Marek's Disease Herpesvirus-Induced DNA Polymerase

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Infection of duck embryo fibroblasts by Marek's disease herpesvirus (MDHV), strain GA, led to the induction of a novel DNA polymerase. This novel DNA polymerase, designated MDHV-induced DNA polymerase, could be distinguished from the DNA polymerase activities of uninfected duck embryo fibroblasts by its chromatographic behavior on phosphocellulose, by its sedimentation coefficient, and by its catalytic properties. The characteristics of MDHV-induced DNA polymerase which had been purified by phosphocellulose chromatography were investigated. The sedimentation coefficient of the enzyme, as determined by sucrose density gradient centrifugation in the presence of 0.25 M KCl, was 5.9S. From this sedimentation coefficient, the molecular weight of MDHV-induced DNA polymerase was estimated to be 100,000. MDHV-induced DNA polymerase could not effectively use either poly(dA)·oligo(dT)₁₂₋₁₈ or poly(dC)·oligo(dG)₁₂₋₁₈ as a template-primer. The DNA polymerases from uninfected duck embryo fibroblasts could use these synthetic template-primers. MDHV-induced DNA polymerase also could not use poly(rA)·oligo(dT)₁₂₋₁₈ or poly(rC)·oligo(dG)₁₂₋₁₈ as template-primers or oligo(dT)₁₂₋₁₈ as a primer, indicating that it was not a polymerase of the type R-DNA polymerase, reverse transcriptase, or a terminal nucleotidyl transferase. In vitro synthesis of DNA by MDHV-induced DNA polymerase was markedly inhibited by the addition of (NH₄)₂SO₄ to the reaction mixture.

Marek's disease is a highly contagious malignant lymphoma of chickens (2, 4, 22). Its etiological agent is a herpesvirus (10, 25, 31). Marek's disease was the first neoplasm that was shown unequivocally to be caused by a herpesvirus and the first neoplasm for which a vaccine was developed (26). In the acute form of Marek's disease, lymphoid tumors develop in the abdominal organs and in the muscles and skin.

Marek's disease herpesvirus (MDHV) is usually grown in culture in chicken kidney cells, chicken embryo fibroblasts, or duck embryo fibroblasts (3, 24). Viral infectivity is cell-associated and infectious virus particles are not recovered from the culture medium. The spread of virus in cultured cells probably is achieved by transfer of the virus from cell to cell via intracellular bridges (16). Cytopathic changes occur in the cultured cells infected with MDHV. Plaques containing both small, rounded, refractile cells and giant multinucleated cells are produced.

Eukaryotic cells contain two main DNA polymerase activities. They are a high-molecular-weight DNA-dependent DNA polymerase (6 to 8S) found in the cytoplasm and a low-molecular-

weight DNA-dependent DNA polymerase (3 to 3.5S) found both in the nucleus and in the cytoplasm (6, 8, 9, 29, 30, 35, 37). In addition to these DNA-dependent DNA polymerase activities, eukaryotic cells contain a DNA polymerase, referred to as R-DNA polymerase, which; under certain assay conditions, prefers poly(rA)·oligo(dT) to activated DNA as a template-primer (5, 12, 13, 21). Another polymerase, terminal deoxyribonucleotidyl transferase, is found only in thymus cells (7). Finally, mitochondria of eukaryotic cells contain a distinct DNA polymerase (14, 33).

Infection of a eukaryotic cell by a DNA virus may lead to the induction of a novel DNA polymerase. Viral-induced DNA polymerases have been detected in cells infected by herpes simplex virus I (18, 19, 20, 34) and by vaccinia virus (11). In this paper we wish to report that infection of duck embryo fibroblasts by MDHV, strain GA, led to the induction of a novel DNA polymerase. The novel DNA polymerase, designated MDHV-induced DNA polymerase, was distinguished from the DNA polymerase activities of uninfected duck embryo fibroblasts by its

chromatographic behavior on phosphocellulose, by its sedimentation coefficient, and by its catalytic properties.

MATERIALS AND METHODS

Reagents. The unlabeled deoxyribonucleoside triphosphates, calf thymus DNA, and the synthetic polynucleotide-oligonucleotides were purchased from P-L Biochemicals. Activated calf thymus DNA (DNase I treated) was prepared by the procedure described by Schlabach et al. (28). [^3H]dTTP and [^3H]dGTP were from ICN Pharmaceuticals and [^3H]dCTP was from Schwarz/Mann. Whatman phosphocellulose (P-11) was obtained from Reeve Angel. *Escherichia coli* alkaline phosphatase, beef heart lactate dehydrogenase, and bovine serum albumin were from Sigma.

