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The DNA polymerases associated with the adenovirus type 2 replication complex : effect of 2'-3'-dideoxythymidine-5'-triphosphate on viral DNA synthesis

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ABSTRACT

An Adenovirus (Ad) DNA replication complex extracted from infected HeLa nuclei could be purified free of the bulk of intracellular DNA polymerase activity by sedimention in neutral sucrose gradients. However, the replication complex still retained some α and γ DNA polymerase activity. Since this complex is inhibited by 2', 3' dideoxythymidine-5'-triphosphate (ddTTP), an inhibitor of DNA polymerase γ, a functional role for this enzyme in Ad DNA replication is suggested. Similar inhibition by ddTTP in intact Ad infected nuclei and comparable inhibition of Ad DNA synthesis in whole cells by dideoxythymidine (ddThy) are consistent with a role for DNA polymerase γ. Uninfected HeLa nuclei or whole cells are not similarly inhibited by ddTTP or ddThy respectively. Such data does not rule out an additional functional role for other DNA polymerases, and recent experiments from this laboratory (1) suggest that DNA polymerase α is also involved in Ad DNA synthesis.

INTRODUCTION

Experiments on Adenovirus (Ad) DNA synthesis in our laboratory have led to the development of an *in vitro* system to study viral DNA replication (2). Ad replication complexes are isolated from the nuclei of infected cells by extraction with ammonium sulfate. Such extracts, which are free of host chromosomal DNA, synthesize viral DNA in a way similar to that *in vivo*. The size of the DNA product formed by the *in vitro* replication complex is full-sized Ad DNA and greater than 95% is complementary to viral DNA. As in whole cells, the *in vitro* DNA replication is bidirectional (2) and the temperature sensitive mutation of the H5*ts*125 virus is biochemically expressed (3). Electron microscopy of Ad DNA replicating intermediates isolated from the *in vitro* system (4) are similar to those isolated from whole cells (5).

Other *in vitro* Ad DNA replication systems have been isolated from nuclei of infected cells (6,7,8). Each of these systems is able to incorporate deoxynucleotide triphosphates into DNA molecules which had been

initiated in vivo. However, there is no evidence for initiation of new rounds of DNA replication in any of the in vitro systems.

The role of the DNA polymerases in Ad replication is not well understood. Since no adenovirus-coded DNA polymerase has been isolated from infected cells, cellular polymerases probably play a functional role in viral replication. DNA polymerases are found in mammalian tissues in 3 major forms, α, β and γ which can be distinguished from each other biochemically (9). Ito et al (10) isolated DNA polymerase γ from a nuclear membrane complex of Ad infected KB cells but this system did not make full-sized Ad DNA. DNA polymerase γ was also isolated by Brison et al (6) from a soluble Ad replication system extracted by ammonium sulfate from nuclei of Ad infected HeLa cells. However, Frenkel (8) detected polymerase α as the major activity in a membrane protein complex, which was isolated from Ad infected cells by a modification of the M-band technique (11). Recently, Arens et al (12) found a mixture of DNA polymerase α and γ activities in a soluble system obtained by heparin extraction of Ad infected KB cell nuclei but they were unable to isolate sufficient amounts for enzyme purification.

The present report demonstrates that both DNA polymerase α and γ activities are present in a high molecular weight Ad DNA replication complex which can be purified free of the bulk of host DNA polymerase on neutral sucrose gradients. This manuscript presents evidence which supports a functional role for DNA polymerase γ in Ad replication. There is similar evidence that DNA polymerase α is also necessary for Ad DNA synthesis (1).

MATERIALS AND METHODS

Cells and Viruses: The sources of HeLa S3 cells and Ad2 have been described (13). Cells were grown in suspension culture and were infected at a multiplicity of 4000 virions per cell (3).

Preparation of Ad2 Infected Nuclear Extract: Nuclei were prepared by Nonidet P-40 (NP40) detergent lysis, of Ad2 infected HeLa cells at 18 hr. post infection and the Ad2 replication system was extracted with 150 mM ammonium sulfate as described previously (2). This system elongates endogenous replicating Ad2 DNA and incorporates added labelled deoxynucleotides.

Sedimentation of Ad2 Nuclear Extract on Neutral Sucrose Gradients: The nuclear extract (0.5-1ml) prepared from Ad2 infected cells was layered onto a 5-20% linear neutral sucrose gradient (11ml). Each gradient contained 20 mM Tris-HCl (pH 7.4), 1.5mM 2-mercaptoethanol, 1 mM EDTA and was prepared over a 1 ml cushion of 60% sucrose in the same gradient buffer. After centrifugation at 35,000 rpm in a Beckman SW41 rotor for 2.5 hr at 4°C,

the gradient was fractionated into 11-12 aliquots.

