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DNA replication and UV-induced DNA repair synthesis in human fibroblasts are much less sensitive than DNA polymerase α to inhibition by butylphenyl-deoxyguanosine triphosphate

Steven L.Dresler and Mark G.Frattini

Department of Pathology, Washington University School of Medicine, St. Louis, MO 63110, USA

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ABSTRACT

In mammalian cells, both semiconservative DNA replication and the DNA repair patch synthesis induced by high doses of ultraviolet radiation are known to be inhibited by aphidicolin, indicating the involvement in these processes of one or both of the aphidicolin-sensitive DNA polymerases, α and/or δ. In this paper, N^2-(p-n-butylphenyl)-2'-deoxyguanosine-5'-triphosphate, a strong inhibitor of polymerase α and a weak inhibitor of polymerase δ, is used to further characterize the DNA polymerase(s) involved in these two forms of nuclear DNA synthesis. In permeable human fibroblasts, DNA replication and ultraviolet-induced DNA repair synthesis are more resistant to the inhibitor than DNA polymerase α by factors of approximately 500 and 3000, respectively. These findings are most consistent with the involvement of DNA polymerase δ in these processes.

INTRODUCTION

In intact and permeable mammalian cells, aphidicolin has been shown to inhibit both semiconservative DNA replication (reviewed in 1) and the DNA repair synthesis induced by high doses of ultraviolet radiation (UV) (for references, see 2). Such results have been regarded as strong evidence for the involvement of DNA polymerase α in these processes. Recently, however, DNA polymerase δ, which like polymerase α is sensitive to aphidicolin and N-ethylmaleimide but which has a 3'-5' exonuclease activity not shown by α (3-5), has been found to be present in sizeable quantities in mammalian cell extracts (5,6). These data suggest that polymerase δ might be involved in cellular DNA replication and repair in addition to or instead of polymerase α. A recently developed compound, N^2-(p-n-butylphenyl)-2'-deoxyguanosine-5'- triphosphate (BuPh-dGTP; 7), which is a strong inhibitor of DNA polymerase α (5,6,8-10) and a weak inhibitor of polymerase δ (5,6,10), provides a means for differentiating between the involvement of polymerases α and δ in cellular DNA synthesis. Using permeable human fibroblasts (11,12), the sensitivity to BuPh-dGTP of semiconservative DNA replication and UV-induced DNA repair synthesis has been examined.

MATERIALS AND METHODS

The tetraammonium salt of BuPh-dGTP, generously provided by Dr. George Wright, was dissolved at 5 mM in 20 mM Tris, pH 7.6, and stored at -20°C.

The permeable cell assays for semiconservative DNA replication and DNA repair synthesis have been described (2,11-13). Diploid human fibroblasts (AG1518; Institute for Medical Research) were grown in glass roller bottles, prelabeled with [^{14}C]thymidine, and either used during exponential growth for studies of semiconservative DNA replication or grown to confluence and used for studies of DNA repair synthesis. The cells were collected, made permeable, washed to remove endogenous nucleotides, irradiated (if required) with 100 J/m^2 UV, and incubated with the appropriate reaction mixture. All reactions contained 40 mM Tris, pH 7.6 at 37°C, 8 mM $MgCl_2$, 5 mM ATP, 2 mM dithiothreitol, 0.67 mM EDTA, and 167 mM sucrose. In addition, replication assays contained 75 mM KCl, 5 μM [^{32}P]dGTP, and 50 μM dATP, dCTP, and dTTP. Repair synthesis assays contained 15 mM KCl, 0.3 μM [^{32}P]dGTP, and 3 μM dATP, dCTP, and dTTP. Where indicated, permeable growth-phase cells in a tube immersed in an ice-water bath were sonicated for 10 sec at a power setting of 1.5 using a Branson 200 sonicator equipped with a microtip, prior to incubation with the replication reaction mixture. Radioactivity incorporated into each sample was determined by liquid scintillation counting of trichloroacetic acid precipitates collected on glass fiber filters. Repair synthesis was determined by taking the difference between specific dGMP incorporation (^{32}P/^{14}C) in corresponding irradiated and unirradiated samples, and replication was measured as total specific dGMP incorporation (^{32}P/^{14}C) in undamaged growth-phase cells.

