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Human Cytomegalovirus
III. Virus-Induced DNA Polymerase

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Infection of WI-38 human fibroblasts with human cytomegalovirus (CMV) led to the stimulation of host cell DNA polymerase synthesis and induction of a novel virus-specific DNA polymerase. This cytomegalovirus-induced DNA polymerase was purified and separated from host cell enzymes by DEAE-cellulose and phosphocellulose column chromatographies. It can be distinguished from host cell enzymes by chromatographic behavior, template primer specificity, sedimentation property, and the requirement of salt for maximal activity. This virus-induced enzyme has a sedimentation coefficient of 9.2*S* and is found in both the nuclei and cytoplasm of virus-infected cells, but not in uninfected cells. This enzyme could efficiently use activated calf-thymus DNA, poly(dA)·oligo(dT)$_{12\text{-}18}$, and poly(dC)·oligo(dG)$_{12\text{-}18}$ as template primers, especially poly(dA)·oligo(dT)$_{12\text{-}18}$, but it could not use poly(rA)·oligo(dT)$_{12\text{-}18}$, poly(rC)·oligo(dG)$_{12\text{-}18}$, or oligo(dT)$_{12\text{-}18}$. The enzyme requires Mg^{2+} for maximal activity, is sensitive to *p*-hydroxymercuribenzoate, and is not a zinc metalloenzyme. In addition, the cytomegalovirus-induced DNA polymerase activity can be enhanced by adding 0.06 to 0.12 M NaCl or 0.03 to 0.06 M $(NH_4)_2SO_4$ to the reaction mixture.

Human cytomegalovirus (CMV) is increasingly recognized as an important pathogen (20). It produces a variety of syndromes including classic cytomegalic inclusion disease to intrauterine death, prematurity, congenital defects, infectious mononucleosis, postperfusion syndrome, and interstitial pneumonia in transplantation patients (14, 20). This virus also exhibits a striking tendency toward latent infection in the human host. The ability to transform hamster embryo fibroblasts (1) suggests that CMV might have oncogenic properties comparable to those of herpes simplex, Epstein-Barr, and other herpes-group viruses.

Although there is a lack of detectable nucleic acid homology between human CMV and herpes simplex virus (10), there are similarities structurally and biologically. As with other members of herpes-group viruses, this double-stranded DNA virus has a genome size around 10^8 daltons (9), which is significant to code for more than 200 virus-specific proteins. Because of the complexity of its genome and the difficulty in obtaining pure virus, little of the molecular virology of this agent has been uncovered.

Infection of baby hamster kidney cells, HEp-2 cells, or HeLa cells with herpes simplex virus leads to the induction of a novel herpes-specific DNA polymerase (19). A virus-induced DNA polymerase has been detected in Marek's disease virus-infected duck embryo fibroblasts (3). We now find that a virus-induced DNA polymerase also exists in a human CMV system. We describe here the purification and characterization of the human CMV-induced DNA polymerase as well as some host cell DNA polymerases.

MATERIALS AND METHODS

Cells and virus. The WI-38 strain of human fibroblasts (Hayflick) obtained from HEM (Rockville, Md.) was used for the entire study. The cells (passages 21 to 28) were subcultured in minimal essential medium (GIBCO, Long Island) supplemented with 10% fetal calf serum, 100 U of penicillin, and 100 μg of streptomycin per ml. For maintenance, 2% fetal calf serum was used. Human CMV strain AD-169 (9, 15) was obtained from the National Heart and Lung Institute, Bethesda.

The virus stock was prepared from cloned virus by infecting WI-38 cells with clarified (8,000 rpm, Sorvall HB-4 rotor) and hypotonically treated (1:20 dilution in water, held at 37 C for 30 min) infected cell lysates to eliminate possible low-level mycoplasma contamination. The virus stock was subcultured once to increase the titer for use in the infections for study of enzyme activity.

Preparation of nuclear and cytoplasmic enzyme extracts. To obtain the enzyme extracts from infected cells, the confluent WI-38 cells in six roller bottles (surface area about 1,000 cm^2 each) were infected with human CMV at 1 to 2 PFU per cell as described before (9). Sixty-eight hours after infection, the cells were washed twice with ice-chilled TBS (0.05 M Tris-hydrochloride [pH 7.4], 0.15 M NaCl) and once with hypotonic buffer A (0.05 M Tris-hydrochloride [pH 7.8], 0.001 M $MgCl_2$, 0.001 M dithiothreitol). The infected cells were then removed from the bottles by scraping with rubber policemen and suspended in hypotonic buffer A (total 60 ml). After 30 min of hypotonic treatment at 4 C, Nonidet P-40 at a final concentration of 0.2% was added to the cell suspension to enhance the disruption of cytoplasmic membranes. The suspension was then mixed in a Vortex mixer for 3 to 5 min and observed by a light microscope to monitor cell disruption (>99%).

The nuclei were collected by centrifugation in a Sorvall HB-4 rotor at 2,000 rpm and washed twice with 10 ml of buffer A containing 0.2% Nonidet P-40. The original and rinsed supernatant fluids were pooled and designated as cytoplasmic crude extract. The isolated nuclei were suspended in 10 ml of buffer B (0.02 M Tris-hydrochloride [pH 7.8], 0.1 M NaCl, 0.015 M $MgCl_2$, 0.008 M dithiothreitol) and lysed by adding 3 ml of a 4 M NaCl solution (final concentration about 1.3 M). This nuclear lysate was kept in an ice bath for 30 min. The cytoplasmic crude extract and nuclear lysate were then clarified by centrifugation in a Spinco SW27 centrifuge at 27,000 rpm for 2 h at 2 C to remove cellular organelles and debris. The clarified cytoplasmic and nuclear extracts were dialyzed overnight against buffer D (0.05 M Tris-hydrochloride [pH 7.8], 0.001 M EDTA, 0.001 M dithiothreitol, and 5% glycerol).

Mock-infected cytoplasmic and nuclear extracts were also prepared simultaneously from the same amount of mock-infected stationary cultures (six roller bottles in 2% maintenance media). Noninfected log-phase cell extracts were prepared from 70% confluent log-phase cultures by an identical procedure.

DEAE-cellulose column chromatography. The cytoplasmic or nuclear extracts after dialysis against buffer D and clarification (7,000 rpm for 20 min in a Sorvall HB-4 rotor) were then loaded on a DEAE-cellulose column (1.3 by 12 cm, DE52, Pharmacia) pre-equilibrated with buffer D. The column was washed with 20 ml of buffer D and then 35 ml of buffer D containing 0.05 M NaCl. A linear gradient of 0.1 to 0.7 M NaCl in buffer D was then applied to the column. The fractions (2 ml) were collected, and a 50-μl volume from each fraction was assayed for DNA polymerase activity with activated calf thymus DNA as template. Each of the peaks of DNA polymerase activity was pooled and frozen in 50% glycerol or dialyzed against buffer D with 0.1 M NaCl and subjected to further purification.

