Nucleosome Rearrangement in Human Cells following Short Patch Repair of DNA Damaged by Bleomycin†

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ABSTRACT: We have examined the structure of newly repaired regions of chromatin in intact and permeabilized human cells following exposure to bleomycin (BLM). The average repair patch size (in permeabilized cells) was six to nine bases, following doses of 1–25 μg/mL BLM, and >80% of the total repair synthesis was resistant to aphidicolin. In both intact and permeabilized cells, nascent repair patches were initially very sensitive to staphylococcal nuclease, analogous to repair induced by "long patch" agents, and are nearly absent from isolated nucleosome core DNA. Unlike long patch repair, however, the loss of nuclease sensitivity during subsequent chase periods was very slow in intact cells, or in permeabilized cells treated with a low dose of BLM (1 μg/mL), and was abolished by treatment with hydroxyurea (HU) or aphidicolin (APC). The rate of repair patch ligation did not correlate with this slow rate of chromatin rearrangement since >95% of the patches were ligated within 6 h after incorporation (even in the presence of HU or APC). In permeabilized cells, repair patches induced by either 5 or 25 μg/mL BLM, where significant levels of strand breaks occur in compact regions of chromatin, lost the enhanced nuclease sensitivity at a rate similar to that observed following long patch repair. This rapid rate of rearrangement was not affected by APC. These results indicate that short patch repair in linker regions of nucleosomes, and/or "open" regions of chromatin, involves much less nucleosome rearrangement than long patch repair or short patch repair in condensed chromatin domains.

Excision repair of DNA damage is a "cut and patch" mechanism, involving recognition of the damaged base(s), excision of these and flanking bases, resynthesis (to fill the gap), and ligation (Hanawalt et al., 1979). This type of repair has been observed to occur in two distinct forms (Regan & Setlow, 1974). "Short patch" repair, as induced by ionizing radiation, occurs via removal and insertion of only a few nucleotides around the damaged base(s) (Painter & Young, 1971; Regan & Setlow, 1974; DiGiuseppe & Dresler, 1989). On the other hand, repair of DNA damaged by UV radiation and bulky chemicals involves removal and insertion of 20–25 bases (Th'ng & Walker, 1985; Dresler, 1985; Smith, 1987; DiGiuseppe & Dresler, 1989) and is known as "long patch" repair.

A number of studies have shown that long patch repair is associated with rearrangements of chromatin structure [reviewed by Smerdon (1989)]. Initially, the newly repaired regions of DNA are rapidly digested by both staphylococcal nuclease and DNase I and lack a native nucleosome structure. Subsequently, rearrangements of chromatin structure occur in these regions in two distinct phases. During the early (fast) phase, a large fraction of the nascent repair patches rapidly ($t_{1/2} \simeq 20$ min) becomes associated with native (or near native) nucleosome structures (Smerdon & Lieberman, 1978, 1980). This phase is followed by a prolonged slow phase, which lasts many hours, and involves the repositioning of nucleosomes along the repaired DNA (Nissen et al., 1986). This phenomenon has been observed for all long patch agents studied, including agents that preferentially bind linker regions of nucleosomes (Smerdon, 1989).

To date, we know very little about the structural rearrangements associated with short patch repair. Clearly, the smaller amount of "DNA processing" by this repair pathway might involve different structural transitions in chromatin than the more extensive long patch repair. Maturation of repair patches induced by the small alkylating agents methyl methanesulfonate and methylnitrosourea involves structural rearrangements analogous to those caused by UV radiation and bulky chemicals (Sidik & Smerdon, 1984). Although such agents were initially thought to induce short patch repair (Regan & Setlow, 1974), more recent evidence suggests that a significant level of the repair induced is of the long patch type (Snyder & Regan, 1982; Th'ng & Walker, 1985). Since it is not clear what the relative proportion of each repair component induced by these agents is, it is not known if the nuclease digestion studies (Sidik & Smerdon, 1984) reflected only the long patch component. Therefore, we have addressed the question of whether chromatin rearrangements occur during short patch repair by examining the nuclease digestion properties of repair patches inserted into chromatin following DNA damage by bleomycin (BLM).[1] These basic glycopeptide-derived antibiotics induce direct strand breaks in DNA in vitro, as well as alkali labile sites [reviewed by Stubbe & Kozarich (1987)]. These breaks cannot be directly ligated, since they contain a phosphoglycolate moiety at the 3′-terminus (Giloni et al., 1981; Uesugi et al., 1984). A possible scheme for the repair of such breaks is the removal of the modified base and adjacent bases by a 3′ → 5′ exonuclease, followed by repair synthesis (to fill the short gap) and ligation (Mosbaugh & Linn, 1984). This is in contrast to the repair of adducts that cause local distortions in the DNA helix, which appear to be recognized by a large multicomponent complex with associated endonuclease activity (Friedberg, 1985; Sancar & Sancar, 1988). Finally, DiGiuseppe & Dresler (1989) have