Preparation and growth of duck embryo fibroblasts and infection with MDHV strain GA. Duck embryo fibroblasts were prepared from 13-day-old embryos. The embryos were decapitated, washed with a sterile solution of phosphate-buffered saline, macerated, and trypsin-treated as described by Solomon et al. (31). The cells were suspended in culture medium at a concentration of 10^4 per ml, and 20-ml samples of the suspension were seeded onto 150 mm-diameter Falcon plastic tissue-culture dishes. The culture medium consisted of 40 volumes of medium 199, 50 volumes of nutrient medium F10, 5 volumes of tryptose phosphate broth, 3 volumes of 2.8% sodium bicarbonate, 4 volumes of inactivated calf serum, 100 units of penicillin per ml, and 100 μg of streptomycin per ml (36). Cultures were incubated at 37 C in a humidified atmosphere containing 5% carbon dioxide. After 48 h of incubation, the culture medium was removed. For the preparation of uninfected cells, 20 ml of fresh culture medium was added to each of the dishes and incubation was continued for another 24 h. The cells were then scraped from the tissue-culture dishes, washed with phosphate-buffered saline, quick-frozen, and stored at -80°C . For the preparation of infected cells, 20 ml of fresh culture medium containing MDHV-infected duck embryo fibroblasts at a concentration of 2×10^4 per ml (2×10^4 PFU per ml) was added to the dishes. After 24 h of incubation, the culture medium was replaced. Twenty-four hours later, the cytopathic effect due to viral infection of the cells was confluent. The infected cells were then scraped from the tissue culture dishes, washed with phosphate-buffered saline, quick-frozen, and stored at -80°C .

Cell fractionation. Frozen uninfected and MDHV-infected duck embryo fibroblasts (0.3 to 1.0 g wet weight) were suspended in buffer A (50 mM Tris-hydrochloride (pH 7.5), 25 mM KCl, 5 mM MgCl_2 , and 0.25 M sucrose). Cytoplasmic and nuclear fractions were prepared by a modification of the procedure described by Chang et al. (9). A cell homogenate was prepared using a Dounce homogenizer and then centrifuged at $1,000 \times g$ for 5 min. The supernatant fraction was made 5 mM in 2-mercaptoethanol, and centrifuged first at $10,000 \times g$ for 10 min and then at $100,000 \times g$ for 60 min. The supernatant fraction from the $100,000 \times g$ centrifugation was termed the cyto-

plasmic fraction. The nuclear pellet from the $1,000 \times g$ centrifugation step was suspended in buffer A, centrifuged, and then resuspended in buffer A containing 1% Triton X-100 (vol/vol). After 15 min at 0 C, the nuclei were collected by centrifugation and washed three times with buffer A. The final nuclear pellet was suspended in buffer A containing 5 mM 2-mercaptoethanol and 0.2 M potassium phosphate (pH 7.5). After gently stirring for 30 min at 0 C to extract the nuclear DNA polymerase activities, the suspension was centrifuged at $100,000 \times g$ for 60 min. The resulting supernatant fraction was made 2 mg per ml in bovine serum albumin and termed the nuclear fraction. The cytoplasmic and nuclear fractions were dialyzed, assayed for DNA polymerase, and then analyzed by sucrose density gradient centrifugation or by phosphocellulose chromatography.

Sucrose density gradient centrifugation. DNA polymerase fractions to be analyzed by sucrose density gradient centrifugation were dialyzed against a buffered solution containing 50 mM Tris-hydrochloride (pH 7.5), 250 mM KCl, and 5 mM 2-mercaptoethanol. Samples (0.2 ml) were layered on 4.8-ml linear 5 to 20% sucrose gradients prepared in the above buffered solution. Centrifugation was at 48,000 rpm for 11 h at 4 C in a Beckman SW 50.1 rotor. After centrifugation, fractions (0.16 ml) were collected from the bottom of the centrifuge tube. Samples (25 μliters) of the sucrose density gradient fractions were assayed for DNA polymerase activity in the reaction mixture with activated DNA. *E. coli* alkaline phosphatase (6.3S) (15) and beef heart lactate dehydrogenase (7.4S) (17) were used as reference proteins for the determinations of the sedimentation coefficients of the DNA polymerases. Alkaline phosphatase was assayed spectrophotometrically at 410 nm by using the substrate *p*-nitrophenyl phosphate. Lactate dehydrogenase was assayed spectrophotometrically at 340 nm by using the substrates NADH and pyruvate.

Chromatography on phosphocellulose. Acid- and base-washed phosphocellulose (P-11) was poured into columns (0.6 by 7.2 cm) and equilibrated with column buffer containing 25 mM Tris-hydrochloride (pH 8), 100 mM KCl, 5 mM 2-mercaptoethanol, and 20% (vol/vol) glycerol. The columns were washed first with 5 ml of column buffer containing 1 mg of bovine serum albumin per ml and then with 20 to 30 ml of column buffer. If the phosphocellulose columns were not washed with the solution of bovine serum albumin, recoveries of DNA polymerase activities after chromatography were about 5%. With the bovine serum albumin washes, recoveries were increased to 75 to 100%. The nuclear fractions and the cytoplasmic fractions of uninfected and MDHV-infected duck embryo fibroblasts were dialyzed against the column buffer and applied to the phosphocellulose columns. All of the DNA polymerase activities applied to the columns were adsorbed. After washing the columns with 8 to 10 ml of column buffer, DNA polymerase activities were eluted with 30-ml gradients of KCl (0.1 to 1 M) in column buffer. Fractions of 0.8 to 1.0 ml were collected. KCl concentrations in the fractions were determined from conductivity measurements. Samples (25 μliters) of the fractions were assayed for

DNA polymerase activity in the reaction mixture with activated DNA.