Phosphocellulose chromatography. A phosphocellulose column (1.3 cm by 6 cm, Whatman P-11, subjected to acid and base washing) was pre-equilibrated with buffer D containing 0.1 M NaCl. Bovine serum albumin (fraction V, 5 mg in 5 ml of buffer D) was first applied to the column to enhance the recovery of enzyme and eliminate nonspecific binding. A similar application was also described in the Marek's disease virus-induced DNA polymerase system (3). After the column was washed with 40 ml of 0.1 M NaCl in buffer D, the dialyzed enzyme from the DEAE-cellulose column (see D2, Fig. 1 and 3) was applied to the phosphocellulose column. Buffer D (15 ml) with 0.1 M NaCl was used for washing after application of the sample. The elution was carried out with a linear gradient of 0.1 to 0.5 M NaCl in buffer D. Fractions of 1.5 ml were collected, and samples (50 μl) from each fraction were assayed for DNA polymerase activity with activated calf thymus DNA as well as poly(dA)·oligo$(dT)_{12-18}$ as template primers.

Glycerol gradient centrifugation. For estimation of the molecular size of virus-induced DNA polymerase, a 5 to 20% glycerol gradient in buffer D was used. DNA polymerase samples (0.3 ml; 0.5 mg of bovine serum albumin was added to stabilize the enzyme) were dialyzed against buffer D without glycerol for 2 h and layered on 5 ml of a linear glycerol gradient. The centrifugation was carried out in a Beckman SW50.1 rotor at 35,000 rpm for 13.5 h at 2 C. Human gamma globulin fraction II and *Escherichia coli* alkaline phosphatase (8) were used as markers in separate tubes (sedimentation values of 7*S* and 6.3*S*, respectively) to determine the sedimentation coefficients of the DNA polymerases. The alkaline phosphatase activity was assayed by the method of Garen and Levinthal (8) by measurement of the absorbancy change at 410 nm with *p*-nitrophenyl phosphate as the substrate. The sedimentation of human gamma globulin fraction II was traced by absorbance at 280 nm.

DNA polymerase assay. The DNA polymerase assays are based on measurement of the incorporation of tritium-labeled nucleoside triphosphate into an acid-precipitable product. In system A, with activated calf-thymus DNA as the template, the stock reaction mixture (×2.5 concentration) contained: Tris-hydrochloride (pH 7.8), 0.1 M; $MgCl_2$, 0.025 M; dithiothreitol, 0.001 M; bovine serum albumin, 1.25 mg/ml; dATP, dCTP, and dGTP, 0.25 mM; and TTP, 0.025 mM. For assay, 0.1 ml of the stock reaction mixture, 0.1 ml of activated calf thymus DNA (200 μg/ml), 0.05 ml (or as stated) of enzyme, and 1 μCi of [^{3}H]TTP (48 Ci/mmol) were incubated at 37 C for 30 min. The reaction was terminated and precipitated by adding 2.5 ml of cold 5% trichloroacetic acid with 12.5 μg of hydrolyzed yeast RNA per ml (the stock solution, 1.25 mg/ml, was hydrolyzed by boiling in 20% trichloroacetic acid for 60 min). The precipitate was collected on a membrane filter (B-6 Schleicher and Schuell) and washed with 5% trichloroacetic acid by filtration. After drying, the filters were counted in toluene scintillation fluid.

In system B, with synthetic poly(dA)·oligo$(dT)_{12-18}$, poly(rA)·oligo$(dT)_{12-18}$, or oligo$(dT)_{12-18}$ as template primer, the reaction mixture contained the same ingredients as in system A, except that dATP, dCTP, and dGTP were omitted. The synthetic template primers in the amount of 2 μg in 0.1 ml per reaction were used instead of activated calf thymus DNA.

In system C, with synthetic poly(dC)·oligo(dG)$_{12-18}$ or poly(rC)·oligo(dG)$_{12-18}$ as template primer, only one substrate, dGTP (0.025 mM for ×2.5 concentration) together with 1 μCi of [^{3}H]dGTP (specific activity 42 Ci/mmol), was used; the template primer concentration was 2 μg per reaction.

Reagents and chemicals. Nonidet P-40 was purchased from BDH Chemicals Ltd. (Poole, England); *p*-hydroxymercuribenzoate, 1,10-phenanthroline, dithiothreitol, deoxyribonucleoside triphosphate, calf thymus DNA, and bovine serum albumin (fraction V) were obtained from Sigma Chemical Co. DEAE-cellulose (DE52) and phosphocellulose (P-11) were purchased from Reeve Angel (Whatman Biochemicals Ltd.). Synthetic polynucleotide-oligodeoxynucleotides were purchased from P. L. Biochemistry. [^{3}H]TTP and [^{3}H]dGTP were obtained from New England Nuclear Corp. 1,7-Phenanthroline, a gift of J. Harrison, was from Alfrea Bader Chemicals.

RESULTS

DNA-dependent DNA polymerase activity in CMV-infected and mock-infected human fibroblasts. Based on the chromatographic pattern of DNA polymerizing activity recovered from the DEAE-cellulose column, two main peaks were found in infected cells, both in cytoplasmic and nuclear extracts (Fig. 1). The first enzyme activity (D1; DEAE-cellulose peak 1) was eluted in low salt (0.05 M NaCl), and the second peak (D2) was eluted between 0.13 and 0.25 M NaCl; the main peak appeared between 0.13 and 0.18 M. Compared with CMV-infected cells, there was little DNA polymerizing activity in either the nuclear or cytoplasmic fractions of mock-infected, stationary-phase cells (elution around 0.16 M salt; Fig. 1). From the observations of Lewis et al. (11), Srivastava (17), and Chang and Bollum (6) in human cells and mouse L cells, it was expected that the activity eluting at low ionic strength (0.05 M NaCl) was the low-molecular-weight form of the DNA polymerase and that the activity eluting at high ionic strength (around 0.15 M NaCl) was the high-molecular-weight form of enzyme (6–8*S*).

The enzymes obtained from peak 2 (D2, fractions 30 to 45) were analyzed by phosphocellulose chromatography (Fig. 2). When activated calf thymus DNA was used as template for activity in the extracts of the infected cytoplasmic fraction, three distinct peaks were resolved. These were designated as D2P1, D2P2, and D2P3 (DEAE-cellulose peak 2 and phosphocellulose peaks 1, 2, and 3). P1 eluted at a salt concentration around 0.17 M, P2 eluted around 0.22 M, and P3 eluted around 0.26 M. In the mock-infected cytoplasmic fraction, only a very low activity could be found at the position corresponding to that of P1 and P2. All three enzymes found in the infected cytoplasmic fraction were also found in the nuclear fraction. In addition, a lesser activity (P4) eluted at higher salt concentrations (around 0.3 M NaCl). The appearance of this peak is not consistent; occasionally it was also found in the cytoplasmic fraction. A more obvious P3 enzyme activity peak was observed when synthetic poly(dA)·oligo(dT)$_{12-18}$ was used as template primer.

