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Accelerated Publications

2′,3′-Dideoxythymidine 5′-Triphosphate Inhibition of DNA Replication and Ultraviolet-Induced DNA Repair Synthesis in Human Cells: Evidence for Involvement of DNA Polymerase δ†

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Received February 24, 1987; Revised Manuscript Received March 30, 1987

ABSTRACT: It is well established that DNA replication and ultraviolet-induced DNA repair synthesis in mammalian cells are aphidicolin-sensitive and thus are mediated by one or both of the aphidicolin-sensitive DNA polymerases, α and/or δ. Recently, it has been shown that DNA polymerase δ is much more sensitive to inhibition by the nucleotide analogue 2′,3′-dideoxythymidine 5′-triphosphate (ddTTP) than DNA polymerase α but is less sensitive than DNA polymerase β [Wahl, A. F., Crute, J. J., Sabatino, R. D., Bodner, J. B., Marraccino, R. L., Harwell, L. W., Lord, E. M., & Bambara, R. A. (1986) *Biochemistry 25*, 7821–7827]. We find that DNA replication and ultraviolet-induced DNA repair synthesis in permeable human fibroblasts are also more sensitive to inhibition by ddTTP than polymerase α and less sensitive than polymerase β. The K_i for ddTTP of replication is about 40 μM and that of repair synthesis is about 25 μM. These are both much less than the K_i of polymerase α (which is greater than 200 μM) but greater than the K_i of polymerase β (which is less than 2 μM). These data suggest that DNA polymerase δ participates in DNA replication and ultraviolet-induced DNA repair synthesis in human cells.

The identity of the DNA polymerase(s) involved in DNA replication and ultraviolet- (UV)[1] induced DNA repair synthesis in mammalian cells has again become a subject of great interest. It is well established that replication and UV-induced repair synthesis are inhibited by aphidicolin (Huberman, 1981; Dresler, 1984), and this fact has been regarded as evidence for the involvement of DNA polymerase α in these two processes. The recent finding that a second aphidicolin-sensitive DNA polymerase, δ, is also present in abundance in mammalian cell extracts (Byrnes, 1985; Crute et al., 1986) indicates, however, that δ should also be regarded as a candidate for participation in DNA replication and repair. The fact that polymerase δ has a 3′–5′ ("proofreading") exonuclease activity has led to suggestions that δ is particularly well suited for high-fidelity cellular DNA synthesis (Crute et al., 1986;

† This work was supported by a grant from the Life and Health Insurance Medical Research Fund, by USPHS Grant CA37261 from the National Cancer Institute, and by Brown and Williamson Tobacco Corp., Phillip Morris, Inc., R. J. Reynolds Tobacco Co., and the United States Tobacco Co.

[1] Abbreviations: UV, ultraviolet; BuPh-dGTP, N^2-(*p*-*n*-butylphenyl)-2′-deoxyguanosine 5′-triphosphate; ddTTP, 2′,3′-dideoxythymidine 5′-triphosphate; dThd, thymidine; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; EDTA, ethylenediaminetetraacetic acid; dATP, 2′-deoxyadenosine 5′-triphosphate; dCTP, 2′-deoxycytidine 5′-triphosphate; dGTP, 2′-deoxyguanosine 5′-triphosphate; dTTP, thymidine 5′-triphosphate; K_m, Michaelis constant; K_i, inhibitor constant.

