Identification of DNA Polymerase δ in CV-1 Cells: Studies Implicating both DNA Polymerase δ and DNA Polymerase α in DNA Replication†

Russell A. Hammond,‡ John J. Byrnes,§ and Michael R. Miller*,‡

Department of Biochemistry, Group W Bench, West Virginia University School of Medicine, Morgantown, West Virginia 26506, and Department of Medicine, Veterans Administration Hospital and Center for Blood Diseases, University of Miami School of Medicine, Miami, Florida 33125

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ABSTRACT: DNA polymerases δ and α were purified from CV-1 cells, and their sensitivities to the inhibitors aphidicolin, (*p*-*n*-butylphenyl)deoxyguanosine triphosphate (BuPdGTP), and monoclonal antibodies directed against DNA polymerase α were determined. The effects of these inhibitors on DNA replication in permeabilized CV-1 cells were studied to investigate the potential roles of polymerases δ and α in DNA replication. Aphidicolin was shown to be a more potent inhibitor of DNA replication than of DNA polymerase α or δ activity. Inhibition of DNA replication by various concentrations of BuPdGTP was intermediate between inhibition of purified polymerase α or δ activity. Concentrations of BuPdGTP which totally abolished DNA polymerase α activity were much less effective in reducing DNA replication, as well as the activity of DNA polymerase δ. Monoclonal antibodies which specifically inhibited polymerase α activity reduced, but did not abolish, DNA replication in permeable cells. BuPdGTP, as well as anti-polymerase α antibodies, inhibited DNA replication in a nonlinear manner as a function of time. Depending upon the initial or final rates of inhibition of replication by BuPdGTP and anti-α antibodies, as little as 50%, or as much as 80%, of the replication activity can be attributed to polymerase α. The remaining replication activity (20–50%) is tentatively attributed to polymerase δ, because it was aphidicolin sensitive and resistant to both anti-polymerase α antibodies and low concentrations of BuPdGTP. A concentration of BuPdGTP which abolished polymerase α activity reduced, but did not abolish, both the synthesis and maturation of nascent DNA fragments. This information suggests that polymerases α and δ are involved in both the synthesis and maturation of nascent DNA. This is the first report to present evidence suggesting that both polymerases α and δ play a significant role in mammalian DNA replication.

Determining the roles of the four mammalian DNA polymerases, α, β, γ, and δ, is a fundamental problem in biology. Because mutants specific for each polymerase are not available, most investigations have relied on inhibitors and correlative studies to determine the functions of these DNA polymerases. Most studies conclude that DNA polymerase α is the primary, or only, polymerase responsible for nuclear DNA replication, based upon the following types of studies: (i) the activity (Baril et al., 1973; Chang & Bollum, 1973; Thommes et al., 1986) and amount (Thommes et al., 1986) of DNA polymerase α increase as cells enter the DNA replicative period. (ii) Aphidicolin, which was initially described as a DNA polymerase α specific inhibitor (Ikegami et al., 1978), inhibits DNA replication (Krokan et al., 1979; Pedrali-Noy & Spadari, 1979; Wist & Prydz, 1979; Yagura et al., 1982). (iii) Monoclonal antibodies which specifically inhibit the activity of DNA polymerase α (Tanaka et al., 1982) reduce DNA replication in permeable cells (Miller et al., 1985a,b) and when microinjected into living cells (Kaczmarek et al., 1986). (iv) Primase, which is assumed to synthesize RNA primers during DNA replication, is associated with many preparations of DNA polymerase α (Chang et al., 1984; Gronostajsko et al., 1984; Gross & Krauss, 1985; Hu et al., 1984; Wang et al., 1984; Yamaguchi et al., 1985), and polymerase α can utilize RNA primers (Gronostajsko et al., 1984; Hu et al., 1984; Spadari & Weissbach, 1975).

Recently, Byrnes (1985) suggested that DNA polymerase δ may be involved in DNA replication. Polymerase δ is established (Byrnes et al., 1976; Goscin & Byrnes, 1982a,b; Lee et al., 1984) to contain intrinsic 3′ → 5′ exonuclease (proofreading) activity; the possible relation between polymerase δ and the exonuclease-containing polymerase α_1, reported by Chen et al. (1979), has not been established. If polymerase δ is involved in DNA replication, the proofreading activity may be fundamental for maintaining the high fidelity of DNA replication. It is therefore essential to determine if polymerase δ is involved in mammalian DNA replication. However, polymerases δ and α exhibit similar sensitivities to most DNA polymerase inhibitors, and it had not been possible to differentiate the functions of these polymerases. (*p*-*n*-Butylphenyl)deoxyguanosine triphosphate (BuPdGTP)[1] (Khan et al., 1984; Wright & Dudycz, 1983) and monoclonal antibodies directed against polymerase α (Tanaka et al., 1982) have been shown to discriminate the activities of DNA polymerases α and δ (Byrnes, 1985; Crute et al., 1986; Lee et al., 1985; Wahl et al., 1986). These inhibitors preferentially inhibit the activity of polymerase α. Dresler and Frattini (1986) recently reported that DNA replication in permeable cells was much less sensitive to BuPdGTP inhibition than was polymerase α. From these observations, Dresler and Frattini (1986) concluded that polymerase δ is responsible for DNA replication. However, that study did not examine the effect of BuPdGTP on po-

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* Correspondence should be addressed to this author.

‡ West Virginia University School of Medicine.

§ University of Miami School of Medicine.

[1] Abbreviations: BuPdGTP, (*p*-*n*-butylphenyl)deoxyguanosine triphosphate; BuPdGMP, (*p*-*n*-butylphenyl)deoxyguanosine monophosphate; BuPdG, (*p*-*n*-butylphenyl)deoxyguanosine; BuPG, (*p*-*n*-butylphenyl)guanine; BrdU, 5-bromo-2′-deoxyuridine; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HF, human fibroblast.

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lymerase δ, nor did it study the effect of anti-α antibodies on DNA replication. In addition, polymerase δ has not been identified in the cells used by Dresler and Frattini (1986). Thus far, polymerase δ has been reported in rabbit bone marrow (Byrnes et al., 1976), calf thymus (Crute et al., 1986; Lee et al., 1984), and human placenta (Lee et al., 1985; Lee & Toomey, 1986). To better dissect the roles of DNA polymerases α and δ in DNA replication, we identified and separated polymerases δ and α from CV-1 cells and determined their sensitivities to different DNA polymerase inhibitors. The effects of these inhibitors on DNA replication were then investigated in detail in permeable CV-1 cells. In contrast to the report of Dresler and Frattini (1986), our results indicate that DNA polymerases α and δ both play a significant role in DNA replication.