To determine whether human CMV induces a new species of DNA polymerase or merely stimulates host cell DNA polymerase synthesis, the enzyme activities in extracts prepared from cytoplasm and nuclei of noninfected log-phase WI-38 cells (70% confluent) were compared. Figure 3 shows the activity elution profile in DEAE-cellulose column chromatography. There was no great difference in the elution profiles of enzyme from growing and infected cells. The DNA polymerase activity of growing cells (Fig. 3) and contact-inhibited stationary-phase cells (Fig. 1) disclosed an obvious quantitative difference. Both DNA polymerase activities were very low in stationary-phase cells. This implies that human CMV infection can induce DNA polymerase synthesis to the same level as that of growing cells.

DNA polymerase activities of infected or log-phase cells eluting from DEAE-cellulose at 0.13 to 0.25 M (D2) were pooled, dialyzed against buffer D with 0.1 M NaCl, and analyzed by phosphocellulose column chromatography (Fig. 4). The lower panel shows the comparison between cytoplasmic fractions of infected and noninfected log-phase cells with activated calf thymus DNA as template primer. In the control noninfected material, two peaks, P1 and P2, were eluted at around 0.17 and 0.22 M NaCl, respectively. In the cytoplasmic extract of CMV-infected cells, an additional activity eluting at 0.26 M was found (P3). An identical result was also obtained with the nuclear fraction (not shown). Because uninfected cells show no detectable activity eluting from phosphocellulose at 0.26 M, it seems that P3 is a new virus-induced DNA polymerase (Fig. 2 and 4). A more obvious difference was found when synthetic template and primer, poly(dA)·oligo(dT)$_{12-18}$, were used as template (Fig. 4, upper panel).

Properties of virus-induced DNA polymerase. (i) Template and primer specificities. Virus-induced enzymes obtained from cytoplasm (CyD2P3) and nucleus (NuD2P3), together with host cell enzymes (CyD1 and NuD2P1), were examined for template primer

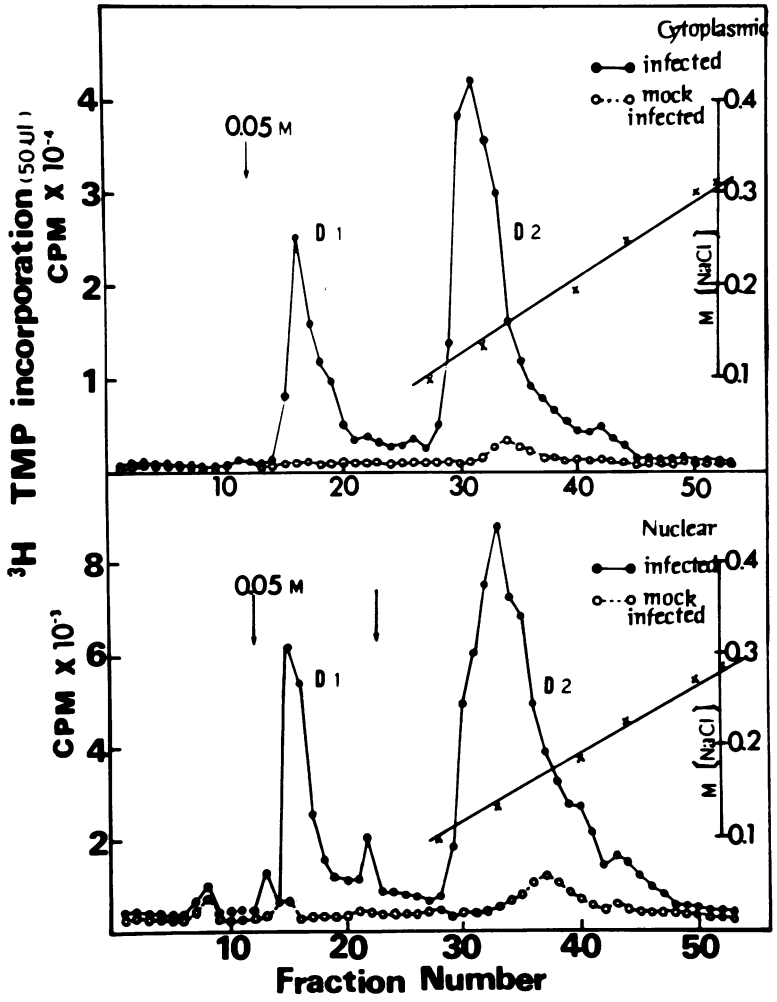

FIG. 1. *DEAE-cellulose chromatography of nuclear and cytoplasmic extracts from CMV-infected and mock-infected WI-38 cells. Cytoplasmic and nuclear extracts were applied to pre-equilibrated DEAE-cellulose columns. Samples were eluted by 0.05 M NaCl and a linear 0.1 to 0.7 M NaCl gradient in buffer D. Each fraction (50 µl) was assayed for DNA polymerase activity with activated calf-thymus DNA as template primer. Top, Elution profile of the enzyme activities from cytoplasmic extract of infected (●) and mock-infected (○) stationary-phase cells. Bottom, Profile of enzymes from the nuclear fraction of infected and mock-infected cells. D1 is designated as DEAE-cellulose peak 1 enzyme obtained by 0.05 M salt elution. D2 is the second peak enzyme eluted at salt concentration between 0.13 and 0.25 M NaCl.*

specificities. Both virus-induced and host cell DNA polymerases can effectively use activated calf thymus DNA as template primer, but can accept denatured and native calf thymus DNA as templates only poorly (Table 1). The virus-induced enzyme can use synthetic poly(dA)·oligo(dT)$_{12-18}$ and poly(dC)·oligo(dG)$_{12-18}$ more efficiently than do host cell enzymes. The virus-induced enzyme is unable to use poly(rA)·oligo-(dT)$_{12-18}$, poly (rC)·oligo(dG)$_{12-18}$, and oligo-(dT)$_{12-18}$ as template primers; this indicates that the virus-induced DNA polymerase is an enzyme with a character distinct from reverse transcriptase (2), R-DNA polymerase (11), and terminal deoxynucleotidyl transferase (4, 5). The viral enzyme prefers synthetic poly(dA)·oligo(dT)$_{12-18}$ and poly(dC)·oligo(dG)$_{12-18}$ over activated calf thymus DNA as template primer.

