Involvement of Eucaryotic Deoxyribonucleic Acid Polymerases α, and γ in the Replication of Cellular and Viral Deoxyribonucleic Acid†

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ABSTRACT: In an effort to identify the deoxyribonucleic acid (DNA) polymerase activities responsible for mammalian viral and cellular DNA replication, the effect of DNA synthesis inhibitors on isolated DNA polymerases was compared with their effects on viral and cellular DNA replication in vitro. DNA polymerase α, simian virus 40 (SV40) DNA replication in nuclear extracts, and CV-1 cell (the host for SV40) DNA replication in isolated nuclei all responded to DNA synthesis inhibitors in a quantitatively similar manner: they were relatively insensitive to 2′,3′-dideoxythymidine 5′-triphosphate (d_2TTP), but completely inhibited by aphidicolin, 1-β-D-arabinofuranosylcytosine 5′-triphosphate (araCTP), and *N*-ethylmaleimide. In comparison, DNA polymerases β and γ were inhibited by d_2TTP but insensitive to aphidicolin and 20–30 times less sensitive to araCTP than DNA polymerase α. Herpes simplex virus type 1 (HSV-1) DNA polymerase and DNA polymerase α were the only enzymes tested that were relatively insensitive to d_2TTP; DNA polymerases β and γ, phages T4 and T7 DNA polymerases, and *Escherichia coli* DNA polymerase I were 100–250 times more sensitive. The results with d_2TTP were independent of enzyme concentration, primer–template concentration, primer–template choice, and the labeled dNTP. A specific requirement for DNA polymerase α in the replication of SV40 DNA was demonstrated by the fact that DNA polymerase α was required, in addition to other cytosol proteins, to reconstitute SV40 DNA replication activity in *N*-ethylmaleimide-inactivated nuclear extracts containing replicating SV40 chromosomes. DNA polymerases β and γ did not substitute for DNA polymerase α. In contrast to SV40 and CV-1 DNA replication, adenovirus type 2 (Ad-2) DNA replication in isolated nuclei was inhibited by d_2TTP to the same extent as γ-polymerase. Ad-2 DNA replication was also inhibited by aphidicolin to the same extent as α-polymerase. Synthesis of CV-1 DNA, SV40 DNA, and HSV-1 DNA in intact CV-1 cells was inhibited by aphidicolin. Ad-2 DNA replication was also inhibited, but only at a 100-fold higher concentration. We found no effect of 2′,3′-dideoxythymidine (d_2Thd) on cellular or viral DNA replication in spite of the fact that Ad-2 DNA replication in isolated nuclei was inhibited 50% by a ratio of d_2TTP/dTTP of 0.02. This was due to the inability of CV-1 and Hela cells to phosphorylate d_2Thd to d_2TTP. These data are consistent with the hypothesis that DNA polymerase α is the only DNA polymerase involved in replicating SV40 DNA and CV-1 DNA and that Ad-2 DNA replication involves both DNA polymerases γ and α.

Mammalian cells contain at least three classes of DNA polymerase activities (α, β, and γ) which are generally identified by their characteristic chromatographic properties, responses to inhibitors, primer–template preferences, salt requirements, and molecular weights. Although DNA polymerase β represents a homogeneous enzyme activity, DNA polymerases α and γ often appear heterogeneous and may be composed of several similar, but distinct, enzymatic activities [reviewed by Weissbach (1977), Falaschi & Spadari (1977), Korn et al. (1977), and Byrnes & Black, 1978)]. The biological roles of these enzymes remain unclear.

Three approaches have been taken to investigate the function of mammalian DNA polymerases. First, correlation studies between the levels of DNA polymerase activity and DNA synthesis have implicated DNA polymerase α in the replication of cellular and papovavirus DNA. A 3–10-fold increase in DNA polymerase α activity consistently appeared concomitant with the onset of DNA synthesis, while DNA polymerase β activity remained relatively constant (Chang & Bollum, 1973; Baril et al., 1973; Spadari & Weissbach, 1974a,b; Chiu & Baril, 1975; Wintersberger & Wintersberger, 1975; Riva et al., 1978). However, results with DNA polymerase γ varied (Spadari & Weissbach, 1974b; Spadari et al., 1978). Second, the fact that DNA polymerase α cosediments with replicating SV40[1] chromosomes (Edenberg et al., 1978; Otto & Fanning, 1978) and DNA polymerase γ copurifies with an Ad-2 DNA replication complex (Ito et al., 1975; Brison et al., 1977; Arens et al., 1978) provides circumstantial evidence that α-polymerase and γ-polymerase are involved in replication of these two genomes, respectively. Finally, the effects of several inhibitors have been studied, but conclusions from these results have been conflicting. Hydroxyurea and 5-fluorodeoxyuridine, which diminish dNTP pools, and araC and araCTP, which preferentially inhibit DNA polymerase α (Yoshida et al., 1977), caused an accumulation of Okazaki fragments. This has been taken as evidence for the involvement of two DNA polymerases, one for elongation of Okazaki fragments and one for gap filling (Magnusson et al., 1973; Laipis & Levine, 1973; Salzman & Thoren, 1973; Hunter & Franke, 1975). However, others did not observe any significant accumulation of Okazaki fragments caused by araC or araCTP (Magnusson et al., 1974;

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[1] Abbreviations used: SV40, simian virus 40; Ad-2, adenovirus type 2; HSV-1, herpes simplex virus type 1; DEAE-cellulose, diethylaminoethylcellulose; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonate; Tris, tris(hydroxymethyl)aminomethane; araCTP, 1-β-D-arabinofuranosylcytosine 5′-triphosphate; d_2TTP, 2′,3′-dideoxythymidine 5′-triphosphate; d_2Thd, 2′,3′-dideoxythymidine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetate; Me_2SO, dimethyl sulfoxide; $NaDodSO_4$, sodium dodecyl sulfate; MalNEt, *N*-ethylmaleimide.

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Wist et al., 1976), and 5-fluorodeoxyuridine is likely to increase the ratio of dUTP to dTTP, which may cause an increase in the number of short nascent DNA chains (Brynolf et al., 1978; Wist et al., 1978). Recently, it was shown that DNA polymerase α is relatively resistant to d_2TTP compared to β- and γ-polymerases which are highly sensitive (Edenberg et al., 1978). Since this analogue had no effect on either SV40 DNA replication (Edenberg et al., 1978; DePamphilis et al., 1978) or Hela cell DNA synthesis (Waqar et al., 1978; Wist, 1979), it was suggested that DNA polymerase α alone is involved in the replication of SV40 and cellular DNA. In contrast, Ad-2 DNA replication in vitro was highly sensitive to d_2TTP, suggesting a role for γ-polymerase in Ad-2 DNA synthesis (van der Vliet & Kwant, 1978; Abboud & Horwitz, 1979). Aphidicolin, a specific inhibitor of DNA polymerase α, inhibited DNA replication in sea urchin embryos, thus supporting the view that the α-polymerase is involved in eucaryotic DNA replication (Ikegami et al., 1978; Ohashi et al., 1978). In contrast, it has been reported that all DNA polymerase α activity can be extracted from nuclei isolated from mouse L929 cells with no effect on the extent or nature of the DNA synthesized in vitro (Butt et al., 1976).

In the absence of structural gene mutations in any of the eucaryotic DNA polymerases except for the one encoded by herpes simplex virus (Aron et al., 1975), the use of specific inhibitors together with in vitro complementation assays provides the most direct and potentially productive approaches to understanding the function of each enzyme. The present investigation provides a direct comparison of the effects of three selective inhibitors of DNA synthesis (d_2TTP, aphidicolin, and araCTP) on the activities of DNA polymerases α, β, and γ from monkey cells with their effects on monkey, SV40, and Ad-2 DNA replication in subcellular systems. In addition, reconstitution experiments were carried out with *N*-ethylmaleimide-inactivated SV40 replicating chromosomes and a cytosol fraction containing DNA polymerases α, β, and γ. Of the three enzymes, DNA polymerase α appears to be solely and specifically responsible for both SV40 and CV-1 nuclear DNA and SV40 DNA replication, while DNA polymerase γ as well as DNA polymerase α is required for Ad-2 DNA replication.

In an effort to confirm the biological roles assigned to DNA polymerases α and γ, we have analyzed in vivo effects of d_2Thd and aphidicolin on viral (SV40, Ad-2, and HSV-1) and cellular (CV-1 and Hela) DNA synthesis and proliferation. Surprisingly, we found that d_2Thd, which has been reported to be rapidly converted into d_2TTP and incorporated into nascent DNA in a variety of cell lines (Byars & Kidson, 1975; Kidson et al., 1975), was taken up by infected and uninfected cells but *not* phosphorylated to the triphosphate and therefore did *not* inhibit DNA synthesis. Aphidicolin, however, did inhibit SV40, HSV-1, and CV-1 DNA replication as expected.

Materials and Methods

Cells and Viruses. A CV-1 cell line (obtained from P. Tegtmeyer) and a Hela cell line that grows in a monolayer (obtained from P. A. Sharp) were grown as previously described (Wilson et al., 1976). Infections with SV40, strain Rh 911 (40 pfu/cell; Wilson et al., 1976), Ad-2 (40 pfu/cell; Flint et al., 1975), or HSV-1, strain KOS (10 pfu/cell; Aron et al., 1975) were done as previously described.

Reagents. [^{3}H]Thymidine, [^{3}H]deoxyadenosine, and carrier-free [^{32}P]phosphate in water were purchased from New England Nuclear. [α-^{32}P]dCTP and [α-^{32}P]dTTP were synthesized according to Symons (1974) as modified by Rigby et al. (1977). [^{3}H]d_2Thd was prepared by Amersham/Searle using a catalytic exchange reaction containing their own tritiated aqueous medium and dideoxythymidine supplied by P–L Biochemicals. The [^{3}H]d_2Thd was then purified from the crude preparation in this laboratory by chromatographing twice on Whatman 3MM paper with water-saturated butanol as the solvent (3% yield). The purified compound cochromatographed with unlabeled d_2Thd in four systems: cellulose thin layers using either water-saturated butanol, isobutyric acid–NH_4OH–H_2O (66:1:33), saturated $(NH_4)_2SO_4$–0.1 M potassium phosphate (pH 6)–2-propanol (70:19:2), or methanol–HCl–H_2O (70:20:10) as the solvent. The first three systems cleanly resolved d_2Thd from dThd, dCyd, Thy, and Ura; the last system separated nucleosides from nucleotides. All nonradioactive nucleotides were purchased from P–L Biochemicals. Aphidicolin was a gift from Dr. B. Hesp, ICI, Macclesfield, England. *E. coli* DNA polymerase I (grade I) was purchased from Boehringer–Mannheim. Purified bacteriophage T4 DNA polymerase was kindly donated by Dr. P. Modrich, and T7 DNA polymerase was donated by Dr. R. Kolodner. Dihydroxyboroylcellulose was purchased from Collaborative Research. Polyethylenimine (PEI) and cellulose thin-layer sheets were purchased from Brinkman. All other reagents were obtained as previously described (Edenberg et al., 1978).

