Bleomycin-Induced DNA Repair Synthesis in Permeable Human Fibroblasts: Mediation of Long-Patch and Short-Patch Repair by Distinct DNA Polymerases†

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ABSTRACT: Treatment of permeable human fibroblasts with bleomycin elicits DNA repair synthesis that is only partially sensitive to aphidicolin, an inhibitor of mammalian DNA polymerases α and δ. Inhibition of long-patch repair synthesis by omission of the three unlabeled deoxyribonucleoside triphosphates (dNTPs) selectively eliminates the aphidicolin-sensitive component. The majority of this residual aphidicolin-resistant repair synthesis is contained in ligated patches as revealed by resistance to exonuclease III. Determination of repair patch length by bromodeoxyuridine-induced density shift under conditions where essentially all of the repair synthesis is sensitive or resistant to aphidicolin yielded values of ~20 and 4 nucleotides per patch, respectively. On the basis of these data and the relative sensitivity of bleomycin-induced repair synthesis to N^2-(*p-n*-butylphenyl)-2′-deoxyguanosine 5′-triphosphate (BuPdGTP), 2′,3′-dideoxythymidine 5′-triphosphate (ddTTP), and *N*-ethylmaleimide (NEM), long-patch repair is attributed to DNA polymerase δ and short-patch repair to DNA polymerase β.

The roles played by different DNA polymerases in the repair patch synthesis step of mammalian DNA excision repair remain unclear. Of the four known mammalian DNA polymerases, two (α and δ) are sensitive to aphidicolin, and two (β and γ) are resistant to this compound (Huberman, 1981; Crute et al., 1986). Although there is substantial evidence indicating that the aphidicolin-sensitive polymerase activity responsible for UV[1] -induced excision repair in human cells is DNA polymerase δ (Dresler & Frattini, 1986, 1988; Dresler & Kimbro, 1987; Nishida et al., 1988), both aphidicolin-sensitive and -resistant components of repair synthesis in human cells have been reported by several groups studying repair induced by DNA strand-breaking agents (Miller & Chinault, 1982b; Seki et al., 1982, Dresler & Lieberman, 1983; Seki & Oda, 1986). To explain the involvement of two distinct polymerase activities in repair, a hypothesis was advanced (Grossman, 1981; Cleaver, 1981) that related the known dichotomy of "long" and "short" repair patch size in human cells (Regan & Setlow, 1974)[2] to the preferred utilization of larger and smaller gaps, respectively, by DNA polymerases α and β (Wang & Korn, 1980). Subsequent studies with purified enzymes in vitro supported this concept (Mosbaugh & Linn, 1983, 1984). The recent evidence that DNA polymerase δ is the aphidicolin-sensitive polymerase activity in UV-induced DNA repair (see references above), a classic long-patch system, does not alter the plausibility of this hypothesis, since the gap-size specificity of polymerase δ is essentially identical with that of polymerase α (Crute et al., 1986). Moreover, the most recent study (Randahl et al., 1988) comparing the activities of all known mammalian DNA polymerases on primer templates containing gaps of different sizes led to the conclusion that both aphidicolin-sensitive polymerases (α and δ) are suited for long-patch repair, while both aphidicolin-resistant polymerases (β and γ) are capable of short-patch repair. The sole study that specifically addressed the relationship between polymerase involvement and patch size in cells (Miller & Lui, 1982) did not include patch size measurements; thus, the hypothesis remains to be tested directly. We have used the antineoplastic DNA strand-breaking agent, bleomycin [reviewed in Stubbe and Kozarich (1987)], in a well-characterized permeable human fibroblast system (Dresler et al., 1982) and report here results indicating that DNA polymerase δ mediates long-patch repair synthesis, whereas DNA polymerase β mediates short-patch repair.

Experimental Procedures

Chemicals. Bleomycin, a gift from Bristol Laboratories, Syracuse, NY, was dissolved in distilled H_2O at 5 mg/mL and stored at −70 °C. Aphidicolin and BuPdGTP were dissolved as described (Dresler et al., 1988a) and stored at −20 °C. BrdUrd was dissolved in water immediately prior to use.