(ii) Effect of divalent metal ions and ribonucleoside triphosphate on virus-induced enzyme activity. The divalent cations are essential for virus-induced DNA polymerase activity (as shown in Table 2). The concentration of Mg^{2+} required for maximal incorporation is around 5 to 10 mM. Only part of the activity can be obtained when the Mg^{2+} is replaced by

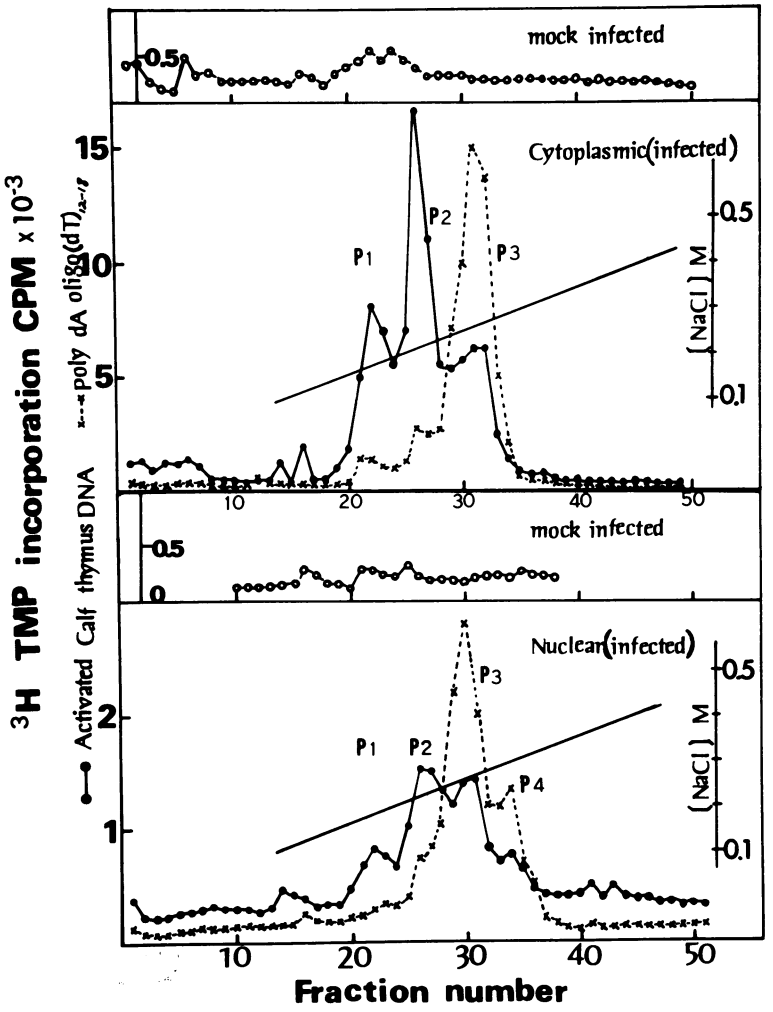

FIG. 2. *Further chromatography of D2 enzymes of infected and noninfected stationary-phase cells in phosphocellulose columns. The enzyme fractions from the second peak (D2) of DEAE-cellulose (Fig. 1, fractions 30 to 45) were pooled, dialyzed against buffer D, and applied to phosphocellulose columns. The elution was carried out with a linear gradient of 0.1 to 0.5 M NaCl in buffer D. Activated calf thymus DNA (●) and poly(dA)·oligo(dT)$_{12-18}$ (×) were used as template primers for enzyme activity assays. Top, Activity profile of D2 enzymes from cytoplasmic fraction of infected and mock-infected cells. Bottom, Profile of enzymes from the nuclear fraction. Very low activity can be seen in either cytoplasmic or nuclear fraction of mock-infected cells (shown in small panels). In the infected-cell fraction, peak 1 enzyme (P1) eluted at the NaCl concentration around 0.17 M, P2 eluted at 0.22 M, and P3 eluted around 0.26 M. P3 was found to be a virus-induced enzyme (see Fig. 4) that prefers poly(dA)·oligo(dT)$_{12-18}$ as template primer.*

Mn^{2+}. Addition of 0.1 mM ribonucleoside triphosphate to the completed reaction mixture did not stimulate or enhance the polymerization activity in vitro (not shown).

(iii) Effect of temperature. The reaction temperature for maximum polymerization by the virus-induced enzyme was between 40 and 45 C (Fig. 5). There is no obvious difference in the temperature preferences of virus-induced and uninfected cell enzymes. The temperature preference of 40 to 45 C can be regarded only as the behavior of the enzyme in vitro and not a property in overall virus DNA synthesis in infected cells.

(iv) Effect of salt concentration. Figure 6 shows the effect of potassium chloride (upper panel) and ammonium sulfate (lower panel) on DNA polymerase activity. The virus-induced enzyme activities, both from infected nuclei (NuD2P3) and from cytoplasm (CyD2P3), can

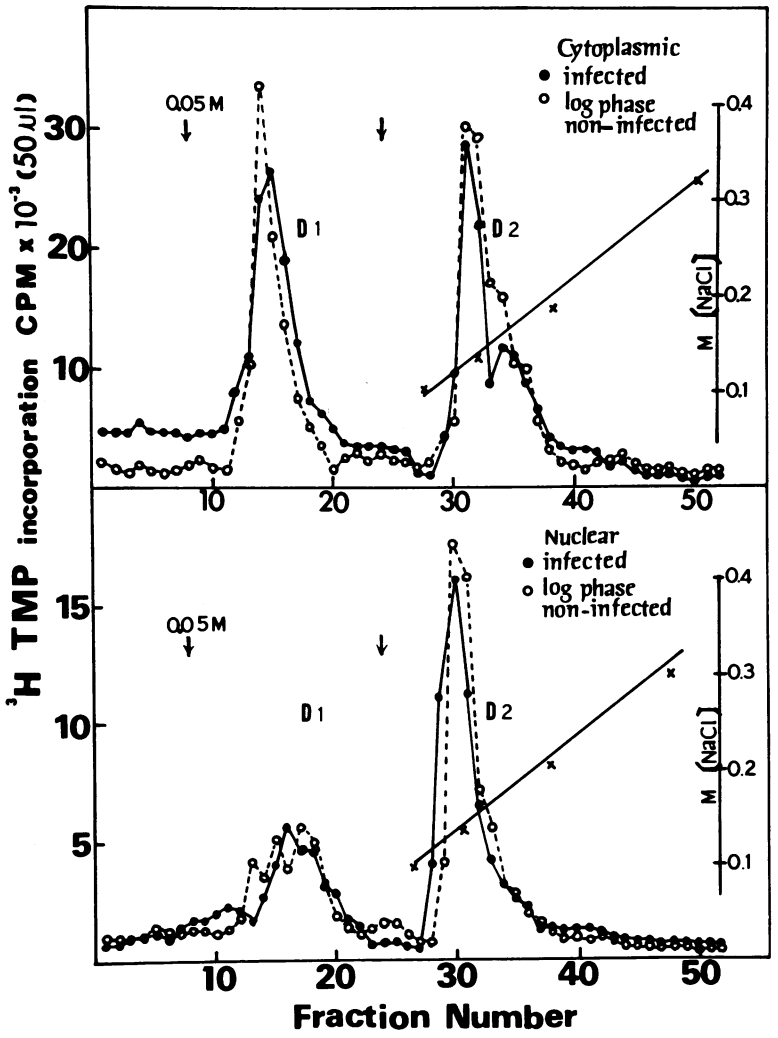

FIG. 3. *DEAE-cellulose chromatography of nuclear and cytoplasmic extracts of log-phase and CMV-infected stationary-phase cells. The cytoplasmic and nuclear extracts from log-phase cells and CMV-infected stationary-phase cells were applied to a DEAE-cellulose column. The elution and assays were described in the text and the legend to Fig. 1. Activated calf thymus DNA was used as template for enzyme assays. Top, Results from the cytoplasmic fraction; bottom, results with the nuclear fraction. Symbols: ●, infected-cell enzyme; ○, log-phase noninfected enzyme.*

be enhanced by adding potassium chloride to concentrations of 0.06 to 0.12 M or $(NH_4)_2SO_4$ to concentrations of 0.03 to 0.06 M, but the enzyme is inhibited when the salt concentration reaches 0.18 M. One of the host enzymes, cytoplasmic D2P2, apparently can also be stimulated by 0.06 M salt, but the remaining two host cell enzymes tested (CyD1, NuD2P1) are both sensitive to the presence of salt. In general, the virus-induced enzyme is more resistant to salt treatment than are host cell enzymes (Fig. 6).