0006-2960/87/0426-2664$01.50/0

Dresler & Frattini, 1986; Wahl et al., 1986). We recently examined the sensitivity of replication and UV-induced repair synthesis in permeable human cells to a nucleotide analogue, N^2-(*p-n*-butylphenyl)-2′-deoxyguanosine 5′-triphosphate (BuPh-dGTP), which differentiates between polymerases α and δ, inhibiting α strongly and δ weakly (Dresler & Frattini, 1986). Both replication and repair synthesis were several hundred fold less sensitive to BuPh-dGTP than was polymerase α, suggesting that polymerase δ may be involved in these two forms of cellular DNA synthesis. The relative resistance of DNA replication and repair synthesis to BuPh-dGTP could have arisen, however, from a relative inaccessibility of repair and replication sites in permeable cells to the inhibitor. To circumvent this difficulty, we have now investigated the sensitivity of DNA replication and UV-induced DNA repair synthesis in permeable human cells to a nucleotide analogue, 2′,3′-dideoxythymidine 5′-triphosphate (ddTTP), which inhibits polymerase δ more strongly than it inhibits polymerase α but less strongly than it inhibits polymerase β (Wahl et al., 1986). We find that ddTTP also inhibits replication and UV-induced repair synthesis more strongly than it inhibits polymerase α, but less strongly than it inhibits polymerase β, supporting the hypothesis that DNA polymerase δ participates in these two forms of cellular DNA synthesis.

Experimental Procedures

Chemicals. Concentrations of nucleotide solutions were determined by UV absorbance. Dideoxythymidine triphosphate, obtained from Pharmacia, was dissolved at 10 mM in 30 mM Tris, pH 7.6, and stored at −20 °C.

Cell Culture. Human diploid fibroblasts (AG1518; Institute for Medical Research) were passed into glass roller bottles, prelabeled with [*methyl*-^{14}C]dThd, and either used during exponential growth for studies of DNA replication or grown to confluence and used for studies of DNA repair synthesis as described (Dresler et al., 1982; Dresler & Lieberman, 1983).

Preparation and Damaging of Permeable Cells. Growth-phase or confluent cells, prelabeled with [^{14}C]dThd, were collected, made permeable in 10 mM Tris (pH 7.6 at 37 °C), 4 mM $MgCl_2$, 1 mM EDTA, 250 mM sucrose, and 3 mM dithiothreitol, and washed twice in the same buffer to remove endogenous nucleotides, as described (Dresler et al., 1982; Dresler, 1984). For studies of repair synthesis, the permeable confluent cell suspension was spread in a layer approximately 1 mm thick in a plastic dish on ice and exposed to 100 J/m^2 of UV radiation from a G15T8 germicidal lamp at a flux of 3 W/m^2.

Measurement of DNA Replication and Repair Synthesis in Permeable Cells. The assays for DNA replication and repair synthesis have been previously described in detail (Dresler et al., 1982; Dresler, 1984). Small portions (0.05–0.1 mL) of the permeable cell suspension were mixed with 0.5 volume of reaction mix to yield 40 mM Tris (pH 7.6 at 37 °C), 8 mM $MgCl_2$, 5 mM ATP, 167 mM sucrose, 2 mM dithiothreitol, and 0.67 mM EDTA and, for replication assays, 75 mM KCl, 50 μM dATP, 50 μM dCTP, and 50 μM dGTP or, for repair synthesis assays, 15 mM KCl, 3 μM dATP, 3 μM dCTP, and 3 μM dGTP. Also present were the indicated concentrations of [α-^{32}P]dTTP and ddTTP. Samples were incubated at 37 °C for either 5 (for measurements of replication) or 15 min (for measurements of repair synthesis). The reactions were stopped, and cells were collected by centrifugation, suspended in 1% sodium dodecyl sulfate, precipitated, and collected on filters, and radioactivity was determined as described (Dresler et al., 1982). Repair synthesis was determined by taking the difference between specific incorporation (^{32}P/^{14}C) in corresponding irradiated and unirradiated samples, and replication was measured as total specific incorporation (^{32}P/^{14}C) in undamaged growth-phase cells.

Assay of Activity of Isolated DNA Polymerases. The polymerase α used was fraction IV prepared from HeLa cells essentially as described by Fisher and Korn (1977). The activity of this polymerase preparation was completely inhibited by aphidicolin and by the anti-polymerase α antibody SJK 287-38 (Tanaka et al., 1982). DNA polymerase β, prepared from Novikoff hepatoma as described (Stalker et al., 1976), was a gift from Drs. Diane Rein and Ralph Meyer, University of Cincinnati.