Cell Culture and Damaging. Human diploid fibroblasts (AG1518; Institute for Medical Research) were grown to confluence in the presence of [*methyl*-^{14}C]dThd (Dresler et al., 1982), and fresh medium was added 7 days after passage. Cells were used 2–4 weeks after passage. For each of the

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[1] Abbreviations: UV, ultraviolet; BrdUrd, 5-bromo-2′-deoxyuridine; dNTP, 2′-deoxyribonucleoside 5′-triphosphate; TTP, thymidine 5′-triphosphate; dCTP, 2′-deoxycytidine 5′-triphosphate; BuPdGTP, N^2-(*p-n*-butylphenyl)-2′-deoxyguanosine 5′-triphosphate; dThd, thymidine; dGTP, 2′-deoxyguanosine 5′-triphosphate; BrdUTP, 5-bromo-2′-deoxyuridine 5′-triphosphate; K_m, Michaelis constant; dNMP, 2′-deoxyribonucleoside 5′-monophosphate; dATP, 2′-deoxyadenosine 5′-triphosphate; nt, nucleotides; EDTA, ethylenediaminetetraacetic acid; ddTTP, 2′,3′-dideoxythymidine 5′-triphosphate.

[2] In an early study (Regan & Setlow, 1974), it was found that DNA damaging agents could be categorized on the basis of the size of repair patches induced. UV and certain chemical agents to which xeroderma pigmentosum (XP) cells were sensitive elicited patches on the order of 100 nucleotides, while ionizing radiation and simple alkylating agents, after which treatment XP cells were competent for repair, induced smaller patches estimated at 3–4 nuclelotides. More recent studies (Smith, 1987; Dresler, 1985) have refined the size of long repair patches to 20–25 nucleotides.

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Table I: Effect on DNA Damage-Induced [^{32}P]dNMP Incorporation of Omitting the Three Unlabeled dNTPs from the Permeable Cell Reaction Mixture[a]

damaging agent	α-^{32}P-labeled dNTP	apparent K_m for dNTP[b] (μM)	incorporation in absence of unlabeled dNTPs (% of control)	aphidicolin-resistant incorporation (% of control)	
				unlabeled dNTPs present	unlabeled dNTPs absent
bleomycin	TTP	0.29	53	63	95
bleomycin	dCTP	0.21	33	39	85
bleomycin	dGTP	0.24	26	20	ND[e]
UV	dCTP	0.11[c]	16[d]		

[a] Confluent AG1518 fibroblasts, prelabeled with [^{14}C]dThd, were permeabilized and incubated on ice with 200 μg/mL bleomycin as indicated for 30 min. After two washes in permeabilization buffer, cells were added to a reaction mixture as described under Experimental Procedures with the indicated α-^{32}P-labeled dNTP. [b] Repair synthesis was determined in duplicate over a range of concentrations of the labeled dNTP, with the three unlabeled dNTPs held constant at 100 μM. Apparent K_m was determined by fitting to the data a curve described by the Michaelis–Menten equation using the multiple regression "fit function" procedure of the RS/1 software package (Bolt, Beranek, and Newman). Determination of apparent K_m was significant at $p < 0.01$ in each case. [c] From Dresler et al. (1988b). [d] From Dresler et al. (1982). [e] ND, not determined.

experiments described, cells were harvested from glass roller bottles and permeabilized on ice (Dresler et al., 1982). To damage cells, bleomycin was added to permeabilization buffer to the final concentration indicated (an equivalent volume of distilled H_2O was added to permeabilization buffer of undamaged controls), and cells were incubated in this damaging solution for 30 min on ice. Cells were then collected by centrifugation and washed twice with 5 mL of permeabilization buffer to remove bleomycin prior to use in repair synthesis reactions.

Measurement of DNA Repair Synthesis in Permeable Cells. Repair synthesis was assayed in duplicate reaction mixtures containing 40 mM Tris (pH 7.6 at 37 °C), 8 mM $MgCl_2$, 15 mM KCl, 5 mM ATP, 167 mM sucrose, 2 mM dithiothreitol, 0.67 mM EDTA, and, as indicated, 1.5 μM of each dNTP, one of which was α-^{32}P-labeled (Dresler et al., 1982). In experiments utilizing aphidicolin or BuPdGTP, the competitor nucleotide (dCTP or dGTP, respectively) was 0.3 μM. Bleomycin-induced repair synthesis, which is the difference in specific incorporation (^{32}P/^{14}C) between corresponding damaged and undamaged samples, is linear with time for at least 30 min when [^{32}P]TTP, [^{32}P]dCTP, or [^{32}P]dGTP is used (data not shown). Therefore, except as noted, repair synthesis reactions were routinely terminated and processed by the glass fiber filter method described in Dresler et al., (1982) after 15 or 20 min at 37 °C during the linear portion of the incorporation reaction.

Measurement of Repair Patch Ligation Using Exonuclease III. The technique used to probe for free 3′-OH termini after bleomycin-induced repair synthesis in permeable cells has been described previously (Hunting et al., 1985a,b). Virtually all nucleotides in unligated repair patches are rendered acid soluble by this treatment (Hunting et al., 1985a).