Preparation of HSV-1 DNA Polymerase. The HSV-1 DNA polymerase activity was extracted from nuclei isolated from HSV-1 infected cells essentially according to a published procedure (Weissbach et al., 1973). Twenty 150-mm diameter dishes confluent with CV-1 cells were infected with HSV-1 (20–50 pfu/mL) for 1 h at 37 °C. At 18 h after infection, the medium was decanted and the cells were washed twice with 10 mL of ice-cold 10 mM Hepes (pH 7.8), 5 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM dithiothreitol, and 0.1 M sucrose per dish. All subsequent procedures were done at 0–4 °C. Cells were scraped from the dishes with a rubber policeman, broken in a Dounce homogenizer with 10 strokes of pestle B, and centrifuged for 5 min at 1400*g* in a Sorvall HB-4 rotor to separate nuclei from cytoplasm. The nuclear pellet was resuspended in 4 mL of the wash buffer described above. The DNA polymerase activity was extracted by addition of 4 mL of 0.8 M potassium phosphate (pH 7.5), 40% glycerol, 0.2% Triton X-100, and 0.5 mM dithiothreitol, and then the tube was slowly rotated for 1 h. The resulting viscous solution was centrifuged in a Beckman Ti 50 rotor (100000*g*, 1 h), and the supernatant was passed through a column of 4 mL of DE-52 resin equilibrated with 0.4 M potassium phosphate (pH 7.5), 20% glycerol, 0.1% Triton X-100, and 0.5 mM dithiothreitol to remove residual nucleic acids. The nonadsorbed material was dialyzed twice against 50 volumes of 0.02 M potassium phosphate (pH 7.5), 20% glycerol, 0.1% Triton X-100, and 0.5 mM dithiothreitol and then adsorbed at a rate of 8 mL/h onto 8 mL of DE-52 resin (1.4 × 5 cm column) equilibrated with the dialysis buffer. The column was then washed with two column volumes of the same buffer before eluting the DNA polymerase activity with an 80-mL linear gradient of 0–0.3 M KCl in the same buffer at a rate of 2 column volumes/h. Fractions of 1.3 mL were collected. The KCl concentration was determined from the conductivity of a diluted aliquot, and DNA polymerase activities were determined as described below.

Preparation of DNA Polymerases α, β, and γ. DNA polymerases α, β, and γ were prepared from uninfected CV-1 cells exactly as described for the preparation of HSV-1 DNA polymerase except that the cells were *not* broken in a Dounce homogenizer. Instead, the cellular pellet was extracted and

chromatographed as described above.

Preparation of Cytosol. The 100000*g* supernatant from uninfected CV-1 cells was prepared as previously described (Anderson et al., 1977).

DNA Polymerase Assays. All incubations were for 30 min at 37 °C. The amount of enzyme activity measured in each case (see figure captions) was always proportional to the amount of enzyme added; the rate of synthesis was linear for at least 30 min. When the effect of d_2TTP on DNA polymerase activity was tested, the dTTP concentration was 10 μM. To keep the concentration of free Mg^{2+} constant, we added $MgCl_2$ in equimolar concentrations with d_2TTP. *E. coli* DNA polymerase I and T4 and T7 polymerase activities were tested by using the α-polymerase assay. One unit of enzyme is defined as 1 nmol of nucleotide incorporated in 60 min.

(*a*) *α-Polymerase Assay* (*Spadari & Weissbach, 1974a*): 50 mM Tris-HCl (pH 8.5), 7.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 50 μg of activated salmon sperm DNA (predigested with pancreatic DNase I until 20% was solubilized), if not otherwise stated 50 μM each dCTP, dTTP, dATP, and dGTP, and 20 μg of bovine serum albumin in a final volume of 0.1 mL. Specific radioactivity and concentration values of the labeled dNTPs are given in the figure captions. Acid-precipitable radioactivity was measured as previously described (Edenberg et al., 1978).

(*b*) *β-Polymerase Assay*: β-Polymerase was treated with 5 mM MalNEt prior to an assay similar to the α-polymerase assay described above. MalNEt treatment had no effect on β-polymerase.

(*c*) *γ-Polymerase Assay* (*Spadari & Weissbach, 1974a*): 50 mM Tris-HCl (pH 7.5), 0.5 mM $MnCl_2$, 100 mM KCl, 0.5 mM dithiothreitol, 0.5 mM poly(rA)–oligo$(dT)_{12-18}$ (ratio of A to T was 25), 10 μM [^{32}P]dTTP or [^{3}H]dTTP (specific activity 2 mCi/μmol), and 35 μg of bovine serum albumin in a final volume of 0.1 mL. Radioactivity incorporated into poly(rA·dT) was measured by selective binding to DE-81 paper (Edenberg et al., 1978) or by measuring acid-precipitable radioactivity after addition of 20 μg of DNA per assay as a coprecipitant. γ-Polymerase was also tested in the HSV polymerase assay.

(*d*) *HSV Polymerase Assay* (*Weissbach et al., 1973*): 100 mM Tris-HCl (pH 8.0), 2 mM $MgCl_2$, 150 mM K_2SO_4, 0.5 mM dithiothreitol, 50 μg of activated salmon sperm DNA, 40 μg of bovine serum albumin, and unless otherwise stated 50 μM each dCTP, dTTP, dGTP, and dATP in a final volume of 0.1 mL. Concentration and specific radioactivity values of the labeled dNTPs are given in the figure captions.

SV40 DNA Synthesis in Nuclear Extracts. A soluble nuclear extract free of cellular DNA but containing replicating SV40 DNA was prepared from SV40 infected CV-1 cells at 36 h after infection when the rate of viral DNA synthesis was maximal (Su & DePamphilis, 1978). Infected cells were prelabeled with [^{3}H]Thd (1 to 2 μCi/mL) from 24 h postinfection to provide an internal standard of SV40 [^{3}H]DNA. The nuclear extract was incubated at 30 °C for up to 30 min in the presence of 46 mM Hepes-Na (pH 7.8), 4.5 mM $MgCl_2$, 2 mM ATP, 65 mM KCl, 0.5 mM dithiothreitol, 280 mM ethylene glycol, 1 mM EGTA, 5 mM phosphoenolpyruvate, 50 μM each dTTP, dATP, and dGTP, 3–10 μM dCTP (1–100 mCi/μmol [α-^{32}P]dCTP), 100 μM GTP, CTP, and UTP, and where indicated the cytosol from uninfected cells. Assays were terminated by addition of 2 mL of ice-cold 1 N HCl containing 0.5% sodium pyrophosphate.

CV-1 Nuclear DNA Synthesis in Isolated Nuclei. Nuclei were isolated from uninfected CV-1 cells in exponential growth as previously described (Anderson et al., 1977). The assay conditions were identical with those for SV40 DNA replication, except that the SV40 nuclear extract was replaced by 10^6 nuclei/assay which contributed 50 mM sucrose to the assay. The cells were prelabeled with [^{3}H]Thd to correct for variation in the number of nuclei per assay. Incubations were for 30 min at 30 °C.

Ad-2 DNA Synthesis in Isolated Nuclei. Hela cells grown to confluency in 100-mm diameter cell culture dishes were infected with Ad-2 (40 pfu/cell in 1 mL of medium) for 1 h at 37 °C. The inoculum was then removed by aspiration, and the cells were washed once with medium. At 19 h after infection, the cells were incubated with [^{14}C]Thd (0.3 μCi/mL, 50 mCi/mmol) for 1 h. All subsequent procedures were performed at 0–4 °C. The cells were washed twice in a hypotonic buffer [10 mM Hepes-Na (pH 7.8), 5 mM KCl, 0.5 mM $MgCl_2$, and 0.5 mM dithiothreitol]. The cells were scraped off with a rubber policeman and broken in a Dounce homogenizer (three strokes with pestle B), and the nuclei were harvested by centrifugation at 1400*g* for 5 min in a Sorvall HB-4 rotor. The nuclear pellet was resuspended in 10 mM Hepes-Na (pH 7.8), 1 mM $MgCl_2$, 50 mM KCl, 0.5 mM dithiothreitol, and 0.25 M sucrose. Nuclei (5×10^6/assay) were incubated under conditions identical with those for CV-1 DNA replication except that the temperature was 37 °C. Ad-2 DNA was selectively extracted (van der Vliet et al., 1977) by adding to each 0.1-mL assay 0.4 mL of an ice-cold solution of 10 mM Tris (pH 7.5) and 10 mM EDTA followed by 10 μL of 10% $NaDodSO_4$ and 10 μL of proteinase K (4 mg/mL). After 30 min at 30 °C, the $NaDodSO_4$ concentration was increased to 1% and the NaCl concentration was increased to 1 M. After 10–24 h, cellular DNA was removed by centrifugation at 17000*g* for 30 min. The amount of acid-precipitable radioactivity in the supernatant (Ad-2 DNA) was compared to the total amount of acid-precipitable radioactivity in an equivalent number of nuclei to determine the fraction of nascent viral DNA synthesized in infected nuclei.

Measurement of Viral and Cellular DNA Synthesis in Intact Cells. The rate of DNA synthesis was measured by incubating either uninfected or virus-infected CV-1 or Hela cells with [^{3}H]dAdo (5 μCi/mL, 20 Ci/mmol) or [^{3}H]dThd (5 μCi/mL, 47 Ci/mol). Experiments involving the addition of either dThd or d_2Thd to the medium also included 10 μM dCyd. Incorporation of labeled substrates into DNA was measured during the period of maximum DNA synthesis: 36-h postinfection for SV40, 20 h for Ad-2, and 9 h for HSV-1.

For CV-1, HSV-1, and Ad-2 DNA synthesis, the incorporation was stopped by removing the medium and washing 3 times with ice-cold buffer [20 mM Tris-HCl (pH 7.4), 137 mM NaCl, 5 mM KCl, and 1 mM Na_2HPO_4]. The cells were then scraped off with a rubber policeman and completely suspended by three strokes of pestle B in a Dounce homogenizer. For DNA synthesis in uninfected cells and HSV-1 infected cells, an aliquot of the suspension was precipitated in 1 N HCl containing 0.5% sodium pyrophosphate, collected on a GF/C filter, washed 3 times with 1 N HCl containing 0.5% sodium pyrophosphate and once with ethanol, dried, and immersed in a standard toluene-based scintillation cocktail, and the radioactivity was measured in a liquid scintillation counter. For Ad-2 DNA synthesis, Ad-2 DNA was selectively extracted as described above.

For SV40 DNA synthesis, where SV40 replicating and mature DNA easily leak out of nuclei, incorporation was stopped by removing the medium and adding 2 mL of 0.6% sodium dodecyl sulfate, 1 M NaCl, 20 mM EDTA, and 10

mM Tris (pH 7.6) directly to the dish. The lysate was decanted, and the supernatant containing the viral DNA was prepared by centrifugation (17000*g*, 30 min at 4 °C) (Hirt, 1967). Aliquots of the supernatant were acid precipitated, and incorporated radioactivity was measured as above.

