is detergent dependent and requires all four deoxynucleotide triphosphates for maximal activity. The product of the reaction is resistant to pancreatic RNase and is sensitive to DNase. As also shown for other leukoviruses, the viral DNA polymerase demonstrates an absolute requirement for divalent cations. The viper viral polymerase exhibits maximum activity at a magnesium concentration of 10 mM. Manganese can partially replace magnesium at an optimum concentration of 2 mM. The enzyme is also stimulated by dithiothreitol, as are the DNA polymerases from other type C viruses. The viper viral DNA polymerase elutes from phosphocellulose at 0.22 M KPO_4 and is thus similar in this respect to the avian polymerase (AMV) (6), but different from the murine viral polymerase (RLV) (9) which elutes at 0.40 M KPO_4. The estimated molecular weight of the viper viral enzyme was determined by glycerol gradient centrifugation to be 109,000. It is therefore much smaller than the AMV DNA polymerase, which has been shown to be composed of two chains with molecular weights of 110,000 and 69,000, respectively (6), and somewhat larger than the RLV enzyme which has a molecular weight of 70,000 (9).

The temperature optimum for endogenous DNA polymerase activity in reptilian type C particles was determined to be 40 to 45 C. A similar temperature optimum has been reported for the avian (AMV) DNA polymerase, whereas the murine (RLV) DNA polymerase demonstrates an optima of 37 to 40 C (10). In this study, cultures of Douglas viper cells (VSW) demonstrated optimal growth and produced highest viral titers when maintained at

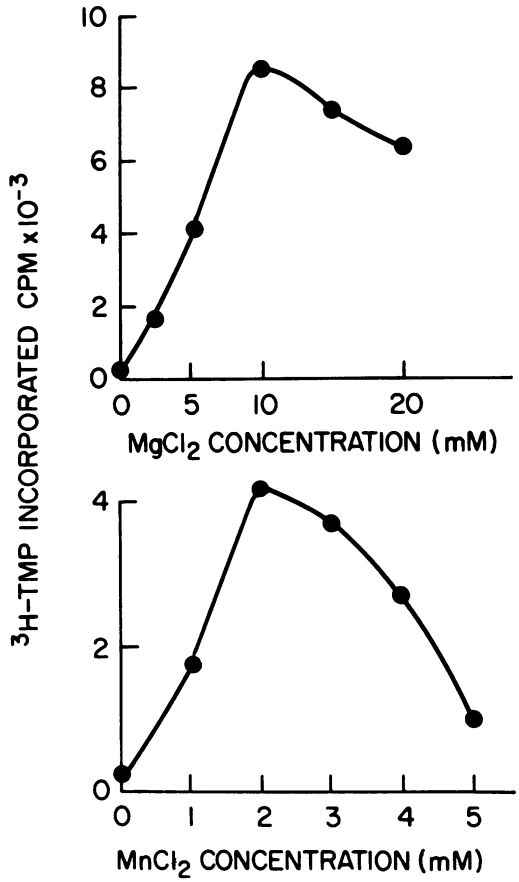

FIG. 5. *Divalent cation requirements of the partially purified viral DNA polymerase. Each reaction mixture (0.1 ml), as described in Fig. 2, contained 2 μg of rA · dT_{10} and 3 μg of viral protein; the amount of magnesium and manganese are as indicated. Reactions were incubated at 37 C for 30 min, and the incorporation of [^3H]TMP in the absence of added divalent cation is subtracted from each value. [^3H]TMP incorporated into acid-insoluble product in presence of varying concentrations (top curve) of magnesium and (bottom curve) manganese.*

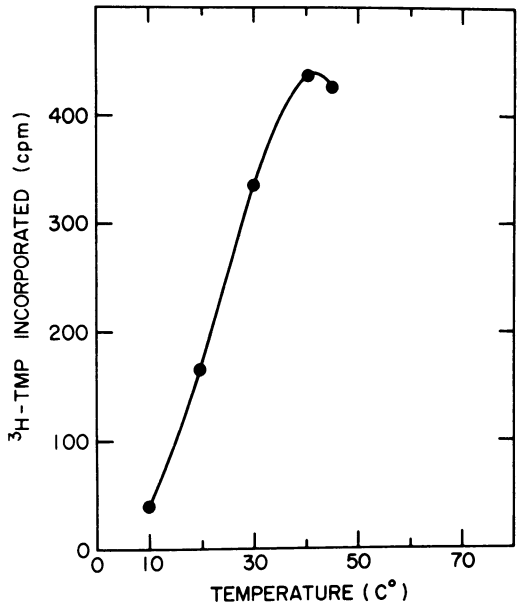

FIG. 6. *Temperature optima for the endogenous DNA polymerization reaction. Virus was disrupted as described in Fig. 1, and samples were incubated for 15 min in reaction mixtures as described in Materials and Methods at the temperature indicated. Reaction mixture temperatures were accurately controlled by using a Lauda circulating water bath. Reactions were stopped by the addition of cold 10% trichloroacetic acid, and the amount of [^{3}H]TMP incorporated into acid-insoluble product was determined.*

30 C; cells would not grow or produce virus at 37 C.

This study provides data on the biochemical and physical properties of the partially purified RNA-dependent DNA polymerase from a reptilian type C virus. Additional data on the DNA polymerases from other type C oncogenic viruses are needed for a comprehensive assessment of the role of viral DNA polymerases and the type C virus in oncogenesis.

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