Assay of DNA polymerase. For the activated DNA directed synthesis of DNA, the reaction mixtures contained, in 200 μ liters: 50 mM Tris-hydrochloride (pH 8), 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.6 mM 2-mercaptoethanol, 25 to 50 mM KCl, 500 μ g of bovine serum albumin per ml, 200 μ g of activated calf thymus DNA per ml, 100 μ M each dATP and dCTP, either 100 μ M dGTP and 20 μ M [^3H]dTTP or 100 μ M dTTP and 20 μ M [^3H]dGTP, and DNA polymerase. The specific radioactivities of the ^3H -labeled deoxyribonucleoside triphosphates were from 400 to 1,000 counts/min per pmol.

For the polymerization of [^3H]dTTP or [^3H]dGTP directed by the synthetic polynucleotides-oligonucleotides, the reaction mixtures contained, in 200 μ liters: 50 mM Tris-hydrochloride (pH 8), 0.5 mM $MnCl_2$, 1 mM dithiothreitol, 0.6 mM 2-mercaptoethanol, 25 mM KCl, 20 μ M [^3H]dTTP or [^3H]dGTP (400 to 1,000 counts/min per pmol), 500 μ g of bovine serum albumin per ml, 2 optical density units at 260 nm per ml of the synthetic polynucleotides-oligonucleotides, and DNA polymerase.

Incubation was at 37 C for 30 min. The reaction was terminated by addition of 2 ml of a cold solution of 10% trichloroacetic acid-1% sodium pyrophosphate. Bovine serum albumin, 50 μ liters of a 5 mg per ml solution, was then added as carrier. After 5 min at 0 C, the mixture was centrifuged at $10,000 \times g$ for 2 min. The supernatant solution was removed and discarded. The pellet was dissolved in 350 μ liters of cold 0.5 N NaOH. After the addition of cold trichloroacetic acid-sodium pyrophosphate and incubation at 0 C for 5 min, the precipitated material was collected on a nitrocellulose membrane filter. The filter was washed, dried, and monitored for radioactivity by liquid scintillation spectrometry.

For the studies reported here, the amounts of DNA polymerase added to the reaction mixtures were within the activity range which gave direct proportionality with the rate of DNA synthesis. One unit of DNA polymerase activity was defined as that amount which, when added to the standard reaction mixture with activated DNA, gave 1 nmol of [^3H]dGMP incorporated into DNA in 30 min at 37 C.

RESULTS

DNA polymerase activities of uninfected duck embryo fibroblasts. The cytoplasmic and nuclear fractions of uninfected duck embryo fibroblasts contained, respectively, 10 and 5 units of DNA polymerase activity per g (wet weight) of cells. The individual DNA polymerase activities present in each of the fractions were resolved by sucrose density gradient centrifugation. As shown in Fig. 1, the nuclear fraction contained a DNA polymerase activity that sedimented at 7.3S and one that sedimented at 3.1S. The cytoplasmic fraction contained DNA polymerase activities that sedimented at 8 and 3.1S.

The characteristics of the activated DNA-directed synthesis of DNA by the cytoplasmic 8S, the nuclear 7.3S, and the nuclear 3.1S DNA polymerase activities were examined. Since the catalytic properties of the nuclear and cytoplasmic 3.0 to 3.5S DNA polymerases from a variety of cells are identical (6, 8), the characteristics of the nuclear 3.1S polymerase only, rather than those of both, were examined. As shown in Table 1, for the three polymerases, if enzyme, activated DNA or Mg^{2+} were omitted from the reaction mixture, no DNA synthesis was observed. For the cytoplasmic 8S and the nuclear 3.1S polymerases, 0.5 mM Mn^{2+} was 22 and 7%, respectively, as effective as 10 mM Mg^{2+} . Incorporation of dGMP decreased for the cytoplasmic 8S polymerase and increased slightly for the nuclear 3.1S polymerase when dATP,

FIG. 1. Sucrose density gradient centrifugation analysis of the DNA polymerase activities of uninfected duck embryo fibroblasts. Samples of the nuclear fraction (upper panel) and the cytoplasmic fraction (lower panel) of uninfected duck embryo fibroblasts were analyzed by sucrose density gradient centrifugation. Sixty-two percent of the DNA polymerase activity of the nuclear fraction that was applied to the sucrose density gradient was recovered in the sucrose density gradient fractions after centrifugation. One hundred percent of the DNA polymerase activity of the cytoplasmic fraction was recovered. Centrifugation was from right to left. The arrow indicates the position of the reference protein alkaline phosphatase (AP) which was centrifuged in a parallel sucrose density gradient.

dCTP, and dTTP were omitted from the reaction mixtures. All three DNA polymerase activities required dithiothreitol for maximal enzyme activity. When the sulfhydryl reagent *p*-hydroxymercuribenzoate was added to the reaction mixture, DNA synthesis by the cytoplasmic 8S and the nuclear 7.3S polymerase decreased markedly, while that by the nuclear 3.1S polymerase decreased only slightly. Finally, the activities of all three polymerases were inhibited by the addition of 50 mM $(\text{NH}_4)_2\text{SO}_4$ to the reaction mixtures. The activities of the cytoplasmic 8S and the nuclear 7.3S polymerases were inhibited, however, to a greater extent by the $(\text{NH}_4)_2\text{SO}_4$ addition than was that of the nuclear 3.1S polymerase.