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[1] Abbreviations: UV, ultraviolet; LPC, L-α-lysophosphatidylcholine; HU, hydroxyurea; APC, aphidicolin; dThd, thymidine; BLM, bleomycin; BrdUrd, 5-bromo-2′-deoxyuridine; dCTP, 2′-deoxycytidine 5′-triphosphate; dTTP, thymidine 5′-triphosphate; dGTP, 2′-deoxyguanosine 5′-triphosphate; BrdUTP, 5-bromo-2′-deoxyuridine 5′-triphosphate; dNTP, 2′-deoxyribonucleoside 5′-triphosphate; PBS, phosphate-buffered saline.

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recently performed direct measurements on the repair patch size following BLM damage to human cells and observed that a substantial portion of the patches inserted are of the short patch type.

We have analyzed the structural features of nascent and mature repair patches incorporated following BLM-induced DNA damage in human diploid fibroblasts. These studies were performed on intact cells, where very low levels of BLM-induced strand breaks occur, and cells permeabilized with lysophosphatidylcholine (LPC), where significantly higher levels of strand breaks occur (Sidik & Smerdon, 1990). Our results indicate that short patch repair of low levels of BLM-induced strand breaks involve significantly less nucleosome rearrangement than repair of DNA damage by UV radiation, "UV-mimetic" chemicals, and small alkylating agents.

Materials and Methods

Chemicals. Bleomycin (Blenoxane) was a gift from Bristol Laboratories (Syracuse, NY) through the courtesy of Dr. William T. Bradner, Linda Sanders, and Daniel T. Elliott. Hydroxyurea and L-α-lysophosphatidylcholine were obtained from Sigma Chemical Co. (St. Louis, MO). Aphidicolin (APC) was obtained from the Natural Products Branch, Division of Cancer Treatment, NCI, or was purchased from Sigma.

Cell Culture. Human diploid fibroblasts (Strain AG1518, Human Genetic Mutant Cell Repository) were grown in culture as described in Smerdon et al. (1982). In most experiments, cells were split 1:3 and prelabeled for 1 week with 5–20 nCi/mL [^{14}C]dThd (50 mCi/mmol; New England Nuclear). The medium was then replaced with fresh medium and the cells were allowed to grow to confluence (2 or 3 weeks). When cells were grown for 3 weeks, the medium was changed again after 2 weeks. The cells were determined to be mycoplasma free by the Gen-Probe test (Gen-Probe).

Labeling of Intact Cells during Repair Synthesis. Confluent AG1518 cells, prelabeled with [^{14}C]dThd, were treated with 2 mM hydroxyurea (HU) or 65 μM APC 1 h prior to BLM treatment. The cells were then damaged by adding 100 μg/mL BLM (final concentration) and 10–20 μCi/mL [^{3}H]dThd (50–80 Ci/mmol, New England Nuclear) followed by incubation at 37 °C for 30 min. BLM was dissolved in 10 mM PIPES buffer, pH 6.8. Control cells were treated identically except they received buffer only. Immediately following the labeling period, the cells were either harvested immediately or the medium was replaced with conditioned medium containing 50 μM unlabeled dThd and the cells were incubated for various chase times prior to harvest. In some cases, the chase medium contained 2 mM HU or 65 μM APC.

In some experiments, confluent cells, prelabeled with [^{14}C]dThd, were incubated with 50 μM bromodeoxyuridine (BrdUrd) for 1.5 h. The cells were then incubated with 100 μg/mL BLM in the presence of BrdUrd or irradiated with 12 J/m^2 UV light (2 W/m^2; predominantly 254 nm). The medium was replaced with condition medium containing 50 μM BrdUrd and 10 μCi/mL [^{3}H]dThd, and the cells were incubated at 37 °C for various times.