Measurement of Repair Patch Size by BrdUrd-Induced Density Shift. The method described in detail by Dresler (1985) was employed with the following modification. Sonicated DNA fragment size was determined by electrophoresis in 2.0% alkaline agarose rather than formamide–polyacrylamide. Number-average length determined by this technique was reproducible (130 ± 5 nucleotides, $n = 6$) and in agreement with values determined by the previous method (Dresler, 1985).

Results and Discussion

Characterization of Bleomycin-Induced DNA Repair Synthesis in Permeable Human Fibroblasts. To examine repair synthesis induced by bleomycin, permeable cells were incubated with the drug for 30 min on ice, washed twice, placed in a repair synthesis reaction mixture, and incubated at 37 °C (Dresler et al., 1982). When TTP in the reaction

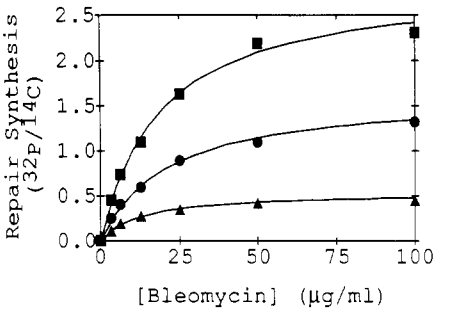

FIGURE 1: Dose dependence of bleomycin-induced repair synthesis using three different [α-^{32}P]dNTPs. Confluent AG1518 fibroblasts, prelabeled with [^{14}C]dThd, were permeabilized and damaged on ice with the indicated concentration of bleomycin for 30 min. After two washes with permeabilization buffer, cells were added to a repair synthesis reaction mixture as described under Experimental Procedures containing 1.5 μM of each dNTP and which differed only in [α-^{32}P]dNTP as follows: (■) [^{32}P]TTP; (●) [^{32}P]dCTP; (▲) [^{32}P]dGTP.

mixture was replaced by BrdUTP and permeable cell DNA isolated and analyzed by isopycnic centrifugation in alkaline CsCl (Dresler et al., 1982), nucleotides incorporated in response to bleomycin were found exclusively in DNA of parental density (data not shown), as expected for DNA repair synthesis. Nucleotide incorporation displayed a saturable dependence on dose of bleomycin (Figure 1). Surprisingly, however, the rate of nucleotide incorporation at a given dose of bleomycin depended on which α-^{32}P-labeled nucleotide was used to monitor the reaction (Figure 1). Overall, the rates of TMP and dCMP incorporation were approximately 5-fold and 3-fold greater, respectively, than the rate of dGMP incorporation. Since repair synthesis was linear with time for each of these three dNTPs at a saturating dose of bleomycin, time-dependent changes in reaction rates appear not to account for the observed differences. Likewise, previous work suggests the absence of significant levels of endogenous TTP, dCTP, and dGTP in the permeable cells (Dresler et al., 1988b); therefore, it is unlikely that differences in reaction rates are due to differences in endogenous dNTP concentrations. Finally, for the experiments shown in Figure 1, all dNTPs were present at approximately 5 times their respective K_m values (Table I), implying that a maximal rate of incorporation was measured in each case.

The data in Figure 1 indicate that the overall nucleotide composition of repair patches synthesized by human fibroblasts following bleomycin damage (TMP:dCMP:dGMP ~ 5:3:1) differs markedly from the overall nucleotide composition of human DNA (TMP:dCMP:dGMP = 3:2:2; Chargaff & Lipshitz, 1953). In this regard, bleomycin-induced repair synthesis is quite different from UV-induced repair synthesis, which produces repair patches with a nucleotide composition

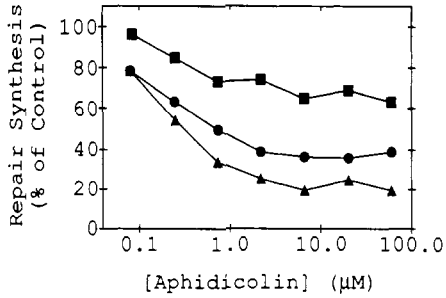

FIGURE 2: Effect of aphidicolin on bleomycin-induced repair synthesis monitored with three different [^{32}P]dNTPs. Confluent AG1518 fibroblasts, prelabeled with [^{14}C]dThd, were permeabilized and damaged on ice for 30 min with 200 μg/mL bleomycin. After two washes in permeabilization buffer, cells were added to a repair synthesis reaction mixture containing the indicated concentration of aphidicolin and labeled as follows: (■) [^{32}P]TTP; (●) [^{32}P]dCTP; (▲) [^{32}P]dGTP.