Analysis of DNA on Alkaline Sucrose Gradient: Endogenous DNA labelled during in vitro or in vivo Ad2 DNA synthesis was analyzed on alkaline sucrose gradients without further purification (2). The 0.5 ml sample in 0.15 M NaCl and 10mM EDTA with added $|^{14}C|$ thymidine labelled Ad2 virion DNA was centrifuged for 16 hr at 24,000 rpm and 4°C in the Beckman SW 27.1 rotor. Each gradient, containing 5-20% sucrose in 0.1N NaOH, 1M NaCl and .01M EDTA, was prepared over 0.5 ml of a CsCl solution (ρ=1.8) as a cushion.

Assay of Endogenous Replication Activity: Ten to twenty µl of an ammonium sulfate extract of Ad2 infected HeLa cells, was incubated in a final volume of 50 µl in 20 mM Tris-HCl (pH 7.5), 2mM dithiothreitol (DTT), 10 mM $MgCl_2$, 0.5mg/ml of bovine serum albumin (BSA), 6 mM ATP , 0.125 mM each of dATP, dCTP, dGTP and 3 µM (^{3}H)dTTP (36,000 cpm/pmol). Unless otherwise stated, incubation was carried out at 30°C for 60 min. The reaction was terminated and the acid precipitable radioactivity was measured as described previously (2).

DNA polymerase assay: The DNA polymerases were assayed as previously described (14). The activity of DNA polymerase α was measured in a final volume of 50 µl containing 50 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$, 1.5 mM DTT, 0.4 mgm/ml BSA, 0.125 mM each of dATP, dCTP and dGTP, 3 µM (^{3}H)dTTP (36,000 cpm/pmol) and 20 µg of activated salmon sperm DNA. The reaction was carried out at 30° for 30 min. Activity was measured as incorporation of radioactivity into acid-precitable material. The DNA polymerase β activity was assayed essentially in the same manner as DNA polymerase α, except that 10 mM N-ethylmaleimide (NEM) was preincubated with the enzyme for 30 min at 4°C prior to the start of the assay. DNA polymerase γ activity was quantitated in a final volume of 50 µl, containing 50 mM Tris-HCl (pH 7.5), 0.5 mM $MnCl_2$, 2.5 mM DTT, 100 mM KCl, 0.4 mgm/ml BSA, 0.5 mM poly(rA)·oligo$(dT)_{12-18}$ and 3µM (^{3}H) TTP (36,000 cpm/pmol). When this assay was carried out with activated DNA as a primer template, 20 ug of activated salmon sperm DNA was included instead of poly(rA)·oligo(dT), 10 mM $MgCl_2$ replaced the $MnCl_2$, and 0.125 mM of dATP, dCTP and dGTP were added. The reaction was carried out at 30°C for 30 minutes.

Fractionation of DNA polymerases from Uninfected HeLa Cells: DNA polymerases α, β and γ were isolated from the nuclei and cytoplasm of HeLa cells, using the procedure described by Spadari and Weissbach (14). The DNA polymerase γ was further purified on a phosphocellulose column (15).

Fractionation of DNA polymerases from Ad2 Infected HeLa Cells: A nuclear extract (15ml) prepared from Ad2 infected HeLa cells was layered onto 6 neutral sucrose gradients (5-20%) and was centrifuged at 24,000 rpm for 5.5 hrs. at 4°C, in the Beckman SW 27 rotor. Each 33 ml gradient contained 50 mM ammonium sulfate, 20 mM Tris-HCl (pH 7.4), 1.5 mM 2-mercaptoethanol, 1 mM EDTA and was prepared over a 3 ml cushion of 60% sucrose in the same buffer. The sucrose cushion fractions were pooled, and dialyzed for 2 hr against 0.4 M potassium phosphate buffer (pH 7.4) containing 0.5 mM DTT. The dialyzed sample was sonicated twice for 15 sec. at a setting of 2 in the Branson sonifier and applied to a DE-52 cellulose column (1x5 cm) prequilibrated with 0.4 M potassium phosphate buffer (pH 7.4, 0.5 mM DTT (14). After a 10 ml wash with the equilibrating buffer, both the pass through and the wash fractions were combined and dialyzed overnight against 20 mM potassium phosphate buffer (pH 7.4) and 0.5 mM DTT. the dialysate was loaded onto another DE-52 cellulose column equilibrated with the dialysis fluid, washed with 25 ml of this buffer and eluted with a gradient of 20-400 mM potassium phosphate buffer (pH 7.4) and 0.5 mM DTT.

