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Effect of 2', 3'-dideoxythymidine-5'-triphosphate on HeLa cell in vitro DNA synthesis: evidence that DNA polymerase α is the only polymerase required for cellular DNA replication

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ABSTRACT

We have studied the effects of the nucleotide analogue, 2',3'-dideoxythymidine-5'-triphosphate (ddTTP) on replicative DNA synthesis in HeLa cell lysates. As previously demonstrated (1), such lysates carry out extensive DNA synthesis in vitro, at rates and in a fashion similar to in vivo DNA replication. We report here that all aspects of DNA synthesis in such lysates (total dNTP incorporation, elongation of continuous nascent strands, and the initiation, elongation, and joining of Okazaki pieces) are only slightly inhibited by concentrations of ddTTP as high as 100-500 μM when the dTTP concentration is maintained at 10 μM. This finding is consistent with the report by Edenberg, Anderson, and DePamphilis (2) that all aspects of replicative in vitro simian virus 40 DNA synthesis are also resistant to ddTTP. We also find, in agreement with Edenberg, Anderson, and DePamphilis (2), that DNA synthesis catalyzed by DNA polymerases β or γ is easily inhibited by ddTTP, while synthesis catalyzed by DNA polymerase α is very resistant. These observations suggest that DNA polymerase α may be the only DNA polymerase required for all aspects of cellular DNA synthesis.

INTRODUCTION

Mammalian cells are known to contain three DNA-dependent DNA polymerases, designated α, β, and γ, which can be distinguished by their enzymatic and physical properties (3). Several experimental approaches have been used in efforts to determine which of these DNA polymerases participate in normal cellular DNA replication. In one approach attempts have been made to correlate the amount of enzyme activity per cell with the rate of cellular division or with the stage of cell cycle. The α polymerase is more abundant in dividing cells than in resting cells (4,5), and both α and γ polymerases have been reported to increase in abundance during S phase, compared to G1 phase (6,7). In some studies the β polymerase has shown little difference in abundance between resting and dividing cells (4,5) or between stages of the cell cycle (6,7), but in other studies a β-like activity was found to increase in dividing cells (8-10). Thus no firm conclusions can be drawn from this approach.

A second method used for assessing the role of the DNA polymerases is examination of their enzymatic properties and correlation of these properties with the properties of DNA replication. For example, α polymerase is the only polymerase capable of elongating RNA primers (11). If RNA primers are required for DNA replication, as they seem to be (12-18), then α polymerase would be a likely candidate for the role of extending such primers. Also, when DNA synthesis is inhibited by drugs which lower deoxyribonucleoside triphosphate (dNTP) pools, such as FdUrd or hydroxyurea, there is an accumulation of short pieces of nascent DNA. One possible hypothetical explanation for this accumulation is that the DNA polymerase which fills in gaps left by excision of RNA primers has a higher K_m for dNTPs than the DNA polymerase which extends RNA primers. The K_m's for α and β DNA polymerases (2-13 μM for the 4 dNTPs; 19) are about 10-fold higher than the K_m's for γ polymerase (0.2-1.0 μM; 19), suggesting that γ polymerase might extend RNA primers and α and β polymerase might fill gaps. In addition, when *in vitro* polyoma DNA synthesis is inhibited by N-ethylmaleimide (NEM) or by 1-β-D-arabinofuranosyl cytosine triphosphate (ara CTP), there is also an accumulation of short pieces of nascent DNA, suggesting (20) that α polymerase, which is more sensitive than β polymerase to those drugs (3,20), might be involved in gap filling.

It is obvious that less ambiguous methods for determining the role of the DNA polymerases are needed. Recently 3 laboratories have attempted to isolate replicating simian virus 40 (SV40) chromosomes and determine the identity of the DNA polymerase(s) associated with them. All 3 laboratories report that α DNA polymerase is the major DNA polymerase associated with replicating SV40 chromosomes (2,21; and Fanning, personal communication). In addition, Edenberg, Anderson, and DePamphilis (2) have reported the important finding that *in vitro* SV40 DNA synthesis is resistant to 2',3'-dideoxythymidine-5'-triphosphate (ddTTP), as is DNA synthesis catalyzed by DNA polymerase α. However, DNA synthesis catalyzed by polymerases β and γ is sensitive to ddTTP (2). As all phases of SV40 DNA synthesis (initiation of Okazaki pieces, elongation of Okazaki pieces, joining of Okazaki pieces, and elongation of continuous strands) are resistant to ddTTP, the implication is that α polymerase is probably responsible for *all* phases of SV40 DNA synthesis (2).