(v) Sedimentation properties of virus-induced DNA polymerase in glycerol gradient centrifugation. The virus-induced DNA polymerases from nuclei (NuD2P3) and cytoplasm (CyD2P3) were analyzed for sedimentation properties by glycerol gradient centrifugation (Fig. 7). Human gamma globulin fraction II and *E. coli* alkaline phosphatase were used as reference markers of 7*S* and 6.3*S*, respectively. The virus-induced enzyme isolated from infected nuclei, NuD2P3, has a major activity at 8.2*S*. Some very minor activities were found in the

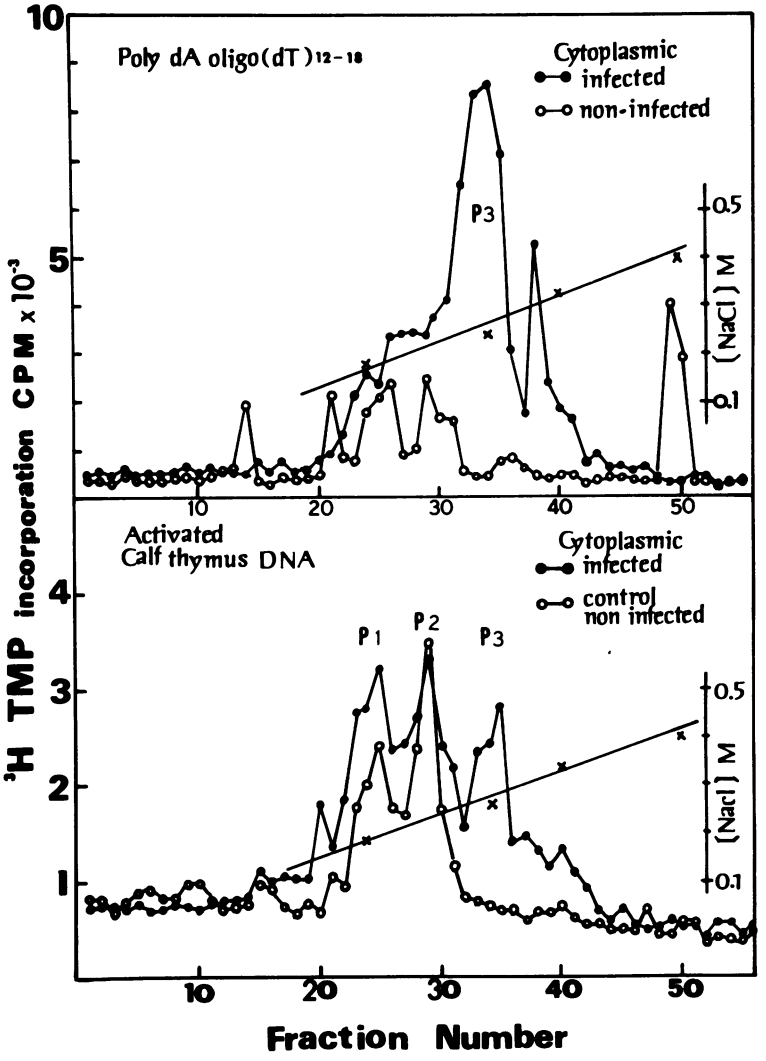

FIG. 4. *Further chromatography of D2 enzymes of log-phase WI-38 and human CMV-infected cells in phosphocellulose column The enzyme fractions from the second peak of the DEAE-cellulose column (Fig. 3, fractions 30 to 38) were pooled and applied to a phosphocellulose column. The enzyme elution and assays were carried out as described in the legend to Fig. 2. Only the results of cytoplasmic enzymes from infected and log-phase cells are shown. Bottom, Activity profile when activated calf-thymus DNA is used as template. P1, P2, and P3 enzymes are designated as described in the legend to Fig. 2. P3 enzyme, eluted at higher-salt (0.26 M NaCl) buffer, is present in the infected cytoplasmic fraction (●) but not in control log-phase cells (○). Top, Activity profile when synthetic poly(dA)·oligo(dT)$_{12-18}$ was used as template primer.*

9.3*S* and 5.3*S* areas (Fig. 7b). The virus-induced enzyme isolated from infected cytoplasmic fraction, CyD2P3, sedimented in the 9.3*S* area (Fig. 7c). The different sedimentation properties of nuclear and cytoplasmic enzymes were unexpected and led us to search for the possible factor(s) involved.

The cytoplasmic enzyme (CyD2P3) was precipitated by 90% saturated $(NH_4)_2SO_4$, dialyzed against buffer D, and sedimented in a glycerol gradient under the same conditions as described above. In this experiment, three distinct peaks of enzyme activity were observed sedimenting at 9.3*S*, 8.2*S*, and 5.3*S*, whereas untreated enzyme still sedimented at 9.3*S*. The procedure for isolating nuclear and cytoplasmic enzymes was identical except that the nuclear enzyme had been exposed to high-salt treatment which was used for lysis and solubilizing the nuclei at the beginning step of enzyme purification. The

TABLE 1. *Template and primer specificities of human CMV-induced and WI-38 host cell DNA polymerases*

Assay system	Template and primer	Substrate	WI-38 enzymes incorporated[a]		Virus-induced enzymes incorporated[a]	
			CyD1	NuD2P1	CyD2P3	NuD2P3
A	Activated calf thymus DNA	[^{3}H]TTP, cold dNTP	85	58	49	53
A	Native calf thymus DNA	[^{3}H]TTP, cold dNTP	7.6	5.8	1.5	4.8
A	Denatured calf thymus DNA	[^{3}H]TTP, cold dNTP	4.2	1.6	0.5	0.5
B	Poly(dA)·oligo(dT)$_{12-18}$	[^{3}H]TTP, cold TTP	232	16	313	329
B	Poly(rA)·oligo(dT)$_{12-18}$[b]	[^{3}H]TTP, cold TTP	11	8.3	<0.1	<0.1
C	Poly(dC)·oligo(dG)$_{12-18}$	[^{3}H]dGTP, cold dGTP	17	13	129	83.2
C	Poly(rC)·oligo(dG)$_{12-18}$[b]	[^{3}H]dGTP, cold dGTP	<0.1	<0.1	<0.1	<0.1
B	Oligo(dT)$_{12-18}$	[^{3}]TTP, cold TTP	<0.1	<0.1	<0.1	<0.1

[a] Picomoles.
[b] In addition to Mg^{2+}, Mn^{2+} at final concentration of 2 mM was added to the reaction.