Assay of 2', 3' Dideoxythymidine-5'-Triphosphate (ddTTP) Inhibition: The effect of ddTTP was determined under the same assay conditions described for the DNA polymerases or the endogenous DNA replication system. The concentration of thymidine triphosphate (TTP) was maintained constant at 60μM, while the concentrations of ddTTP was varied from 0 to 180μM. (^{3}H)dCTP (19,000 cpm/pmol) at a concentration of 3μM was used as the labelled precursor, and dATP and dGTP were used at a concentration of 60μM each. When DNA polymerase γ was assayed with poly(rA)·oligo(dT) as a primer template, (^{3}H)dTTP (36,000 cpm/pmol) was used at a concentration of 3μM. The concentration of ddTTP was varied from 0 to 10μM.

In Vivo Inhibition by Dideoxythymidine: Ad2 infected HeLa cells at 18 hr post infection and control, uninfected, HeLa cells were resuspended with dialyzed fetal calf serum. One ml aliquots of the resuspended cells were incubated in the presence of various concentrations of dideoxythymidine at 37°C for 20 min. (^{3}H)thymidine (10μCi) was added and the incubation was continued for an additional hour.

Heat Inactivation: A purified DNA polymerase or viral nucleoprotein complex was preincubated at 45°C, in the presence of 1 mg/ml BSA, and 50 mM Tris-HCl (pH 7.5) as described by Dube et al (16). At various times, 10 μl aliquots were removed and assayed for polymerase activity as described above.

Materials: (^{3}H)thymidine (19 Ci/mmol) and (^{3}H)TTP (52 Ci/mmol) were purchased from Schwarz-Mann, Inc. All other nucleotides and synthetic polynucleotides were purchased from P.L. Biochemicals.

RESULTS

Sedimentation of Ad2 Nuclear Extract on Neutral Sucrose Gradient: The ammonium sulfate extract of the nuclei of Ad2 infected HeLa cells was sedimented on 5-20% neutral sucrose gradient Approximately 50-60% of the Ad2 endogenous replicating activity sedimented into a 60% sucrose cushion at the bottom of the gradient (Fig. 1). The elongation activity in the cushion fraction was 3-4 fold purified compared to that in the unfractionated nuclear extract. When DNA in the cushion fraction was subsequently labeled in the elongation reaction and analyzed on an alkaline sucrose gradient, much of the Ad2 DNA product was found to be full sized (4). Further attempts to purify an intact Ad2 replication complex from the cushion

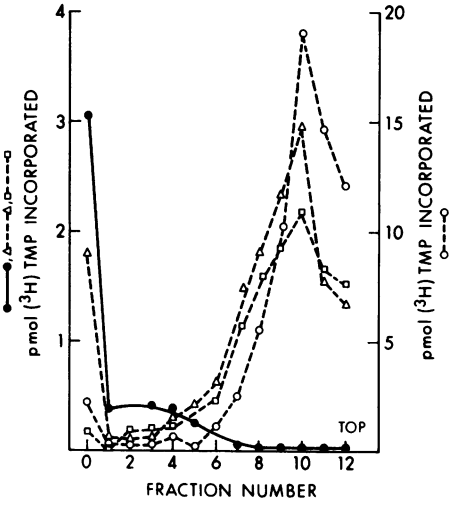

Figure 1. Sedimentation of the Ad DNA Replication Complex on a Neutral Sucrose Gradient: A nuclear extract (0.5 ml) was prepared from Ad2 infected HeLa cells and centrifuged on a 5-20% neutral sucrose gradient in the Beckman SW41 rotor as described in Materials and Methods.

An aliquot (20 µl) of each fraction was assayed for endogenous Ad2 DNA synthesis activity (●—●), and an equal aliquot was assayed for α (○—○) and β (□——□) DNA polymerase activities using activated salmon sperm DNA as a primer-template. DNA polymerase γ (Δ——Δ) activity was measured using poly(rA)·oligo(dT) as a primer-template. Fraction "0" represents the 60% sucrose cushion.

fraction, either by using gel filtration or another neutral sucrose gradient resulted in a complex which produced only small DNA fragments.