The results of a study of the template-primer specificities for the three DNA polymerases are

presented in Table 2. All three DNA polymerases were active in the poly(dA)·oligo(dT)₁₂₋₁₈-directed polymerization of dTTP, and to a lesser extent in the poly(dC)·oligo(dG)₁₂₋₁₈-directed polymerization of dGTP. For the cytoplasmic 8S polymerase and the nuclear 3.1S polymerase, the two polymerases tested, little activity was observed in the poly(rA)·oligo(dT)₁₂₋₁₈-directed polymerization of dTTP. No activity was observed in the oligo(dT)₁₂₋₁₈-primed polymerization of dTTP or in the poly(rC)·oligo(dG)₁₂₋₁₈-directed polymerization of dGTP.

The DNA polymerase activities of the cytoplasmic and nuclear fractions of uninfected duck embryo fibroblasts were also resolved by chromatography on phosphocellulose (Fig. 2). For the nuclear fraction, two DNA polymerase

TABLE 1. *Characteristics of the activated DNA-directed synthesis of DNA by the cytoplasmic 8S, the nuclear 7.3S, and the nuclear 3.1S DNA polymerase activities of uninfected duck embryo fibroblasts*

Composition of the reaction mixtures ^a	Relative incorporation of [³ H]dGMP by		
	Cytoplasmic 8S polymerase	Nuclear 7.3S polymerase	Nuclear 3.1S polymerase
Complete	100 (48)	100 (3)	100 (7)
Minus enzyme	0	0	0
Minus activated DNA	0	0	0
Minus Mg ²⁺	0	0	0
Minus Mg ²⁺ , plus 0.5 mM Mn ²⁺	22		7
Minus dATP, dCTP, dTTP	42		110
Minus dithiothreitol	21	25	63
Plus 1 mM <i>p</i> -hydroxymercuribenzoate	2	7	79
Plus 50 mM $(\text{NH}_4)_2\text{SO}_4$	30	30	52

^aThe composition of the complete reaction mixture was described in Materials and Methods. The DNA polymerase activities used for these experiments were prepared by sucrose density gradient centrifugation. For each DNA polymerase, the picomoles of [³H]dGMP incorporated into DNA after 30 min of incubation are given in the parentheses.

TABLE 2. *Template-primer specificities of the cytoplasmic 8S, the nuclear 7.3S, and the nuclear 3.1S DNA polymerase activities of uninfected duck embryo fibroblasts^a*

Template-primer	d-Nucleoside triphosphate substrate	Relative incorporation of [³ H]d-nucleoside monophosphate by		
		Cytoplasmic 8S polymerase	Nuclear 7.3S polymerase	Nuclear 3.1S polymerase
Activated DNA	[³ H]dGTP, dTTP, dCTP, dATP	100 (48)	100 (3)	100 (7)
Poly(dA)·oligo(dT) ₁₂₋₁₈	[³ H]dTTP	370	330	480
Poly(rA)·oligo(dT) ₁₂₋₁₈	[³ H]dTTP	5		2
Oligo(dT) ₁₂₋₁₈	[³ H]dTTP	0		0
Poly(dC)·oligo(dG) ₁₂₋₁₈	[³ H]dGTP	130	170	20
Poly(rC)·oligo(dG) ₁₂₋₁₈	[³ H]dGTP	0		0

^aThe compositions of the reaction mixtures were described in Materials and Methods. The DNA polymerase activities used for these experiments were prepared by sucrose density gradient centrifugation. For each DNA polymerase, the picomoles of [³H]d-nucleoside monophosphate incorporated into DNA after 30 min of incubation are given in the parentheses.

activities were resolved. One polymerase activity, which corresponded in relative amount and in catalytic properties to the nuclear 7.3S polymerase, eluted from the phosphocellulose column at about 0.3 M KCl. The other DNA polymerase activity which corresponded to the nuclear 3.1S polymerase eluted at about 0.5 M KCl. For the cytoplasmic fraction, DNA polymerase activities which eluted at about 0.3 M and 0.5 M KCl and which corresponded to the cytoplasmic 8 and 3.1S polymerase activities, respectively, were resolved.

DNA polymerase activities of MDHV-infected duck embryo fibroblasts. The cytoplasmic and nuclear fractions of MDHV-infected duck embryo fibroblasts contained, respectively, 20 and 15 units of DNA polymerase activity per g (wet weight) of cells. These activities were higher than those found in the corresponding fractions of the uninfected cells. The DNA polymerase activities of the two fractions from the infected cells were resolved by chromatography on phosphocellulose (Fig. 3). For the nuclear fraction, three DNA polymerase activities were resolved. One polymerase activity eluted from the phosphocellulose column at about 0.2 M KCl. A second one eluted at about 0.3 M KCl and a third one at about 0.5 M KCl. No polymerase activity which eluted at 0.2 M KCl was detected in either the nuclear fraction or the cytoplasmic fraction of uninfected duck embryo fibroblasts (see Fig. 2). This polymerase activity was, therefore, unique to MDHV-infected duck embryo fibroblasts and,

accordingly, was designated MDHV-induced DNA polymerase. A DNA polymerase activity eluting at about 0.2 M KCl was also detected in the cytoplasmic fraction of the infected cells (Fig. 3). Subsequent experiments have shown that the properties of this polymerase activity and that of MDHV-induced DNA polymerase of the nuclear fraction were identical. Consequently, only those experiments dealing with MDHV-induced DNA polymerase from the nuclear fraction are reported.