For fully BrdUrd-substituted DNA, AG1518 cells were split 1:3 and grown in the presence of [^{14}C]dThd for 48 h. The medium was replaced with fresh medium and the cells were allowed to grow for 5 days. The cells were pretreated with 50 μM BrdUrd for 1.5 h and then incubated in both BrdUrd and 10 μCi/mL [^{3}H]dThd for 5 h prior to harvest.

Preparation and Labeling of Permeable Cells during Repair. Confluent cells were permeabilized with LPC as previously described (Lorenz et al., 1988). Briefly, confluent cells were rinsed twice with ice cold PBS (16 mM phosphate buffer, pH 7.2, 135 mM NaCl, and 5 mM KCl) containing 1 mM $CaCl_2$. An 80 μg/mL solution of LPC (dissolved in PBS and 1 mM $CaCl_2$) was added to the cells and the cells were kept on ice for 2 min. The solution was carefully removed and replaced with repair mix (35 mM HEPES buffer, pH 7.4, 50 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$, 7.5 mM KH_2PO_4, 1 mM $CaCl_2$, 5 mM ATP, 3 μM dATP, 3 μM dGTP, and 3 μM dCTP), containing various concentrations of BLM (held at 37 °C). For nuclease digestion experiments, 10–20 μCi/mL [^{3}H]dThd was included with the repair mix to label repair patches. [Labeled dThd could be used in this case because the majority of cells reseal their membranes within 15 min (Lorenz et al., 1988) and can replenish their dTTP pools with [^{3}H]dThd.] The cells were incubated at 37 °C for 30 min. Immediately following the labeling period, the cells were either harvested or the medium was replaced with conditioned medium containing 50 μM unlabeled dThd and the cells incubated for varying times prior to harvest. In some experiments, the cells were treated with 65 μM APC prior to permeabilization and during the repair labeling step.

Measurement of Patch Size. Patch size measurements were performed by using the BrdUrd density-shift method (Smith et al., 1981). In most experiments, permeabilized cells were treated with BLM as above, except the repair mix contained 3 μM BrdUTP (instead of dThd) and 20 μCi/mL [^{3}H]dCTP (18 Ci/mmol, ICN). In other experiments, confluent cells were irradiated with 12 J/m^2 UV light, permeabilized with LPC, and labeled during repair with repair mix containing 3 μM BrdUTP and 40 μCi/mL [^{35}S]dCTP (1800 Ci/mmol, New England Nuclear). In this case, cold dCTP was added to bring the concentration of dCTP up to the concentration present when [^{3}H]dCTP was used (i.e., when cells were damaged with BLM). In each of these experiments, cells were repair labeled for 3 h.

Cells were harvested immediately following the repair labeling period and the DNA was isolated essentially as described in Smerdon et al. (1978). At the same time, confluent cells, prelabeled with [^{14}C]dThd, were harvested and the DNA was isolated along with the repair labeled samples. For comparison with normal density DNA, aliquots of DNA from BLM-treated cells or from UV-irradiated cells (labeled during repair with [^{3}H]dCTP) were added to aliquots of the DNA prelabeled with [^{14}C]dThd. For comparison of DNA from BLM-treated cells and UV-irradiated cells on the same CsCl gradient, we combined DNA from BLM-treated cells with DNA from the UV-irradiated cells (labeled during repair with [^{35}S]dCTP) prior to sonication and CsCl gradient centrifugation. DNA samples from intact cells were first isolated as large fragments on neutral CsCl gradients (pooled normal density fractions) to remove DNA labeled by replicative synthesis (Smith et al., 1981). These DNA samples, and DNA from permeabilized cells, were placed in ice water and sonicated by exposing the samples to 10, 30-s bursts from a Tekmar Sonicator using a microtip and a power setting of 4, as described by Dresler (1985).