Polymerase α was assayed under standard polymerase reaction conditions by incubating portions of enzyme (0.04 unit/assay) with 20 mM Tris (pH 7.6 at 37 °C), 10 mM $MgCl_2$, 30 mM KCl, 2 mM 2-mercaptoethanol, 100 μM dATP, 100 μM dCTP, 100 μM dGTP, 200 μg/mL bovine serum albumin, 200 μg/mL activated DNA [prepared as described by Fisher et al. (1979)], and the indicated concentrations of [α-^{32}P]dTTP and ddTTP for 10 min at 37 °C. The reactions were stopped by adding 4 volumes of ice-cold 10% trichloroacetic acid, the precipitates were collected on glass fiber filters and washed, and radioactivity was determined by liquid scintillation counting. In some experiments, polymerase α was assayed under permeable cell replication or repair synthesis reaction conditions by omitting the permeable cells and including enzyme (0.04 unit/assay) along with 100 μg/mL bovine serum albumin and 200 μg/mL activated DNA. Polymerase β was assayed under permeable cell repair synthesis reaction conditions by omitting permeable cells and including enzyme (0.07 unit/assay), 15% glycerol, and 200 μg/mL activated DNA. Samples were incubated for 10 min at 37 °C and incorporated radioactivity was determined as described above. Both the α and β polymerase assays showed linear nucleotide incorporation for at least 15 min (data not shown).

Results

Using our previously described permeable human fibroblast systems (Dresler et al., 1982; Dresler, 1984), we compared inhibition by ddTTP of DNA replication and UV-induced DNA repair synthesis with ddTTP inhibition of isolated DNA polymerases α and β assayed under equivalent reaction conditions (Figure 1). DNA replication in permeable cells was substantially more sensitive to ddTTP than isolated DNA polymerase α assayed under the same reactions conditions but was much less sensitive than polymerase β. In contrast, UV-induced DNA repair synthesis in permeable cells appeared to have a sensitivity to ddTTP similar to that of isolated human DNA polymerase α assayed under the same conditions.

Previous studies (Dresler, 1984) have indicated that simple inhibitor curves may not be adequate for assessing the characteristics of complex cellular processes such as repair and replication. Thus, we extended our analysis by determining the apparent K_i's for ddTTP of DNA replication and UV-induced DNA repair synthesis in permeable cells and of isolated DNA polymerases α and β. For these determinations, we used the standard technique of measuring activity at a number of concentrations of the inhibitor ddTTP and its competitive substrate dTTP and displaying the data as Dixon plots (i.e., inverse of activity vs. inhibitor concentration; Figure 2). As expected for competitive inhibition, lines drawn through points assayed at equal substrate (dTTP) concentrations intersected at single points to the left of the ordinate. The apparent K_i's for ddTTP (determined by dropping perpendiculars from the points of intersection to the abscissas) for the two forms of cellular DNA synthesis were quite

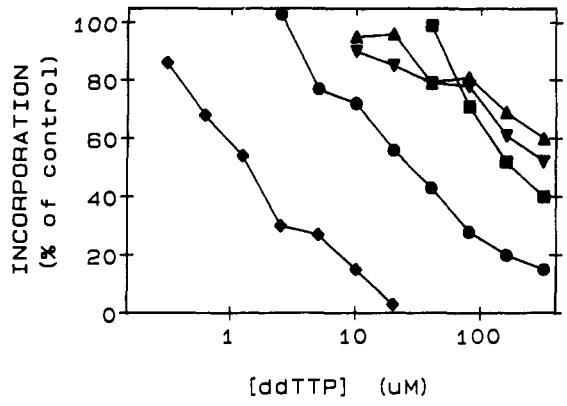

FIGURE 1: Inhibition by ddTTP of isolated DNA polymerases α and β and of semiconservative DNA replication and UV-induced DNA repair synthesis in permeable human cells. DNA replication (●) was assayed in the presence of 3 μM dTTP. UV-induced DNA repair synthesis (▲) was assayed in the presence of 1 μM dTTP. DNA polymerase α was assayed under both permeable cell replication (■) and repair synthesis (▼) reaction conditions. DNA polymerase β (◆) was assayed under permeable cell repair synthesis reaction conditions. Each assay contained the indicated concentration of ddTTP. Each point is the average of two determinations.