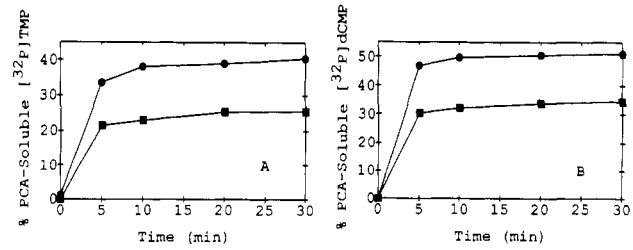

FIGURE 3: Exonuclease III sensitivity of aphidicolin-resistant repair patches containing only [^{32}P]TMP or [^{32}P]dCMP. Confluent AG1518 fibroblasts, prelabeled with [^{14}C]dThd, were damaged on ice for 30 min with 200 μg/mL bleomycin. After two washes in permeabilization buffer, cells were added to a repair synthesis reaction mixture containing 1.5 μM [^{32}P]TTP and no other dNTPs (A) or 1.5 μM [^{32}P]dCTP and no other dNTPs (B). DNA isolated from cells was incubated with exonuclease III for the indicated times as described under Experimental Procedures, with fresh enzyme added after 15 min. The fractional solubilization of ^{32}P label is shown. In all cases, less than 5% of the ^{14}C prelabel was solubilized by exonuclease III after 30 min (not shown). (●) 15-min pulse only; (■) 15-min pulse followed by 15-min chase with 100-fold excess of unlabeled TTP (A) or dCTP (B).

similar to that of bulk DNA (Dresler, 1985). A possible explanation for the biased nucleotide incorporation seen after bleomycin damage is that a substantial fraction of the repair patches synthesized are very short (e.g., one nucleotide long). In such a situation, the overall nucleotide composition of the repair patches would be weighted heavily in favor of those nucleotides that are actually damaged. Bleomycin has been shown to attack pyrimidines in certain sequences preferentially in vitro (D'Andrea & Haseltine, 1978; Takeshita et al., 1978, 1981), in isolated chromatin (Murray & Martin, 1985a), and in intact cells (Murray & Martin, 1985b). Therefore, the relative preference seen in Figure 1 for incorporation of these three nucleotides into DNA repair patches may reflect the sequence specificity of the damaging agent, bleomycin.

If a substantial fraction of bleomycin-induced DNA repair patches are very short, one would predict that omission of the three unlabeled dNTPs from the repair synthesis reaction mixture would have little effect on incorporation of the remaining α-^{32}P-labeled dNTP. For example, if TMP were incorporated exclusively into repair patches one nucleotide long, then omitting dATP, dCTP, and dGTP from the repair synthesis reaction mixture would have no effect on TMP incorporation. If, on the other hand, TMP were incorporated exclusively into long repair patches, most TMP residues would be located 3′ to other dNMPs in the repair patch, and omission of the three unlabeled dNTPs would produce substantial reductions in TMP incorporation. The latter effect has been clearly documented in this permeable cell system for UV-induced DNA repair synthesis (Dresler et al., 1982), which yields a repair patch approximately 25 nucleotides long (Dresler, 1985). We therefore examined the effect of omission of unlabeled dNTPs on bleomycin-induced TMP, dCMP, and dGMP incorporation. As shown in Table I, repair synthesis in the absence of the three unlabeled dNTPs was reduced to 53%, 33%, and 26% of control, respectively. For comparison, we have included in Table I our previous data which show that omitting the three unlabeled dNTPs from the repair synthesis reaction mixture reduces UV-induced incorporation (a "long-patch" process) to 16% of control. These data suggest that a substantial portion of the dNMPs incorporated in response to bleomycin are contained in short repair patches.