Materials and Methods

Cell Culture and Extract Preparation. CV-1 cells and human fibroblast (HF) cells were cultured in Dulbecco's modified Eagle's medium (Gibco) with 10% fetal calf serum (Microbiological Associates) and 10 μg of gentamycin/mL (Sigma). Exponential cultures of CV-1 cells were collected by trypsinization, counted with a Coulter particle counter, and washed twice in phosphate-buffered saline containing 0.2 mM phenylmethanesulfonyl fluoride (Sigma) by centrifugation and resuspension. Centrifugation and all subsequent manipulations were performed at 2 °C. CV-1 cells were then used either to prepare extracts for identifying and purifying DNA polymerases or to study DNA replication. To prepare extracts, cells were suspended in 5 volumes of 2 mM $MgCl_2$, 1 mM sodium bisulfite, 1 mM phenylmethanesulfonyl fluoride, 5 mM potassium phosphate (pH 7.5), 1 mM ethylenediaminetetraacetic acid, and 1 mM 2-mercaptoethanol and gently homogenized in a Teflon–glass homogenizer until ≥95% of the cells were disrupted. The homogenate was centrifuged at 30000*g* for 15 min to pellet nuclei and cellular debris. The supernatant was assayed for DNA polymerase activity, in the presence or absence of inhibitors.

Hybridoma cells producing anti-polymerase α antibodies (Tanaka et al., 1982) were obtained from the ATCC and propagated in Dulbecco's modified Eagle's medium, as indicated above for CV-1 cells. Antibodies were purified from spent culture medium by protein A–Sepharose chromatography (Tanaka et al., 1982).

DNA Polymerase Purification and Assays. DNA polymerases δ and α were purified from 30 mL of packed CV-1 cells, as previously described in detail (Goscin & Byrnes, 1982a,b; Byrnes & Black, 1978), through step V. The peak II DNA polymerase activity from step V, a DEAE-Sephadex column, was then subjected to glycerol gradient centrifugation, as described (Goscin & Byrnes, 1982a,b; Byrnes & Black, 1978). The sensitivities of CV-1 polymerases α and δ to different inhibitors, as well as association with 3′ → 5′ exonuclease activity, were determined as described (Byrnes, 1985). However, because only very small amounts of CV-1 cell polymerases were available, more detailed studies with inhibitors were performed with highly purified polymerases α_1 and δ prepared from rabbit erythroid hyperplastic bone marrow. These polymerases were purified from rabbit bone marrow by procedures described in detail (Goscin & Byrnes, 1982a,b; Byrnes & Black, 1978), through step IX, at which point polymerases α and δ are resolved. The specific activities of polymerases α and δ at this step are 30 000–50 000 and approximately 200 000 units/mg of protein, respectively. The DNA polymerase assays contained the following in a final volume of 0.1 mL: 180 μg/mL DNase-activated DNA, or 0.75 A_{260} unit of poly(dA) and 1.88×10^{-2} A_{260} unit of oligo(dT), 20 μM TTP, 0.01–0.1 mCi/mL [^{3}H]TTP (70 Ci/mmol, from ICN), 50 mM Tris buffer (pH 7.4), 30% glycerol, 5 mM $MgCl_2$, 2 mg of albumin/mL, 1 mM dithiothreitol, inhibitors as indicated, and DNA polymerase (purified DNA polymerases or cell extracts); 25 μM dGTP, 100 μM dATP, and 100 μM dCTP were included in assays with DNase-treated DNA. Albumin was from Calbiochem; the other reagents were from Sigma. The reactions were incubated 15–30 at 37 °C, unless indicated otherwise, after which incorporation of [^{3}H]TTP into DNA was determined, as described in detail (Goscin & Byrnes, 1982a,b; Byrnes & Black, 1978). Although these are not the optimum reaction conditions for either DNA polymerase δ or DNA polymerase α, they were chosen because they approximate the conditions under which DNA replication was studied in permeable cells.

BuPdGTP, BuPdGMP, BuPdG, and BuPG (Wright & Dudycz, 1983) were the generous gift of Dr. George Wright. Aphidocolin was purchased from Sigma. For antibody studies, assays were preincubated with control monoclonal antibodies, directed against rat liver pyruvate kinase (the generous gift of Dr. James Blair) or bromodeoxyuridine (Miller et al., 1986), or with anti-polymerase α monoclonal antibodies SJK-132 or SJK-287 (Tanaka et al., 1982) for 30 min at 2 °C, before incubation at 37 °C.

DNA Replication. Procedures for permeabilizing cultured animal cells with lysolecithin have previously been described (Miller et al., 1978, 1985a; Miller & Chinault, 1982). Nucleotides readily enter permeabilized cells and are incorporated into replicated DNA in a normal, semiconservative, discontinuous fashion. Cells were suspended in 150 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$, and 35 mM Hepes (pH 7.2) (solution A) at 8×10^7 cells/mL, 2 °C, and permeabilized by the addition of one-third volume of 1.5 mg of lysolecithin/mL (Sigma, type I) in solution A. The cells were diluted to a final concentration of 2×10^7 cells/mL in solution A containing 20 μM TTP, 0.1 mCi/mL [^{3}H]TTP, 25 μM dGTP, 100 μM each of dATP, dCTP, GTP, and UTP, 5 mM phosphoenolpyruvate, and 1.25 mM ATP, in the presence or absence of DNA polymerase inhibitors. Fifty-microliter aliquots were incubated at 37 °C for 30 min, and the incorporation of [^{3}H]TTP into replicated DNA was determined. To study the effect of monoclonal antibodies on DNA replication, permeable cells were preincubated with antibodies for 1 h at 2 °C, before incubation at 37 °C, as described (Miller et al., 1985a,b).

Alkaline Sucrose Gradients. Procedures for studying the synthesis and maturation of Okazaki DNA in permeable cells have been described previously, in detail (Miller et al., 1978, 1985a). Briefly, permeable cells were incubated with high specific activity [^{3}H]TTP (9–18 μM, 50 Ci/mmol) for 20 s at 37 °C, in the presence or absence of inhibitors (pulse). Excess TTP (400 μM) was then added, and the 37 °C incubation was continued for an additional 40 min (chase). Following the pulse and chase periods, DNA was isolated and analyzed on alkaline sucrose gradients as described (Tseng & Goulian, 1975). Phage fd DNA (20 S) was used as a marker.