similar—approximately 40 μM for replication (Figure 2B) and approximately 25 μM for UV-induced repair synthesis (Figure 2C). These values were much smaller than the apparent K_i for ddTTP of isolated polymerase α (greater than 200 μM; Figure 2A) but were greater than the apparent K_i of isolated polymerase β (less than 2 μM; Figure 2D).

A question raised by the data in Figure 2 is why replication and repair synthesis, which have such similar apparent K_i's for ddTTP, seem to have such different ddTTP sensitivities in the simple inhibition experiments depicted in Figure 1. To address this question, we measured the apparent K_m's of each process for dTTP, the substrate nucleotide that is competitive with ddTTP. The apparent K_m for dTTP of UV-induced repair synthesis was approximately 0.33 μM (Figure 3A) and that for replication was approximately 2.7 μM (Figure 3B). Thus, in Figure 1, UV-induced repair synthesis was studied at a dTTP concentration (1 μM) approximately 3-fold greater than its K_m for that nucleotide, while replication was studied at a dTTP concentration (3 μM) approximately equal to its K_m. The greater relative concentration of dTTP in the repair synthesis assays made it a more effective competitor for the inhibitor and decreased the apparent ddTTP sensitivity of the process.

DISCUSSION

We have measured the sensitivity of DNA replication and UV-induced DNA repair synthesis in permeable human cells to inhibition by the nucleotide analogue ddTTP and have compared these data with the ddTTP sensitivities of isolated DNA polymerases α and β. Both replication and repair synthesis are more sensitive to ddTTP than polymerase α but less sensitive than polymerase β. The apprent K_i's for ddTTP of replication (approximately 40 μM) and repair synthesis (approximately 25 μM) are 5–10-fold lower than the apparent K_i of polymerase α (greater than 200 μM) but are 10–20-fold greater than the apparent K_i of polymerase β (less than 2 μM). The simplest interpretation of these data is that DNA polymerase δ, which is also more sensitive to ddTTP than DNA polymerase α but less sensitive than polymerase β (Wahl et al., 1986), is involved in DNA replication and UV-induced DNA repair synthesis in human cells. Because these two forms