The maximal initial rate (V_{max}) for replicative DNA synthesis in permeable growth-phase AG1518 cells is about 73 pmol/hr/10^6 cells (unpublished data). Under our culture conditions, the doubling time for these fibroblasts is about 72 hrs, which, assuming a DNA content of 6 pg/cell, corresponds to a DNA replication rate (for unsynchronized cells) of 250 pmol/hr/10^6 cells. Thus, the maximal initial rate of replicative DNA synthesis in the permeable cells is approximately 30% of the rate seen in intact cells. The maximal initial rate of nucleotide incorporation into DNA repair patches in permeable confluent cells irradiated with 100 J/m^2 UV is about 1.6 pmol/hr/10^6 cells (11,13) which, assuming a repair patch length of 30 nucleotides (13), represents synthesis of about 500 repair patches/cell/min. This is similar to the maximal rate of repair of UV damage (approximately 750 pyrimidine dimers removed/cell/min) observed in intact

human fibroblasts irradiated with either 20 or 40 J/m^2 UV (14).

The polymerase α used was Fraction IV prepared from HeLa cells essentially as described (15). Reaction conditions for the truncated DNA synthesis assay (lacking dGTP; Figure 2) were as described (16). Polymerase activity was also assayed under permeable cell replication and repair synthesis reaction conditions (Figure 1) by omitting permeable cells and including isolated polymerase α and 200 μg/ml activated calf thymus DNA.

RESULTS

Inhibition by BuPh-dGTP of Semiconservative DNA Replication and UV-Induced DNA Repair Synthesis in Permeable Human Fibroblasts

Using previously described permeable human fibroblast systems (2, 11-13), inhibition of DNA replication and repair synthesis by BuPh-dGTP was investigated (Figure 1). Both replicative (Figure 1A) and repair (Figure 1B) synthesis were inhibited essentially completely by the compound with 50% inhibition values of 14 μM and 4.5 μM, respectively. The inhibition curve

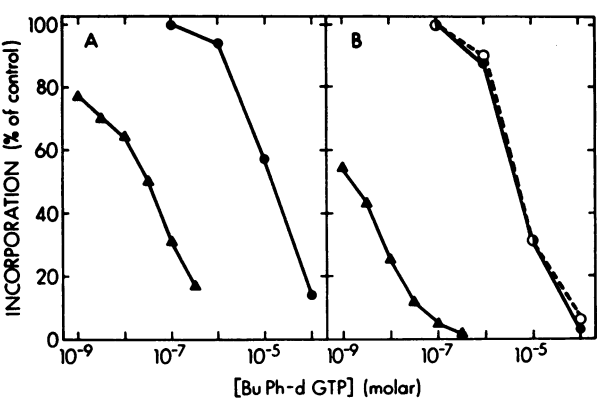

Figure 1. Inhibition by BuPh-dGTP of semiconservative DNA replication and UV-induced DNA repair synthesis in permeable fibroblasts, compared with inhibition of DNA polymerase α. (A) Semiconservative DNA replication (●) was measured in permeable growth-phase cells and the activity of isolated HeLa DNA polymerase α (▲) was assayed under permeable cell replication conditions in the presence of the indicated concentrations of inhibitor. (B) DNA repair synthesis (●) induced by 100 J/m^2 UV was measured in permeable confluent cells in the presence of the indicated concentrations of inhibitor. Semiconservative DNA replication in permeable growth-phase cells (○) and the activity of isolated HeLa DNA polymerase α (▲) were assayed under permeable cell repair synthesis reaction conditions in the presence of the indicated concentrations of inhibitor. The data are presented as percentages of the activity measured in the absence of inhibitor. Each point is the average of two determinations.

for repair synthesis was shifted slightly to the left relative to the curve for inhibition of replicative synthesis, however, when replication was studied under the same reaction conditions as are used for the repair synthesis assay, an inhibition curve was obtained which was identical to the inhibition curve for repair synthesis (Figure 1B). This result is consistent with the concept that the same DNA polymerase is involved in both replication and UV-induced repair synthesis.

Inhibition by BuPh-dGTP of Isolated DNA Polymerase α, Assayed Under Permeable Cell DNA Replication and Repair Synthesis Reaction Conditions

Reports from several laboratories have established that DNA polymerase α is very sensitive to inhibition by BuPh-dGTP (5,6,8-10). To permit a direct comparison with the permeable cell data presented above, inhibition of isolated HeLa DNA polymerase α was studied under permeable cell DNA replication and repair synthesis reaction conditions (Figure 1). As expected, the polymerase was strongly inhibited by BuPh-dGTP, with 50% inhibition values of 30 nM under replication conditions (Figure 1A) and 1.5 nM under repair synthesis conditions (Figure 1B). [The greater sensitivity of the polymerase to BuPh-dGTP under repair synthesis conditions is probably due to the low concentration of the competitive substrate, dGTP, present in the repair synthesis reaction mixture (0.3 μM compared with 5 μM in the replication reaction mixture)]. Clearly, both semiconservative DNA replication and UV-induced DNA repair synthesis are much less sensitive to BuPh-dGTP than DNA polymerase α.