We were interested in determining whether the conclusions of Edenberg, Anderson, and DePamphilis (2) with respect to the effect of ddTTP on SV40 DNA synthesis could be extended to cellular DNA synthesis in uninfected cells. We decided to test this question using the well-characterized *in vitro* HeLa cell DNA synthesizing system of Fraser and Huberman (1). In this system replicative DNA synthesis proceeds at sites which were replicating *in vivo*

prior to cell disruption, at a fork movement rate 50-60% of the in vivo rate during the first h of incubation at 37°C, and at lower rates thereafter. Using this system we have found that all aspects of cellular DNA synthesis proceed in nearly normal fashion even in the presence of high concentrations of ddTTP. We have also confirmed the finding of Edenberg, Anderson, and DePamphilis (2) that DNA synthesis catalyzed by α polymerase is selectively resistant to ddTTP. We conclude that α polymerase is probably the only DNA polymerase required for cellular DNA replication.

MATERIALS AND METHODS

Cell culture and labeling. The HeLa cell S3 stock (from Dr. Sheldon Penman) was maintained free of mycoplasma contamination in spinner culture at 37°C in Joklik-modified Minimal Essential Medium supplemented with 10% (v/v) fetal calf serum (Grand Island Biological Co.). All experiments reported in this paper were carried out with unsynchronized, exponentially growing cells in spinner culture. The DNA in unsynchronized cultures was bulk-labeled with 10 μCi of [^{3}H]-thymidine (47 Ci/mmole; Amersham-Searle Corp.)/300 ml of medium for 16 h before harvesting.

Lysate preparation. HeLa cell lysate was prepared as described previously (1) by harvesting the cells and suspending the pellet in buffer T (0.137 M NaCl, 0.005 M KCl, 0.007 M Na_2HPO_4, 0.025 M Tris-HCl, pH 7.8). The cells were centrifuged for 5 min at 800 g and resuspended in hypotonic buffer H (2 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 1 mM EDTA, 10 mM Na_2HPO_4, adjusted to pH 8.0 with KOH) at 10^7 cells/ml. The cells were centrifuged again and resuspended in an equal volume of buffer H by determining the volume of the resultant pellet.

The lysate was prepared by Dounce homogenization with a tight-fitting pestle (Kontes Glass Co.), using no more than 10 strokes. The extent of cell lysis was determined by microscopic examination in a phase contrast microscope. The nuclear concentration was determined by hemocytometer and was in the same range (5.0×10^7 to 7.5×10^7 nuclei/ml) as reported previously (1).

In vitro incubation, preparation of the nuclei for radioactivity determination, preparation of DNA for gradient centrifugation and collecting and assaying of gradient fractions for radioactivity determination were the same as previously described (1). Final concentrations during in vitro DNA synthesis were: 36 mM KCl, 9 mM Na_2HPO_4, 45 mM HEPES (pH 7.9), 8 mM glucose, 2 mM DTT, 1 mM EDTA, 0.45 mM EGTA, 12 mM $MgCl_2$, 30 μM spermine, 5 mM ATP, 0.1 mM each of GTP, UTP, and CTP, and 10 μM each of dATP, dCTP, dGTP, and dTTP.

ddTTP was purchased from P-L Biochemicals, Inc. [α-^{32}P]-dTTP was purchased from Amersham-Searle Corp.