Measurement of Virus Production. Virus production was quantitated by standard plaque assay procedures for SV40 (Mertz & Berg, 1974), Ad-2 (Williams, 1970), and HSV-1 (Dreesman & Benyesh-Melnick, 1967).

Measurement of Cell Proliferation and Cell Death. The cell proliferation was measured by counting trypsinized cells from monolayers in a hemocytometer. Cell death was estimated by the trypan blue exclusion test.

Uptake of d_2Thd by SV40-Infected Cells. Thirty-six hours after CV-1 cells were infected with SV40, the medium was replaced with 2 mL of medium containing 10% fetal calf serum and 6 μM [^{3}H]d_2Thd (4 μCi/mL, 330 μCi/μmol). The dishes were incubated for up to 1 h at 37 °C in a CO_2 incubator. The medium was removed by aspiration, and the cells were washed 4 times with ice-cold 20 mM Tris-HCl (pH 7.4) containing 137 mM NaCl, 5 mM KCl, and 1 mM Na_2HPO_4. The cells were then drained, scraped off the dish with a rubber policeman, and broken in a Dounce homogenizer (pestle B). The lysate was quickly adjusted to 0.5 M perchloric acid and centrifuged for 10 min at 5000 rpm, 2 °C, in a Sorvall HB-4 rotor. The supernatant was immediately neutralized with KOH, stored for 2 h on ice, and then centrifuged as above to remove $KClO_4$. Aliquots were transferred to GF/C paper and immersed in a standard toluene-based scintillation cocktail, and radioactivity was measured in a liquid scintillation counter.

Measurement of Phosphorylation of [^{3}H]d_2Thd and [^{3}H]Thd by Intact Cells. A neutralized $HClO_4$ supernatant was obtained from six 100-mm dishes of SV40-infected CV-1 cells that had been incubated with either 6 μM [^{3}H]d_2Thd or 6 μM [^{3}H]Thd (4 μCi/mL, 330 mCi/μmol) for 15–120 min as described above. This supernatant was either analyzed directly for [^{3}H]dideoxy- or [^{3}H]deoxythymidine nucleotides by thin-layer chromatography on PEI-cellulose or cellulose alone as described in the caption to Figure 12. Alternatively, the supernatant (2.5 mL) was diluted threefold with water, and the nucleotides were adsorbed to DEAE-Sephadex A-25 (HCO_3^- form) equilibrated with water. The labeled nucleoside and 70–80% of the nucleoside monophosphate were eluted with 0.15 M NH_4HCO_3. The remaining nucleotides were eluted with 0.4 M NH_4HCO_3, lyophilized to remove the volatile salt, dissolved in 100 μL of water, and chromatographed on polyethyleniminecellulose thin layers. The sheet was cut into 14 fractions and each slice placed in a scintillation vial with 200 μL of 0.7 M $MgCl_2$ and 20 mM Tris-HCl (pH 7.4) to extract the nucleotides. Ten milliliters of Biofluor was added and the radioactivity measured in a liquid scintillation counter. This extraction procedure increases counting efficiency about fourfold.

Measurement of Phosphorylation of d_2Thd and dThd in Intact Cells by Incorporation of [^{32}P]Phosphate into Nucleotides. At 34 h after infection with SV40, the medium in six 100-mm dishes of CV-1 cells was replaced with 3 mL of phosphate-free medium supplemented with [^{32}P]phosphate (166 μCi/mL) and 10% fetal calf serum. Three hours later, when the appearance of [^{32}P]DNA was increasing at a linear rate, d_2Thd was added to a final concentration of 1 mM and the cells were incubated for 2 more hours at 37 °C in a CO_2 incubator. Cells were then washed and broken, and a perchloric acid extract was prepared as described. The final extract contained 344 μCi of ^{32}P in 2.5 mL. The extract was diluted threefold with 0.015 M NH_4HCO_3 to give a final pH of 7.5 before addition of 5 μCi of [^{3}H]dTTP and then applied to a 5-mL column of DEAE-Sephadex A-25 (HCO_3^-) equilibrated with water. The column was washed with 10 mL of water, and then the nucleotides were eluted with a 40-mL linear gradient of 0.15–0.4 M NH_4HCO_3 (pH 7.5). Fractions of 20 drops were collected and assayed for radioactivity and conductivity. [^{3}H]dTTP eluted between 0.22 and 0.28 M NH_4HCO_3. Control experiments showed that [^{3}H]dTTP and unlabeled d_2TTP (detected by A_{260}) chromatographed together (d_2TTP eluted at 0.24 M salt and dTTP at 0.25 M salt). Fractions containing [^{3}H]dTTP were pooled, and the NH_4HCO_3 was removed by lyophilization followed by rotary evaporation. The sample was redissolved in 50 mM *N*-ethylmorpholine (pH 7.5) containing 0.1 M $MgCl_2$ and adsorbed onto an 8-mL column of dihydroxyboroylcellulose equilibrated with the same buffer (Rosenberg et al., 1972). The column was eluted at a rate of 0.1 mL/min, and 10-drop fractions were collected. Radioactivity was assayed in 10-μL aliquots. The first peak (fraction 10–16) contained all the [^{3}H]dTTP radioactivity and 25% of the ^{32}P radioactivity. The rest of the ^{32}P radioactivity eluted as a broad peak between fractions 17 and 60. Control experiments showed that dTTP and d_2TTP cochromatographed in this step. The first peak was rechromatographed in the same system, and all the ^{32}P eluted with [^{3}H]TTP. To avoid hydrolysis of nucleotides by $MgCl_2$, we added EDTA (pH 7.5) to a final concentration of 0.12 M. The sample was diluted threefold with water and applied to a 3-mL column of DEAE-Sephadex A-25 (HCO_3^- form) equilibrated with water. The column was washed with 5 volumes of water followed by 5 volumes of 0.15 M NH_4HCO_3 to remove nonvolatile salts and any remaining nucleoside mono- and diphosphates, and then the nucleoside triphosphates were eluted with 0.4 M NH_4HCO_3. Volatile salts were removed by rotory evaporation, and the sample was further desalted by gel filtration on Bio-Gel P2 in 1% NH_4OH. NH_4OH was removed by rotary evaporation, and the sample was dissolved in water and then applied to Whatman 3MM paper (56-cm long). High-voltage chromatography was carried out with 0.05 M sodium citrate (pH 3.5) as buffer at 60 V/cm. Initial experiments showed that dTTP, d_2TTP, and UTP cochromatographed but were well separated from all other nucleoside di- and triphosphates. The spot containing UV markers for dTTP and d_2TTP was cut out and eluted with 0.015 M NH_4HCO_3. The sample was desalted by chromatography on DEAE-Sephadex A-25 and gel filtration was described above and then dissolved in water. Aliquots were analyzed by thin-layer chromatography as described in Figure 13.

Sedimentation Analysis. Neutral and alkaline sucrose gradients were prepared as previously described (DePamphilis et al., 1975). For analysis of SV40 DNA, 200 μL of a Hirt supernatant was layered on top of the gradient and centrifuged in a Beckman SW50.1 rotor at 50 000 rpm for 2 h at 4 °C. For analyses of cellular DNA, nuclei were isolated as described by Su & DePamphilis (1978) and 5×10^4 nuclei were lysed with 1% Sarkosyl, 0.1 M NaCl, 10 mM EDTA, and 0.4 M NaOH as described by Krokan et al. (1975) and centrifuged at 50 000 rpm for 80 min at 4 °C. Acid-precipitable radioactivity was measured in each fraction as described above.

Results

Preparation of DNA Polymerases α, β, γ, and HSV-1. In order to study the effects of several inhibitors of DNA synthesis on mammalian DNA polymerases, we extracted DNA polymerases α, β, and γ from a lysate of uninfected CV-1

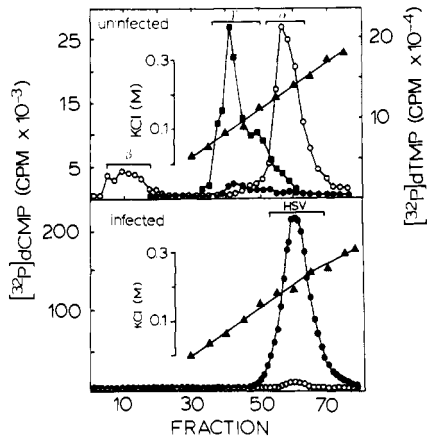

FIGURE 1: Fractionation of eucaryotic DNA polymerases. Cell extracts were prepared from uninfected or HSV-1 infected CV-1 cells and chromatographed on DEAE-cellulose as described under Materials and Methods. Fractions were collected and assayed for DNA polymerase activity. Labeled precursor was [α-^{32}P]dTTP (10 μM, 2 mCi/μmol) in the γ-polymerase assay and [α-^{32}P]dCTP (50 μM, 0.2 mCi/μmol) for the other assays. (○) α and β assays; (■) γ assay; (●) HSV-1 DNA polymerase assay; (▲) KCl concentration. Fractions were pooled as indicated by brackets.

monkey cells and fractionated them by chromatography on DEAE-cellulose (Figure 1). As expected from previous work in this (Edenberg et al., 1978) and other laboratories (Weissbach, 1977), DNA polymerase β was not retained by DEAE-cellulose in 0.02 M potassium phosphate (pH 7.5) and was insensitive to 5 mM *N*-ethylmaleimide. DNA polymerase γ was eluted as a peak at 0.10 M KCl with a shoulder at 0.15 M KCl, and DNA polymerase α was eluted at 0.21 M KCl. Fractions containing each DNA polymerase were pooled as indicated. In addition, HSV-1 DNA polymerase was extracted from nuclei isolated from HSV-1 infected CV-1 cells. The nuclei contained ~80% of the total HSV-1 DNA polymerase activity found in a lysate of virus-infected cells. When the dialyzed nuclear extract was chromatographed on DEAE-cellulose under the same conditions used for cellular DNA polymerases, HSV-1 DNA polymerase eluted at the same ionic strength as DNA polymerase α, 0.20 M KCl (Figure 1).

Each of the four partially purified DNA polymerases (α, β, γ, and HSV-1) could be uniquely identified under one of the assay conditions employed (Materials and Methods). HSV-1 DNA polymerase was ~10 times more active in the HSV-1 polymerase assay, which contained 150 mM K_2SO_4, than in the α-polymerase assay. Conversely, α-polymerase was 97% inhibited in the HSV-1 DNA polymerase assay (Figure 1). In contrast to α-polymerase, DNA polymerase γ, which is known to be stimulated by high salt and which also can use activated DNA as template–primer (Spadari & Weissbach, 1974b), was active in the HSV-1 DNA polymerase assay. When correcting for the difference in specific radioactivity of the labeled dNTP substrate and the fact that only one of the four substrates was labeled, we calculated that the activity of DNA polymerase γ in the HSV-1 DNA polymerase assay was 37% of that found in the γ assay. Because of the striking difference between DNA polymerases γ and α in their response to high salt, DNA polymerase γ could be distinguished from DNA polymerase α on activated DNA in partially purified preparations.