TABLE 2. *Factors influencing the polymerization activity of human CMV-induced and host cell DNA polymerases*

Reaction mixture (mM)	^{3}H-labeled deoxyribonucleotide incorporated[a] (counts/min)				
	Host cell enzymes			Virus-induced enzymes	
	CyD1[b]	CyD2P1[c]	CyD2P2[c]	NuD2P3[b]	CyD2P3[b]
Complete reaction mixture without template primer (with 10 mM Mg^{2+})	160	240	217	155	118
Without Mg^{2+} and Mn^{2+}	144	2,812	6,751	210	140
Mg^{2+}, 20	3,147	2,198	2,179	11,739	9,010
Mg^{2+}, 10	13,904	3,845	5,464	20,558	16,796
Mg^{2+}, 5	16,877	3,640	6,710	22,676	19,540
Mg^{2+}, 1	2,206	3,989	6,281	10,848	10,090
Mn^{2+}, 5	12,187	5,023	10,244	5,325	5,733
Mn^{2+}, 2	11,973	5,093	11,973	5,975	6,961
Mn^{2+}, 1	9,551	4,843	10,355	5,723	5,977
Mn^{2+}, 0.5	1,127	4,071	7,980	3,363	2,730

[a] Reaction was carried out at 37 C for 60 min.
[b] Assay system B; poly(dA)·oligo(dT)$_{12-18}$ was used as template primer.
[c] Assay system A; activated calf thymus DNA was used as template primer.

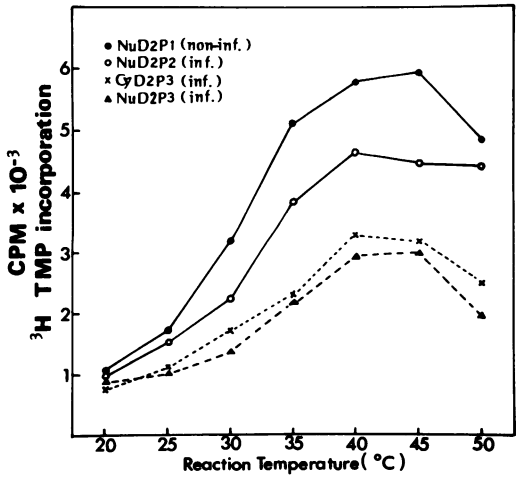

FIG. 5. *Effect of temperature on the activity of virus-induced DNA polymerase. Human CMV-induced DNA polymerase isolated from the cytoplasm (CyD2P3, ×) and the nucleus (NuD2P3, ▲), together with two host cell DNA polymerases, NuD2P1 (●) and NuD2P2 (○), was examined in vitro for temperature optima. The activity assays were carried out with activated calf-thymus DNA as template primer (system A). The temperature for maximum polymerization of nucleotides by both virus-induced and host cell DNA polymerase is between 40 and 45 C.*

resolution of 5.3*S* and 8.2*S* components from 9.3*S* cytoplasmic enzyme might be explained by the dissociation of a subunit(s) or factor(s) by high-salt treatment. We speculate that 8.2*S* is the dimer of 5.3*S*, and a factor(s) is added to 8.2*S* enzyme to generate a 9.3*S* enzyme. More

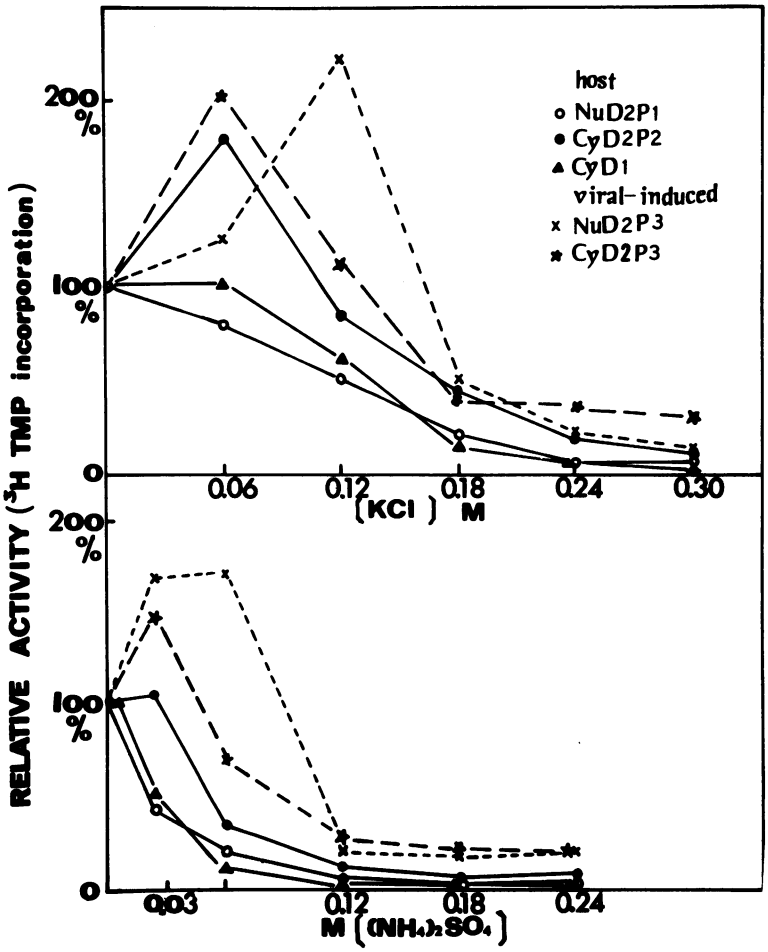

FIG. 6. *Effect of salt concentration on virus-induced DNA polymerase activity. The reaction and polymerase assays were carried out as described in the text, except that KCl or $(NH_4)_2SO_4$ was added to the reaction mixture at a final concentration of 0.06 to 0.30 M or 0.03 to 0.24 M, respectively. Activated calf thymus DNA was used as template for host cell enzyme assays (▲, CyD1; O, NuD2P1; ●, CyD2P2), and poly(dA)·oligo$(dT)_{12\text{-}18}$ was used as template primer for the virus-induced enzyme (×, NuD2P3; ★, CyD2P3). The activity is expressed as relative activity using the activity obtained in the absence of KCl or $(NH_4)_2SO_4$ as 100% control.*

investigation is necessary before final conclusions can be made. Further examination of the sedimentation property of virus-induced enzyme is under way.

(vi) Effect of p-hydroxymercuribenzoate. Both virus-induced and host cell DNA polymerases are remarkably sensitive to *p*-hydroxymercuribenzoate, an inhibitor for enzymes with SH groups (Table 3). The polymerization activity of the virus-induced enzyme is almost completely inhibited in the presence of 0.25 mM *p*-hydroxymercuribenzoate. As compared with the virus-induced enzyme, one of the host cell enzymes, CyD1, is somewhat resistant to 0.25 M *p*-hydroxymercuribenzoate treatment, but its activity is almost completely suppressed when the drug concentration reaches 0.5 M.