DNA Polymerase Activity: Using exogenous primer templates, the three DNA polymerase activities α, β and γ were detected in the nuclear extract of Ad2 infected HeLa cells. The bulk of these activities sedimented at the top of the neutral sucrose gradient (Fig. 1). However, small amounts of α and γ were also associated with the sucrose cushion fraction. Table 1 shows that in the cushion fraction 24 pmol of the DNA polymerase α activity and 7 pmol of the DNA polymerase γ activity were found, whereas only 0.11 pmol of the DNA polymerase β activity were present. Consistent with the failure to show significant levels of DNA polymerase β activity in Ad DNA replication complexes is our finding that 4 mM NEM severely inhibited the endogenous activity in the cushion fraction (data not shown) whereas polymerase β is resistant at such NEM concentrations (17).

Purification of DNA Polymerase from the Sucrose Cushion Fraction: Sonication of the sucrose cushion fraction sedimented from Ad2 nuclear extracts did not significantly affect the activities of DNA polymerases α and γ on exogenous primer-templates; however, the endogenous Ad2 DNA synthesizing activity was almost completely abolished (Table 1). When this sonicated homogenate was applied to DE-52 cellulose columns, 2

TABLE 1 DNA POLYMERASE ACTIVITIES IN CRUDE FRACTIONS OF SUCROSE GRADIENTS

	α DNA POLYMERASE		β DNA POLYMERASE		γ DNA POLYMERASE		AD2 ENDOGENOUS ACTIVITY	
FRACTION	TOTAL UNITS	% RECOVERY	TOTAL UNITS	% RECOVERY	TOTAL UNITS	% RECOVERY	PMOL	% RECOVERY
Nuclear Extract	864	100	10.9	100	50	100	2250	100
Sucrose Gradient Top (Fr 7-12)*	774	90	9.0	83	38	77	5	5
Cushion (Fr 0)*								
Intact	32	3.7	0.15	1.3	9	18	1430	63
Sonicated	24	2.7	0.11	1.1	7	14	45	2

* See Figure 1 for profiles of similar gradients

Legend to Table 1: Nuclear extracts (15ml) from Ad2 infected HeLa cells were applied to six neutral sucrose gradients as described in Methods. Following centrifugation for 5.5 hrs at 24,000 rpm in the Beckman SW27 rotor, the 33 ml gradients over 3 ml 60% sucrose cushions were fractionated into aliquots. The three DNA polymerase activities were measured both in the cushion fractions and in the fractions from the tops of the gradients without further extraction. The activity of DNA polymerase in the cushion was measured before and after sonication of the replication complex.

peaks of DNA polymerase activity were eluted at 0.14M and .24M potassium phosphate respectively (Fig. 2). The first peak was more active with poly (rA)·oligo(dT) and the second with nicked salmon sperm DNA. Further characterization of these two peaks suggested that the first peak represents DNA polymerase γ and the second is DNA polymerase α. There was no DNA polymerase β activity in the flow through fractions of the column elute.

Effect of ddTTP: In order to investigate the functional role of the DNA polymerase α and γ associated with the sucrose cushion fraction, we tested the effect of the DNA polymerase γ inhibitor ddTTP on endogenous Ad2 DNA synthesis activities. The assay of the ddTTP inhibitory effect is based on maintaining a constant concentration of TTP and increasing the concentration of ddTTP (18). The DNA polymerase α, β and γ were fractionated from uninfected HeLa cells (14). These DNA polymerases were resolved on DE-52 cellulose chromatography, under conditions where DNA polymerase β passed through in the loading buffer and α and γ were bound and subsequently eluted at higher salt concentrations. We found, like others (9, 14) that

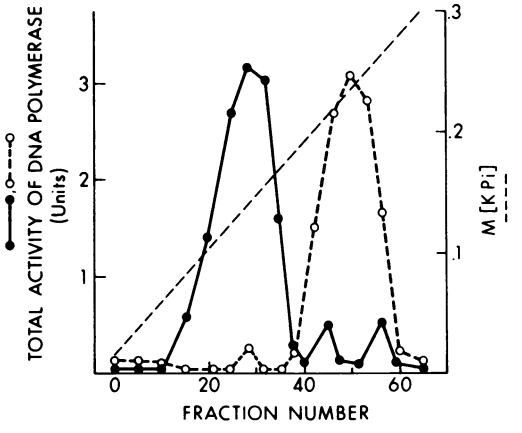

Figure 2. Extraction of DNA Polymerases from Ad2 Replication Complex: A nuclear extract (15 ml) prepared from Ad2 infected HeLa cells was layered onto 6 neutral sucrose gradients as described in Materials and Methods and in Table 1. The sucrose cushion fractions from the 6 gradients were pooled, sonicated, dialyzed and applied to a DE-52 cellulose column. The DNA polymerases were eluted with a gradient of 20-400 mM potassium phosphate buffer (pH 7.4) containing 0.5 mM DTT and the activities were assayed using the primer-template poly(rA)·oligo(dT) (●—●) or activated salmon sperm DNA (0—0) as described in Materials and Methods. One unit of DNA polymerase represents the amount of activity required to incorporate one nmol of ^{3}H-TMP into acid precitable material in a one hour reaction at 30°C and the activity is expressed as units per 1 ml aliquot.