Characterization of Aphidicolin-Sensitive and Aphidicolin-Resistant Components of Bleomycin-Induced DNA Repair Synthesis. To evaluate the possibility discussed previously (Grossman, 1981; Cleaver, 1981; Mosbaugh & Linn, 1983, 1984; Randahl et al., 1988) that short-patch repair synthesis is mediated by DNA polymerase β, we examined the sensitivity of bleomycin-induced repair synthesis to aphidicolin (Huberman, 1981; Lee et al., 1981), an inhibitor of nuclear DNA polymerases α and δ but not β. As seen in Figure 2, an aphidicolin-resistant component of repair synthesis was observed with each labeled precursor and amounted to approximately 60%, 40%, and 20% of total for TTP, dCTP, and dGTP, respectively. Note that for each of the α-^{32}P-labeled dNTPs the fraction of incorporation resistant to aphidicolin is similar to the fraction of repair synthesis remaining when the three unlabeled dNTPs are omitted from the reaction mixture (Table I). Furthermore, repair synthesis assayed in the absence of unlabeled dNTPs was almost completely resistant to aphidicolin; incorporation of TMP and dCMP was inhibited only 5% and 15%, respectively, by 60 μM aphidicolin under these conditions (Table I). These results are consistent with the hypothesis that an aphidicolin-resistant DNA polymerase is responsible for short DNA repair patch synthesis in response to bleomycin and, conversely, that an aphidicolin-sensitive polymerase is responsible for long-patch synthesis.

Extent of Ligation of Aphidicolin-Resistant Repair Patches Containing Only TMP or dCMP As Determined by Resistance to Exonuclease III. We have employed *Escherichia coli* exonuclease III, a 3′→5′ exonuclease, to determine whether the repair patches synthesized in the presence of only [^{32}P]TTP or [^{32}P]dCTP are completed and ligated (Hunting et al., 1985a,b). As shown in Figure 3A, after a 15-min pulse labeling and a 15-min chase period, 75% of the repair label incorporated in the presence of only [^{32}P]TTP is resistant to exonuclease III digestion. Similarly, 65% of dCMP incorporated in the absence of any other dNTP resisted exonuclease III digestion (Figure 3B). Since residual levels of endogenous dNTPs in the permeable cells are negligible (Dresler et al., 1988b), these ligated repair patches should contain only TMP or only dCMP. Because bleomycin shows no preference for damaging in tracts of poly(T) or poly(dC) (see references above), we conclude that complete repair patches as short as one nucleotide long are synthesized by an aphidicolin-resistant polymerase.

Determination of Repair Patch Size of Aphidicolin-Sensitive and -Resistant Repair Synthesis Systems by Bromodeoxyuridine-Induced Density Shift. To test directly the hypothesis that aphidicolin-sensitive and -resistant polymerase activities synthesize long and short repair patches, respectively, in response to bleomycin, we sought to identify conditions appropriate for patch size measurement in which repair synthesis was either completely sensitive or resistant to aphidicolin. Repair synthesis monitored with[^{32}P]dGTP displays the

Table II: Systems for Examining Aphidicolin-Sensitive and Aphidicolin-Resistant DNA Repair Synthesis in Bleomycin-Damaged Permeable Cells[a]

system	bleomycin dose (μg/mL)	α-^{32}P-labeled dNTP	aphidicolin present (60 μM)	aphidicolin sensitivity (%)	repair patch ligation[b] (%)		repair patch length[c] (nt)	
					15-min pulse	pulse + 15-min chase	15-min pulse	pulse + 15-min chase
I	5	dGTP	-	94	80	90	18	19
II	200	dCTP	+	(0)	70	85	4	4

[a] Confluent AG1518 fibroblasts, prelabeled with [^{14}C]dThd, were permeabilized and damaged on ice with the indicated concentration of bleomycin. After two washes in permeabilization buffer, cells were added to a reaction mixture containing α-^{32}P-labeled dNTP as indicated. [b] Determined as in Figure 3. [c] Determined as in the figure legend to Figure 5. Values are based on the difference in mean density between repair-labeled (^{32}P) DNA and bulk (^{14}C) DNA, assuming a density shift of 0.12 g/cm^3 for DNA fully substituted with BrdUrd [see text and Dresler (1985)].

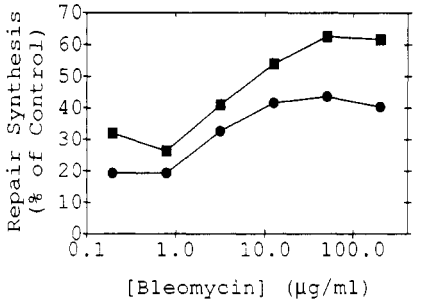

FIGURE 4: Variation in aphidicolin sensitivity with bleomycin dose. Confluent AG1518 fibroblasts, prelabeled with [^{14}C]dThd, were permeabilized and damaged on ice with the indicated concentration of bleomycin for 30 min. After two washes in permeabilization buffer, cells were added to repair synthesis reaction mixtures containing (■) [^{32}P]TTP or (●) [^{32}P]dCTP and a full complement of unlabeled dNTPs, in the absence or presence of 60 μM aphidicolin. Repair synthesis in the presence of aphidicolin is expressed as a percentage of that obtained in the absence of aphidicolin.

greatest aphidicolin sensitivity (Figure 2). Furthermore, regardless of which [^{32}P]dNTP was used to monitor the reaction, the aphidicolin sensitivity of repair synthesis increased at lower doses of bleomycin (shown for dCTP and TTP in Figure 4). Thus, by using 5 μg/mL bleomycin and [^{32}P]dGTP as the labeled nucleotide (system I, Table II), we obtained repair synthesis that was 94% sensitive to aphidicolin. As shown in Table II, dGMP incorporated by aphidicolin-sensitive system I was almost exclusively in ligated patches, a condition essential for accurate patch size determination.