(vii) Effect of 1,10-phenanthroline. One of the DNA polymerases of chicken embryos (18), the terminal deoxynucleotidyl transferase (4, 5), and some other DNA polymerases (16) have been demonstrated to contain Zn. We examined whether human cytomegalovirus-induced enzyme is also a zinc metalloenzyme.

The Zn-chelating compound 1,10-phenanthroline and its nonchelating derivative 1,7-phenanthroline were added to the reaction mixture to final concentrations of 0.25 to 2 mM (Fig. 8). In the presence of 2 mM 1,10-phenanthroline, the activity of virus-induced polymer-

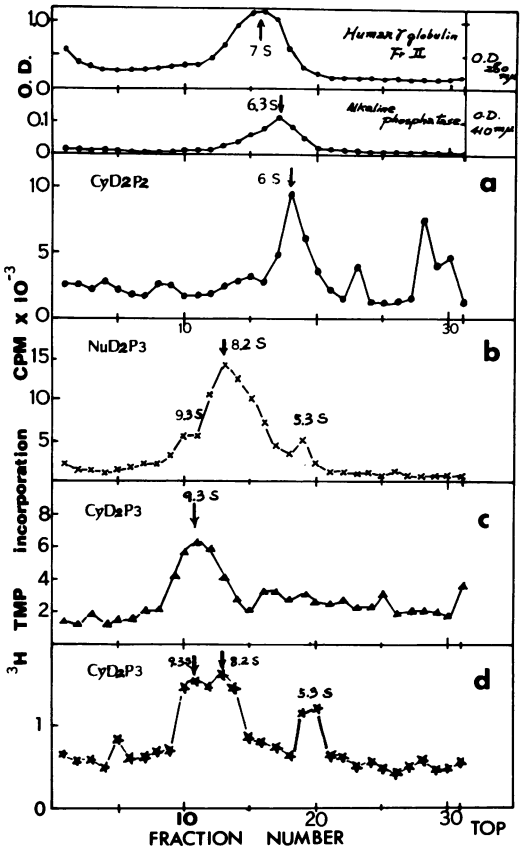

FIG. 7. *Glycerol density gradient centrifugation of human CMV-induced DNA polymerase. Virus-induced and host cell DNA polymerases obtained after phosphocellulose chromatography (in a volume of 0.3 ml) were dialyzed and centrifuged through linear 5 to 20% glycerol gradients in buffer D at 35,000 rpm in a Beckman SW50.1 rotor for 13.5 h at 2 C. The fractions (0.17 ml) were collected from the bottom and assayed for enzyme activities. Human gamma globulin fraction II (7S) and alkaline phosphatase (6.3S), centrifuged in a parallel tube, served as markers. A host cell DNA polymerase, CyD2P2, sedimented at 6S (a). (b and c) Sedimentation profiles of virus-induced enzymes isolated from the nucleus and cytoplasm, NuD2P3 and CyD2P3, respectively. (d) Sedimentation profile of the cytoplasmic virus-induced enzyme that has been subjected to 90% saturated ammonium sulfate precipitation and then redissolved and dialyzed.*

ase was reduced by 30 to 35% (Fig. 8c and d). However, the same degree of inhibition was found when the nonchelating 1,7-phenanthroline was used (Fig. 8c and d). Complete inhibition of a chicken embryo DNA polymerase, a Zn metalloenzyme, by 1,10-phenanthroline occurred at a concentration of 0.4 mM, whereas 1,7-phenanthroline did not inhibit the polymerase, according to Stavrianopoulos et al. (18). Thus, the slight inhibition of CMV-induced DNA polymerase by 1,10- and 1,7-phenanthroline could be due not to zinc chelation but rather to hydrophobic interactions of 1,10- and 1,7-phenanthroline with the enzyme (13). This observation suggests that human CMV-induced DNA polymerase might not be a zinc metalloenzyme. The degree of inhibition of a host cell enzyme, CyD1 (Fig. 8b), by 1,7-phenanthroline was even higher than with 1,10-phenanthroline; this indicates that CyD1 low-molecular-weight host DNA polymerase is also not a zinc metalloenzyme. Another host cell enzyme tested, CyD2P2, is much more sensitive to 1,10-phenanthroline than to the nonchelating 1,7-derivative; it might be a zinc metalloenzyme.

DISCUSSION

Infection of human fibroblastic cells by human CMV leads to the stimulation of the overall synthesis of host cell DNA polymerase as well as induction of a new virus-specific DNA polymerase. Contact-inhibited stationary-phase WI-38 cells that exhibit very low DNA polymerase activity in both cytoplasm and nuclei (Fig. 1 and 2) gain a tremendous ability to synthesize DNA polymerizing enzymes after

TABLE 3. *Inhibition of host cell and virus-induced DNA polymerases by p-hydroxymercuribenzoate*

Enzymes	Relative polymerization activity[a] (%)				
	0[b]	0.25	0.5	1	2
Host cell					
CyD1[c]	100 (13,263)	51.0 (6,764)	3.2 (424)	1.6 (212)	1.2 (159)
CyD2P1[d]	100 (5,310)	4.1 (217)	6.5 (345)	6.4 (340)	6.4 (339)
CyD2P2[d]	100 (6,690)	12.0 (803)	0 (0)	0 (0)	1.8 (120)
Virus induced					
CyD2P3[c]	100 (10,744)	1.1 (119)	2.3 (427)	0.9 (97)	1.1 (118)
NuD2P3[c]	100 (9,040)	1.0 (90)	0.5 (46)	2.5 (226)	1.9 (172)

[a] Polymerization activity obtained without *p*-hydroxymercuribenzoate treatment was used as 100% control. The number in parentheses represents counts per minute; background of 260 counts/min was subtracted.

[b] Concentration of *p*-hydroxymercuribenzoate (millimolar).

[c] Poly(dA)·oligo(dT)$_{12-18}$ was used as the template primer (system B).

[d] Activated calf thymus DNA was used as the template primer (system A).