Results

Identification of DNA Polymerase δ in CV-1 Cells. To initially determine if DNA polymerase δ activity was present in CV-1 cells, the effects of various DNA polymerase inhibitors on DNA polymerase activity in CV-1 cell extracts were investigated. Table I shows that *N*-ethylmaleimide, which inhibits all mammalian DNA polymerases except β, inhibited the DNA polymerase activity in CV-1 cell extract by 84%.

Table I: Effect of BuPdGTP and Monoclonal Antibody SJK-287 on CV-1 Cell DNA Polymerase Activity and on DNA Replication[a]

inhibitor	extract	replication
NEM	84	≥97
aphidicolin	78	≥95
BuPdGTP	41	34
SJK-287	42	71
SJK-287 + BuPdGTP	46	73

[a] CV-1 cell extract was prepared as described, and aliquots of approximately 1 unit (Byrnes, 1985) of DNA polymerase activity were assayed in triplicate in the presence or absence of the indicated inhibitors: 10 mM *N*-ethylmaleimide (NEM); 50 μg of aphidicolin/mL; 10 μM BuPdGTP (extract). Permeable CV-1 cells were prepared as described, and DNA replicative activity was determined in triplicate in the presence and absence of the same inhibitors (replication). For antibody studies, both permeable cells and extracts were preincubated with a saturating level (0.1 mg/mL) of polymerase α specific antibody SJK-287 (Tanaka et al., 1982) for 1 h at 2 °C, before being assayed at 37 °C, in the presence or absence of 10 μM BuPdGTP. Control samples were not incubated with either inhibitor but were preincubated with or without a monoclonal antibody directed against BrdU (Miller et al., 1986). The control antibody did not alter DNA polymerase or replication activity. Results are presented as percent inhibition by the inhibitors, relative to control samples, and are the average of three different experiments.

Aphidocolin inhibits the activity of DNA polymerases δ and α, and this inhibitor reduced DNA polymerase activity in the extract 78%. These data suggest that polymerases α and/or δ account for approximately 80–85% of the total DNA polymerase activity in CV-1 cell extract, and the remaining activity is largely due to polymerase β; 10 μM BuPdGTP essentially abolishes DNA polymerase α activity and only reduces polymerase δ activity 15–40% (Byrnes, 1985; Lee et al., 1985). The DNA polymerase activity of CV-1 cell extract was reduced 41% by 10 μM BuPdGTP (Table I). SJK-287 and SJK-132 are monoclonal antibodies directed against KB cell DNA polymerase α (Tanaka et al., 1982). These antibodies are potent and specific inhibitors of DNA polymerase α activity (Tanaka et al., 1982; Byrnes, 1985; Crute et al., 1986). Saturating concentrations of antibody SJK-287 inhibited the total DNA polymerase activity of CV-1 cell extract 42%. Inhibition of DNA polymerase activity in the cell extract by both SJK-287 and 10 μM BuPdGTP was not significantly reduced more than that by either inhibitor alone. These patterns of inhibition indicate that approximately 40% of the total DNA polymerase of the cell extract is attributed to DNA polymerase α. Approximately 40% of the total DNA polymerase activity in CV-1 cell extract is tentatively attributed to DNA polymerase δ, because it is aphidicolin sensitive and resistant to 10 μM BuPdGTP and to antibody SJK-287; these are characteristics only of DNA polymerase δ.

We then attempted to identify and separate DNA polymerases δ and α from CV-1 cells and determine if they exhibited the anticipated sensitivities to different DNA polymerase inhibitors. DNA polymerases were purified, as described in detail by Byrnes and Black (1978) and Goscin and Byrnes (1982a,b), through step V, a DEAE-Sephadex column. Two peaks of polymerase activity were identified, designated I and II (Figure 1A); 3′ → 5′ exonuclease activity was associated with both. Peak I polymerase activity was reduced ≥75% by 50 μg of antibody SJK-132/mL (not shown) and nearly abolished by 10 μM BuPdGTP. In contrast, peak II DNA polymerase activity was resistant to inhibition by BuPdGTP (Figure 1A) and was inhibited ≤20% by 50 μg of antibody SJK-132/mL (not shown). Both peak I and peak II were inhibited ≥92% by 50 μg of aphidicolin/mL. The highest activity fractions of peaks I and II were concentrated and analyzed on 15–30% glycerol gradients, as described (Byrnes & Black, 1978). Figure 1B shows that peak II DNA polymerase migrated as an approximately 6S enzyme, which was resistant to inhibition by both anti-polymerase α antibodies (not shown) and 10 μM BuPdGTP. Most of the 3′ → 5′ exonuclease activity comigrated with DNA polymerase activity, and a small amount of exonuclease appeared not to be associated with polymerase (fractions 3 and 4). Analysis of peak I DNA polymerase on glycerol gradients resulted in a rather broad peak of DNA polymerase activity which was nearly abolished by 10 μM BuPdGTP or antibody SJK-132; 3′ → 5′ exonuclease activity did not cosediment with this DNA polymerase activity (not shown). The association with 3′ → 5′ exonuclease and sensitivities to aphidicolin, BuPdGTP, and anti-α antibody SJK-132 indicate that peak II polymerase (Figure 1A) is predominantly δ and peak I is predominantly α (Byrnes, 1985; Byrnes & Black, 1978; Lee et al., 1985).