To calculate the average size of the sonicated DNA, a fraction of the sonicated samples was electrophoresed on a 2.8% agarose gel, stained with ethidium bromide, and photographed through a red filter with Polaroid positive/negative film (type 55) (Smerdon et al., 1978). The negatives were scanned with a laser densitometer (LKB Model 2222). The scans were analyzed on an IBM PS/2 (Model 60) computer equipped with a digitizing tablet (Jandel Scientific) and corrected for the nonlinear film response to exposure (Pul-

leyblank et al., 1977; Willis & Holmquist, 1985). The absorbance scans were transformed by usig the equation $10^{(A/\gamma)} - 1$ where A is the absorbance, above background, and γ is the film contrast, which was taken to be 0.65 for Polaroid type 55 film (Polaroid specifications). The corrected values were then plotted against the log of the corresponding DNA length (log L). To obtain the values for log L, we first plotted the log values of *Hpa*II fragments of pBR322 (run on the same gel) against the distance migrated by these fragments. The conversion of log L was then made by using the equation

$$\log L = a - bD$$

where a and b are the y intercept and slope, respectively, obtained from the migration of the marker fragments, and D is the distance migrated by the sonicated fragments. The median length of the DNA fragments (L_{med}) was determined from the value of log L corresponding to half the area of the peak in the $10^{(A/\gamma)} - 1$ versus log L plot using the Sigma Scan program (Jandel Scientific) written for this digitizing system.

Another portion of the sonicated sample was banded in an alkaline CsCl gradient. The samples were added to a CsCl solution (1.74 g/cm^3) containing 0.1 M K_2HPO_4, pH 12.5, and were centrifuged at 40 000 rpm at 20 °C for 40 h, in a Beckman VTi65 rotor and a Beckman L8-70 ultracentrifuge. The gradients were fractionated and assayed for radioactivity as previously described (Smerdon et al., 1979). The refractive index measurements of individual fractions were determined on a Bausch and Lomb refractometer. The DNA isolated from cells labeled with BrdUrd during replicative synthesis (fully substituted) was sheared and directly banded in an alkaline CsCl gradient (1.79 g/cm^3) (i.e., with no sonication or "prebanding" in a neutral CsCl gradient). These samples were centrifuged in a VTi65 rotor at 50 000 rpm for 20 h. (The rotor speed was increased in this case to allow banding of both peaks on the same gradient.) The separation of the heavy- and light-density peaks (in fractions) was adjusted for the difference between the 40 000 and 50 000 rpm runs by multiplying this difference by the ratio of slopes obtained for the refractive index gradient in each case.

Nuclei Preparation and Nuclease Digestion. Nuclei were prepared as described previously (Smerdon et al., 1979) and suspended in B3 buffer (10 mM Tris, pH 7.8, 0.1 mM $CaCl_2$, and 0.25 M sucrose) by Dounce homogenization. Staphylococcal nuclease (14.9 units/μg, Worthington) digestion was performed according to Smerdon et al. (1978). The concentration of nuclease was adjusted to give an initial digestion rate of 1% of the total DNA rendered acid soluble per minute.

Exonuclease III (Bethesda Research Laboratory) digestion was also performed as described earlier (Smerdon, 1986). Briefly, nuclei were suspended in B3 buffer and incubated with 100 μg/mL proteinase K (E. Merck) at 37 °C overnight. The DNA was precipitated with ethanol and redissolved in B4 buffer (50 mM Tris, pH 7.8, 5 mM dithiothreitol, and 0.1 mM EDTA). Prior to the addition of exonuclease III, the solution was brought up to a final concentration of 1.2 mM $MgCl_2$. The DNA samples were then incubated with exonuclease III at a concentration of 0.5 unit/μg of DNA. For both staphylococcal nuclease and exonuclease III digestion, aliquots of the digestion mixture were removed and assayed for acid-soluble radioactivity at various times (Smerdon et al., 1978).

Autoradiography. Cells to be analyzed by autoradiography were grown on four-well glass tissue culture slides (Lab-Tek). The medium was changed after 1 week and the cells were allowed to grow to confluence. Confluent cells were incubated with 2 mM HU for 45–60 min prior to the addition of BLM. The cells were then incubated with 100 μg/mL BLM for 30 min. Following damage, the medium was replaced with conditioned medium containing 10 μCi/mL [^{3}H]dThd with or without 2 mM HU. Following the labeling period, cells were rinsed twice with ice-cold 0.15 M NaCl, fixed, coated with nuclear track emulsion (Kodak NTB2), exposed, and developed according to Cleaver and Thomas (1981). Silver grains were visualized and counted by using an Olympus phase-contrast microscope (model IMT).