From the results of the experiment reported in Fig. 3, the total amount of MDHV-induced DNA polymerase activity in the nuclear and cytoplasmic fractions of MDHV-infected duck embryo fibroblasts was estimated to be about 15 units per g (wet weight) of cells. About one half of this amount was detected in each of the fractions. Again, from the results of the experiment reported in Fig. 3 dealing with the nuclear fraction, it was estimated that there were approximately 5 and 3 units of activity per g (wet weight) of cells, respectively, for the DNA polymerase activities that eluted from the phosphocellulose column at 0.3 M KCl and 0.5 M KCl. The nuclear fraction of uninfected duck embryo fibroblasts contained about 2 and 3 units of activity per g (wet weight) of cells, respectively, for the polymerase activities that eluted at 0.3 M KCl and 0.5 M KCl (see Fig. 2). Thus, although the nuclear fraction of infected cells contained the same amount of the DNA polymerase that eluted at 0.5 M KCl as did the nuclear fraction of uninfected cells, it contained

FIG. 2. Phosphocellulose chromatography of the DNA polymerase activities of uninfected duck embryo fibroblasts. The nuclear fraction (left panel) and the cytoplasmic fraction (right panel) of uninfected duck embryo fibroblasts (0.3 g wet weight of cells) were chromatographed on phosphocellulose. DNA polymerase activity (●) and KCl concentration (■) were measured. All of the DNA polymerase activities applied to the columns were adsorbed. Seventy-six percent of the DNA polymerase activity of the nuclear fraction was recovered after chromatography. Eighty-eight percent of the DNA polymerase of the cytoplasmic fraction was recovered.

FIG. 3. Phosphocellulose chromatography of the DNA polymerase activities of MDHV-infected duck embryo fibroblasts. The nuclear fraction (left panel) and one-half of the cytoplasmic fraction (right panel) of MDHV-infected duck embryo fibroblasts (0.85 g wet weight of cells) were chromatographed on phosphocellulose. DNA polymerase activity (●) and KCl concentration (■) were measured. All of the DNA polymerase activities applied to the columns were adsorbed. Seventy-four percent and 100% of the DNA polymerase activities of the nuclear fraction and the cytoplasmic fraction, respectively, were recovered after chromatography.

2.5 times more of the DNA polymerase activity that eluted from the phosphocellulose column at 0.3 M KCl.

Sucrose density gradient centrifugation of MDHV-induced DNA polymerase. A sample of MDHV-induced DNA polymerase from the phosphocellulose column was analyzed by sucrose density gradient centrifugation. As shown in Fig. 4, MDHV-induced DNA polymerase sedimented as a single symmetrical peak. By using as reference proteins *E. coli* alkaline phosphatase and beef heart lactate dehydrogenase, a sedimentation coefficient of 5.9S was determined for MDHV-induced DNA polymerase. From a sedimentation coefficient of 5.9S, the molecular weight of MDHV-induced DNA polymerase was estimated to be 100,000 (23).

The DNA polymerase activities of the nuclear fraction of MDHV-infected duck embryo fibroblasts which eluted from the phosphocellulose column at 0.3 M KCl and at 0.5 M KCl were also analyzed by sucrose density gradient centrifugation. The results are presented in Fig. 5. The DNA polymerase activity which eluted from the phosphocellulose column at 0.3 M KCl had a sedimentation coefficient of 7.3S and the polymerase activity which eluted at 0.5 M KCl had a sedimentation coefficient of 3.1S. Thus, these two DNA polymerase activities corresponded in chromatographic behavior on phosphocellulose and in sedimentation velocity characteristics to the two DNA polymerases found in the nuclear fraction of uninfected duck embryo fibroblasts (see Fig. 1 and 2).

Characteristics of the activated DNA-

directed synthesis of DNA by MDHV-induced DNA polymerase. The characteristics of the activated DNA-directed synthesis by MDHV-induced DNA polymerase and, for comparison, those of the nuclear 7.3 and 3.1S polymerases from infected duck embryo fibro-

FIG. 4. Sucrose density gradient centrifugation of MDHV-induced DNA polymerase. A sample (0.2 ml) of MDHV-induced DNA polymerase of the peak fraction of a phosphocellulose column was made 1.3 mg/ml in bovine serum albumin, dialyzed, and centrifuged through a sucrose gradient. Sucrose density gradient centrifugation was as described in Materials and Methods except that the buffer used to make the sucrose gradient also contained 0.5 mg of bovine serum albumin per ml. Sixty percent of the DNA polymerase activity was recovered after centrifugation. Centrifugation was from right to left. The arrows indicate the positions of the reference proteins alkaline phosphatase (AP) and lactate dehydrogenase (LDH) which were centrifuged in a parallel gradient.