DNA polymerase α was prepared by a combination of methods reported previously (22,23). The temperature was maintained at 0-4°C throughout extraction and purification. Sixty gm of simian liver tissue were thawed, minced into small fragments, and washed in Hank's phosphate buffered saline by centrifugation. Fragments were resuspended in 50 ml cold TKM (.25 M sucrose, 50 mM Tris·HCl, pH 7.8, 25 mM KCl, 5 mM $MgCl_2$, 5 mM phenyl methyl sulfonylfluoride, PMSF) and homogenized in a Sorvall "Omnimixer" until microscopic examination revealed no intact cells (5-30 sec bursts at rheostat setting of 75). The homogenate was centrifuged at 1750 rpm, 15 min, in an International PR-2 centrifuge. The supernatant was collected and the pellet was resuspended in 50 ml TKM, centrifuged again and the second supernatant combined with the first supernatant as the source for chromatographic purification of DNA polymerase α. The extract was dialyzed overnight against 100 volumes of TEMG (50 mM Tris·HCl, pH 7.8, 1 mM EDTA, 1 mM 2-mercaptoethanol, 20% glycerol) and applied to a 2.4 x 6 cm column of DEAE cellulose (Whatman DE-52). The column was eluted with a 200 ml salt gradient from 0-0.8 M KCl in TEMG. Fractions eluting from the salt front to .15 M KCl, containing a peak of activity detected in the α polymerase assay, were pooled, dialyzed against TEMG and applied to a 2.4 x 7 cm phosphocellulose column (Whatman P-11). The column was eluted with a 150 ml gradient of 0-0.8 M KCl in TEMG. A single peak of α polymerase activity, eluting at .1 M KCl, was pooled, excluding a shoulder of activity eluting at .15 M KCl which contained traces of γ polymerase activity. Velocity gradient centrifugation on 10-30% glycerol in gradient buffer (25 mM Tris·HCl, pH 7.8, 1 mM EDTA, 1 mM 2-mercaptoethanol, .5 M KCl, 500 μg/ml bovine serum albumin) of a sample from this pool revealed a single peak of polymerase activity sedimenting at 8S. Activity detectable with polymerase β and γ assays was less than 1% of the α polymerase activity.

DNA polymerase β was obtained from an extract of simian liver (60 gm) prepared essentially as described by Bolden, Noy and Weissbach (24). Briefly, the liver was minced in Hank's buffered saline and the fragments washed twice by centrifugation. Fragments were resuspended and washed twice in extraction buffer (250 mM sucrose, 1 mM EDTA, pH 7.0, 0.5 mM DTT, 0.5 mM PMSF) and homogenized in a Sorvall Omnimixer in 5-30 sec bursts at a rheostat setting of 70. The extract was centrifuged at 1000 x g, 15 min and the supernatant was divided into 6 aliquots and centrifuged at 85,000 x g for 10 min in the Beckman #30 rotor. The resulting pellets were washed 6 times in extraction buffer, 25 ml per aliquot, and finally resuspended in 50 mM Tris·HCl, pH 7.8, 0.5 mM DTT, 1

M KCl and sonicated 3 times for 25 sec at maximum cavitation. The suspension was centrifuged at 150,000 x g for 30 min and the supernatant dialyzed against TEMG. The extract was chromatographed on DEAE cellulose as described above for DNA polymerase α and the flow-through fractions were collected and applied to a phosphocellulose column. DNA polymerase β eluted from phosphocellulose at .25 M KCl and was further characterized by velocity gradient centrifugation on 10-30% glycerol in gradient buffer. A single peak of NEM-resistant activity was observed sedimenting at 3-4S.

DNA polymerase γ recovery from the extract of simian liver just described was extremely low; therefore the DNA polymerase γ used in these experiments was obtained from an extract of murine liver (30 gm) prepared as described for polymerase β except that tissue fragments were Dounce homogenized by 10 strokes of a tight-fitting pestle and chromatography was performed with 1 mg/ml BSA in all buffers. The murine liver extract was chromatographed on DEAE cellulose, fractions eluting at .15 M KCl, detected in assays with $rA_n \cdot dT_{12-18}$, were pooled and chromatographed on phosphocellulose. Fractions eluting at .22-.25 M KCl were collected from the phosphocellulose column and characterized by velocity gradient sedimentation. A single peak of DNA polymerase activity sedimenting at 7S and exhibiting a 4-fold preference for $rA_n \cdot dT_{12-18}$ over activated DNA was observed.

DNA polymerase assays. DNA polymerase activity was assayed in 3 reaction mixtures optimized for the specific detection of DNA polymerases α, β, and γ and in the reaction mixture used for *in vitro* DNA synthesis (see above) with the addition of activated DNA or $rA_n \cdot dT_{12-18}$. Each reaction was performed in a total volume of 50 µl containing 25 µl of 2 x reaction mixture and 25 µl of enzyme. Incubation of DNA polymerase α and β assays was at 37°C for 60 min and DNA polymerase γ assays were incubated 60 min at 30°C. Reactions were terminated by absorbing the entire reaction mixture onto 2.5 cm filter paper discs (Whatman 3 MM) and transferring filters to 10% trichloroacetic acid (TCA), 1% sodium pyrophosphate for 15 min. Filters were further washed 6 times, 15 min each, in 5% TCA, twice in 75% ethanol, 10 min, and finally in acetone, 5 min. Radioactivity was determined by liquid scintillation counting at an efficiency for ^3H of 8.5%. All reactions were linear with time for 60 min.