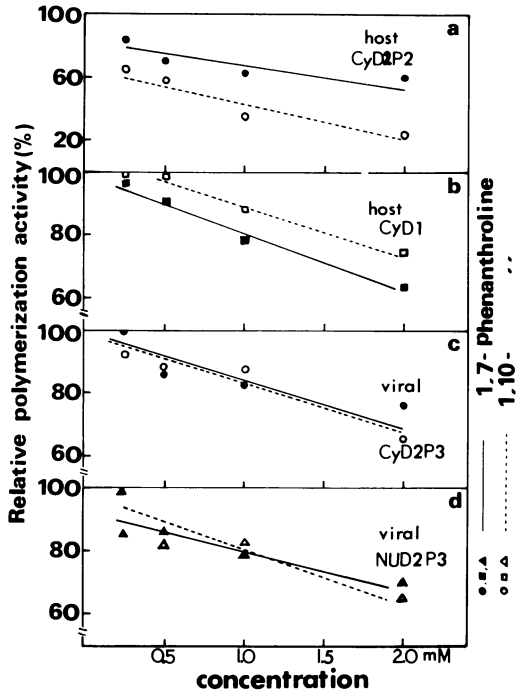

FIG. 8. *Effect of 1,10-phenanthroline and 1,7-phenanthroline on the polymerizing activities of virus-induced and host-cell DNA polymerases. The reaction and polymerization assays were performed as described in the text, except that 1,10-phenanthroline and its nonchelating 1,7-derivative were added to the reaction mixture at a final concentration of 0.25 to 2.0 mM. The activity is expressed as relative activity using the activity obtained in the absence of phenanthroline as 100% control. Activated calf thymus DNA was used as the template primer for the host cell enzyme (a and b), and poly(dA)·oligo(dT)$_{12-18}$ was used in virus-induced DNA polymerase assays (c and d). Symbols:* ○, □, △, *1,10-phenanthroline;* ●, ■, ▲, *1,7-phenanthroline.*

infection with human CMV. The stimulation patterns of host cell enzymes upon infection appeared no different in the cytoplasmic and nuclear fractions. Both low- (D1 enzyme, Fig. 3) and higher- (D2 enzyme, Fig. 3) molecular-weight enzymes were found distributed in parallel in the cytoplasm and nuclei of growing-phase and virus-infected stationary-phase cells.

This novel virus-induced DNA polymerase can be distinguished easily from uninfected host cell DNA polymerases by its different behavior in phosphocellulose chromatography, template specificity, salt sensitivity, and sedimentation property. Separation of the virus-induced DNA polymerase from host cell DNA polymerizing enzymes was accomplished by DEAE-cellulose and phosphocellulose chromatography. In the DEAE-cellulose column step, virus-induced enzyme and two of three host cell enzymes were eluted at salt concentrations between 0.13 and 0.25 M NaCl. In the phosphocellulose column, virus-induced enzyme eluted at 0.26 M, whereas host cell enzymes (D2P1, D2P2) eluted earlier at around 0.17 and 0.22 M NaCl, respectively. The elution property of CMV-induced DNA polymerase in the phosphocellulose column is somewhat different from that of herpes simplex virus and Marek's disease virus. In the herpes simplex system, virus-induced DNA polymerase eluted from phosphocellulose at a lower salt concentration (0.085 M KPO_4) than did the host cell enzyme (0.14 M KPO_4) (19). Marek's disease virus-induced DNA polymerase eluted from phosphocellulose at 0.2 M KCl, whereas two host cell enzymes eluted at higher ionic strengths: 0.3 and 0.5 M KCl, respectively (3).

CMV-induced DNA polymerase can efficiently use activated calf thymus DNA and synthetic deoxy oligomer-homopolymers, poly(dA)·oligo(dT)$_{12-18}$ and poly(dC)·oligo(dG)$_{12-18}$, as primer templates, but native and denatured calf thymus DNAs were relatively inactive. The efficiency of virus-induced enzyme with poly(dA)·oligo(dT)$_{12-18}$ as template primer is greater than with activated calf thymus DNA and poly(dC)·oligo(dG)$_{12-18}$. This property somewhat resembles that of one of the host cell enzymes CyD1 or NuD1 (low-molecular-weight enzyme). Similar phenomena were found in a herpes simplex system, except that in this case the virus-induced enzyme had a higher efficiency with poly(dC)·oligo(dG)$_{12-18}$. Marek's disease virus-induced DNA polymerase could not effectively use either poly(dA)·oligo(dT)$_{12-18}$ or poly(dC)·oligo(dG)$_{12-18}$ as template primer (3). No reverse transcriptase or terminal deoxynucleotidyl transferase-type activities can be detected in CMV-induced and Marek's disease virus-induced DNA polymerases (3).

Weissbach et al. (19) reported that the maximal activity of herpes simplex virus-induced DNA polymerase could be obtained by adding potassium sulfate to the reaction mixture to a final concentration of 100 to 200 mM. Human CMV-induced DNA polymerase activity can also be stimulated, up to twofold, by addition of KCl (final concentration of 0.06 to 0.12 M) or $(NH_4)_2SO_4$ (0.03 to 0.06 M) to the reaction mixture. However, a marked inhibition phenomenon was observed when the KCl concentration was higher than 0.12 M or that of $(NH_4)_2SO_4$ was higher than 0.06 M. Compared with CMV-induced DNA polymerase, WI-38 host cell enzymes appeared much more sensi-

tive to $(NH_4)_2SO_4$ inhibition. It is worth mentioning that this kind of salt stimulation effect does not appear in the Marek's disease virus-induced DNA polymerase system; 50% inhibition was observed even with the $(NH_4)_2SO_4$ concentration at a concentration as low as 0.02 M.

We have not examined the effect of omitting dithiothreitol from the reaction mixture. From the results with *p*-hydroxymercuribenzoate (Table 3), we have come to the conclusion that CMV-induced DNA polymerase might contain an —SH group(s), probably near an active site(s), that is essential for polymerizing activity. Host cell DNA polymerases are also sensitive to *p*-hydroxymercuribenzoate (Table 3). It is of interest that Marek's disease virus-DNA polymerase is somewhat resistant to *p*-hydroxymercuribenzoate even at a concentration of 1 mM, whereas the human CMV-induced enzyme activity is completely eliminated by 0.25 mM *p*-hydroxymercuribenzoate.

A low degree of inhibition of cytomegalovirus-induced DNA polymerase by both chelating and nonchelating phenanthrolines was found. The fact that the nonchelating 1,7-compound could inhibit the enzyme to the same extent as did the chelating 1,10-phenanthroline suggests that the inhibition is not due to the binding of 1,10-phenanthroline to zinc required for enzymatic activity but rather to a hydrophobic interaction (13).

Virus-induced enzymes isolated from nuclei (NuD2P3) and cytoplasm (CyD2P3) appear to be identical. They have similar template primer specificity, chromatographic behavior, and salt, Mg^{2+}, and sulfhydryl requirements for maximal activity. Although the enzyme isolated from nuclei, NuD2P3, has its major activity sedimenting at 8.2*S* and the enzyme from cytoplasm sedimented at 9.3*S*, the 8.2*S* enzyme can be generated from cytoplasmic 9.3*S* enzyme by subjecting it to high-salt $(NH_4)_2SO_4$ precipitation.

The facts of different chromatographic behavior, the efficiency of poly(dA)·oligo$(dT)_{12\text{-}18}$ and poly(dC)·oligo$(dG)_{12\text{-}18}$ as template primers, and the non-detectability in uninfected cells are all consistent with the hypothesis that this virus-induced DNA polymerase is coded by the virus genome. These observations do not, however, rule out the possibility that the appearance of this new DNA polymerase is due to the derepression of a host cell enzyme that is not detectable in normal cells or to a modification of a preexisting host enzyme as a result of virus infection.

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