Preparation of in Vitro Systems That Replicate Cellular and Viral DNA. The object of determining the specificity of DNA synthesis inhibitors on isolated DNA polymerases was to allow a comparison with their effects on in vitro DNA replication. Previous work in this laboratory has shown that

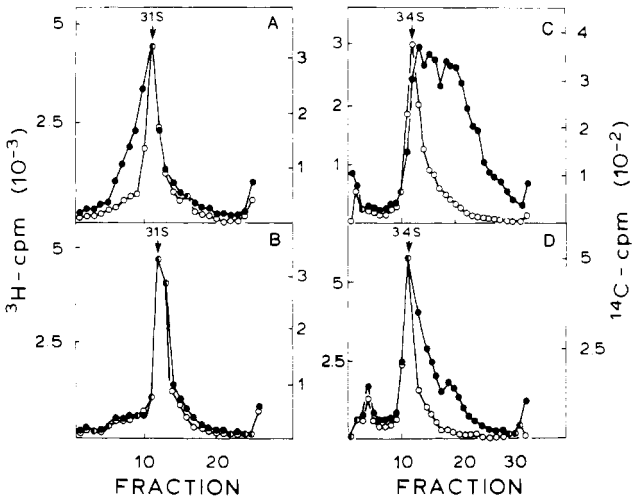

FIGURE 2: Synthesis of Ad-2 DNA in isolated Hela cell nuclei. Hela cell nuclei from Ad-2 infected cells prelabeled for 1 h with [^{14}C]-thymidine were pulse labeled for 5 min with [^{3}H]dTTP (4 μM, 12.5 Ci/mmol), and then the reaction was either immediately stopped or continued for 55 min in the presence of a 100-fold excess of unlabeled dTTP (see Materials and Methods). Adenovirus DNA was selectively extracted and analyzed by sedimentation through either neutral or alkaline sucrose gradients. Panel A: 5-min pulse, neutral sucrose. Panel B: 5-min pulse followed by 55-min chase, neutral sucrose. Panel C: 5-min pulse, alkaline sucrose. Panel D: 5-min pulse followed by 55-min chase, alkaline sucrose. (●) ^{3}H; (○) ^{14}C. Sedimentation is from right to left. The sedimentation coefficients of full length Ad-2 DNA are indicated.

SV40 DNA replication already in progress is faithfully continued in either isolated nuclei or nuclear extracts, resulting in the synthesis of mature forms of viral DNA (DePamphilis & Berg, 1975; DePamphilis et al., 1975; Su & DePamphilis, 1978; Anderson et al., 1977). Nuclei isolated from uninfected CV-1 cells or nuclei from SV40-infected CV-1 cells harvested prior to the onset of SV40 DNA synthesis and incubated under the same conditions also synthesize Okazaki fragments, join them to longer nascent DNA chains, and assemble nascent cellular DNA into chromatin (Shelton et al., 1978; data not shown).

Ad-2 DNA replication was also continued in nuclei isolated from Hela cells 20 h after infection and incubated under the same conditions used for SV40 and CV-1 DNA synthesis. At that time more than 90% of the DNA synthesized in vitro and in vivo was viral as judged from its sedimentation properties in alkaline and neutral sucrose gradients and from the finding that more than 90% of the labeled DNA was recovered by a procedure that specifically extracted viral DNA. These conditions closely resemble those reported for the synthesis of adenovirus DNA in nuclei isolated from KB cells except for the absence of 0.4 mM $CaCl_2$ and the presence of 100 μM GTP, CTP, and UTP (van der Vliet et al., 1977). The rate of Ad-2 DNA synthesis in nuclei was linear for ~20 min, after which it gradually declined until no synthesis was observed after 60 min. The newly synthesized viral DNA was not degraded since there was no loss of acid-precipitable radioactivity upon further incubation in the presence of excess unlabeled substrates. Single-stranded Ad-DNA chains synthesized in the first 5 min in the presence of [^{3}H]TTP were heterogeneous in length (Figure 2C), but a significant proportion matured to full length during continued incubation in an excess of unlabeled dTTP (Figure 2D). No loss of [^{3}H]DNA was observed during the chase period, and no further maturation of nascent DNA chains was observed when the chase period was extended to 115 min. Sedimentation analysis of nondenatured Ad-2 DNA showed that replicating

Table I: Inhibitor Concentration That Gives 50% Inhibition of DNA Synthesis

	DNA polymerases				DNA synthesis in subcellular systems ± cytosol[a]		
inhibitor	HSV-1	α	β	γ	CV-1	SV40	Ad-2
d_2TTP/dTTP	20	20	0.2	0.08	>20 (±)	>20 (±)	0.02 (–)
aphidicolin (μg/mL)	0.38	1.8	>125	>125	0.47 (–)	2.4 (–)	3.0 (–)
					0.16 (+)	2.0 (+)	
araCTP/dCTP		0.8	23	14	0.6 (±)	1.0 (±)	
MalNEt (mM)		0.5	>10			0.7 (+)	

[a] The presence (+) or absence (–) of cytosol is indicated in parentheses.

DNA (greater than 31 S, Figure 2A) was converted into mature DNA (31 S, Figure 2B). When replicating Ad-2 DNA was briefly labeled with [^{14}C]Thd in intact cells, DNA strands shorter than one genome length were converted to full length molecules during a subsequent in vitro incubation (data not shown). These data confirmed that Ad-2 DNA strands initiated in vivo were elongated in vitro.

Effects of d_2TTP on DNA Polymerase Activities. The unexpected observation (Edenberg et al. 1978) that a potential DNA chain terminator, d_2TTP, inhibits mammalian DNA polymerases β and γ without significantly inhibiting DNA polymerase α was first confirmed (Figure 3A). These and other enzymes were then assayed under several conditions to determine whether the discriminatory ability of d_2TTP was simply the result of either a particular primer–template combination or a set of assay conditions. For example, d_2TTP should terminate chains more effectively on poly(rA)–oligo(dT)$_{12-18}$ than on activated DNA. However, DNA polymerases β and γ were equally sensitive to d_2TTP on either primer–template (Figure 3A; Edenberg et al., 1978). One would also expect that a processive enzyme would be more sensitive to chain termination than a distributive enzyme which can more readily seek out new primer termini. However, *E. coli* polymerase I, a processive enzyme (Bambara et al., 1978), was slightly less sensitive to d_2TTP than DNA polymerase β, a distributive enzyme (Bambara et al., 1978), when assayed under the same conditions. The replication of intact T7 DNA by purified DNA polymerase and gene 4 protein was highly sensitive to d_2TTP (Kolodner & Richardson, 1977). This same degree of sensitivity was also observed for T7 DNA polymerase activity on activated salmon sperm DNA containing frequent single-stranded gaps; both T7 and T4 DNA polymerases were as sensitive to d_2TTP as DNA polymerase γ. In contrast, HSV-1 DNA polymerase, which is known to be required in replicating the HSV-1 genome (Aron et al., 1975), was as insensitive to d_2TTP as DNA polymerase α (Figure 3A). The HSV-1 DNA polymerase was slightly more sensitive in the α-polymerase assay than in the HSV-1 DNA polymerase assay and slightly less sensitive on activated DNA under the conditions used for SV40 DNA replication in vitro. Similarly, DNA polymerase α was less sensitive to d_2TTP when assayed on activated DNA under the in vitro replication conditions described for SV40 DNA than when assayed in the standard α-polymerase assay (Figure 3A). The reason for these slight variations in sensitivity under different assay conditions is not known, but irrespective of the assay conditions employed, the relative processivity of the enzyme, or the absence of a 3′- to 5′-exonuclease, DNA polymerase α and HSV-1 were 100–250-fold *less* sensitive to d_2TTP than were DNA polymerases β, γ (Table I), T4 and T7 and *E. coli* DNA polymerase I (data not shown).

The relative insensitivity of DNA polymerase α was not significantly affected by enzyme concentration, primer–template concentration, or the particular labeled substrate

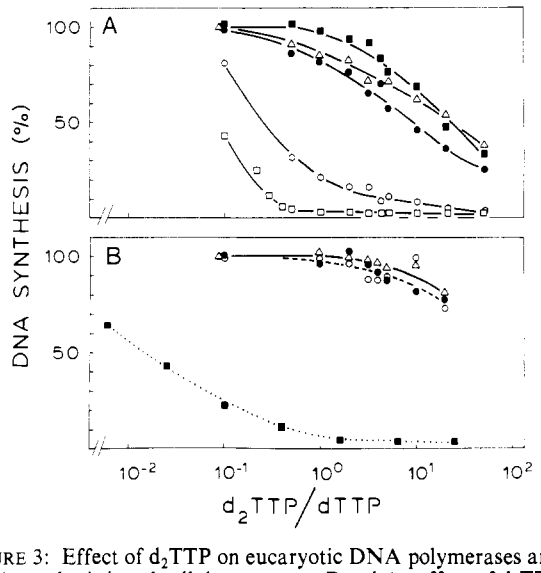

FIGURE 3: Effect of d_2TTP on eucaryotic DNA polymerases and on DNA synthesis in subcellular systems. Panel A: effect of d_2TTP on α, β, γ, and HSV-1 DNA polymerases. (●) α-Polymerase on activated DNA in α-polymerase assay; (■) α-polymerase on activated DNA in the in vitro DNA replication assay; (○) β-polymerase on activated DNA in β-polymerase assay; (□) γ-polymerase on activated DNA in HSV-1 DNA polymerase assay; (△) HSV-1 DNA polymerase on activated DNA in HSV-1 DNA polymerase assay. The final concentration of dTTP was 20 μM, and the specific activity of [α-^{32}P]dCTP was 0.2 mCi/μmol for the α, β, and HSV-1 DNA polymerase assays. In the in vitro DNA replication assay with α-polymerase the specific radioactivity was 1 mCi/mol, and in the γ-polymerase assay, [^{3}H]dTTP was 2 mCi/mol. The amount of incorporation defined as 100% was 35 675 cpm for α-polymerase in the α-polymerase assay, 65 550 cpm for α-polymerase in the in vitro assay, 20 305 cpm for β-polymerase in the β-polymerase assay, 10 523 cpm for γ-polymerase in the HSV-1 DNA polymerase assay, and 126 950 cpm for HSV-1 DNA polymerase in the HSV-1 DNA polymerase assay. Panel B: effect of d_2TTP on in vitro synthesis of cellular and viral DNA. CV-1 DNA synthesis in isolated nuclei in the absence (○) or presence (●) of added cytosol, SV40 DNA synthesis in a soluble nuclear extract supplemented with cytosol (△), and Ad-2 DNA synthesis in isolated nuclei without cytosol present (■). The dTTP concentration was always 10 μM. Results were expressed as a percentage of the amount of [^{32}P]dCMP (●, ○, and △) or [^{3}H]dTMP (■) incorporated in the absence of d_2TTP. The specific radioactivity for [α-^{32}P]dCTP was 2 Ci/mmol, and that for [^{3}H]dTTP was 12.5 Ci/mmol. The amount of incorporation defined as 100% was 28 234 cpm for SV40, 20 106 cpm for CV-1 nuclei + cytosol, 7300 cpm for CV-1 nuclei – cytosol, and 184 284 cpm for Ad-2. All assays in panels A and B were done in duplicate.