in some preparations the elution profile of DNA polymerases α and γ from DE-52 cellulose were partially overlapping (data not shown). These preparations required further purification of DNA polymerase γ by phosphocellulose column chromatography which gave a single peak of γ activity. We used the selective assay conditions described to measure the activities of cellular DNA polymerases (14). We found that in our preparations of polymerase α and β purified from DEAE cellulose and γ run through a second column of phosphocellulose, that each DNA polymerase contained little contamination by the other two activities.

The effect of ddTTP on the activities of DNA polymerase α, β and γ was determined. Figure 3a shows that when the ratio of (ddTTP/TTP) is increased from 0 to 3, the activity of DNA polymerase γ is inhibited. This inhibition reaches more than 90% at a ratio of (ddTTP/TTP) equal to 2.0, irrespective of the primer template used. The activity of DNA polymerase β which is essentially undetectable in Ad2 replication complexes is also inhibited, but less extensively. In contrast, the activity of DNA polymerase α is very resistant to the inhibitor.

When the same ratios of (ddTTP/TTP) were used with the endogenous Ad2 replication system, the DNA elongation activity was decreased (Figure 3b). The inhibition of the unfractionated Ad2 nuclear extract reached 75% whereas the further purified replication complex from the sucrose cushion was inhibited more than 90%. For comparison, the replication activities of intact Ad2 infected or uninfected HeLa nuclei were measured. HeLa nuclei were not inhibited by these levels of ddTTP whereas Ad2 infected intact nuclei were inhibited to the same extent as the infected nuclear extracts.

The Effect of Dideoxythymidine on DNA Replication in Whole Cells: The effect of dideoxythymidine (ddThy) on Ad2 DNA synthesis was investigated. At 18 hr after infection, the cells were exposed for 20 min to ddThy at concentrations up to 3.5×10^{-4} M, and then pulsed with (^{3}H) thymidine for one hour to determine the amount of Ad2 DNA synthesized. Uninfected HeLa cells were treated with the drug under similar conditions. Figure 4a shows that DNA synthesis from Ad2 infected cells was considerably inhibited by ddThy while uninfected HeLa cells were not inhibited.

In order to test the effect of ddThy on the size of DNA produced during either HeLa or Ad2 DNA synthesis, the cells were treated with various concentrations of ddThy and analyzed on alkaline sucrose gradients. Figure 4b shows that most of the 40% residual Ad2 DNA formed in the presence of

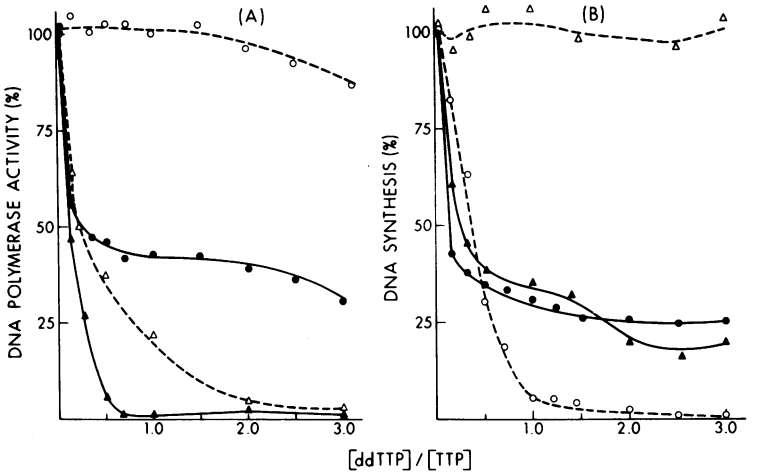

Figure 3. Effect of Dideoxythymidine Triphosphate (ddTTP) on Isolated DNA Polymerases and on Intact Nuclei or Nuclear Extracts: The activities of the three DNA polymerases from uninfected HeLa cells were assayed using constant amounts of TTP and increasing concentrations of ddTTP.