Stability of BuPh-dGTP during Incubation with Permeable Cells

A possible explanation for the relative insensitivity of repair and replicative synthesis in permeable human cells to BuPh-dGTP is that the inhibitor is rapidly inactivated, either physically or chemically, during the permeable cell incubations. To explore this possibility, permeable cell repair and replication reaction mixtures containing 2 μM BuPh-dGTP were prepared. Portions of these reactions mixtures, taken prior to incubation with permeable cells, were diluted and added to truncated HeLa DNA polymerase α assays (Figure 2) to yield the indicated final concentrations of inhibitor. Other portions of these reaction mixtures were then incubated at 37°C with permeable cells as for a typical repair synthesis or replication assay, following which the cells were removed by centrifugation and the post-incubation supernatants were diluted into HeLa polymerase α assays (Figure 2) to give the indicated expected BuPh-dGTP concentrations. Neither the replication (Figure 2A) nor the repair synthesis (Figure 2B) incubation

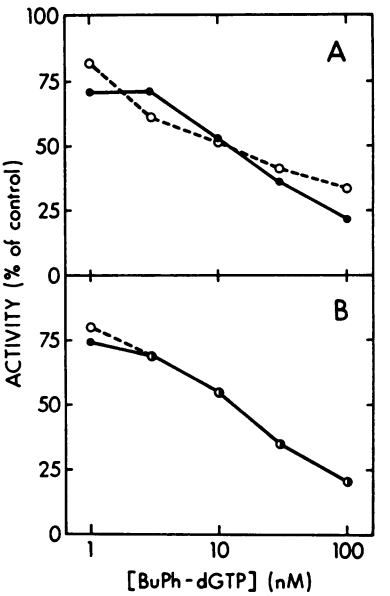

Figure 2. Stability of BuPh-dGTP during incubation with permeable cells under semiconservative replication and repair synthesis reaction conditions. (A) A reaction mix for the permeable cell replication assay was prepared which contained 2 μM BuPh-dGTP and no radioactive label. A portion of this permeable cell reaction mix was diluted into truncated DNA polymerase assay mix to give the indicated final concentrations of BuPh-dGTP (○) and the polymerase α activity was determined. Another portion of the permeable cell reaction mix was incubated for 5 min at 37°C with twice-washed, permeable growth-phase cells. The cells were then removed by centrifugation at 4°C, the post-incubation permeable cell mix was diluted into DNA polymerase mix to give the indicated expected concentrations of BuPh-dGTP (●), and the polymerase α activity was determined. (B) A reaction mix for the permeable cell repair synthesis assay was prepared which contained 2 μM BuPh-dGTP and no radioactive label. This reaction mix was diluted into truncated DNA polymerase assay mix before (○) and after (●) incubation with twice-washed, permeable confluent cells for 15 min at 37°C and polymerase α activity was determined. For both experiments, the polymerase activity is expressed as a percentage of the activity in control samples to which were added equal amounts of permeable cell reaction mix not containing BuPh-dGTP. Each assay sample contained 0.04 units of HeLa polymerase α.

caused a loss of BuPh-dGTP inhibitory potency.

Inhibition by BuPh-dGTP of DNA Synthesis in Sonicated Permeable Growth-Phase Fibroblasts

Another possible explanation for the insensitivity to BuPh-dGTP of repair synthesis and replication is the existence of a permeability barrier which limits entry of the inhibitor into the nuclei of permeable cells. A priori this seems unlikely because i) the protocol used in these studies is known to

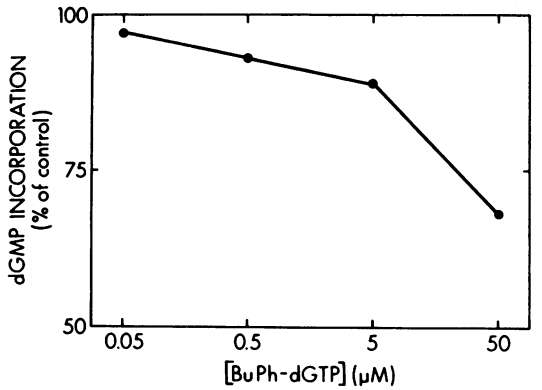

Figure 3. Inhibition by BuPh-dGTP of DNA synthesis in sonicated, permeable growth-phase fibroblasts. Cells were made permeable, washed, and sonicated gently until no intact cells or nuclei could be seen by phase-contrast microscopy. The sonicated cell suspension was incubated for 5 min at 37°C under replicative synthesis reaction conditions in the presence of the indicated concentrations of BuPh-dGTP. Incorporation is expressed as a percentage of the incorporation in sonicated cells incubated without inhibitor. Each point is the average of two determinations.