As indicated above, aphidicolin-resistant repair synthesis is elicited by damaging with 200 μg/mL bleomycin, using [^{32}P]TTP as the labeled nucleotide, and omitting unlabeled dNTPs from the reaction mixture (Table I). However, utilization of this system for patch size measurement would require an assumption about the frequency of incorporation of the density label BrdUMP vs the radioactive label [^{32}P]TMP. Such assumptions have led to considerable uncertainty in previous determinations of DNA repair patch size [e.g., Th'ng & Walker (1985)]. Moreover, we wished to avoid the possibility that omission of unlabeled dNTPs might also eliminate a population of aphidicolin-resistant long patches. Therefore, as an aphidicolin-resistant repair synthesis system suitable for patch size measurement, we employed [^{32}P]dCTP as the labeled precursor in the presence of a saturating dose of aphidicolin (system II, Table II). As shown in Table II, repair patch ligation was quite efficient under these conditions, indicating that the use of aphidicolin did not prevent completion and ligation of patches.

The BrdUrd-induced density shift technique (Edenberg & Hanawalt, 1972) was used to determine the size of repair patches synthesized by the aphidicolin-sensitive and aphidicolin-resistant repair systems characterized in Table II. The aphidicolin-sensitive repair synthesis system (system I, Table II) yielded a mean density shift of 1.72 (±0.03) × 10^{-2} g/cm^3 (Figure 5A), consistent with a repair patch of roughly 20 nucleotides (Table II). By contrast, sheared fragments containing repair patches synthesized by aphidicolin-resistant system II shifted only slightly from the density of parental DNA: 0.38 (±0.01) × 10^{-2} g/cm^3 (Figure 5B), implying a patch length of approximately 4 nucleotides (Table II). [In the absence of aphidicolin, an intermediate shift by [^{32}P]-dCMP-containing repair patches is observed (not shown),