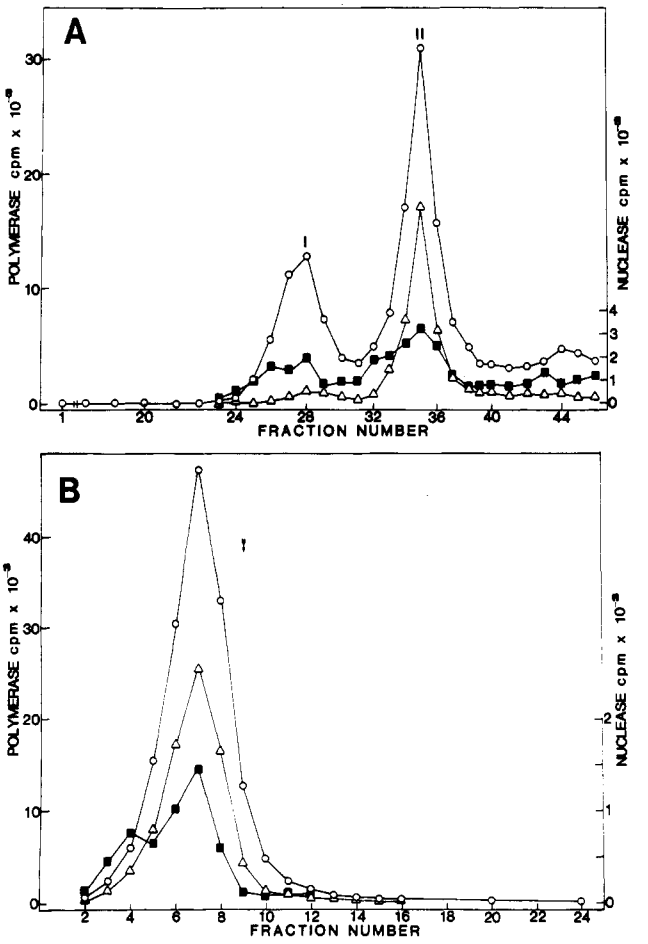

FIGURE 1: Purification of DNA polymerase δ from CV-1 cells. DNA polymerases were purified from 30 g of CV-1 cells, through step IV (561 units), as described in detail (Byrnes & Black, 1978; Goscin & Byrnes, 1982a,b). (A) The DNA polymerase activity from step IV was applied to a 20-mL DEAE-Sephadex column as described (Goscin & Byrnes, 1982a,b) and eluted with buffer containing the following concentrations of KCl, in 10-mL stepwise increments: 0 KCl; 50 mM KCl; 0.1 M KCl; 0.15 M KCl; 0.2 M KCl; 0.5 M KCl. Approximately 1.2-mL fractions were collected. (B) the highest activity fraction of peak II was concentrated with a Centricon 30 membrane and analyzed on a 15–30% glycerol gradient as described (Byrnes & Black, 1978). The arrow indicates the position of the molecular weight marker enzyme aldolase; fractions were collected from the bottom (fraction 1). Both the DEAE-Sephadex (A) and glycerol gradient (B) fractions were assayed for DNA polymerase activity using poly(dA)/oligo(dT) in the absence (○) or presence (△) of 10 μM BuPdGTP, presented as cpm of [^{3}H]TTP (2.5 pmol/cpm) incorporated into DNA. Fractions were also assayed for 3′ → 5′ exonuclease activity (■) as described (Byrnes, 1985), presented as cpm of [^{3}H]TMP released from 3′-labeled substrate.

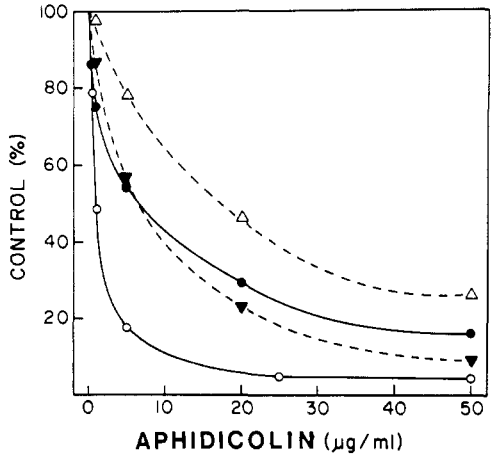

FIGURE 2: Effect of aphidicolin on purified DNA polymerase δ and α activities and on CV-1 cell DNA replication. DNA replication in permeable CV-1 cells (○) and also the activities of purified DNA polymerases δ with poly(dA)/oligo(dT) or with DNase-treated DNA (●) and α with poly(dA)/oligo(dT) (△) and with DNase-treated DNA (▼) were assayed in the presence or absence (control) of the indicated concentrations of aphidicolin. DNA polymerases α and δ were purified from rabbit bone marrow, through step IX, as described (Goscin & Byrnes, 1982a,b). One unit of each polymerase was used in the assays. Results are presented as the percent inhibition of each activity by aphidicolin, relative to control samples.

Furthermore, purified CV-1 cell DNA polymerases δ and α appear to be very similar to, if not identical with purified rabbit bone marrow (Goscin & Byrnes, 1982a,b; Byrnes & Black, 1978) DNA polymerases δ and α, on the basis of their migration in glycerol gradients, their degree of association with 3′ → 5′ exonuclease, and the effect of aphidicolin, BuPdGTP, and anti-polymerase α antibodies (Tanaka et al., 1982) on their activities.

Effect of Inhibitors on DNA Polymerases δ and α and on DNA Replication. Figure 2 shows that the activities of purified DNA polymerases δ and α are inhibited by aphidicolin, in a dose-dependent manner, confirming earlier studies (Goscin & Byrnes, 1982a; Lee et al., 1984). Polymerase δ exhibits essentially identical sensitivities with aphidicolin inhibition on poly(dA)/oligo(dT) and on DNase-activated DNA, whereas the aphidicolin inhibition of polymerase α is more pronounced on DNase-activated DNA. DNA replication is somewhat more sensitive to aphidicolin inhibition than are the activitites of polymerases α or δ. Nonetheless, Figure 2 suggests that DNA polymerase(s) α and/or δ are involved in DNA replication.

Figure 3a shows the effects of low concentrations of BuPdGTP on the activities of purified DNA polymerases δ and α. With both DNase-activated DNA and poly(dA)/oligo(dT), polymerase α is much more sensitive to BuPdGTP inhibition than is polymerase δ. At 10 μM BuPdGTP, polymerase α activity is essentially abolished, and polymerase δ activity is reduced approximately 30% or 50% with poly(dA)/oligo(dT) or DNase-activated DNA, respectively. Figure 3B shows the effects of higher BuPdGTP concentrations on DNA polymerases δ and α and on DNA replication in permeable CV-1 and HF cells. BuPdGTP inhibition of polymerase δ produces an apparent biphasic curve (Figure 3B) with both DNase-activated DNA and poly(dA)/oligo(dT). This pattern was noted and discussed by Byrnes (1985). The BuPdGTP inhibition of DNA replication in CV-1 cells and HF cells is very similar and is intermediate between that exhibited by polymerases δ and α; 10 μM BuPdGTP abolishes polymerase α activity, reduces polymerase δ activity about 30% or 50% with poly(dA)/oligo(dT) or DNase DNA, respectively,