(A) The effect of ddTTP on DNA polymerase α activity (O—O) or DNA polymerase β activity (●—●) was measured on activated salmon sperm DNA and DNA polymerase γ activity, on the primer-template poly(rA)·oligo(dT) (▲——▲) or activated salmon sperm (Δ——Δ) as described in Materials and Methods. 100% activity for polymerase α is 125 pmol; for polymerase β, 24 pmol; and for polymerase γ, 10 pmol with poly(rA·oligo(dT) and 4 pmol with activated salmon sperm DNA.

(B) Ammonium sulfate extracts of infected nuclei (●—●) or the cushion fraction (O—O) obtained from partially purifying these extracts on neutral sucrose gradients were assayed for endogenous Ad2 replication activity and inhibition by ddTTP. In addition, the effect of ddTTP on similar activity using intact nuclei prepared from infected cells (▲——▲) was measured. Uninfected HeLa cell nuclei served as controls (Δ——Δ). Nuclei were prepared from cells with 0.5% NP40, and washed as for extraction, however, the pelleted nuclei were resuspended in 20 mM Tris pH 7.5, 5 mM EDTA and 10% sucrose and maintained on ice for assay of activity.

100% activity for 10^7 Ad2 nuclei was 16 pmol; for HeLa nuclei 6 pmol, for the Ad2 nuclear extract 13 pmol; and for the Ad2 cushion 7 pmol.

10^{-5}MddTTP in infected cells was full-sized 34S molecules. When the ddTTP concentrations was increased to $7x10^{-5}$M, the size of the recently synthesized Ad2 DNA was considerably diminished. However, all the DNA produced in the uninfected HeLa cell even at $7x10^{-5}$M sedimented to the bottom of the gradient, indicating the continued synthesis of large sized DNA.

Heat Inactivation: As another approach to determine the function of the DNA polymerases associated with the sucrose cushion fraction from Ad2

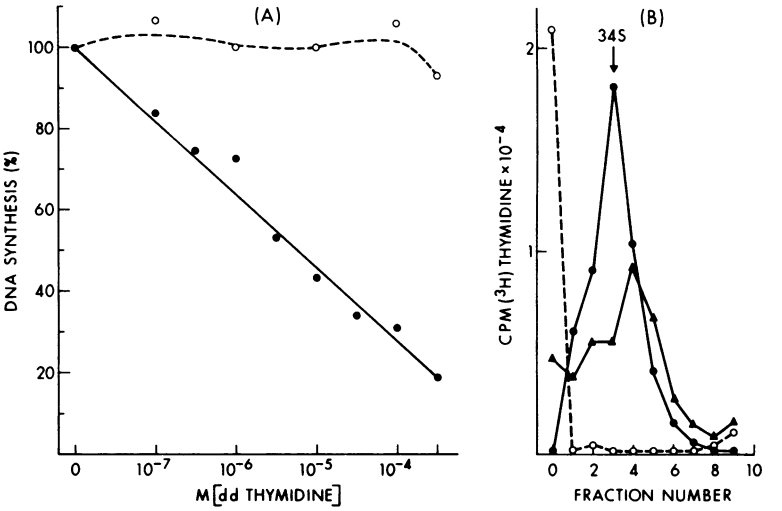

Figure 4. Effect of Dideoxythymidine on DNA Replication in Whole Cells:
(A) DNA Synthesis in uninfected (0—0) or Ad2 infected (●—●) HeLa cells was measured in the presence of increasing concentrations of dideoxythymidine as described in the Methods. 100% synthesis for uninfected HeLa cells was $6x10^4$ cpm and for Ad2 infected cells $12x10^4$ cpm.
(B) The $|^3H|$ thymidine labeled DNA synthesized in the presence of 10^{-5}M dideoxythymidine from uninfected (0—0) and Ad2 infected (●—●) HeLa cells was analyzed on 5-20% alkaline sucrose gradients. Similar studies on Ad2 infected cells in the presence of $7x10^{-5}$M ddthy are shown (Δ—Δ). The results for uninfected HeLa cells with $7x10^{-5}$M ddthy are essentially identical to the $1x10^{-5}$M results (0--0). The arrow represents the position of 34S $|^{14}C|$ Ad2 DNA which was included in the gradient as a marker.

infected cells, we compared the effect of heat treatment on purified DNA polymerase activity with that on the in vitro Ad2 replication system. Figure 5a shows that when the isolated DNA polymerases were preincubated at 45°C for 30 min, DNA polymerase γ is rapidly inactivated. DNA polymerase β is also inactivated but at a slower rate while the DNA polymerase α is considerably more resistant to heat.