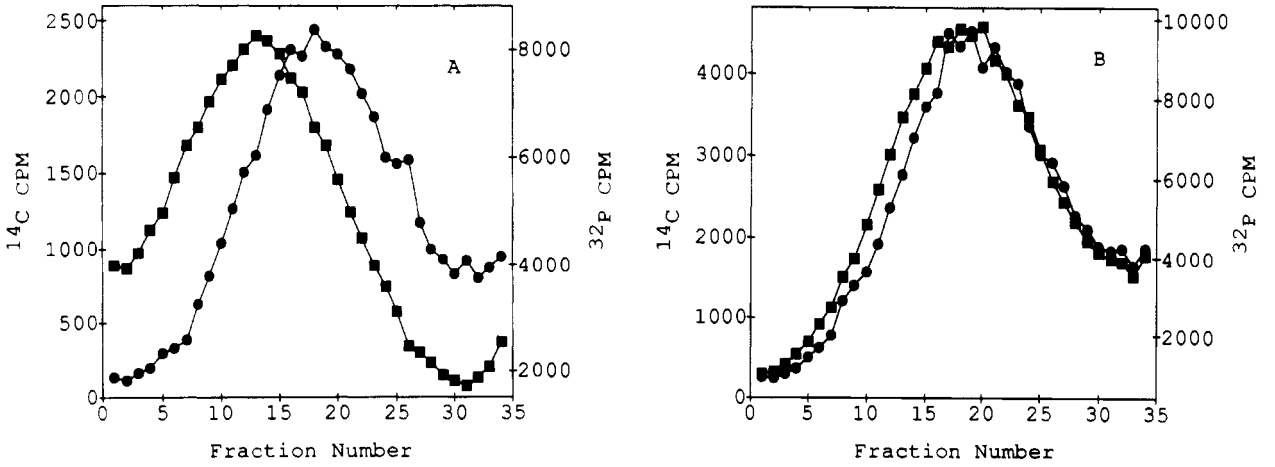

FIGURE 5: Patch size analysis of aphidicolin-sensitive (A) and -resistant (B) bleomycin-induced repair synthesis by BrdUrd-induced density shift. Confluent AG1518 fibroblasts, prelabeled with [^{14}C]dThd, were permeabilized and damaged on ice with 5 μg/mL (A) or 200 μg/mL (B) bleomycin. After two washes in permeabilization buffer, cells were added to repair synthesis reaction mixtures labeled with [^{32}P]dGTP (A) or [^{32}P]dCTP (B), in which TTP was replaced with BrdUTP, and incubated for 15 min at 37 °C. In (B), 60 μM aphidicolin was present; therefore (A) and (B) represent conditions under which repair synthesis is completely sensitive or resistant to aphidicolin, respectively (systems I and II, Table II). DNA was isolated from cells, sonicated to a number average of 130 nucleotides, and subjected to equilibrium density centrifugation in alkaline CsCl as described under Experimental Procedures. Density decreases from left to right. (■) ^{32}P label; (●) ^{14}C prelabel.

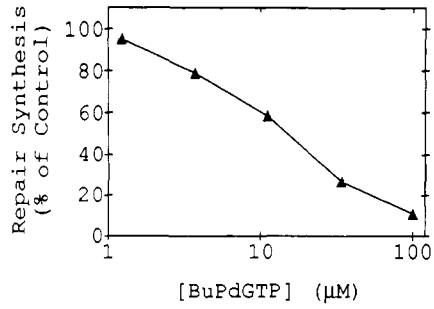

FIGURE 6: Inhibition of aphidicolin-sensitive bleomycin-induced repair synthesis by BuPdGTP. Confluent AG1518 fibroblasts, prelabeled with [^{14}C]dThd, were permeabilized and damaged on ice for 30 min with 5 μg/mL bleomycin. After two washes in permeabilization buffer, cells were added to reaction mixtures labeled with [^{32}P]dGTP and incubated at 37 °C in the presence of the indicated concentration of BuPdGTP.

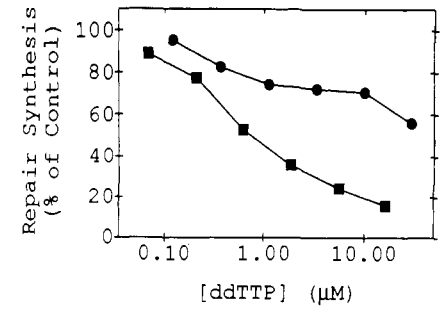

FIGURE 7: Inhibition of aphidicolin-sensitive and -resistant repair synthesis by ddTTP. Confluent AG1518 fibroblasts, prelabeled with [^{14}C]dThd, were permeabilized and damaged on ice for 30 min with (●) 5 μg/mL or (■) 200 μg/mL bleomycin. After two washes in permeabilization buffer, cells were added to reaction mixtures containing (●) [^{32}P]dGTP and the three unlabeled dNTPs or (■) [^{32}P]TTP only. In both cases, TTP was 1 μM. ddTTP was varied as indicated.

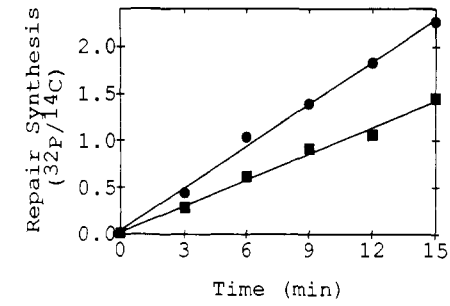

FIGURE 8: Inhibition of aphidicolin-resistant bleomycin-induced repair synthesis by NEM. Confluent AG1518 fibroblasts, prelabeled with [^{14}C]dThd, were permeabilized and damaged on ice for 30 min with 200 μg/mL bleomycin. After two washes in permeabilization buffer, cells were added to reaction mixtures containing 1.5 μM [^{32}P]TTP only, in the (●) absence or (■) presence of 8 mM NEM.

consistent with significant components of both aphidicolin-sensitive and -resistant repair synthesis under these conditions (Figure 2).] Finally, note that measurement of BrdUrd-induced density shift after a 15-min chase yielded essentially identical results for both systems (Table II), supporting the conclusion based on exonuclease III studies that these patches are full-length.

Calculation of patch length by BrdUrd-induced density shift assumes that the distribution of T residues in repair patches is identical with their genomic distribution (Dresler, 1985). In the case of UV-induced repair patches in permeable cells, which we have previously determined as about 25 nucleotides using the same procedure (Dresler, 1985), this assumption is met reasonably well. Our data indicate, however, that T residues are significantly overrepresented in bleomycin-induced repair patches (Figure 1 and unpublished data). Therefore, our patch size estimates probably represent upper limits in both cases.