FIG. 5. Sucrose density gradient centrifugation of the DNA polymerase activities of the nuclear fraction of MDHV-infected duck embryo fibroblasts which eluted from the phosphocellulose column at 0.3 M KCl and at 0.5 M KCl. Samples (0.2 ml) of the peak fractions from a phosphocellulose column for the two DNA polymerase activities were made 1.3 mg/ml in bovine serum albumin, dialyzed, and centrifuged through sucrose gradients. Sucrose density gradient centrifugation was as described in Materials and Methods except that the buffer used to make the sucrose gradients also contained 0.1 mg of bovine serum albumin per ml. The sedimentation profile of the nuclear DNA polymerase activity which eluted from the phosphocellulose column at 0.3 M KCl is presented in the upper panel and that for the DNA polymerase activity that eluted at 0.5 M KCl is

blasts were studied. The DNA polymerase activities used in these studies and in the subsequent studies were prepared by phosphocellulose chromatography. As shown in Table 3, for the three polymerases, no DNA synthesis was detected if enzyme, activated DNA or Mg^{2+} ions were omitted from the reaction mixtures. Mn^{2+} , at a concentration of 0.5 mM, was 17% as effective as 10 mM Mg^{2+} for MDHV-induced DNA polymerase and 22 and 8% as effective, respectively, for the 7.3S and 3.1S polymerases. When dATP, dCTP, and dTTP were omitted from the reaction mixtures, dGMP incorporation decreased for the MDHV-induced DNA polymerase and for the 7.3S polymerase, but increased slightly for the 3.1S polymerase. If dithiothreitol was not added to the reaction mixtures, DNA synthesis by the 7.3S and by the 3.1S polymerase, but not by MDHV-induced DNA polymerase, decreased. The activities of MDHV-induced DNA polymerase and the 3.1S polymerase were more resistant to inhibition by *p*-hydroxymercuribenzoate than was that of the 7.3S polymerase.

The K_m value for dGTP for MDHV-induced DNA polymerase was determined. For this determination, the concentrations of dATP, dCTP, and dTTP were fixed at 100 μ M, and the concentration of dGTP was varied. DNA synthesis was measured as dGMP incorporation. In Fig. 6, the data for MDHV-induced DNA po-

presented in the lower panel. Forty percent of each of the DNA polymerase activities was recovered after centrifugation. Centrifugation was from right to left. The arrow indicates the position of the reference protein alkaline phosphatase (AP) which was centrifuged in a parallel gradient.

TABLE 3. Characteristics of the activated DNA-directed synthesis of DNA by MDHV-induced DNA polymerase, and by the 7.3S DNA polymerase and the 3.1S DNA polymerase activities of the nuclear fraction of MDHV-infected duck embryo fibroblasts

Composition of reaction mixtures ^a	Relative incorporation of [³ H]dGMP by		
	MDHV-induced DNA polymerase	7.3S polymerase	3.1S polymerase
Complete	100 (49)	100 (14)	100 (9)
Minus enzyme	0	0	0
Minus activated DNA	0	0	0
Minus Mg^{2+}	0	0	0
Minus Mg^{2+} , plus 0.5 mM Mn^{2+}	17	22	8
Minus dATP, dCTP, dTTP	24	40	105
Minus dithiothreitol	100	46	78
Plus 1.0 mM <i>p</i> -hydroxymercuribenzoate	96	25	85

^aThe composition of the complete reaction mixture was described in Materials and Methods. The DNA polymerase activities used for these experiments were from the phosphocellulose column. For each DNA polymerase, the picomoles of [³H]dGMP incorporated into DNA after 30 min are given in the parentheses.

lymerase are presented in the form of a Lineweaver-Burk double reciprocal plot. An apparent K_m of 5 μM was determined. K_m values for dGTP for the nuclear 7.3 and 3.1S polymerases from infected duck embryo fibroblasts, and the cytoplasmic 8S and nuclear 3.1S polymerases from uninfected cells, determined in a similar manner, were all between 5 and 10 μM . Thus, the K_m value for dGTP for MDHV-induced DNA polymerase did not vary significantly from those of the other DNA polymerases.

The activated DNA-directed synthesis of DNA by herpes simplex virus-induced DNA polymerase was optimal when 150 mM Na_2SO_4 or $(\text{NH}_4)_2\text{SO}_4$ was added to the reaction mixture (34). The DNA polymerase was 25- to 50-fold more active in the presence of the added salt than in its absence. Accordingly, the effect of $(\text{NH}_4)_2\text{SO}_4$ on the activity of MDHV-induced DNA polymerase was examined. As shown in Fig. 7, the activity of MDHV-induced DNA polymerase was markedly inhibited by the addition of $(\text{NH}_4)_2\text{SO}_4$ to the reaction mixture. Fifty percent inhibition was observed at a concentration of about 20 mM. The activities of the nuclear 7.3 and 3.1S polymerases were also inhibited by $(\text{NH}_4)_2\text{SO}_4$, but less markedly. Fifty percent inhibition was observed at 80 mM $(\text{NH}_4)_2\text{SO}_4$ for the 7.3S enzyme and at 45 mM for the 3.1S enzyme.