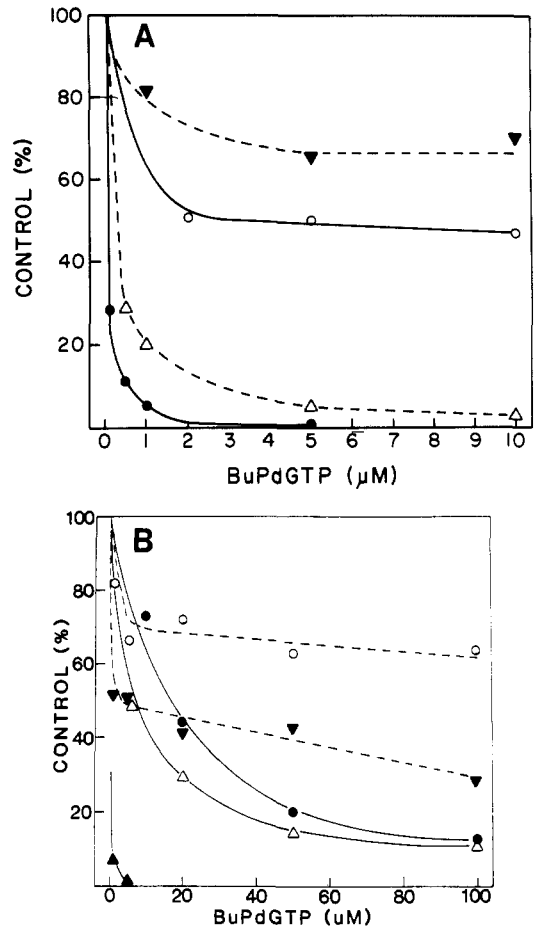

FIGURE 3: Effect of BuPdGTP on CV-1 cell DNA replication and on the activities of purified DNA polymerases δ and α. (A) DNA polymerase δ (step IX, from rabbit bone marrow; Goscin & Byrnes, 1982a,b) was assayed in the presence and absence of low concentrations of BuPdGTP with poly(dA)/oligo(dT) (▼) or with DNase-activated DNA (○); polymerase α (step IX, from rabbit bone marrow; Goscin & Byrnes, 1982a,b) was assayed in the presence and absence of BuPdGTP with poly(dA)/oligo(dT) (△) or with DNase-activated DNA (●). (B) DNA replication was assayed in permeable CV-1 cells (△) and in permeable HF cells (●) in the presence and absence of BuPdGTP. DNA polymerases α and δ were assayed as in (A): DNA polymerase α (▲); DNA polymerase δ with poly(dA)/oligo(dT) (○) and with DNase-activated DNA (▼). Results are presented as the percent inhibition of each activity by BuPdGTP, relative to control samples assayed in the absence of BuPdGTP.

and inhibits DNA replication 35–50%. At 100 μM BuPdGTP, polymerase δ activity is reduced 40% or 70% with poly(dA)/oligo(dT) or DNase-activated DNA, respectively, and replication is inhibited 80–90%. A significant fraction of the DNA polymerase δ activity and of the DNA replication activity of CV-1 cells is active in the presence of concentrations of BuPdGTP which abolish the activity of polymerase α, suggesting a role for polymerase δ in replication. The pattern of BuPdGTP inhibition of replication in permeable cells (Figure 3B) may indicate that both polymerases α and δ are responsible for replication.

A different interpretation of Figure 3B is that in permeable cells BuPdGTP is adsorbed to cellular structures or degraded, thereby decreasing its effective concentration available to inhibit polymerases. To investigate this possibility, the effect of several BuPdGTP analogues (BuPdGMP, BuPdG, and BuPG) on DNA polymerase α activity and DNA replication in permeable cells was determined; 20 μM BuPdG or BuPG did not inhibit polymerases α or δ or replication in permeable cells; much higher concentrations were not soluble; 10 μM BuPdGMP did not inhibit polymerases α or δ or replication,

Table II: Effect of BuPdGTP, Incubated with Permeable Cells, on DNA Polymerase α Activity[a]

inhibitor	% inhibition of polymerase α
10 μM BuPdGTP	96
1 μM BuPdGTP	72
filtrate: 10 μM BuPdGTP	92
filtrate: 1 μM BuPdGTP	77

[a] Permeable CV-1 cells were incubated in the presence of 100 μM BuPdGTP for 20 min at 0 and 37 °C. The cells were centrifuged at 1000*g* for 10 min, and the supernatants were applied to Centricon-30 microconcentrators and centrifuged at 1400*g* for 20 min. The solutions passing through the membranes, filtrates, as well as BuPdGTP not incubated with permeable cells, were tested for ability to inhibit polymerase α at dilutions that gave the final concentrations listed in the table, assuming no loss or degradation of BuPdGTP in permeable cells. Filtrate from cells incubated with BuPdGTP at 0 °C inhibited polymerase α to the same extent as filtrate from cells incubated with BuPdGTP at 37 °C. Results are expressed as percent polymerase α inhibition by the inhibitors, relative to control samples not treated with BuPdGTP or filtrate.

while 100 μM BuPdGMP inhibited polymerase α 10–25%, did not inhibit polymerase δ, and reduced replication in permeable CV-1 cells about 30% (not shown). This indicates that if BuPdGTP is degraded in permeable cells, the analogues generated will likely not be potent inhibitors of polymerases α or δ or DNA replication. Our results are consistent with those of Khan et al. (1984), who reported that BuPdGTP is approximately 100-fold more potent than BuPdGMP and approximately 1000-fold more potent than BuPdG or BuPG in inhibiting polymerase α activity. The reason BuPdGMP was relatively potent in inhibiting DNA replication in permeable cells may be attributed to its phosphorylation to BuPdGDP or BuPdGTP in permeable cells. An assessment of possible BuPdGTP degradation or adsorption to cellular constituents in permeable cells was determined as follows. Permeable CV-1 cells were incubated in the presence of 100 μM BuPdGTP for 20 min at 0 or 37 °C. The cells were centrifuged, and the supernatants were applied to a Centricon-30 microconcentrator and centrifuged, allowing compounds smaller than 30 000 daltons to pass through the membrane (filtrate). The filtrate was then tested for ability to inhibit polymerase α. Table II indicates the filtrate from cells incubated with BuPdGTP at 0 or 37 °C was just as effective in inhibiting polymerase α as BuPdGTP which had not been incubated with permeable cells. This indicates that in permeable cells BuPdGTP is not significantly degraded to a less potent polymerase inhibitor or absorbed to cell structures.