Identification of Aphidicolin-Sensitive and -Resistant DNA Polymerase Activities Responsible for Bleomycin-Induced Repair Synthesis. To characterize further the aphidicolin-sensitive repair synthesis activity seen following bleomycin damage (system I, Table II), we used the nucleotide analogue BuPdGTP, which inhibits polymerase α at nanomolar concentrations and polymerase δ, the other aphidicolin-sensitive mammalian DNA polymerase, at high micromolar concentrations (Byrnes, 1985; Lee et al., 1985). Inhibition of aphidicolin-sensitive repair synthesis (system I, Table II) by BuPdGTP (Figure 6) is consistent with that reported for purified polymerase δ (Byrnes, 1985; Lee et al., 1985) and identical with that seen previously for UV-induced repair synthesis (Dresler & Frattini, 1986).

A second nucleotide analogue that has been used to distinguish the two aphidicolin-sensitive polymerases in vitro (Wahl et al., 1986; Syvaoja & Linn, 1989) and in permeable cells (Dresler & Kimbro, 1987) is ddTTP. Whereas polymerase α is unaffected by ddTTP:TTP ratios of up to 40 (Wahl et al., 1986) or 50 (Waqar et al., 1978), polymerase δ displays sensitivity intermediate between that of polymerase α and the aphidicolin-resistant polymerases, β and γ, which are inhibited 50% at a ddTTP:TTP ratio of ~1 (Fry & Loeb, 1986). As shown in Figure 7, aphidicolin-sensitive repair synthesis is inhibited significantly at a ddTTP:TTP ratio of 1 and ~50% at a ddTTP:TTP ratio of 30. Studies with both BuPdGTP and ddTTP, therefore, suggest that DNA polymerase δ mediates long-patch repair synthesis in response to bleomycin.

As noted above, both aphidicolin-resistant polymerases, β and γ, are inhibited 50% by ddTTP:TTP ratios on the order of 1. As expected, aphidicolin-resistant repair synthesis displays comparable sensitivity to ddTTP (Figure 7). Note also that short-patch repair is more sensitive to ddTTP than long-patch repair (Figure 7). This finding argues against the formal possibility that long-patch synthesis is preferentially inhibited over short-patch synthesis, despite mediation of both processes by the same polymerase. If such a mechanism were operative, the resistance of short-patch repair to aphidicolin might only be apparent.

Although polymerase β is the major, if not exclusive, aphidicolin-resistant nuclear DNA polymerase, small amounts of extramitochondrial polymerase γ, which is also aphidicolin resistant, have been detected by some investigators (Hubscher et al., 1977; Bertazzoni et al., 1977). To distinguish β- from γ-polymerase activity, we used *N*-ethylmaleimide (NEM), which, at millimolar concentrations, inhibits polymerase γ potently but inhibits polymerase β only weakly (Fry & Loeb, 1986). As shown in Figure 8, aphidicolin-resistant repair synthesis was inhibited 40% by 8 mM NEM, a concentration that completely inhibits polymerase γ (Fry & Loeb, 1986). Since we have determined the effect of NEM on the initial velocity of repair synthesis (Figure 8), we can exclude the possibility that this inhibition is artifactually low due to slow onset in the permeable cells. The slight inhibition of aphidicolin-resistant repair synthesis seen in the presence of 8 mM NEM could be interpreted as due to a minor (i.e., ~40%) role for extramitochondrial polymerase γ in short-patch repair. However, preparations of β-polymerase have been found to be partially sensitive to such high concentrations of NEM [reviewed in Fry and Loeb (1986)]. Some inhibition of β-polymerase by NEM is therefore expected and probably accounts for our observations. Studies using BuPdGTP support this interpretation. Whereas polymerase γ is completely re-

sistant to BuPdGTP (Khan et al., 1984), inhibition of aphidicolin-resistant repair synthesis by BuPdGTP is essentially identical with that reported for purified DNA polymerase β (DiGiuseppe et al., 1989).

Conclusion. As pointed out in a recent review of DNA repair mechanisms (Dresler, 1989), the biochemical basis for repair patch size heterogeneity in human cells has not previously been defined. The data presented here are consistent wiht the proposal made nearly a decade ago (Grossman, 1981; Cleaver, 1981) that long-patch and short-patch repair are mediated by distinct DNA polymerases. Further study is required to elucidate the steps in excision repair preceding synthesis by either the long-patch or short-patch polymerase.

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