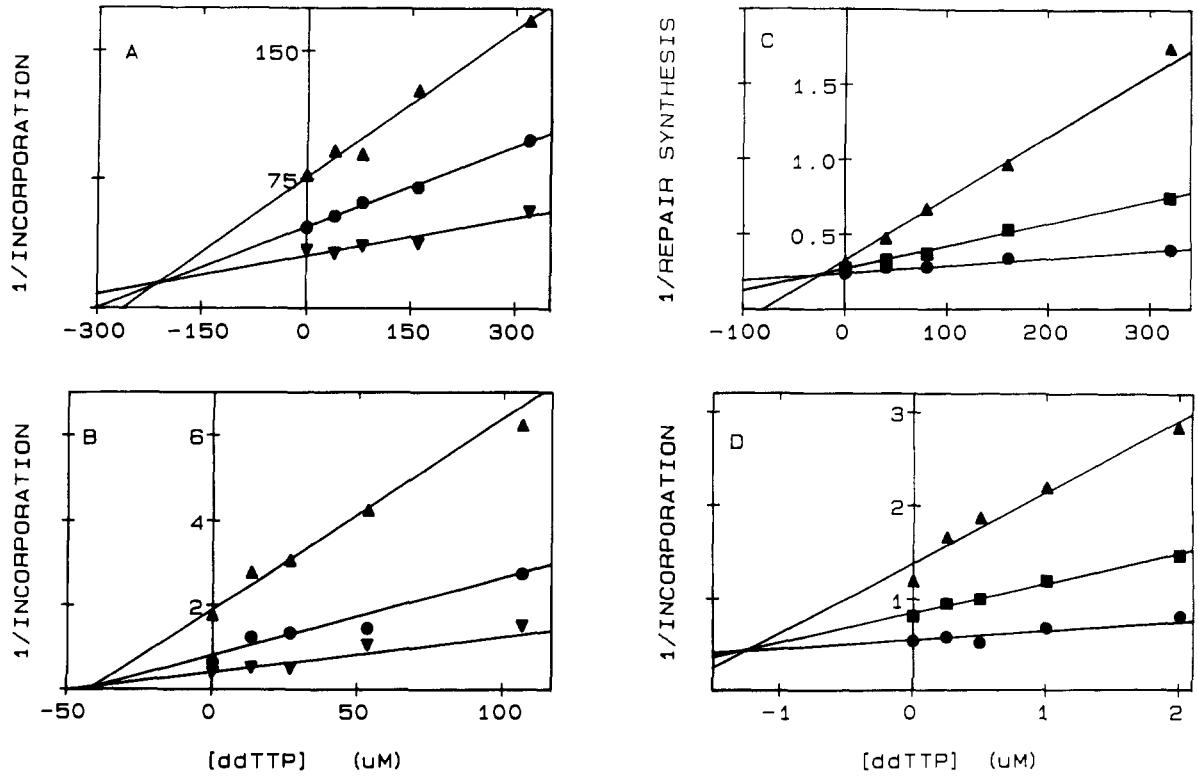

FIGURE 2: Determination of K_i's for ddTTP of DNA polymerase α, DNA replication and UV-induced DNA repair synthesis in permeable cells, and DNA polymerase β. (A) DNA polymerase α was assayed under standard reaction conditions in the presence of either 1.25 (▲), 2.5 (●), or 5.0 μM (▼) dTTP. Incorporation is expressed as ^{32}P cpm × 10^{-6}. (B) DNA replication in permeable cells was determined in the presence of either 1.25 (▲), 2.5 (●), or 5.0 μM (▼) dTTP. Incorporation is expressed as $^{32}P/^{14}C$ (see Experimental Procedures). (C) UV-induced DNA repair synthesis in permeable cells was determined in the presence of either 0.125 (▲), 0.375 (■), or 1.0 μM (●) dTTP. Repair synthesis is expressed as $^{32}P/^{14}C$ (see Experimental Procedures). (D) DNA polymerase β was assayed under permeable cell repair synthesis reaction conditions in the presence of either 0.25 (▲), 0.5 (■), or 1.0 μM (●) dTTP. Incorporation is expressed as ^{32}P cpm × 10^{-5}. Each assay contained the indicated concentration of ddTTP. Each point is the average of two determinations.