The involvement of polymerases δ and α in DNA replication in permeable cells was further investigated by studying the effects of anti-polymerase α antibodies SJK-287 and SJK-132. Table I indicates that the maximum inhibition of replication by SJK-287 was 71%; in most experiments, replication was inhibited 60–65% by SJK-287 or SJK-132. Both antibodies SJK-132 and SJK-287 were potent inhibitors of CV-1 cell DNA replication and DNA polymerase α; however, only antibody SJK-132 inhibited rabbit bone marrow polymerase α (not shown). This finding is consistent with the observation that SJK-132 reacts well with DNA polymerases from different species but SJK-287 does not (Dr. T. Wang, personal communication). The replication activity which was not inhibited by the antibodies was aphidicolin sensitive (Miller et al., 1985a; data not shown). DNA replication was inhibited 37% by 10 μM BuPdGTP, and the addition of both SJK-287 and 10 μM BuPdGTP did not inhibit replication significantly more than did SJK-287 alone. Thus, a significant fraction of the DNA replicative activity of permeable CV-1 cells is resistant to the simultaneous action of two polymerase α inhibitors, BuPdGTP and antibody SJK-287. However, it was surprising that these two potent α-polymerase inhibitors appeared to inhibit replication in permeable cells to somewhat different extents; 10 μM BuPdGTP inhibited replication 35–50%, and anti-α antibodies inhibited replication 60–70% (Table I and Figure 3B).

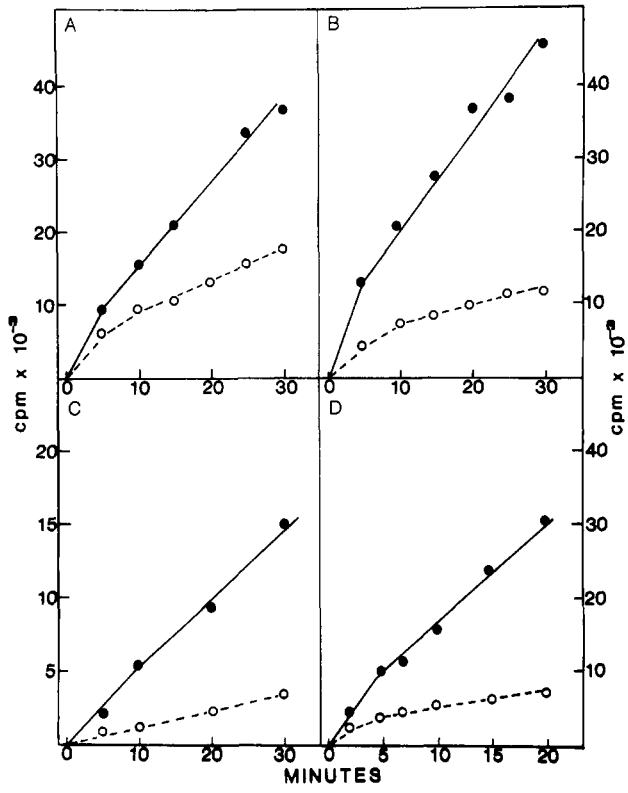

FIGURE 4: Time courses of inhibition of DNA replication in permeable cells and of DNA polymerase activity. (A–C) DNA replication was measured in permeable CV-1 cells in the absence (●) or presence (○) of 10 μM BuPdGTP (panel A), 50 μg of antibody SJK-132-mL (panel B), or 2 μg of aphidicolin/mL (panel C). (D) DNA polymerase α (step IX, from rabbit bone marrow; Goscin & Byrnes, 1982a,b) measured in the absence (●) or presence (○) of 0.5 μM BuPdGTP. Results are presented as total cpm of [^{3}H]TTP incorporated into DNA at 37 °C for the indicated times.

To gain insight into this apparent discrepancy, the effects of these inhibitors on DNA replication and DNA polymerase activity were further studied. Figure 4A shows the time course for 10 μM BuPdGTP inhibition of DNA replication. In this and other experiments, the rate of replication was only inhibited about 30% during the first 5 min; however, the rate of replication was inhibited 60–83% during the 20–30-min period. Preincubating permeable cells with 10 μM BuPdGTP at 0 °C for 30–60 min before initiating the time course did not alter the pattern of inhibition shown in Figure 4A. The time course of replication inhibition by antibody SJK-132 was also nonlinear (Figure 4B). In this and other experiments, replication was inhibited 50–60% during the first 5 min and inhibited 72–85% during the 20–30-min period. These experiments demonstrate that the final rates of inhibition of DNA replication produced by antibody SJK-132 and 10 μM BuPdGTP are similar. Not all DNA polymerase inhibitors exhibit the nonlinear time course of replication inhibition produced by SJK-132 and 10 μM BuPdGTP. Figure 4C demonstrates that 2 μg of aphidicolin/mL produced a linear inhibition of replication with time; other concentrations of aphidicolin also exhibited a linear time course of inhibition (not shown). The effects of these inhibitors on purified DNA polymerases as a function of time were examined. Figure 4D demonstrates that 0.5 μM BuPdGTP did not inhibit polym-