make the nuclei permeable even to Micrococcus luteus UV endonuclease (11), a protein of about 15,000 daltons, ii) electron microscopy of these permeable cells reveals numerous small breaks in the plasma membrane and in the nuclear membranes (data not shown), and iii) repair and replication in permeable cells are much more resistant than DNA polymerase α to inhibition not only by the charged molecule BuPh-dGTP, but also by the uncharged nucleoside, butylphenyl-deoxyguanosine, and base, butylphenyl-guanine (data not shown). The possible existence of a permeability barrier was, however, addressed directly by studying the inhibition by BuPh-dGTP of replicative DNA synthesis in growth-phase fibroblasts which were made permeable, washed to remove endogenous nucleotides, and then disrupted by sonication. Examination by phase-contrast microscopy revealed the permeable cells and their nuclei to be extensively fragmented; no intact cells or nuclei were seen. These fragmented permeable cells performed DNA synthesis at about 20% of the rate seen in permeable cells which were not fragmented (data not shown). The DNA synthesis in fragmented cells had a BuPh-dGTP inhibition curve (Figure 3) similar to the curve for inhibition of replicative synthesis in undisrupted permeable cells (compare Figure 3 with Figure 1A), suggesting that there is no significant barrier to entry of BuPh-dGTP into the nuclei of permeable cells. DNA synthesis in fragmented cells (Figure 3) was, however, slightly less sensitive

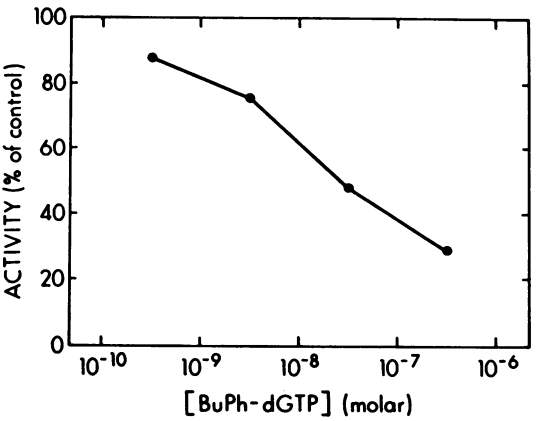

Figure 4. Inhibition by BuPh-dGTP of isolated DNA polymerase α, assayed under permeable cell replication conditions in the presence of sonicated growth-phase cells. Cells were made permeable, washed, and sonicated as in Figure 3. Portions of the sonicated cell suspension were added to permeable cell replication reaction mixture supplemented with a large excess (0.2 units) of isolated polymerase α, 100 μg/ml activated calf thymus DNA, and the indicated concentrations of BuPh-dGTP. Polymerase activity is expressed as a percentage of the activity in samples incubated without inhibitor. Each point is the average of two determinations. Incorporation in the control sample (containing 0.2 units of isolated polymerase α but no inhibitor) was 580,000 cpm, almost 10 times the incorporation seen in controls in which the sonicated cells were incubated with reaction mixture not containing polymerase α (60,000 cpm).

to BuPh-dGTP than DNA synthesis in non-fragmented permeable growth-phase cells (Figure 1A), suggesting that sonication may release a cellular factor which alters the sensitivity of the DNA polymerase to the inhibitor. To test this possibility, we prepared a suspension of sonicated, permeable growth-phase cells, added replication reaction mixture supplemented with isolated DNA polymerase α and activated DNA, and measured the activity of the added polymerase at various concentrations of BuPh-dGTP. The inhibition curve for polymerase α activity assayed in the presence of the sonicated cell preparation (Figure 4) is essentially identical to that for polymerase α assayed under the same reaction conditions without the sonicated cells (compare with Figure 1A). We find no evidence for a factor in the sonicated cell preparation which decreases the sensitivity of polymerase α to BuPh-dGTP. Overall, the data presented here indicate that DNA repair synthesis and semiconservative DNA replication in permeable human cells are much less sensitive than DNA polymerase α to inhibition by BuPh-dGTP.