Table I: Size of Repair Patches Incorporated during Repair Synthesis

damaging agent	av shift (in fractions)	av DNA size (bp)[b]	patch size (bases)[c]
UV (intact)[a]	3.0	290	22
UV (permeable)[a]	2.7	300	20
1 μg/mL BLM	1.4	250	9
5 μg/mL BLM	1.0	275	7
25 μg/mL BLM	1.0	250	6

[a] Cells were exposed to 12 J/m^2 UV light. Both the UV- and BLM-damaged cells were labeled for 3 h with [^{3}H]dCTP. [b] The average DNA size (median length) was calculated from agarose gels as described under Materials and Methods. [c] The average patch size ($\langle B \rangle$) was calculated from the expression (Smith et al., 1981): $\langle B \rangle = L_{med}(\Delta\rho/\Delta\rho_0)$ where L_{med} is the median length of sonicated DNA, $\Delta\rho$ is the separation (in fractions) of repair labeled DNA and parental density DNA, and $\Delta\rho_0$ is the separation (in fractions) of completely substituted DNA and parental density DNA. This latter difference ($\Delta\rho_0$) was 48 fractions after correction for the different rotor speeds used (Materials and Methods).

Gel Electrophoresis. Electrophoresis of staphylococcal nuclease resistant fragments on 2.8% agarose gels and determination of the radioactive profile were carried out as described previously (Smerdon et al., 1978).

Isopycnic Centrifugation for Determination of Replicative and Repair Synthesis. Preparation of BrdUrd-labeled DNA for determination of replicative and repair synthesis by CsCl gradient centrifugation was carried out as described previously (Smerdon et al., 1979, 1982). The DNA was banded in alkaline CsCl by using a Beckman L5-65 ultracentrifuge and a 70.1 Ti rotor at a speed of 45 000 rpm. The gradients were fractionated and the fractions assayed for radioactivity as described in Smerdon et al. (1982).

Results

Repair Patch Size Induced by BLM. We have measured the size of patches incorporated during excision repair of BLM-induced damage in human diploid fibroblasts using the BrdUrd density-shift technique (Smith et al., 1981). These measurements were performed on cells permeabilized with 80 μg/mL LPC in order to significantly increase the amount of DNA damage and repair synthesis in the majority of the cell population (Sidik & Smerdon, 1990). Under these conditions, the cells are *reversibly* permeabilized to small molecules and remain viable (Lorenz et al., 1988). As a test of our procedure using permeabilized cells, we first examined the patch size of DNA isolated from cells that were irradiated with 12 J/m^2 UV light. As indicated earlier, several laboratories have recently reported the patch size for UV-induced damage in human cells by using the BrdUrd density-shift method and obtain values of 20–25 bases (Th'ng & Walker, 1985; Dresler, 1985; Smith, 1987; DiGiuseppe & Dresler, 1989). In our studies, the UV-damaged DNA from permeabilized cells was repair labeled with [^{3}H]dCTP (in the presence of BrdUTP), combined with ^{14}C labeled (normal desity) DNA, sonicated, and banded in an alkaline CsCl gradient. The median positions of the ^{3}H- and ^{14}C-labeled DNA peaks (Walker & Th'ng, 1982) were shifted by 2.7 fractions (Table I). By also measuring the shift in density for totally BrdUrd-substituted