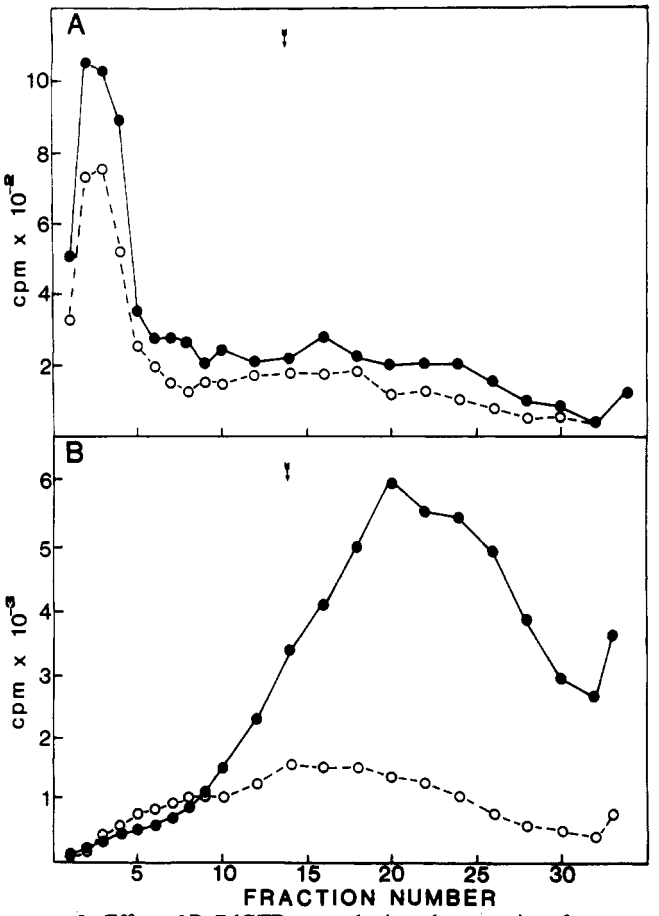

FIGURE 5: Effect of BuPdGTP on synthesis and maturation of nascent DNA. (A) Permeable cells were incubated in the replication solution containing high specific activity [^{3}H]TTP for 20 s, in the presence (○) or absence (●) of 20 μM BuPdGTP. DNA was then isolated and analyzed on 5–20% alkaline sucrose gradients. Fractions were collected, and the amount of [^{3}H]DNA in each fraction (cpm) was determined. Total [^{3}H]DNA in samples incubated in the absence and presence of BuPdGTP was 8420 and 4090 cpm, respectively. (B) Following incubation for 20 s with high specific activity [^{3}H]TTP, excess TTP was added to permeable cells, in the presence (○) or absence (●) of 20 μM BuPdGTP, and incubation was continued for 40 additional min at 37 °C. DNA from each sample was then isolated and analyzed on 5–20% alkaline sucrose gradients. Total [^{3}H]DNA in samples incubated in the absence and presence of BuPdGTP was 96 630 and 29 845 cpm, respectively. In panels A and B, fraction 1 is the top of the gradient, and the arrow indicates the position of 20S marker DNA.

erase α in a linear fashion; the polymerase activity was reduced 50% in the first 2 min and 83% during the 10–20-min period. At higher concentrations of BuPdGTP, which inhibited polymerase α about 90%, the residual activity appeared to be inhibited in a more linear fashion with time. Similar results were obtained with polymerase α using DNase-activated DNA as the substrate, with polymerase δ using poly(dA)/oligo(dT), and with a mixture of polymerases α (60%) + δ (40%). Inhibition of polymerase activity by antibody SJK-132 was linear with time (not shown).

Effect of BuPdGTP on Okazaki DNA Synthesis and Maturation. Figure 5 shows the effects of 20 μM BuPdGTP on the synthesis and maturation of nascent DNA. Both in the absence and in the presence of BuPdGTP, approximately 50% of the DNA synthesized in 20 s (pulse) was associated with small, Okazaki DNA fragments (4S DNA). Although BuPdGTP reduced [^{3}H]TTP incorporation into DNA 51% during the pulse period, the relative size distribution of radioactive DNA was unaltered (Figure 5A). In the absence of BuPdGTP, essentially all of the ^{3}H-labeled Okazaki DNA became associated with much larger DNA during the chase period, demonstrating normal maturation of the nascent DNA (Figure 5B). Most of the Okazaki DNA also became associated with larger DNA during the chase period in the presence of BuPdGTP. However, a larger fraction of [^{3}H]DNA remained associated with small DNA (Figure B, fractions 1–7) in the presence of BuPdGTP (12%) than in the absence of BuPdGTP (2%). In addition, following the chase period, the average size of [^{3}H]DNA was somewhat smaller in the presence of BuPdGTP than in the absence of BuPdGTP. The results in Figure 5 demonstrate that BuPdGTP reduced both the synthesis of Okazaki DNA and also the maturation of nascent DNA to larger DNA.

Discussion

Our studies identify for the first time DNA polymerase δ in CV-1 cells and demonstrate that polymerases α and δ are physically distinguishable in these cells. This finding establishes that polymerase δ is not restricted to the few tissues (thymus, bone marrow, placenta) in which δ has thus far been identified. Futhermore, polymerases δ and α from CV-1 cells exhibit sensitivities to the inhibitors aphidicolin, BuPdGTP, and anti-polymerase α antibodies comparable to those reported for these polymerases isolated from other tissue (Byrnes, 1985; Lee et al., 1985). The preferential inhibition of polymerase α by BuPdGTP and anti-polymerase α antibodies is proving useful in identifying and purifying polymerase δ in CV-1 cells and in other tissues (Byrnes, 1985; Crute et al., 1986; Lee et al., 1985).

The differential inhibition of polymerases α and δ by BuPdGTP and anti-polymerase α antibodies also provides one means of investigating the function of these polymerases in DNA metabolism. Dresler and Frattini (1986) reported that DNA replication in permeable human cells was approximately 100- fold less sensitive to BuPdGTP inhibition than polymerase α and concluded that δ was responsible for DNA replication. Figure 3B in this paper confirms that replication is indeed much less sensitive to BuPdGTP inhibition than is the activity of purified DNA polymerase α. Furthermore, the sensitivity of replication to BuPdGTP is shown to be different than that exhibited by polymerase δ. The intermediate level of BuPdGTP inhibition of replication (Figure 3B) suggests that neither polymerase α nor polymerase δ is solely responsible for CV-1 DNA replication but rather that replication requires both polymerases. The experiments presented in Table II indicate the resistance of DNA replication to BuPdGTP is not simply attributed to adsorption of BuPdGTP to cell components or to degradation of BuPdGTP. In addition, BuPdGTP inhibition of replication increases as a function of time (Figure 4A), further indicating that BuPdGTP is not being inactivated in the permeable cells. The fact that antibodies specific for DNA polymerase α (Tanaka et al., 1982) inhibit DNA replication in intact (Kaczmarek et al., 1986) and permeable cells (Table I; Miller et al., 1985a,b) strongly supports a role of polymerase α in replication. The replication activity not inhibited by anti-α antibodies is both aphidicolin sensitive (Miller et al., 1985b) and insensitive to 10 μM BuPdGTP (Table I); these are characteristics only attributed to polymerase δ and also suggest that polymerase δ is involved in mammalian DNA replication. Very recent studies further support the idea that DNA polymerase δ is involved in DNA replication. Prelich (1987b) and Bravo (1987) demonstrated that proliferating cell nuclear antigen (PCNA or cyclin) is apparently identical with an auxiliary protein for polymerase δ tht stimulates polymerase δ activity on specific templates (Tan, 1986). This same protein is also shown to be required for SV40 DNA replication in an

in vitro system (Prelich, 1987a).