Similar treatment of nuclei or nuclear extracts obtained from Ad2 infected cells suggested that the elongation activity of Ad2 is thermosensitive, (Fig. 5B). In order to demonstrate that such treatment is affecting the activity of the DNA polymerase γ present in the Ad2 replication system, nuclear extracts of Ad2 infected cells were sedimented on neutral sucrose gradients and the cushion fraction was divided into 2 portions. One was heat inactivated at 45°C for 30 min. Both portions were sonicated

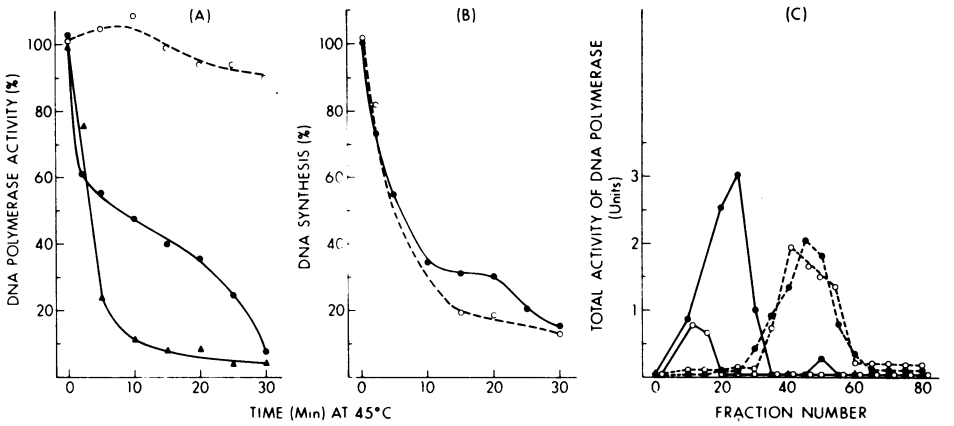

Figure 5. Heat Inactivation of DNA Polymerases: Heat inactivation of the purified DNA polymerases was compared to that of the Ad2 replication system.

(A) DNA polymerase α, β and γ were incubated at 45°C, in the presence of 1 mg/ml BSA and 20 mM Tris-HCl (pH 7.5). At various times, 10 µl aliquots were removed and assayed for DNA polymerase activity. The activity remaining after heating was measured for DNA polymerase α (0—0); DNA polymerase β (●—●); and DNA polymerase γ (▲——▲)

(B) Ammonium sulfate extracts of Ad2 infected nuclei were prepared and sedimented on neutral sucrose gradients. The nuclear extract before sedimentation and the sucrose cushion fraction, dialyzed for 2 hrs. against 20 mM Tris-HCl (pH 7.4), 1.5 mM 2-mercaptoethanol and 1 mM EDTA, were incubated at 45° as in Panel A and assayed for endogenous Ad2 DNA synthesis. (●—●) activity of the nuclear extract; activity of the sucrose cushion fraction (0—0).

(C) A nuclear extract (15 ml) prepreared from Ad2 infected cells was centrifuged as described in Figure 2. The sucrose cushion fractions were pooled, dialyzed as in Panel A and divided into 2 equal parts, one of which was heat inactivated at 45°C for 30 minutes. Each sample was fractionated by 2 successive steps of DE-52 cellulose chromatography as described in Methods. Activity of DNA polymerase γ isolated from the intact Ad2 replication system (●—●); or from heat inactivated Ad2 replication system (0—0). Activity of DNA polymerase α isolated from untreated Ad2 replication system (●--●) or from heat inactivated Ad2 replication system (0--0). One unit of DNA polymerase is the activity needed to incorporate one nmol of $|^{3}H|$TTP into acid insoluble counts in a 1 hr reaction at 30°C.

and chromatographed on DEAE-cellulose. Figure 5C shows that the activity of DNA polymerase γ extracted from the heat inactivated replication complex is greatly reduced when compared to that from the untreated replication complex.