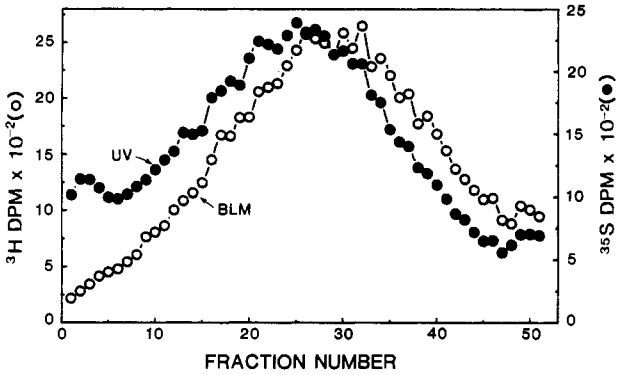

FIGURE 1: Alkaline CsCl density gradient profiles of repair-labeled DNA from permeable AG1518 cells that were either irradiated with 12 J/m^2 UV light or incubated with 5 μg/mL BLM. UV-irradiated, confluent cells were permeabilized with LPC and labeled with [^{35}S]dCTP and BrdUTP during repair (●). BLM-treated cells were incubated with repair mix containing [^{3}H]dCTP, BrdUTP, and 5 μg/mL BLM (○) following permeabilization. The DNA isolated from these cells was combined, sonicated, and centrifuged to equilibrium in an alkaline CsCl gradient at 40 000 rpm. The gradients were fractionated and the ^{3}H and ^{35}S DPM profiles were determined as previously described (Smerdon et al., 1982).

DNA (48 fractions) and the average size of the sonicated DNA fragments, the average size of the repair patches could be determined (Smith et al., 1981). As shown in Table I, this calculation yields an average patch size of 20 bases and is very close to the value obtained for intact cells (Table I). This result indicates that any dilution of density label that occurs during the latter stages of the repair incubation (i.e., after the cells reseal) has little effect on the average density of the fragment population.

In the case of BLM-induced repair in permeabilized cells, the shift in density between [^{3}H]dCTP repair-labeled DNA fragments and normal-density DNA fragments (^{14}C) was significantly less than observed for UV-induced repair (Table I). Average density shifts of 1.0–1.4 fractions were obtained for three different BLM concentrations (Table I). In this case, the calculated patch size ranged from nine to six bases for BLM concentrations of 1 to 25 μg/mL, respectively. As discussed in DiGiuseppe and Dresler (1989), these values are probably overestimates of the patch size due to the bias toward thymine residues in BLM-induced repair patches shown by these authors.

Since the above calculations represent comparisons between different sets of DNA populations, having small differences in average density, we wished to demonstrate the difference in average density of repair patches induced by BLM and UV radiation directly. Therefore, DNA from BLM treated cells and UV-irradiated cells was combined *prior* to sonication and centrifugation on the same CsCl gradient. In this case, permeabilized cells were treated with either 12 J/m^2 UV light or 5 μg/mL BLM and repair labeled with [^{35}S]dCTP or [^{3}H]dCTP, respectively, in the presence of BrdUTP. The results of this experiment (Figure 1) show a clear difference in average density between the two DNA populations.

Another "test" for short patch repair has been resistance to the polymerase α and polymerase δ inhibitor aphidicolin (APC) (Miller & Chinault, 1982; Seki et al., 1982; Dresler & Lieberman, 1983). Therefore, we compared the levels of repair synthesis in permeable cells, exposed to UV radiation or BLM, following pretreatment with APC. As shown in Table II, the level of total repair incorporation (labeled with [^{3}H]dThd) in intact and permeabilized cells irradiated with UV light was reduced by >80% following pretreatment with 65 μM APC for 1 h. On the other hand, <20% of the overall

Table II: Repair Synthesis in the Presence of Aphidicolin

treatment[a]	repair synthesis (^{3}H/^{14}C) −APC	+APC	% inhibition
UV (intact)	10.3	1.6	84.5
UV (permeable)	7.1	1.2	83.1
BLM (permeable)	2.2	1.8	18.2

[a] Cells (prelabeled with [^{14}C]dThd) were pretreated with or without 65 μM APC, exposed to 12 J/m^2 UV light or 5 μg/mL BLM, and labeled with [^{3}H]dThd for 30 min during repair, in the presence or absence of 65 μM APC.