DNA polymerase α assays contained 30 mM sodium phosphate, pH 7.2, 100 µg/ml activated calf thymus DNA (25), 100 µM each dATP, dCT, dGTP, 9 mM $MgCl_2$, 1.2 mM β-mercaptoethanol and 10 µM [^3H]-dTTP, specific activity 722 cpm/pmole (New England Nuclear).

DNA polymerase β assays contained 50 mM Tris·HCl, pH 8.3, 0.1 M KCl, 100 μg/ml activated DNA, 100 μM each dATP, dCTP, dGTP, 9 mM $MgCl_2$, 5 mM N-ethyl maleimide (NEM) and 10 μM [^{3}H]-dTTP, specific activity 722 cpm/pmole (New England Nuclear).

DNA polymerase γ assays contained 50 mM Tris·HCl, pH 8.3, 0.1 M KCl, 6 μM rA_n (Miles Laboratories), 6 μM dT_{12-18} (Collaborative Research), 0.8 mM $MnCl_2$, 12 mM β-mercaptoethanol, and 10 μM [^{3}H]-dTTP, specific activity 722 cpm/pmole (New England Nuclear).

ddTTP inhibition assays. ddTTP (PL Biochemicals, Inc.) was added to reaction mixtures in duplicate at the concentrations indicated. Control reactions, also in duplicate, were adjusted to equivalent volume. $MgCl_2$ was present in the ddTTP stock solution in equimolar concentration with the ddTTP to insure that Mg^{2+} concentration did not limit the reaction. The concentration of dTTP was constant at 10 μM.

RESULTS

Effect of ddTTP on in vitro replicative DNA synthesis. Following the procedure of Fraser and Huberman (1), we prepared a whole HeLa cell lysate, and then we incubated the lysate at 37°C along with the ingredients necessary for in vitro DNA synthesis (1). We used dATP, dCTP, and dGTP at 10 μM and [α-^{32}P]-dTTP at 10 μM in each incubation; ddTTP was added at concentrations from 0 to 500 μM, along with an equimolar concentration of $MgCl_2$, as indicated in figure legends.

The data in Figure 1 show that very high concentrations of ddTTP were required to inhibit cellular DNA synthesis. Even when the ratio [ddTTP]/[dTTP] was as high as 50:1, total incorporation was inhibited by less than 50%.

In order to see whether the size of the nascent DNA strands, and the conversion of short strands to long strands, were affected by ddTTP, we measured strand size by alkaline sucrose gradient sedimentation. The gradients shown in Figure 2 were centrifuged under conditions previously used by Fraser and Huberman (1), in which 35S marker DNA sedimented about half way down the gradient and Okazaki pieces remained near the top of the gradient. In the absence of ddTTP (Fig. 2a), a large proportion (but no more than 50%) of the radioactivity incorporated during 1 min and 2 min incubations sedimented slowly, in a peak near the top of the gradient. The rest of the incorporated radioactivity sedimented heterogeneously throughout the gradient. Notice that the slow strands labeled for 2 min sedimented detectably faster than those labeled for only 1 min. After a 15 min labeling period,

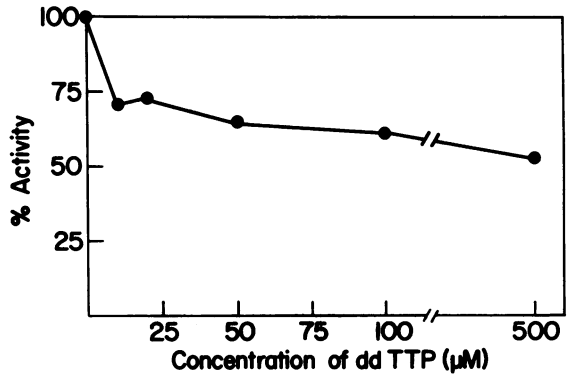

Figure 1. Effect of ddTTP on DNA synthesis in the HeLa cell lysate. The lysate was prepared and assays were done in duplicate as described in reference (1) and in Materials and Methods. To maintain a constant free Mg^{++} concentration in our reaction mixture, we added $MgCl_2$ in equimolar amounts with ddTTP. All assays contained 10 μM [α-^{32}P]-dTTP (specific activity 0.45 Ci/mmole). Bulk DNA was prelabeled with [^{3}H]-dThd (see Materials and Methods). Samples were incubated for 30 min at 37°C, then processed for determination of radioactivity as described (1). The activity of the system was plotted after calculating the $^{32}P/^{3}H$ ratio; 100% activity was assumed when there was no ddTTP in the reaction mixture.