DISCUSSION

BuPh-dGTP is a useful agent for differentiating between mammalian α and δ DNA polymerases (5,6,8,10), enzymes which show similar responses to other inhibitors. Human polymerase α isolated from HeLa cells (Figure 1), like the α polymerases isolated from several other mammalian sources (6,8,9,10), is strongly inhibited by nanomolar concentrations of the drug. Although the α DNA polymerases used in the studies described here and in a number of previous reports (6,8,10) were isolated by traditional methods which yield enzymes of relatively low molecular weight (apparently as a result of proteolysis during isolation), a high degree of BuPh-dGTP sensitivity is also characteristic of high molecular weight α polymerases purified by immunoaffinity chromatography (5,9). For example, the high molecular weight α polymerase from African green monkey cells is 95% inhibited by 1 μM BuPh-dGTP in the presence of the competitive substrate, dGTP, at 50 μM (9). In contrast, mammalian δ DNA polymerases are relatively insensitive to inhibition by BuPh-dGTP, with concentrations required for 50% inhibition in the high micromolar range (5,6,8,10). Lee et al. (10) found, for example, that 50% inhibition of DNA synthesis catalyzed by human placental polymerase δ (with activated calf thymus DNA as template) required 120 μM BuPh-dGTP, while, in the same assay, 50% inhibition of human polymerase α required only 40 nM BuPh-dGTP. Thus the data reported here, showing that inhibition by BuPh-dGTP of semiconservative DNA replication and UV-induced DNA repair synthesis requires doses of inhibitor 500 to 3000 times the doses required to inhibit polymerase α, suggest that DNA polymerase δ is involved in cellular DNA replication and repair.

Miller et al. (17,18) have shown that monoclonal antibodies prepared against DNA polymerase α inhibit DNA replication in permeable human fibroblasts, with maximal inhibition of 70%. [We have confirmed this inhibition in our permeable growth-phase cell system using SJK 287-38, the antibody found by Miller et al. (17,18) to be the most potent replication inhibitor, however, the maximal inhibition we have seen is 40% (unpublished data)]. Inhibition of replication by anti-polymerase α antibodies takes place, however, at concentrations several orders of magnitude higher than those which inhibit isolated DNA polymerase α (18). Because high concentrations of at least one anti-polymerase α antibody have been shown to inhibit polymerase δ (5), we feel that previous antibody inhibition results are not inconsistent with the suggestion that DNA polymerase δ is involved in DNA replication.

For several reasons, polymerase δ seems well suited for involvement in cellular DNA replication and repair. First, the enzyme is present in abundance in mammalian cell extracts (5,6). Second, δ has a constitutive DNA-dependent RNA polymerase or primase activity (5) which might be involved in initiation of replication. Third, although initial reports suggested that polymerase δ has low activity on DNA templates containing all four deoxynucleotides (4,5,19), it has recently been found that in the presence of low concentrations of histones or spermidine, polymerase δ efficiently utilizes activated native DNA as primer-template (20). Finally, polymerase δ possesses an intrinsic 3'-5' exonuclease activity (3-5) which could serve a proofreading function similar to that of the 3'-5' exonucleases of prokaryotic DNA polymerases (21). This latter point is of particular importance because purified mammalian α polymerase, which has been regarded as the polymerase responsible for mammalian nuclear DNA replication, has a misincorporation frequency many orders of magnitude higher than the error frequency of _in vivo_ mammalian DNA replication (22,23). Proofreading exonucleases apparently contribute substantially to fidelity of cellular DNA synthesis in prokaryotes (22) and the exonuclease of polymerase δ might make a similar, substantial contribution to the fidelity of mammalian DNA replication and repair.

In closing, we should note that there are at least two other possible explanations for the relative resistance to BuPh-dGTP of DNA replication and repair synthesis. It is possible that DNA polymerase α (i) is involved in these processes, and (ii), in its native form, is relatively resistant to BuPh-dGTP, but (iii) is rapidly altered during existing isolation protocols in a way which causes it to become very sensitive to BuPh-dGTP. The known sensitivity of DNA polymerase α to proteolysis during isolation (24) adds plausibility to this scenario. Or perhaps DNA polymerase α mediates cellular DNA replication and repair synthesis but is altered _in situ_, possibly by association with accessory proteins, in a way which reduces its sensitivity to BuPh-dGTP. We previously proposed a similar explanation for the finding that UV-induced DNA repair synthesis and DNA replication have similar responses to inhibitors but very different K_m's for deoxynucleotides (2). For teleologic reasons, at least, we favor the proposal that DNA polymerase δ is involved in DNA repair synthesis and replication in human cells. Current experiments employing antibodies against polymerase δ should more clearly resolve the issue.

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