Template-primer specificity of MDHV-induced DNA polymerase. The template-primer specificity of MDHV-induced DNA polymerase, and those for the nuclear 7.3 and 3.1S polymerases of infected duck embryo fibro-

FIG. 6. Lineweaver-Burk plot of the effect of varying the concentration of dGTP on the activity of MDHV-induced DNA polymerase. The reaction mixtures with activated DNA contained 100 μM each of dATP, dCTP, and dTTP and various concentrations of $[^3\text{H}]\text{dGTP}$. Incorporation of $[^3\text{H}]\text{dGMP}$ into DNA was measured after 30 min of incubation. The data is plotted as a double reciprocal Lineweaver-Burk plot.

FIG. 7. Effects of $(\text{NH}_4)_2\text{SO}_4$ on the activity of MDHV-induced DNA polymerase, and of the 7.3S DNA polymerase and the 3.1S DNA polymerase of the nuclear fraction of MDHV-infected duck embryo fibroblasts. The effects of $(\text{NH}_4)_2\text{SO}_4$ on the activities of MDHV-induced DNA polymerase (●), the nuclear 7.3S DNA polymerase (▲) and the nuclear 3.1S DNA polymerase (■) were measured. DNA polymerase activity of 100% corresponded to the incorporation of 23, 8, and 5 pmol of $[^3\text{H}]\text{dTTP}$ in 30 min for MDHV-induced DNA polymerase, the nuclear 7.3S DNA polymerase, and the nuclear 3.1S DNA polymerase, respectively.

blasts, were examined (Table 4). MDHV-induced DNA did not use effectively as template-primers either poly(dA)·oligo(dT)₁₂₋₁₈ or poly(dC)·oligo(dG)₁₂₋₁₈. Both the 7.3S and the 3.1S polymerases used these synthetic template-primers, but to varying degrees. None of the polymerases used poly(rA)·oligo(dT)₁₂₋₁₈ or poly(rC)·oligo(dG)₁₂₋₁₈ effectively as template-primers, or oligo(dT)₁₂₋₁₈ as a primer. Thus, MDHV-induced DNA polymerase, like the 7.3 and 3.1S polymerases, is not a polymerase of the type R-DNA polymerase (5, 12), reverse transcriptase (1, 32), or terminal nucleotidyl transferase (7, 37).

The reaction of MDHV-induced DNA polymerase with poly(dA)·oligo(dT)₁₂₋₁₈ was studied in more detail. As reported by Brun et al. (6) and Rougeon et al. (27), DNA polymerase activity on synthetic polynucleotides is markedly influenced by the ionic strength of the reaction mixture. Accordingly, $[^3\text{H}]\text{dTTP}$ incorporation with poly(dA)·oligo(dT)₁₂₋₁₈ was examined in reaction mixtures which contained

TABLE 4. *Template-primer specificities of MDHV-induced DNA polymerase, and of the 7.3S DNA polymerase and the 3.1S DNA polymerase activities of the nuclear fraction of MDHV-infected duck embryo fibroblasts^a*

Template-primer	d-Nucleoside triphosphate substrate	Relative incorporation of [³ H]-nucleoside monophosphate by		
		MDHV-induced DNA polymerase	7.3S DNA polymerase	3.1S DNA polymerase
Activated DNA	[³ H]dTTP, dGTP, dCTP, dATP	100 (41)	100 (12)	100 (7)
Poly(dA)·oligo(dT) ₁₂₋₁₈	[³ H]dTTP	3	76	672
Poly(rA)·oligo(dT) ₁₂₋₁₈	[³ H]dTTP	1	5	15
Activated DNA	[³ H]dGTP, dTTP, dCTP, dATP	100 (40)	100 (16)	100 (9)
Poly(dC)·oligo(dG) ₁₂₋₁₈	[³ H]dGTP	1	43	50
Poly(rC)·oligo(dG) ₁₂₋₁₈	[³ H]dGTP	0	0	1
Oligo(dT) ₁₂₋₁₈	[³ H]dGTP	0	0	2

^aThe compositions of the reaction mixtures were described in Materials and Methods. The DNA polymerase activities used for these experiments were from the phosphocellulose column. For each DNA polymerase, the picomoles of [³H]-nucleoside monophosphate incorporated into DNA after 30 min are given in the parentheses.

KCl in concentrations ranging from 25 to 200 mM. No increase in [³H]TTP incorporation over that low level of activity observed at 25 mM KCl (as in Table 4) was observed. In fact, this low level of activity was reduced with increasing KCl concentrations. Mg²⁺ ions at 10 mM produced the same low level of activity as did 0.5 mM Mn²⁺ ions. Finally, MDHV-induced DNA polymerase apparently did not contain an inhibitor, such as a nuclease or a phosphatase, which might block the poly(dA)·oligo(dT)₁₂₋₁₈-directed polymerization of TTP. When MDHV-induced DNA polymerase was mixed with the 3.1S polymerase, the amount of [³H]-TTP incorporation observed was not reduced over that observed with the 3.1S DNA polymerase alone. The inability of MDHV-induced DNA polymerase to effectively use as template-primers either poly(dA)·oligo(dT)₁₂₋₁₈ or poly(dC)·oligo(dG)₁₂₋₁₈ can be used to distinguish this polymerase not only from the nuclear 7.3S and 3.1S polymerase of infected cells (see Table 4) but also from the DNA polymerases of the uninfected cells (see Table 2).