the slow peak was no longer evident and the radioactivity was distributed in heterogeneously sedimenting strands throughout the gradient, as previously observed by Fraser and Huberman (1). In the presence of 100 μM ddTTP, the sedimentation patterns of DNA strands labeled for 2 min or 15 min were unaltered, although total incorporation was slightly reduced (Fig. 2b). These data suggest that the synthesis of continuous strands and the synthesis and joining of Okazaki pieces are not selectively inhibited by ddTTP.

In order to test the effect of ddTTP on the joining of Okazaki pieces in a more sensitive way, we did the following experiment. We labeled DNA strands for 1 min, either in the absence (Fig. 3a) or in the presence of 100 μM (Fig. 3c) or 500 μM (Fig. 3e) ddTTP. Then we added excess unlabeled dTTP (952 μM) and continued incubation for 2 min, in the absence (Fig. 3b) or presence of 100 μM ddTTP (Fig. 3d) or 500 μM ddTTP (Fig. 3f). Centrifugation of alkaline sucrose gradients was carried out for a longer time than in Figure 2, so that Okazaki pieces of about 180 nucleotides would sediment about ½ of the length of the gradient (1). Both in the presence and absence of ddTTP, more than 90% of the radioactivity detected after 1 min labeling was recovered after a 2 min chase with cold dTTP, but much of it was recovered near the bottom of the gradient. In all cases the result of the cold chase was conversion of most of the radioactivity in the peak of Okazaki pieces (Figs. 3a, 3c, and 3e) into longer strands (Figs. 3b, 3d, and 3f). In the presence of

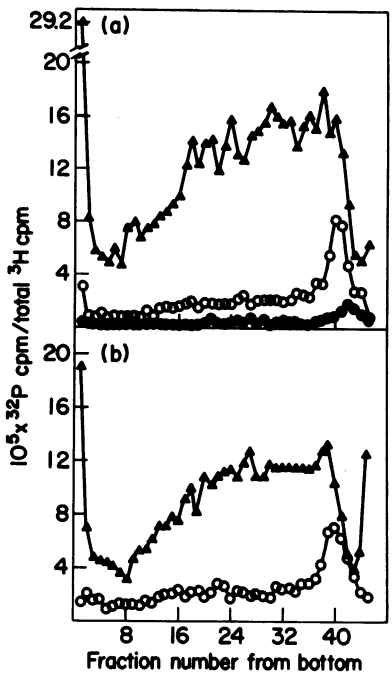

Figure 2. Alkaline sucrose gradients of DNA labeled in vitro in the presence and absence of ddTTP. Unsynchronized cells were prelabeled overnight with [^{3}H]-dThd, harvested and lysed. Assays were carried out as in Figure 1, in the presence of 10 µM [α-^{32}P]-dTTP (0.45 Ci/mmole) and in the absence (a) or presence (b) of 100 µM ddTTP. The cell lysate (0.18 ml) and the assay mix (0.02 ml; 10 x concentrated) were prewarmed at 37°C before mixing, and incubations at 37°C were for 1 (●), 2 (○), or 15 min (▲). Incubations were stopped by addition of 10 ml ice-cold buffer A (10 mM Tris, 10 mM EDTA, 10 mM NaCl, adjusted to pH 7.5). Nuclei were pelleted, then suspended in 0.2 ml buffer A. The nuclei were lysed by addition of 50 µl of 10% Sarkosyl (K & K Laboratories, Inc.), and the lysate was digested 16 h at 37°C with 100 µg/ml proteinase K (EM Laboratories, Inc.). Proteinase K was autodigested at 37°C for 2 h before use. DNA was denatured with NaOH at 0.2 N, 50°C for 30 min, then layered onto a linear 5 to 20% (w/v) alkaline sucrose gradient (0.9 M NaCl, 1 mM EDTA, 0.1% Sarkosyl, pH 12.3 with NaOH). Gradients were centrifuged in the SW 41 rotor, 40,000 rpm, 3.5 h, 23°C. Fractions were collected and assayed for radioactivity as described (1). The ^{32}P radioactivity in each fraction was divided by the total ^{3}H radioactivity recovered in each gradient.