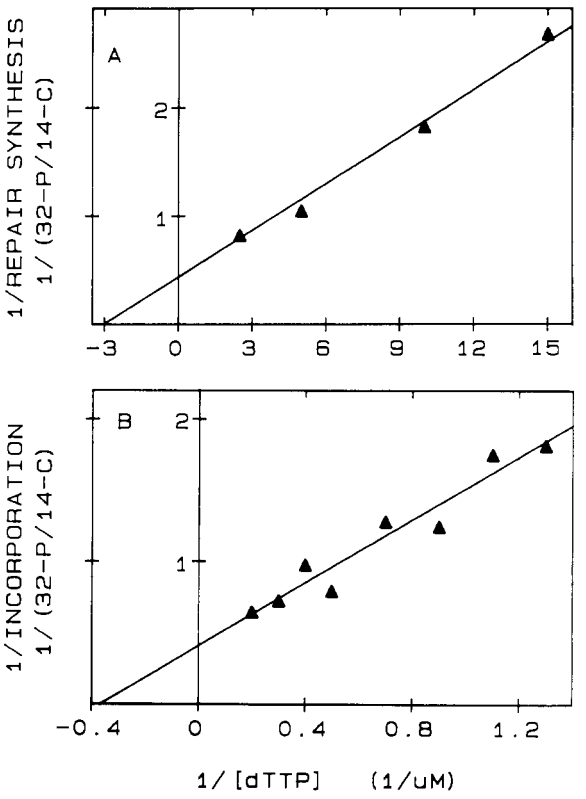

FIGURE 3: Determination of the K_m's for dTTP of UV-induced DNA repair synthesis (A) and DNA replication (B) in permeable cells. DNA repair synthesis and replication were assayed in the presence of 100 μM dATP, 100 μM dCTP, 100 μM dGTP, and the indicated concentrations of dTTP. The K_m for dTTP is given by $-(1/x$ intercept) of a line fit to the data points. Each point is the average of two determinations.

of cellular DNA synthesis are *more* sensitive to ddTTP than isolated DNA polymerase α, this conclusion is not susceptible to the criticism that the inhibitor sensitivities of these processes might differ from that of isolated polymerase α as a result of impeded access of the inhibitor to sites of replication and repair in the permeable cells.

It could be objected that our results are contradicted by previous studies of the ddTTP sensitivity of mammalian DNA replication, which concluded that the process is mediated largely or entirely by DNA polymerase α (Edenberg et al., 1978; Waqar et al., 1978; Abboud & Horowitz, 1979; Krokan et al., 1979; van der Vliet & Kwant, 1981). In fact, one of these studies (Waqar et al., 1978) did show that DNA replication in lysed HeLa cells was more sensitive to ddTTP than isolated DNA polymerase α. Under the reaction conditions used, replication was inhibited by 40% at 100 μM ddTTP, while polymerase α was inhibited by only 10%. At the time, however, neither the abundance of polymerase δ in mammalian cells nor the relative ddTTP sensitivity of polymerase δ was recognized. In that situation, the fact that DNA replication was much less sensitive to ddTTP inhibition than the other known mammalian DNA polymerases, β and γ (which were each inhibited by greater than 60% at 10 μM ddTTP under the conditions used), led the authors to conclude that polymerase α mediates semiconservative DNA replication.

In several other previous ddTTP studies, the competitive substrate dTTP was used at concentrations sufficiently high [20 μM dTTP (Edenberg et al., 1978); 20 μM dTTP (Krokan et al., 1979); 50 μM dTTP (van der Vliet & Kwant, 1981)] to obscure possible differences between the sensitivities to ddTTP of semiconservative replication and isolated polymerase α. This phenomenon is analogous to the deceptively low sensitivity to ddTTP shown by UV-induced repair synthesis in Figure 1, where assays were conducted at a dTTP concentration severalfold greater than the K_m for that nucleotide. In the other previous ddTTP study of which we are aware (Abboud & Horowitz, 1979), the concentration of competitive substrate used was low (3 μM) but the maximal ddTTP concentration used was sufficiently low (9 μM) that no difference between the ddTTP sensitivities of replication and DNA polymerase α was detected. In contrast to these studies, we have used high concentrations of ddTTP and concentrations of dTTP that are close to the K_m's for the two processes analyzed, enabling us to clearly demonstrate that DNA replication and UV-induced DNA repair synthesis are more sensitive to ddTTP than DNA polymerase α.