employed. DNA polymerase α remained insensitive to d_2TTP from 0.005 to 0.1 units of enzyme per assay. Even when the concentration of activated DNA was varied 250-fold, a 10-fold excess of d_2TTP over dTTP never inhibited DNA synthesis more than 50% as measured by the simultaneous incorporation of both [α-^{32}P]dCMP and [^{3}H]dTMP (Figure 4). Despite the complex but reproducible relationship between percent inhibition and primer–template concentration, DNA polymerase α was relatively insensitive to levels of d_2TTP that

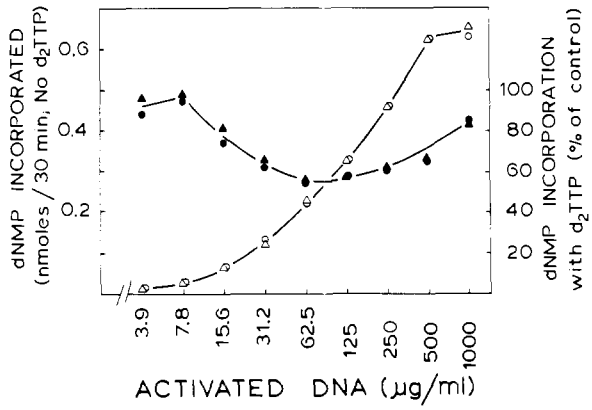

FIGURE 4: Effect of d_2TTP on DNA polymerase α as a function of template–primer concentrations. DNA polymerase α activity as a function of template–primer concentration was measured by the incorporation of [^{32}P]dCMP (○) and [^{3}H]dTMP (△); both dNTPs were present in the same assay mixture. Inhibition of DNA synthesis by d_2TTP was measured at each concentration of activated DNA [(●) [^{32}P]dCMP and (▲) [^{3}H]dTMP]. The results are expressed as percentage of the amount of nucleotide incorporated in the presence of d_2TTP relative to the amount of incorporation in the absence of the inhibitor. The ratio d_2TTP/dTTP was 10. The concentration of dTTP was 20 μM.

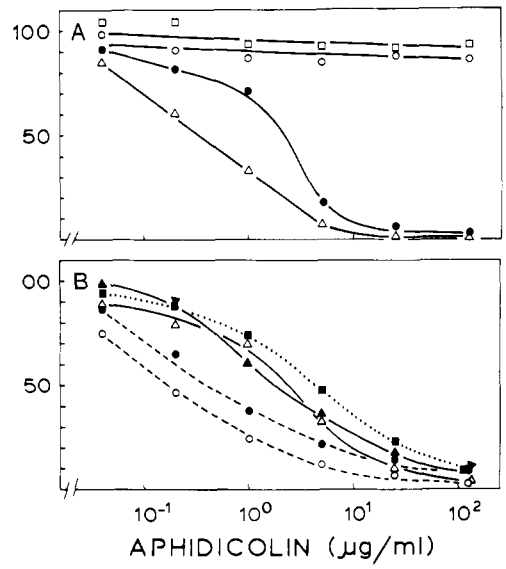

FIGURE 5: Effects of aphidicolin on DNA polymerase activities and on DNA synthesis in subcellular systems. Panel A: effect of aphidicolin on α-polymerase activity in α-polymerase assay (●), β-polymerase activity in β-polymerase assay (○), γ-polymerase activity in HSV-1 polymerase assay (□), and HSV-1 DNA polymerase activity in HSV-1 polymerase assay (△). Activated DNA was the template–primer in each case. Panel B: effect of aphidicolin on synthesis of CV-1 DNA in isolated nuclei in the absence (●) and presence (○) of cytosol, SV40 DNA synthesis in the soluble nuclear extract in the absence (▲) and presence (△) of cytosol, and Ad-2 DNA synthesis in isolated nuclei (■). Assay conditions and the assay of incorporated radioactivity are described under Materials and Methods. All incubations in both panels were for 30 min and were performed in duplicate. Specific radioactivities were the same as those described in Figure 3. The actual amount of incorporation in each case was equivalent to those amounts in Figure 3.

inhibited DNA polymerases β and γ at least 90%, even when the rate of DNA synthesis was limited by the amount of primer–template. If α-polymerase were highly distributive under these conditions and if d_2TTP were inhibited by terminating chains, then inhibition of [^{3}H]dTMP incorporation would have been expected to be more pronounced than inhibition of [α-^{32}P]dCMP incorporation. Such was the case for araCTP inhibition of β-polymerase, a distributive enzyme, where araCTP chain termination inhibited incorporation of dCTP more effectively than incorporation of dTTP (Yoshida et al., 1977). Therefore, it appeared that α-polymerase did not avoid the inhibitory effects of chain termination by rapidly jumping from one primer terminus to the next, but instead discriminated between d_2TTP and dTTP at the level of substrate recognition. This conclusion was also supported by the finding that d_2TTP had virtually no effect on the synthesis of CV-1 or SV40 DNA in subcellular systems where the primer–template concentration was limiting and DNA synthesis presumably processive (see below).

Effects of d_2TTP on Cellular and Viral DNA Synthesis. The surprising discovery that d_2TTP fails to inhibit any phase of SV40 DNA replication in isolated nuclei, nuclear extracts, or isolated replicating chromosomes (Edenberg et al., 1978) was confirmed (Figure 3B). In addition, d_2TTP failed to inhibit DNA synthesis in uninfected CV-1 cell nuclei incubated under the same conditions, with or without the addition of cytosol (Figure 3B), suggesting that both virus and host share the same mechanism for DNA replication. In contrast, Ad-2 DNA synthesis in isolated Hela cell nuclei was as sensitive to d_2TTP as was DNA polymerase γ (Figure 3B), although uninfected Hela cell nuclei, like CV-1 cell nuclei, were as insensitive to d_2TTP as DNA polymerase α. These results suggest that both SV40 and cellular DNA replication use DNA polymerase α exclusively, while Ad-2 DNA replication involves DNA polymerase γ (Table I).

Effects of Aphidicolin on DNA Polymerase Activities and DNA Replication. In agreement with a recent report (Ikegami et al., 1978), we found that aphidicolin can completely inhibit DNA polymerase α with no apparent effect on the activity of either DNA polymerase β or γ (Figure 5A). Furthermore, HSV-1 DNA polymerase was even more sensitive to aphidicolin than α-polymerase (Figure 5A). Aphidicolin inhibited SV40, CV-1, and Ad-2 DNA synthesis in a manner similar to its inhibition of α-polymerase regardless of the presence or absence of cytosol (Figure 5B), which stimulated SV40 and CV-1 DNA replication three- to fourfold. Me_2SO, which was used to dissolve aphidicolin, had no effect on DNA synthesis at the highest concentration present (0.6%) in any of the assays. These data suggest that DNA polymerase α is required for SV40, CV-1, and Ad-2 DNA replication (Table I).

Effects of araCTP on DNA Polymerase Activities and DNA Replication. The dCTP analogue araCTP inhibited DNA polymerase α activity from 20 to 30 times more effectively than either DNA polymerase γ or β, respectively (Figure 6A). AraCTP inhibited SV40 and CV-1 DNA synthesis in essentially the same manner as it inhibited DNA polymerase α (Figure 6B). DNA polymerase α and β activities were tested under conditions identical with those for in vitro synthesis of DNA in the subcellular systems, except that activated DNA was substituted for nuclei or nuclear extracts. DNA polymerase γ was also assayed on activated DNA, but in the presence of 150 mM K_2SO_4 to suppress any contaminating α-polymerase activity. AraCTP did not inhibit γ-polymerase on a poly(rA)–oligo(dT) template–primer. [^{3}H]dTTP was used rather than labeled dCTP to avoid direct competition between araCTP and the labeled substrate (Wist et al., 1976; Yoshida et al., 1977). These data again suggest that DNA polymerase α is involved in replicating both SV40 and CV-1 DNA (Table I).

Reconstitution of SV40 DNA Replication in Nuclear Extracts Inactivated with N-Ethylmaleimide (MalNEt). DNA polymerase α is highly sensitive to MalNEt, whereas DNA polymerase β is resistant (Figure 7A); DNA polymerase

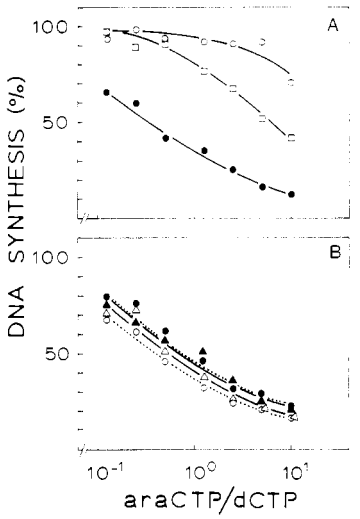

FIGURE 6: Effect of araCTP on DNA polymerase activities and on DNA synthesis in subcellular systems. Panel A: effect of araCTP on α-polymerase activity on activated DNA in the in vitro DNA replication assay (●), β-polymerase activity on activated DNA in the in vitro DNA replication assay (○), and γ-polymerase activity on activated DNA in the HSV-1 DNA polymerase assay (□). β-Polymerase was first treated with 5 mM *N*-ethylmaleimide. The concentration of dCTP was 20 μM, and that of [^{3}H]dTTP was 20 μM (1 mCi/μmol). Panel B: effect of araCTP on synthesis of CV-1 DNA in isolated nuclei in the absence (●) and presence (○) of added cytosol and on SV40 DNA synthesis in the nuclear extract in the absence (▲) and presence (△) of added cytosol. All assays were done in duplicate. Specific radioactivities and the amounts of radioactivity incorporated were as described in Figure 3.