500 µM ddTTP a slightly greater proportion of Okazaki pieces was not converted to long strands. The reason for this slight inhibition is not clear, but it is consistent with the extent of inhibition of total incorporation by 500 µM ddTTP (Fig. 1). If ddTTP had been incorporated during the 1 min pulse, it should have prevented joining of the Okazaki pieces during the chase. The fact that efficient joining was observed shows either that ddTTP is not incorporated into Okazaki pieces when present in 10- or 50-fold excess over dTTP, or that it is rapidly excised after incorporation.

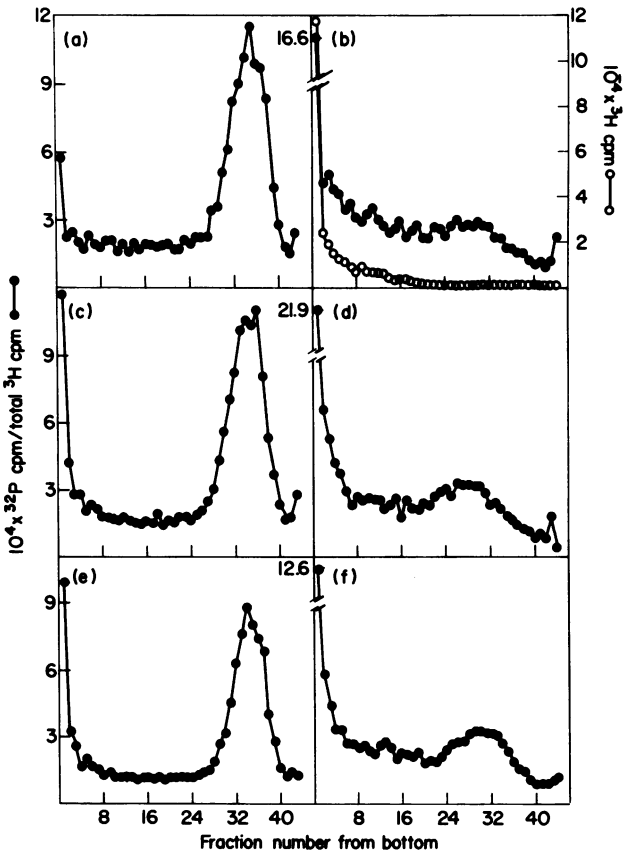

Figure 3. Alkaline sucrose gradients of DNA labeled and chased *in vitro* in the presence and absence of ddTTP. Unsynchronized cells were prelabeled overnight with [^{3}H]-dThd, harvested and lysed. Nuclei (1.2 x 10^7/ml) were assayed in complete assay mix with 10 µM [α-^{32}P]-dTTP (1.1 Ci/mmole) in the absence (a,b) or presence (c,d,e & f) of ddTTP. The concentration of ddTTP was 100 µM in c & d and 500 µM in e & f. Unlabeled dTTP (952 µM) was added after 1 min of incubation in samples b,d, & f and the reaction was allowed to proceed for an additional 2 min. Reactions a,c, & e were stopped after 1 min. The samples were processed exactly as described in Figure 2. Centrifugation was for 13 h at 34,000 rpm, 23°C in an SW 41 rotor. The average recovery of ^{3}H label was 96% and the average recovery of [^{32}P]label was 89%. ●—● DNA labeled *in vitro* with [α-^{32}P]-dTTP (normalized to total ^{3}H cpm recovered in gradient); o—o DNA prelabeled *in vivo* with [^{3}H]-dThd (shown only in (b)).

In order to sensitively test the effect of ddTTP on the initiation of Okazaki pieces we preincubated our cell lysate for 5 min *in vitro* with unlabeled dNTPs and with or without 100 µM ddTTP in order to chase all preformed Okazaki pieces into longer strands. We then added [α-^{32}P]-dTTP and continued incubation for 1 min more. As shown by the data in Figure 4, a significant proportion of the incorporated radioactivity was found to sedi-