DISCUSSION

Infection of duck embryo fibroblasts by MDHV, strain GA, led to the induction of a novel DNA polymerase. This novel DNA polymerase, designated MDHV-induced DNA polymerase, could be distinguished from the DNA polymerase activities of uninfected duck embryo fibroblasts by its chromatographic behavior on phosphocellulose, by its sedimentation coefficient, and by its catalytic properties. MDHV-induced DNA polymerase eluted from a phosphocellulose column at 0.2 M KCl. The

DNA polymerase activities of uninfected duck embryo fibroblasts eluted at 0.3 M KCl and at 0.5 M KCl. The sedimentation coefficient of MDHV-induced DNA polymerase, as determined by sucrose density gradient centrifugation in the presence of 0.25 M KCl, was 5.9S. The sedimentation coefficients of the polymerases from the uninfected cells were 8 and 3.1S for those found in the cytoplasmic fraction and 7.3 and 3.1S for those found in the nuclear fraction. The *in vitro* synthesis of DNA by MDHV-induced DNA polymerase was more resistant to inhibition by the sulphydryl reagent *p*-hydroxymercuribenzoate than was DNA synthesis by either the cytoplasmic 8S or the nuclear 7.3S DNA polymerase activities of uninfected duck embryo fibroblasts. MDHV-induced DNA polymerase could be differentiated from the 3.1S DNA polymerase activity of uninfected cells by the fact that the incorporation of dGMP by MDHV-induced DNA polymerase decreased markedly, while that of the 3.1S DNA polymerase increased slightly when dATP, dCTP, and dTTP were omitted from the activated DNA reaction mixtures. Finally, MDHV-induced DNA polymerase could be distinguished from the DNA polymerase activities of uninfected duck embryo fibroblasts by the fact that it could not effectively use either poly(dA)·oligo(dT)₁₂₋₁₈ or poly(dC)·oligo(dG)₁₂₋₁₈ as a template-primer, whereas the DNA polymerase activities of uninfected cells could use these synthetic template-primers.

MDHV-induced DNA polymerase could possibly arise in infected duck embryo fibroblasts by *de novo* synthesis of the polymerase protein. The structural gene for the polymerase might be either a viral gene or a cellular gene which

becomes derepressed as a result of MDHV infection. Alternatively, MDHV-induced DNA polymerase might not arise by de novo synthesis but by a modification of a pre-existing cellular polymerase. No direct evidence which bears on these alternatives has yet been obtained.

Infection of susceptible cells by herpes simplex virus I (HSV) leads to the induction of a new DNA polymerase (18, 19, 20, 34). The DNA polymerases induced by infection by HSV and MDHV have quite different molecular weights and catalytic properties. Weissbach et al. (34) reported that the molecular weight of HSV-induced DNA polymerase was 180,000 to 200,000. MDHV-induced DNA polymerase has a molecular weight of about 100,000. The activity of HSV-induced DNA polymerase was 25- to 50-fold greater in the presence of 150 mM Na_2SO_4 or $(\text{NH}_4)_2\text{SO}_4$ than in its absence. The activity of MDHV-induced DNA polymerase was markedly inhibited by the addition of $(\text{NH}_4)_2\text{SO}_4$ to the reaction mixture. HSV-induced DNA polymerase could effectively use poly(dC)·oligo(dG) but not poly(dA)·oligo(dT) as a template-primer. MDHV-induced DNA polymerase could not effectively use either of these synthetic polydeoxyribonucleotide-oligonucleotides as template-primers.

Although it has not been established, MDHV-induced DNA polymerase presumably plays a role in viral DNA synthesis. There is some evidence to suggest that the nuclear 7.3S DNA polymerase of infected cells may also be involved in MDHV DNA synthesis. The amount of the 7.3S DNA polymerase detected in the nuclear fraction of infected cells was greater than that detected in uninfected cells. Approximately 2.5 times more nuclear 7.3S DNA polymerase per g (wet weight) of cells was detected in infected cells than in uninfected cells. Although other interpretations are possible, the occurrence of the nuclear 7.3S polymerase in amounts greater in the infected cells than in the uninfected cells may indicate that this DNA polymerase plays a role in MDHV DNA synthesis. Furthermore, there is some evidence to suggest that the nuclear 7.3S polymerase detected in MDHV-infected cells may, in fact, be different from that detected in uninfected duck embryo fibroblasts. Although the two polymerases are similar in chromatographic behavior on phosphocellulose, sedimentation coefficients, and in many of their catalytic properties, the two polymerases seemed to differ in their sensitivities to $(\text{NH}_4)_2\text{SO}_4$. The addition of about 75 mM $(\text{NH}_4)_2\text{SO}_4$ to the reaction mixture produced a 50% inhibition in DNA

synthesis by the nuclear 7.3S polymerase of infected duck embryo fibroblasts (see Fig. 7). The addition of about 20 mM $(\text{NH}_4)_2\text{SO}_4$ to the reaction mixture produced a 50% inhibition for the nuclear 7.3S polymerase of uninfected duck embryo fibroblasts (data not shown but see Table 1). Additional experimental data are needed, however, before a decision should be made as to whether or not the two 7.3S polymerases are the same or different. All of our data indicate that the nuclear 3.1S polymerases of infected and uninfected cells are identical.

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