If polymerases α and δ are involved in replicating mammalian DNA, it will be important to determine in more detail the roles of each polymerase. α and δ may form a physical or functional complex to coordinately replicate DNA, or these polymerases may independently synthesize DNA. The possibility that polymerases α and δ have different functions in the synthesis and maturation of nascent DNA was examined. A concentration of BuPdGTP which abolishes the activity of polymerase α reduced, but did not abolish, both the synthesis and maturation of nascent DNA. Very similar findings were obtained with anti-polymerase α antibodies (Miller et al., 1985a). These findings indicate that the normal synthesis and maturation of nascent DNA require the activity of both polymerases α and δ. In prokaryotes, pol III is thought to be involved in synthesizing Okazaki DNA from RNA primers, and pol I is proposed to be responsible for the maturation of Okazaki DNA (Kornberg, 1982).

The reason(s) BuPdGTP and anti-polymerase α antibodies inhibit DNA replication in permeable cells in a nonlinear manner as a function of time (Figure 4A,B) is (are) not yet clear. Possible explanations include the following: (1) The inhibitors may slowly enter nuclei, and the "lag" in inhibition reflects the time required for the inhibitors to equilibrate within nuclei. (2) The inhibitors may be concentrated within nuclei. Although nucleotides may be concentrated, it is unlikely any mechanism would concentrate antibodies within nuclei. (3) The inhibitors may initially inhibit polymerase α without affecting polymerase δ, and the more pronounced inhibition seen at later times could reflect decreased polymerase δ activity because it has become dependent upon synthesis by polymerase α (discussed above). (4) BuPdGTP may be incorporated into DNA strands, decreasing the efficiency of their subsequent utilization. This seems unlikely, because studies by Wright's lab (Wright & Dudyz, 1983; Khan et al., 1984) have not been able to detect incorporation of BuPdGTP into DNA. (5) The inhibitors may freely penetrate nuclei, but some time could be required for equilibration or binding to polymerase. Tucker et al. (1986) demonstrated that the exchange or equilibration time between free and protein-bound nucleotide can be quite slow. Slow binding of BuPdGTP by DNA polymerase to the dGTP site, or a subsequent change in conformation which affects polymerase activity, could cause the nonlinear kinetics observed in Figure 4A. Because purified polymerases also exhibit a nonlinear inhibition of activity as a function of time with BuPdGTP (Figure 4D), this latter explanation may contribute to the nonlinear kinetics observed for replication.

Comparing the rates of replication in the presence of BuPdGTP and anti-polymerase α antibodies provides information which cannot be obtained by studying individual time points. The final rates of DNA replication are inhibited 60–83% by 10 μM BuPdGTP and 72–85% by anti-polymerase α antibodies. If these values best reflect specific inhibition of polymerase α in permeable cells, α may be responsible for approximately 75% of mammalian DNA replication. Polymerase δ is tentatively proposed to be responsible for at least 25% of the DNA replication activity. However, if initial rates of inhibition of replication by 10 μM BuPdGTP (approximately 30%) and by anti-polymerase α antibodies (approximately 55%) most accurately reflect the rates of DNA replication when polymerase α is specifically inhibited, polymerase δ may be responsible for replicating 50% or more of CV-1 cell DNA. In cells temperature sensitive for polymerase α, Eki et al. (1986) demonstrated [^{3}H]dT incorporation was decreased 80% at the nonpermissive temperture; synthesis and maturation of Okazaki DNA occurred at a reduced rate. Residual DNA synthesis in these temperature- sensitive cells may be attributed to polymerase δ.

The idea that DNA polymerase α is the only replicative polymerase must be reexamined. Clearly, the fact that aphidicolin inhibits DNA replication cannot by interpreted to indicate that polymerase α is the primary replication enzyme, since both polymerases δ and α are inhibited by this compound. One reason replication is more sensitive to aphidicolin than either purified polymerase α or purified polymerase δ may be that the DNA polymerases are more sensitive to aphidicolin when replicating DNA in situ than when utilizing artificial template/primers. In addition, the native form of the polymerases, with associated proteins, may be more sensitive to aphidicolin than the highly purified polymerases (Ikegami et al., 1978; Plevani et al., 1980). Alternatively, aphidicolin may inhibit the function of other proteins involved in DNA replication, which would increase the sensitivity of replication to aphidicolin inhibition. In fact, aphidicolin inhibits the exonuclease activity of polymerase δ (Goscin & Byrnes, 1982; Lee et al., 1984), indicating that aphidicolin effects are not strictly limited to inhibition of polymerase activity. The subcellular location (Bensch et al., 1982) and changes in polymerase α activity during the cell cycle (Chang & Bollum, 1973; Baril et al., 1973; Thommes et al., 1986) are consistent with a replicative role but in no way exclude a significant role of polymerase δ. Primase activity is presumed to synthesize RNA primers used in discontinuous DNA replication, and this activity has recently been shown to be associated with DNA polymerase δ (Crute et al., 1986), as well as polymerase α (Wang et al., 1984; Chang et al., 1984; Gross & Krauss, 1985; Gronostajsko et al., 1984; Hu et al., 1984; Yamaguhi et al., 1985). Antibodies which specifically inhibit DNA polymersae α activity reduce DNA replication in mammalian cells (Miller et al., 1985a,b; Kaczmarek et al., 1986), supporting a replicative role of polymerase α. However, these antibodies do not abolish DNA replication at the highest concentrations tested, indicating that DNA polymerase δ could be responsible for a significant portion of DNA replication. Antibodies specific for polymerase δ (Lee & Toomey, 1987) should prove useful in better defining the degree to which polymerase δ may be involved in replication, when they are available.

Lee and Toomey (1987) reported human placenta polymerases α and δ are immunologically distinct. However, Bambara's group (Crute et al., 1986; Wahl et al., 1986) has recently identified similarities between α, δ-1, and δ-2 from calf thymus and proposed that polymerases α and δ may be structurally related. Although the present studies do not address this intriguing possibility, they do indicate that polymerases α and δ may well be functionally related.

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Registry No. BuPdGTP, 87782-05-6; aphidicolin, 38966-21-1; DNA polymerase, 9012-90-2.

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