The results of our studies with ddTTP and with another DNA polymerase inhibitor, BuPh-dGTP (Dresler & Frattini, 1986), strongly suggest that DNA polymerase δ participates in DNA replication and UV-induced DNA repair synthesis in human cells. There are, however, several qualifications to this conclusion and alternate explanations of our data that should be mentioned. First, although the data indicate that DNA polymerase δ is involved in DNA replication and repair, they do not provide a basis for excluding the involvement and repair, they do not provide a basis for excluding the involvement of DNA polymerase α in these processes. Nucleotide incorporation in our permeable cell replication system primarily reflects elongation of previously initiated replicons (unpublished data); thus, our results do not address the question of which DNA polymerase, α or δ, is involved in replicon initiation. Also, monoclonal antibodies directed against polymerase α (SJK 287-38 and SJK 132-20; Tanaka et al., 1982) have been shown to inhibit DNA replication when added to the reaction mixture of permeable human fibroblasts (Miller et al., 1985a,b; M. G. Frattini and S. L. Dresler, unpublished data) or when microinjected into the nuclei of intact human, hamster, and mouse cells (Kaczmarek et al., 1986), suggesting that polymerase α does participate in some way in replication. Unfortunately, the degree to which these antibodies differentiate between polymerases α and δ is unclear. Some authors (Byrnes, 1985; Lee & Toomey, 1987) report that the SJK 287-38 and SJK 132-20 antibodies do not inhibit polymerase δ, but Wahl et al. (1986) found that both antibodies were capable of inhibiting one of the two forms of δ that they isolated from calf thymus. The results of anti-polymerase antibody inhibition experiments are thus somewhat ambiguous, but they do require that one remain open to the possible involvement of polymerase α along with polymerase δ in DNA replication.

An alternate explanation of our data is the possibility that DNA replication and UV-induced repair synthesis in human cells are entirely mediated by DNA polymerase α but that the characteristics of the cellular environment are such that the polymerase behaves very differently in situ than it does as an isolated enzyme. There is precedent for such variations in the attributes of DNA polymerases. The K_m for dNTP's of bacteriophage T4 DNA polymerase is dramatically altered, for example, when it associates with polymerase accessory proteins (Mathews & Sinha, 1982). Similarly, although DNA replication and UV-induced DNA repair synthesis in permeable human fibroblasts appear to be mediated by the same DNA polymerase, the K_m's for dNTP's of these two processes differ by about 10-fold (Dresler, 1984), suggesting that in one or both situations the characteristics of the polymerase are altered by association with specific accessory factors. Yet another example is the incorporation of dNTP's at SV40

replication origins by the polymerase α–primase complex, a process that is quite insensitive to aphidicolin, in contrast to the activity of purified polymerase α on in vitro templates such as activated DNA, which is very sensitive to the inhibitor (Decker et al., 1986). Thus, it is possible that the BuPh-dGTP and ddTTP sensitivities of DNA replication and UV-induced DNA repair synthesis differ from those of polymerase α not because a different polymerase (δ) is involved in these processes but simply because the characteristics of polymerase α are altered when it functions in the cell.

Finally, we must consider the possibility that DNA polymerases α and δ are not totally distinct enzymes but are, in fact, closely related polypeptides. Wahl et al. (1986) examined the characteristics of two forms of polymerase δ isolated from calf thymus and concluded that one, δ_1, has the properties of DNA polymerase α with a loosely associated nuclease, while the other, δ_2, has the properties of DNA polymerase α with a tightly associated or covalently attached nuclease. These results suggest that the various polypeptides which have been isolated and characterized as forms of polymerases α and δ may all be derived from a single native cellular polymerase that mediates replication and UV-induced repair synthesis in vivo. Such a situation could explain the fact that these processes have some characteristics (e.g., antibody inhibition) that are similar to DNA polymerase α and some characteristics (e.g., BuPh-d-GTP and ddTTP sensitivities) that are similar to polymerase δ. Although our data do not allow us to precisely identify the DNA polymerase(s) involved in DNA replication and UV-induced DNA repair synthesis, they do compel a reassessment of the previously accepted conclusion that DNA polymerase α alone mediates these processes in mammalian cells.

ACKNOWLEDGMENTS

We thank Rona M. Robinson-Hill for technical assistance, Drs. Diane Rein and Ralph Meyer for generously providing isolated DNA polymerase β, Dr. Robert Bambara for helpful discussions about polymerases α and δ and for providing his manuscript prior to publication, and Mark G. Frattini and Brenda Jo Mengeling for their comments on the manuscript.

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