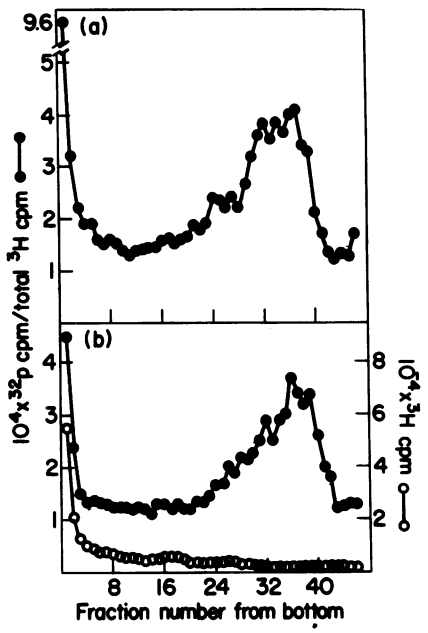

Figure 4. Alkaline sucrose gradients demonstrating initiation of short nascent strands (Okazaki pieces) in the presence and absence of ddTTP. HeLa cells were labeled in vivo with [^{3}H]-dThd, harvested and lysed as in Figure 1. Nuclei (1.2 x 10^7/ml) were incubated with 2.5 mM ATP, 50 µM each of GTP, UTP, CTP; 5 µM each of dCTP, dGTP, dATP and 5 µM dTTP; the concentrations of other chemicals in the reaction mixture were the same as described in Figure 1. Nuclei were incubated at 37°C for 5 min in the presence (b) or absence (a) of 100 µM ddTTP. [α-^{32}P]-dTTP (2.2 Ci/mmole, 0.29 µM) was added after 5 min in each case and the incubation was carried out for an additional 1 min. The samples were processed as described in Figure 3. The actual proportion of ^{32}P in the Okazaki piece peaks in the absence and presence of ddTTP was 50% and 55% respectively. ●—● DNA labeled in vitro with [α-^{32}P]-dTTP (normalized to total ^{3}H cpm recovered in gradient); o—o DNA prelabeled in vivo with [^{3}H]-dThd (shown only in (b)).

ment in a slow peak. The presence of ddTTP had no obvious effect on the proportion of radioactivity in this slow peak. We conclude that the initiation of Okazaki pieces is also not selectively sensitive to ddTTP.

Effect of ddTTP on the activity of fractionated DNA polymerases. DNA synthesis catalyzed by all 3 DNA polymerases, α, β, and γ, is inhibited to some extent by ddTTP (Fig. 5). However, under normal polymerase assay conditions (Fig. 5a), synthesis catalyzed by polymerases β and γ is inhibited > 50% by 5 µM ddTTP, while at the same ddTTP concentration synthesis catalyzed by α polymerase is inhibited only 5%.

Under conditions of in vitro replicative DNA synthesis assay (Fig. 5b), synthesis catalyzed by the α polymerase is barely inhibited even by 100 µM

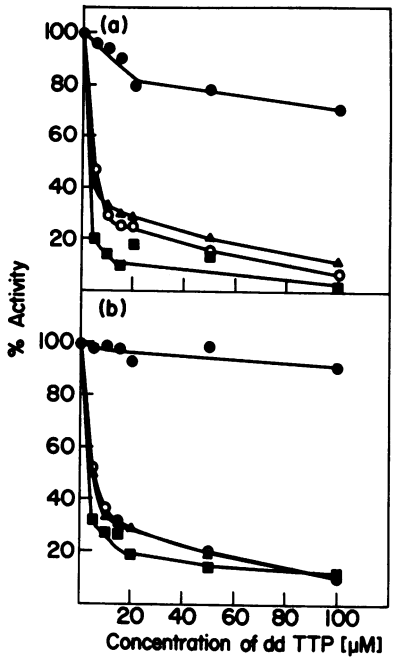

Figure 5. DNA polymerases α, β, and γ assayed in the presence of increasing concentrations of ddTTP. (a) Effect of ddTTP on DNA polymerases assayed under conditions optimized for detection of individual enzymes. (b) Effect of ddTTP on DNA polymerases assayed under same conditions used to determine in vitro DNA synthesis (Fig. 1-4), with activated DNA or $rA_n \cdot dT_{12-18}$ added as template. ●, DNA polymerase α; o, DNA polymerase β; ▲, DNA polymerase γ, activated DNA; ■, DNA polymerase γ, $rA_n \cdot dT_{12-18}$. Assay conditions were as described in Materials and Methods.

ddTTP. The sensitivity to ddTTP of synthesis catalyzed by the other 2 polymerases is also decreased, but is still far greater than that of α polymerase. Synthesis catalyzed by the β polymerase (± ddTTP) is not affected by the presence or absence of N-ethyl-maleimide (NEM) (data not shown).