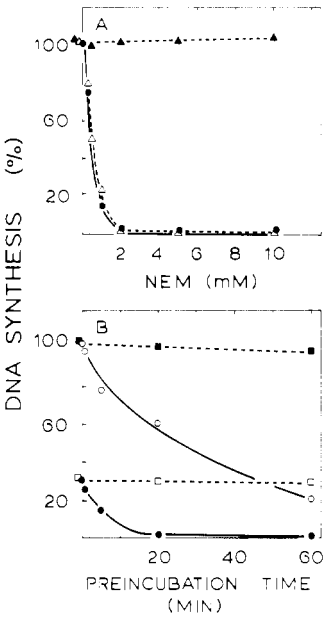

FIGURE 7: MalNEt sensitivities of DNA polymerases α and β and SV40 DNA synthesis in nuclear extracts. Panel A: effect of MalNEt on DNA polymerase α in the α-polymerase assay (△), DNA polymerase β in the β-polymerase assay (▲), and SV40 DNA synthesis in nuclear extracts (●) during a 30-min incubation at 37 (polymerases) or 30 °C (nuclear extract). The results are expressed as a percentage of the amount of [^{32}P]dCMP incorporated into DNA in the absence of MalNEt. Panel B: nuclear extracts were incubated at 0 °C either with or without 3 mM MalNEt for the times indicated. Unreacted MalNEt was then inactivated by addition of 15 mM 2-mercaptoethanol, and the incubation was continued for at least 10 min. 2-Mercaptoethanol was also added to the controls. SV40 DNA synthesis was then measured during a 30-min incubation at 30 °C: MalNEt-treated nuclear extract minus cytosol (●) and plus cytosol (○); control nuclear extract minus cytosol (□) and plus cytosol (■). Results are expressed as a percentage of the amount of [^{32}P]dCMP incorporated in unlabeled nuclear extracts in the presence of cytosol.

γ is intermediate in its sensitivity [for a review, see Weissbach (1977)]. Inactivation of DNA synthesis by MalNEt in nuclear extracts containing replicating SV40 chromosomes was indistinguishable from the inactivation curve for DNA polymerase α (Figure 7A). This suggested that inactivation of α-polymerase was the primary reason for loss of DNA synthesis, although other proteins were likely to be modified by MalNEt as well. Since excess MalNEt could be destroyed by addition of dithiothreitol or 2-mercaptoethanol, we attempted to reconstitute MalNEt-inactivated nuclear extracts by addition of cell extracts and/or partially purified enzymes from uninfected cells. Isolated nuclei or nuclear extracts rapidly lost their ability to replicate SV40 DNA when incubated at 37 °C even in the absence of DNA synthesis. However, at 0 °C they were stable for several hours. Therefore, nuclear extracts were inactivated at 0 °C by treating then with 3 mM MalNEt for varying lengths of time. The unreacted MalNEt was then destroyed by addition of 15 mM 2-mercaptoethanol, and the inactivated nuclear extract was assayed for its ability to continue SV40 DNA replication after addition of cytosol (Figure 7B). Limited treatment with MalNEt (20 min) inactivated 90% of the DNA synthesis normally observed in the absence of cytosol. In 10 different experiments addition of cytosol reconstituted 40–70% of the endogenous SV40 DNA synthesis activity observed in the control with cytosol. Longer MalNEt treatment reduced the ability to reconstitute DNA synthesis. The products of viral DNA synthesis in the reconstituted system were normal as judged from sedimentation analysis in neutral and alkaline sucrose gradients (Figure 8). All of the newly synthesized [^{32}P]DNA in both reconstituted MalNEt-treated nuclear extracts (Figure 8A) and controls (Figure 8D) was associated with native SV40 replicating DNA. Short nascent DNA fragments (4–5 S) were observed when the in vitro incubations were limited to 1.5–3 min (parts B and E of Figure 8). The rate of DNA replication in reconstituted nuclear extracts was ~50% less than that in the control. These Okazaki fragments were joined to longer DNA chains when the in vitro incubations were continued with an excess of unlabeled substrates (parts C and F of Figure 8).

MalNEt inactivation of SV40 replicating chromosomes was consistent with a specific involvement of DNA polymerase α in SV40 DNA replication (Figure 7A). This hypothesis was supported by the finding that aphidicolin, but not d_2TTP, prevented reconstitution of MalNEt-inactivated nuclear extracts by cytosol (Table II). Furthermore, DNA polymerases β and γ did not substitute for DNA polymerase α when these enzymes were added together with cytosol and aphidicolin. The cytosol activity, required for in vitro synthesis of covalently closed, superhelical SV40 form I DNA (DePamphilis & Berg, 1975; Su & DePamphilis, 1978; Anderson et al., 1977), was eluted from DEAE-cellulose and then fractionated on phosphocellulose to separate DNA polymerase α from a nonbinding protein fraction (the pass-through) that contains all of the nuclei complementing activity (B. Karas, unpublished experiments). The DEAE-adsorbed material containing α-polymerase was as active as cytosol in restoring activity to MalNEt-inactivated viral chromosomes (Table II). However, the phosphocellulose pass-through material, which did not contain any detectable DNA polymerase activity, failed to reconstitute the MalNEt-inactivated nuclear extract but was still able to complement controls as effectively as cytosol. DNA polymerase α, partially purified from DEAE-cellulose as described in Figure 1 or further purified by phosphocellulose

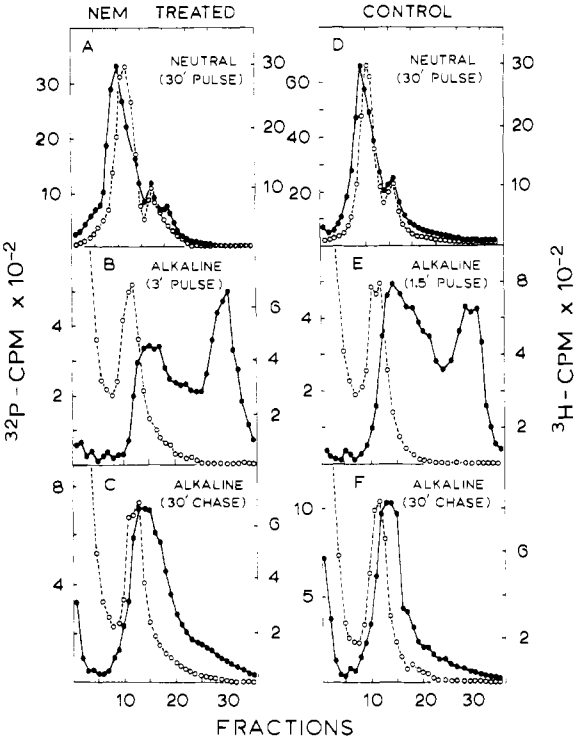

FIGURE 8: Sedimentation velocity analysis of SV40 synthesized in MalNEt-inactivated nuclear extracts reconstituted with cytosol. Nuclear extracts prepared from cells preincubated with [^{3}H]Thd were inactivated by incubation with 3 mM MalNEt for 20 min at 0 °C, and unreacted MalNEt was destroyed by 15 mM 2-mercaptoethanol. Control nuclear extracts were not treated. Nuclear extracts were then pulse labeled with [α-^{32}P]dCTP (6 M, 12 Ci/mmol) in an assay mixture supplemented with cytosol. Incubations were terminated with 1% Sarkosyl, 1 M NaCl, and 10 mM EDTA, and each sample was incubated with proteinase K (200 μg/mL) at 30 °C for 20 min. MalNEt-treated (panel A) and control nuclear extracts (panel D) were pulse labeled for 30 min and the DNA products analyzed by sedimentation in neutral sucrose gradients. MalNEt-treated (panel B) and control nuclear extracts (panel E) were pulse labeled for 3 and 1.5 min, respectively, or MalNEt-treated (panel C) and control nuclear extracts (panel F) were pulse labeled for a period of 3 or 1.5 min, respectively, after which the incubation was continued for 30 min in the presence of a 100-fold excess of unlabeled dCTP ("chase") and the DNA products were sedimented in alkaline sucrose gradients. Neutral sucrose gradients in SW60 tubes were run in a Beckman SW60 rotor at 55 000 rpm for 4.5 h (4 °C), and alkaline gradients were run at 55 000 rpm for 6 h (4 °C). Fractions were collected from the bottom of the tubes, and the DNA in each fraction was precipitated with acid onto a GF/C filter, washed, and analyzed in a scintillation counter. (○) [^{3}H]DNA; (●) [^{32}P]DNA.

chromatography, neither reconstituted the MalNEt-treated nuclear extract nor stimulated the control. Since DNA polymerase α is already present in SV40 replicating chromosomes (Edenberg et al., 1978), further addition of α-polymerase was not expected to stimulate DNA synthesis in control nuclear extracts. However, the failure of the phosphocellulose pass-through material to efficiently aid α-polymerase in reconstituting MalNEt-inactivated nuclear extracts suggests that an additional MalNEt-sensitive component originally present in the cytosol and DEAE-adsorbed material is required for complete reconstitution. The inability to achieve complete reconstitution of DNA synthesis may have been due to inactivation of chromosomal proteins that could not easily be replaced. Addition of nuclear extracts from uninfected cells together with cytosol did not improve reconstitution.

Effects of Aphidicolin on DNA Synthesis in Intact Cells. As expected, aphidicolin inhibited SV40, HSV-1, and CV-1 DNA synthesis in living cells, although inhibition of Ad-2 DNA synthesis required higher concentrations. These results

Table II: Reconstitution of SV40 DNA Synthesis in MalNEt-Inactivated Nuclear Extracts[a]

addition	SV40 DNA synthesis (%) control	MalNEt treated
buffer	35	3
cytosol	100	56
cytosol + aphidicolin (25 μg/mL)	8	8
cytosol + d_2TTP (d_2TTP/dTTP = 10)	92	54
cytosol + aphidicolin + DNA polymerases $\beta + \gamma$	8	11
DEAE-adsorbed material	100	52
phosphocellulose pass-through material	92	4
DNA polymerase α	36	5
DNA polymerase α + phosphocellulose pass-through	98	9

[a] Both control and MalNEt-treated nuclear extracts were incubated for 30 min at 30 °C. The incorporation in control was defined as 100%. "Buffer" refers to the dialysis solution used in preparing cytosol [(20 mM Hepes-Na (pH 7.8), 0.4 M ethylene glycol, 50 mM KCl, 0.5 mM $MgCl_2$, and 0.5 mM dithiothreitol]. "DEAE-adsorbed material" is the fraction of cytosol that adsorbed to DEAE-cellulose equilibrated with dialysis buffer minus 0.5 mM $MgCl_2$ and that eluted between 0.05 and 0.35 M KCl. "Phosphocellulose pass-through material" refers to the fraction of the DEAE-adsorbed material that did not adsorb to phosphocellulose equilibrated with 20 mM potassium phosphate (pH 7.5), 75 mM KCl, 1 M ethylene glycol, and 0.5 mM dithiothreitol. The nonadsorbed material was precipitated with 80% $(NH_4)_2SO_4$, dissolved, and dialyzed against cytosol buffer. The amount of DNA polymerase α in cytosol or DEAE-adsorbed material was 0.6–1 unit/assay. The amount of DNA polymerase added to MalNEt-inactivated nuclear extracts was 0.1–0.6 unit/assay. Each experiment was performed at least twice with duplicate assays, and the results of all experiments were averaged together.