DISCUSSION

The Ad2 DNA replication complex extracted from infected nuclei and

purified on neutral sucrose gradients can make full size viral DNA. This purification procedure separated the bulk of soluble host DNA polymerases from the Ad2 DNA elongation activity in the replication complex. Characterization of the DNA polymerases remaining with the Ad2 replication complex suggested that these enzymes belonged to the classes of DNA polymerases α and γ. These results are consistent with the finding of Arens et al (12) who detected α and γ-like activities in their in vitro preparation from Ad2-infected KB cells. However, there was insufficient DNA polymerase activity present in their in vitro systems to attempt isolation and purification. Frenkel (8) extracted DNA polymerase α as the predominant activity from an in vitro system of Ad2 which was prepared by a modification of the M-band technique. Previously, DNA polymerase γ was isolated from a nuclear membrane complex of Ad2 infected cells (10); however, the relationship of either of these membrane complex systems to Ad DNA synthesis is unclear. Although only DNA polymerase γ was isolated from another Ad2 replication complex making full size DNA (6), the authors speculated that some DNA polymerase α activity may have been lost during their isolation procedure.

By direct inhibition of Ad2 DNA synthesis in vitro utilizing ddTTP, we demonstrated a functional role for the γ DNA polymerase associated with the Ad DNA replication complex. As a control, we used isolated enzymes to show that at ddTTP/TTP ratios of 0.5,DNA polymerase γ is extensively inhibited and DNA polymerase β is partially inhibited, while DNA polymerase α is resistant. This effect of ddTTP on isolated DNA polymerases from mammalian tissues is entirely consistant with previous observations (18,19). Since β activity was detected in exceedingly small quantities in DNA polymerase preparations partially purified from the Ad2 replication complex, it suggests that the decrease in Ad2 elongation activity by ddTTP is due to an inhibition of the DNA polymerase γ present in the replication complex. This conclusion is based on the assumption that the ddTTP inhibition of DNA polymerase in the replication complex is similar to that observed with the isolated enzymes.

Similar inhibitor experiments with ddTTP were done with intact nuclei isolated either from the Ad2 infected or control HeLa cells. The inhibition of Ad DNA synthesis in whole nuclei by ddTTP was similar to the results in the soluble Ad DNA replication complex. However, uninfected nuclei were resistant to the drug, consistent with previous reports for HeLa (19) and CV1 cells (18). Van der Vliet and Kwant have recently

reported similar results with Ad infected nuclei (20).

Although the data on ddThy inhibition of Ad DNA synthesis in whole cells is consistent with the inhibition in nuclei and the soluble DNA replication complex, the in vivo data are more difficult to interpret bec of the obvious difficulty in monitoring the endogenous thymidine concentrations. However, we have demonstrated clearly that concentrations of ddThy which could inhibit (^{3}H)thymidine incorporation into Ad2 DNA, had little effect on host DNA synthesis. This differential effect of ddThy on Ad2-infected cells most likely is due to a selective interference with Ad2 DNA replication. Other changes in uninfected HeLa cells such as a lack of uptake of ddThy, a low rate of ddThy phosphorylation, rapid degradation of ddThy or effective repair mechanisms to remove the ddThy incorporated into DNA could produce similar results. However, in mouse myeloma cells, Byars and Kidson (21) have demonstrated a rapid phosphorylation of ddThy and also that the radioactive labeled ddThy was incorporated into DNA. Although there was some partial repair of the ddThy incorporated into DNA of uninfected mouse cells, about 40% of the labelled ddThy was retained for periods greater than 2 cell generation times (21).

The heat inactivation experiment, which showed that the Ad2 replication complex at 45^{o} and DNA polymerase γ purified from it are heat labile, is supportive of our findings with ddTTP. We also examined the effect of heat treatment on isolated DNA polymerases and confirmed the results of others (16, 22) that DNA polymerase γ is very sensitive, polymerase β is less sensitive and DNA polymerase α is relatively stable when heated to 45^{o}.

These conclusions do not exclude a concomitant role for the DNA polymerase α. Recently we have shown that aphidicolin, a specific inhibitor of DNA polymerase α also inhibits Ad replication both in HeLa cells and in the Ad DNA replication complex (1). The extent of inhibition with either aphidicolin or ddTTP alone is greater than ninety per cent and suggests a concomitant need for both DNA polymerases α and γ in the elongation process.

Recent work with extracts of SV40 infected cells or uninfected HeLa cells have suggested that the DNA polymerase α is the predominant activity associated with the replication complex. In contrast to our findings with the Ad2 system, in vitro SV40 or HeLa DNA synthesis is insensitive even to high ratios of ddTTP/TTP (18,19). At the present time, we do not know why DNA polymerases from mammalian tissues show different sensitivity towards ddTTP. One possibility is that the active site of each of these enzymes

has a different affinity for ddTTP binding. The recent development of adenosine 2'-3'-riboepoxide 5'-triphosphate as an active site directed reagent for DNA polymerase (23) might help in our understanding of the active site of these mammalian polymerases.

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