DISCUSSION

The data of this paper show that all aspects of replicative DNA synthesis carried out in a HeLa cell lysate under well characterized conditions (1) are only slightly inhibited by ddTTP. All aspects measured (total incorporation of [α-^{32}P]-dTTP, elongation of continuous strands, and initiation, elongation, and joining of Okazaki pieces) proved to be only slightly sensitive to ddTTP concentrations as high as 100 or 500 μM and [ddTTP]/[dTTP] ratios as high as 10:1 or 50:1. These data are completely consistent with the finding of Edenberg, Anderson, and DePamphilis (2) that all aspects of in vitro repli-

cative SV40 DNA synthesis are also resistant to ddTTP, and they reinforce the conclusion that papovavirus DNA synthesis uses the same enzymes as cellular DNA synthesis (reviewed in 12).

We also confirm in this paper the finding of Edenberg, Anderson, and DePamphilis (2) that the activity of DNA polymerases β and γ is very sensitive to ddTTP while that of polymerase α is relatively resistant. Therefore it is tempting to conclude that DNA polymerase α is the only DNA polymerase responsible for cellular and papovavirus DNA synthesis. We feel that this conclusion is a valid working hypothesis, but we point out that interaction of DNA polymerases with other components of the cellular of papovaviral DNA replication machinery may change the properties of the polymerases. In particular, the changed sensitivities to ddTTP of synthesis catalyzed by the 3 DNA polymerases as a result of changes in assay conditions (Fig. 5) emphasize the need for caution in extrapolating data obtained with relatively purified enzymes to more complicated *in vitro* systems (such as our HeLa cell lysates) or to *in vivo* situations.

The facts that α polymerase increases in concentration when cells are replicating DNA (4-6), is the only DNA polymerase capable of elongating an RNA primer (11), and is the major polymerase activity associated with replicating simian virus 40 chromosomes (2,21; and Fanning, personal communication) are also consistent with the idea that α polymerase is the only polymerase involved in cellular or papovaviral DNA replication. But, as pointed out in the Introduction, these facts are open to alternative explanations. Additional evidence for the role of α polymerase comes from the observation that certain lower eukaryotic organisms have only *one* DNA polymerase (26). This polymerase, which is almost certainly involved in DNA replication, has properties similar to the mammalian α polymerase (26).

More conclusive evidence for the replicative function of α polymerase might come from two sources. If mutants of any of the 3 DNA polymerases were available they could provide information on the *in vivo* functions of the polymerases. Also, if it were possible to selectively dissociate and reassociate the α polymerase from nuclei or replicating chromosomes, then by correlation of *in vitro* replicative activity with degree of association of α polymerase it might be possible to draw conclusions about the role of α polymerase in *in vitro* replicative DNA synthesis.

The findings in this paper and those of Edenberg, Anderson, and DePamphilis (2) raise the possibility that mammalian cells might prove to be much more resistant to 2',3'-dideoxythymidine (ddThd) than certain viruses which do not use the α DNA polymerase to replicate their DNA. Indeed, Byars

and Kidson (27) have shown that, although ddThd is phosphorylated to ddTTP by mammalian cells, very high concentrations (∿ 1 mM) of ddThd are required to significantly inhibit cellular DNA synthesis; this finding was confirmed by Edenberg, Anderson, and DePamphilis (2). DNA synthesis catalyzed by at least one form of viral DNA polymerase, the reverse transcriptase of avian myeloblastosis virus or of Rous sarcoma virus, has been reported to be very sensitive to ddTTP (28,29). The implcation that dideoxynucleosides might prove useful in therapy of certain viral diseases is worth further exploration.

Also worth further exploration is the actual mechanism of inhibition of the activity of mammalian DNA polymerases by ddTTP. If ddTTP is incorporated onto the 3' end of a growing DNA chain it will prevent further chain growth. This mechanism of inhibition has been demonstrated for E. coli DNA polymerase I (30) and for viral reverse transcriptase (28,29). Additional possible inhibitory mechanisms include competition with dTTP for binding to the polymerase and irreversible binding of DNA polymerase to DNA chains terminated with a dideoxythymidylate residue (30). Knowledge of mechanism of inhibition will allow further use of ddThd and ddTTP as tools for studying the roles of the cellular polymerases.

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