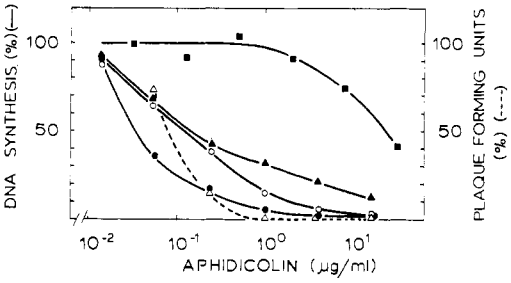

FIGURE 9: Effect of aphidicolin on SV40, Ad-2, HSV-1, and CV-1 replication in intact cells. Uninfected CV-1 cells (●), SV40 infected CV-1 cells (○), Ad-2 infected CV-1 cells (■), or HSV-1 infected CV-1 cells (▲) were incubated for 1 h with [^{3}H]Thd in the presence of aphidicolin during the period of maximum DNA synthesis in each case. Incorporation of [^{3}H]TMP into the appropriate DNA was measured as described under Materials and Methods. HSV-1 plaque-forming units (△) were measured at 19 h after infection. Aphidicolin was added with the [^{3}H]Thd, but in the infectious particle assay it was added 1 h after infection. The results are expressed as the percent of DNA synthesis relative to a control without aphidicolin. Actual incorporation defined as 100% was 284 189 cpm/dish for CV-1, 297 677 cpm/dish for SV40, 109 243 cpm/dish for Ad-2, and 215 860 cpm/dish for HSV-1. Each dish contained ~2 × 10^6 cells. The plaque assay control contained 2.3 × 10^8 pfu/dish. In the presence of 16 μg/mL aphidicolin, 1.2 × 10^5 pfu was obtained. All assays were done in duplicate.

were based both on changes in the rates of incorporaton of DNA precursors and by demonstrating a 10^3-fold reduction in HSV-1 plaque-forming units (Figure 9). Aphidicolin (2–32 μg/mL) had no effect on the incorporation of [^{14}C]leucine into acid-precipitable material, which was consistent with its specificity for DNA polymerase α and HSV-1 polymerase. Ad-2 DNA synthesis was examined both during its abortive infection of CV-1 cells (Figure 9) as well as during a prod-

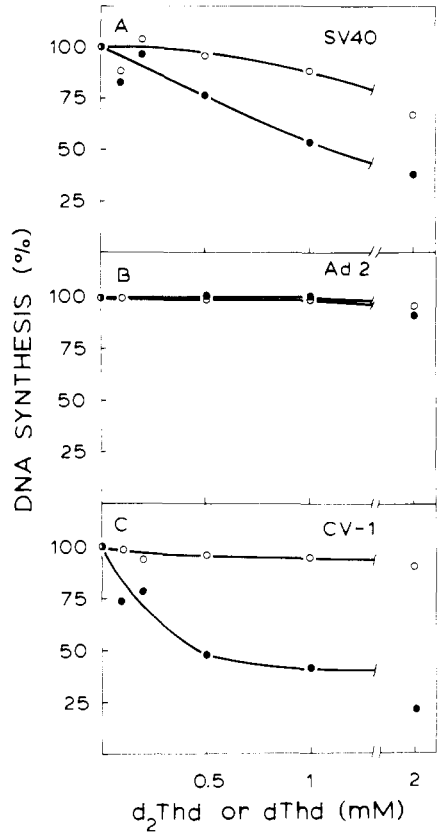

FIGURE 10: Effect of d_2Thd and dThd on viral and cellular DNA synthesis. CV-1 cells infected with either SV40 (A) or Ad-2 (B) and uninfected exponentially growing CV-1 cells (C) were incubated for 1 h at 37 °C with [^{3}H]dAdo and dCyd in the presence of d_2Thd (○) or dThd (●), which were added 30 min prior to the radiolabeled substrate. Incorporation of the radiolabeled substrate was measured as described under Materials and Methods.

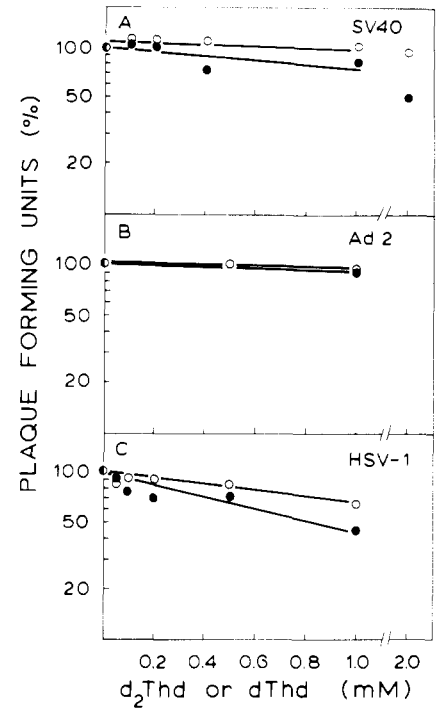

FIGURE 11: Effect of d_2Thd and dThd on virus replication. CV-1 cells were infected with SV40 (A) or HSV-1 (C) and Hela cells were infected with Ad-2 (B), and the excess virus was washed off the cells. At 1-h postinfection, either d_2Thd (○) or dThd (●) was added to the medium. At either 19 (HSV-1), 32 (Ad-2), or 48 h (SV40), the cells were scraped off the plate into the medium and subjected to three cycles of freeze–thawing. Cell debris was removed by centrifugation (2000*g*, 10 min, 2 °C). Infectivity was then titrated by plaque formation.

uctive infection of Hela cells (data not shown). Although the rate of Ad-2 DNA synthesis in these two cell lines was equivalent, aphidicolin had no effect on viral DNA synthesis in Hela cells. Similarly, HSV-1 DNA synthesis was also resistant to aphidicolin in these Hela cells. These apparent inconsistencies may stem from the fact that ~40% of the Hela cell DNA synthesis appeared to be completely resistant to aphidicolin whereas at least 98% of CV-1 cell DNA synthesis was sensitive. However, 50% of the DNA synthesis in either cell line was inhibited by 0.05 μg of aphidicolin per mL.

Effects of d_2Thd on DNA Synthesis in Intact Cells. d_2Thd was tested for its effects on DNA replication in living cells. Concentrations of up to 2 mM d_2Thd in the cell culture medium for 1 h prior to addition of [^{3}H]dAdo had little or no effect on the rate of either SV40, Ad-2, or CV-1 cell DNA synthesis (Figure 10). In fact, increasing concentrations of the normal DNA substrate, dThd, were generally more inhibitory than the corresponding amount of d_2Thd. In these experiments, 10 μM dCyd was added to the medium to counteract the effect of high concentrations of dThd on the intracellular dCTP pool (Bjursell & Reichard, 1973).

Prolonged incubation with 1 mM d_2Thd had no apparent effect on the structure of nascent DNA. Addition of this nucleotide analogue to SV40-infected CV-1 cells did not change either the size distribution of nascent DNA chains in replicating SV40 DNA or the fraction of newly synthesized covalently closed, superhelical SV40 DNA. In a similar experiment, the size distribution of nascent DNA chains in exponentially growing uninfected CV-1 cells was unaffected by the presence of 0.5 mM d_2Thd. Therefore, d_2Thd did not terminate the growth of DNA strands because there was no accumulation of either viral or cellular short nascent DNA chains.

Effects of d_2Thd on Virus and Cell Growth. The complex allosteric controls that regulate deoxyribonucleotide pool sizes may complicate interpretation of DNA synthesis studies involving labeled DNA precursors (Bjursell & Reichard, 1973). Therefore, the effect of d_2Thd on the production of infectious virus was also measured. Consistent with the failure of d_2Thd to inhibit DNA synthesis (Figure 10), this nucleoside, when added immediately after infection, was also unable to inhibit the production of either infectious SV40, HSV-1, or Ad-2 (Figure 11). In contrast, 1 mM d_2Thd caused a 1000-fold decrease in the production of phage T7 in *E. coli* (data not shown) in spite of the fact that phosphorylation of d_2Thd is slow in *E. coli* (Atkinson et al., 1969). CV-1 cells continued to replicate in the presence of 0.5 mM d_2Thd with little effect on their growth rate. However, after ~20-h exposure to the same levels of either d_2Thd or dThd at concentrations greater than 0.5 mM, dead cells began to accumulate in the culture. Although these results were consistent with the failure of d_2Thd to inhibit either HSV-1 DNA polymerase activity or SV40 or CV-1 DNA replication in vitro, the failure of d_2Thd to inhibit the synthesis of Ad-2 DNA and infectious virus was completely unexpected since Ad-2 DNA replication in isolated nuclei was inhibited 50% at a d_2TTP/dTTP ratio of 0.02. This paradox prompted an investigation of the ability of CV-1 and Hela cells to convert d_2Thd to d_2TTP.

Uptake and Phosphorylation of d_2Thd by Intact Cells. The ability of CV-1 cells to convert d_2Thd into d_2TTP, the active form of the potential inhibitor of DNA synthesis, was measured by two different methods. In the first method,

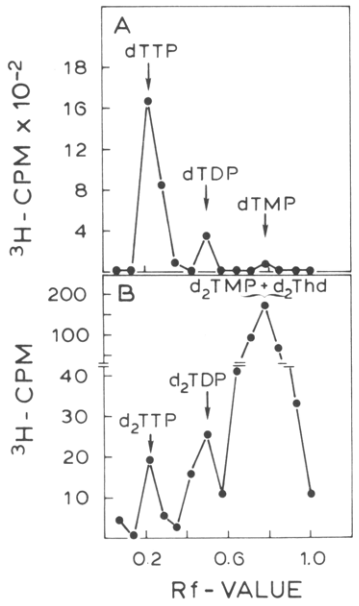

FIGURE 12: Phosphorylation of [^{3}H]d_2Thd and [^{3}H]dThd in SV40-infected cells. SV40-infected CV-1 cells were labeled for 15 min with either [^{3}H]dThd or [^{3}H]d_2Thd and the acid-soluble nucleotides recovered by DEAE-Sephadex chromatography. In the case of [^{3}H]d_2Thd, about 99% of the free nucleoside and 70–80% of [^{3}H]d_2TMP were removed by this step. Only the sample from [^{3}H]d_2Thd-treated cells was concentrated 22-fold. Aliquots of 5 μL of ^{3}H-labeled deoxynucleotides (panel A) and 20 μL of ^{3}H-labeled dideoxynucleotides (panel B) were analyzed on polyethyleniminecellulose thin layers using 1 M LiCl–1 M HCOOH as the solvent. The positions of UV-absorbing standards are indicated in the figure.

SV40-infected CV-1 cells were incubated with [^{3}H]d_2Thd for varying periods of time. The ^{3}H label was rapidly taken up by the cells (80% in the first 20 min). At least 90% of the radioactivity recovered from cells at 2 h was [^{3}H]d_2Thd; complete separation of d_2Thd from its phosphorylated forms was accomplished by chromatography on polyethyleniminecellulose using water as the solvent. Experiments with [^{3}H]dThd showed that ~90% of the acid-soluble radioactivity appeared in [^{3}H]dTTP within 15 min. In order to detect small amounts of [^{3}H]d_2TTP, we first isolated the ^{3}H-labeled nucleotides by chromatography on DEAE-Sephadex, concentrated them 20-fold, and then chromatographed them on polyethyleniminecellulose. Almost all of the nucleoside and ~80% of the nucleoside monophosphate were removed in the DEAE-Sephadex step. Of the *remaining radioactivity*, 91% was [^{3}H]d_2TMP, 6% was [^{3}H]d_2TDP, and 3% was [^{3}H]d_2TTP (Figure 12B). The analogous control experiments with [^{3}H]dThd resulted in 3% of the radioactivity recovered as [^{3}H]dTMP, 12% as [^{3}H]dTDP, and 85% as [^{3}H]dTTP (Figure 12A). On the basis of only the specific radioactivities of the added nucleosides, the intracellular concentration of dTTP was at least 8000-fold greater than that of d_2TTP. The identity of the small amount of [^{3}H]d_2TTP (~20 cpm above background) in Figure 12B was confirmed by thin-layer chromatography of another aliquot from the DEAE-Sephadex column on cellulose using isobutyric acid–NH_4OH–water (66:1:33) as the solvent which separates d_2TTP from dTTP. About 20 cpm again cochromatographed with an added d_2TTP standard. Therefore, the amount of d_2TTP synthesized was negligible. A similar experiment, carried out with uninfected Hela and CV-1 cells, also failed to detect significant phosphorylation of d_2Thd.

The second method measured the synthesis of [^{32}P]dTTP by incubating SV40-infected CV-1 cells with [^{32}P]phosphate for 3 h before adding 1 mM d_2Thd to the medium and

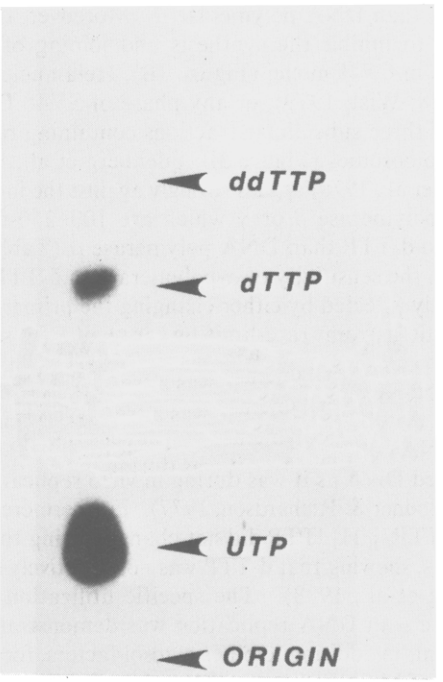

FIGURE 13: TLC of fractionated ^{32}P-labeled nucleoside triphosphates from cell extracts. ^{32}P-labeled nucleoside triphosphates from [^{32}P]phosphate-labeled cells were fractionated sequentially on DEAE-Sephadex and dihydroxyboroylcellulose and by high-voltage paper electrophoresis to separate dTTP, d_2TTP, and contaminating UTP from other nucleotides. An aliquot of the sample which also contained unlabeled UTP, dTTP, and d_2TTP as UV-absorbing standards was fractionated on cellulose thin layers using isobutyric acid–NH_4OH–H_2O (66:1:33) as the solvent. Location of the radioactivity was visualized by autoradiography using Kodak XR-5 film with a Kodak regular intensifying screen for 2 days at −70 °C. To avoid merging of overexposed spots, we cut the thin layer between UTP and dTTP and separated the spots by 2 cm prior to autoradiography. [^{32}P]d_2TTP was not detected even after 14 days of exposure. The centers of the UV-absorbing standards are indicated: d_2TTP (R_f 0.36), dTTP (R_f 0.24), and rUTP (R_f 0.13).

continuing the incubation for 2 h. The ^{32}P-labeled nucleotides were first isolated by chromatography on DEAE-Sephadex, and ribonucleotides were then separated from deoxyribonucleotides by chromatography on dihydroxyboroylcellulose followed by high-voltage paper electrophoresis. A reconstitution experiment using [^{3}H]dTTP in a 100-fold excess of d_2TTP (detected by its UV absorbtion) showed that d_2TTP and dTTP comigrated through these three steps. Thin-layer chromatography was then used to resolve dTTP, d_2TTP, and contaminating UTP. No [^{32}P]d_2TTP was detected even when the [^{32}P]dTTP and [^{32}P]UTP spots were greatly overexposed (Figure 13). Therefore, CV-1 cells were unable to convert d_2Thd to d_2TTP even in the presence of high d_2Thd concentrations.

Discussion

The combined results from inhibition studies with d_2TTP, aphidicolin, and araCTP and reconstitution studies with MalNEt-inactivated SV40 chromosomes offer the most direct evidence available that CV-1 cell nuclei (and presumably other mammalian cells) and SV40 (and presumably polyoma virus) use DNA polymerase α exclusively in replicating their DNA. DNA replication in vitro was inhibited by aphidicolin and araCTP in essentially the same manner as isolated DNA polymerase α was inhibited (Figures 5 and 6, Table I). DNA polymerases β and γ were not inhibited by aphidicolin and were about 30- and 20-fold less sensitive to araCTP, re-

spectively, than DNA polymerase α. Moreover, the failure of d_2TTP to inhibit the synthesis and joining of Okazaki fragments in CV-1 nuclei (Figure 3B), Hela nuclei (Waqar et al., 1978; Wist, 1979), or any phase of SV40 DNA replication of three subcellular fractions containing replicating SV40 chromosomes (Figure 3B; Edenberg et al., 1978; DePamphilis et al., 1978) argues strongly against the involvement of DNA polymerase β or γ which are 100–250-fold more sensitive to d_2TTP than DNA polymerase α (Table I). We found that the sensitivity of α-polymerase to d_2TTP was not significantly affected by either changing the primer-template concentration from rate-limiting amounts to saturating amounts or changing the labeled substrate from dTTP to dCTP. DNA polymerases β and γ were severely inhibited by d_2TTP on either activated DNA or poly(rA)–oligo$(dT)_{12-18}$, and T7 DNA polymerase was as sensitive to d_2TTP inhibition on activated DNA as it was during in vitro replication of T7 DNA (Kolodner & Richardson, 1977). Furthermore, the ratio [α-^{32}P]d_2TTP/[^{3}H]dTTP did not change during the in vitro incubations, showing that d_2TTP was not selectively destroyed (Edenberg et al., 1978). The specific utilization of DNA polymerase α in DNA replication was demonstrated by its requirement, in addition to other cytosol factors, for restoring the ability of MalNEt-inactivated nuclear extracts to continue SV40 DNA replication (Figures 7B and 8, Table II). DNA polymerases β and γ did not substitute for α-polymerase when added to aphidicolin-treated cytosol.

Ad-2 DNA replication was sensitive to both aphidicolin and d_2TTP and therefore involves both DNA polymerases α and γ (Figures 3 and 5, Table I). Neither SV40 nor adenovirus appears to encode their own DNA polymerase (Weissbach, 1977). Adenovirus DNA replication is different from cell nuclear and papovavirus DNA replication in three ways: Ad DNA replication requires both γ- and α-polymerases, it proceeds unidirectionally from either end by a strand displacement mechanism similar to mitochondrial DNA, and it does not appear to rely on discontinuous DNA synthesis (Winnacker, 1978). Mitochondrial DNA synthesis may also use γ-polymerase (Bertazzoni et al., 1977; Hübscher et al., 1977). It would therefore be of interest to study the effect of aphidicolin and d_2TTP on mitochondrial DNA replication.

Inhibition of SV40 and CV-1 DNA synthesis by aphidicolin suggests that DNA polymerase α is required for DNA replication in intact cells as well as in subcellular systems. This conclusion is based on the specificity of aphidicolin for DNA polymerase α compared to DNA polymerases β and γ as previously described. In addition, we found no effect of aphidicolin on protein synthesis, in agreement with an earlier report (Ikegami et al., 1978). Aphidicolin is also a strong inhibitor of HSV-1 DNA polymerase, which explains the earlier observation that multiplication of herpes virus is strongly inhibited by this drug (Bucknall et al., 1973). This observation was confirmed for HSV-infected CV-1 cells, where we found a strong inhibition of DNA synthesis with a concomitant decrease in the synthesis of infectious virus. However, Ad-2 DNA synthesis in intact cells was more resistant to aphidicolin than expected from the relative sensitivities of CV-1, SV40, and Ad-2 DNA synthesis in subcellular systems. The reason for this apparent discrepancy may stem from the fact that inhibition of DNA synthesis by aphidicolin required $\sim$5 times lower concentration in vivo than in vitro. This suggests that aphidicolin is either concentrated and/or metabolically activated by the cell. Therefore, some cell lines, such as Hela, may be less sensitive to aphidicolin, and some viruses, such as Ad-2, may alter one of these mechanisms during productive infection. On the basis of a molecular weight of 338 for aphidicolin (Bucknall et al., 1973), 50% inhibition of CV-1, SV40, and HSV-1 DNA synthesis occurred between 0.15 and 0.5 μM aphidicolin; 50% inhibition of Ad-2 DNA synthesis occurred at $\sim$60 μM aphidicolin.

In contrast to earlier studies (Byars & Kidson, 1975; Kidson et al., 1975), we found no evidence that d_2Thd, a potential DNA chain terminator, affected DNA synthesis in either uninfected mammalian cells or HSV-1 or SV40 infected cells. Neither a reduction in the rate of nucleotide incorporation nor an increase in the amount of short pieces of nascent DNA was observed in the presence of d_2Thd. Furthermore, analysis of the ratio of covalently closed, circular SV40 DNA to SV40 DNA containing at least one phosphodiester bond interruption demonstrated that incorporation of d_2TMP occurred at less than 10^{-5}/nucleotide. Although these results were consistent with the failure of d_2TTP to inhibit either SV40 or CV-1 DNA replication in vitro or HSV-1 DNA polymerase activity, the lack of effect of d_2Thd on Ad-2 DNA replication or virus production was completely unexpected since synthesis of this genome is very sensitive to low concentrations of d_2TTP in vitro (50% inhibition by a d_2TTP/dTTP ratio of 0.02). The explanation to this apparent inconsistency was offered by the finding that d_2Thd was not phosphorylated to d_2TTP in some mammalian cells. This could also explain the lack of sensitivity of Rous sarcoma virus to d_2Thd, although the virus-coded reverse transcriptase is highly sensitive to d_2TTP (Smoler et al., 1971). Since the degree of phosphorylation of a nucleoside analogue depends on the structure of the carbohydrate moiety as well as the base (Schaeffer et al., 1971), we can not rule out the possibility that other dideoxynucleoside analogues may be phosphorylated to their triphosphate derivatives and thus become specific inhibitors of growth of some viruses such as adenovirus and Rous sarcoma virus.

Acknowledgments

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