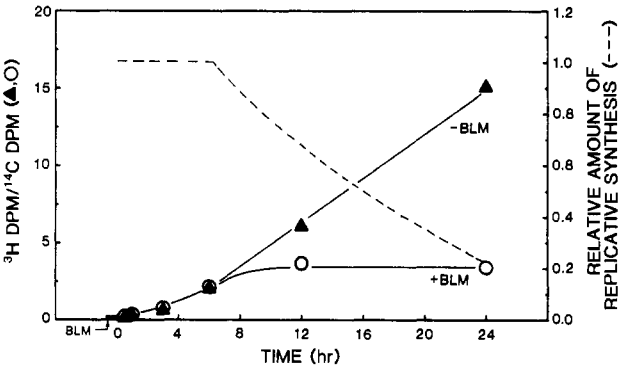

FIGURE 2: DNA replicative synthesis in cells that were treated with or without BLM. Confluent cells, prelabeled with [^{14}C]dThd, were incubated with (○) or without (▲) 100 μg/mL BLM for 30 min and labeled with [^{3}H]dThd and BrdUrd for the times shown. At each time point, the DNA was isolated and banded in an alkaline CsCl gradient. In each case, the ratio of total ^{3}H dpm in the heavy peak (newly replicated DNA) and the total ^{14}C dpm in the light peak was determined (e.g., Sidik & Smerdon, 1984). The small increase in slope at early times most likely resulted from changes in temperature during the addition of label to the cells. The amount of replicative synthesis in the BLM-treated cells relative to the untreated (control) cells is denoted by the dashed line.

repair synthesis was inhibited in permeable cells exposed to BLM. Thus, each of the above experiments indicates that the majority of repair of BLM-induced damage in LPC permeabilized human fibroblasts is of the short patch type.

Effect of BLM on Replicative Synthesis. In order to accurately account for the contribution of (residual) replicative synthesis to the total amount of label incorporated during repair synthesis in intact cells, we measured the effect of BLM on the level of DNA replicative synthesis. This was done by determining the difference between replicative synthesis measured in untreated cells and the actual level of replicative synthesis in cells treated with BLM. In these experiments, the BrdUrd density-shift method was used to separate newly repaired DNA from newly replicated DNA (Smith, 1981). (In this case, the DNA was not sonicated as for the patch size measurements.) The data indicate that the level of replicative synthesis in BLM-treated cells is similar to that in the untreated cells for the first 6 h after BLM treatment (Figure 2). After 6 h, the level of replicative synthesis in the BLM-treated cells is reduced, presumably due to a decrease in both DNA replicon initiation and chain elongation (Cramer & Painter, 1981; Edwards et al., 1981). The amount of replicative synthesis in the BLM-treated cells relative to the control cells following each chase time was used to correct the nuclease sensitivity values determined for repair-labeled DNA (Smerdon, 1983; Sidik & Smerdon, 1984).

Nucleosome Rearrangement in Intact Cells. Analysis of the nuclease digestion characteristics of repair-incorporated nucleotides in chromatin of intact cells was performed on confluent cells that were prelabeled with [^{14}C]dThd and incubated with 100 μg/mL BLM in the presence of [^{3}H]dThd

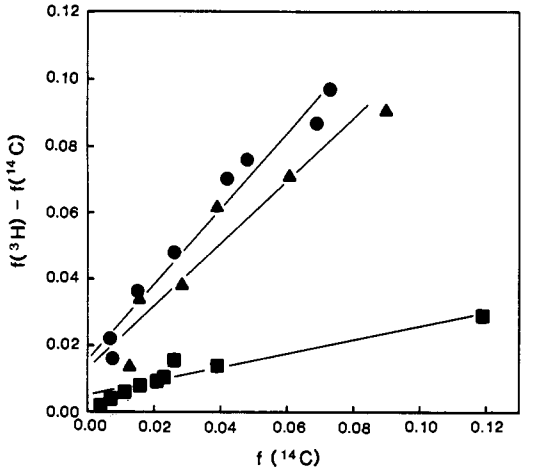

FIGURE 3: Normalized difference curves for staphylococcal nuclease digestion of repair-labeled nuclei. Confluent cells, prelabeled with [^{14}C]dThd and treated with 2 mM HU, were incubated with 100 μg/mL BLM for 30 min in the presence of [^{3}H]dThd. The cells were then harvested immediately (●) or incubated for 24 h in chase medium containing (▲) or not containing (■) 2 mM HU. Nuclei, isolated from these cells, were suspended in digestion buffer and incubated for varying times with staphylococcal nuclease (Materials and Methods). The data represent the difference between the fraction of ^{3}H dpm [f(^{3}H), repaired DNA] and ^{14}C dpm [f(^{14}C), bulk DNA] rendered acid soluble for different extents of digestion of bulk DNA [f(^{14}C)].

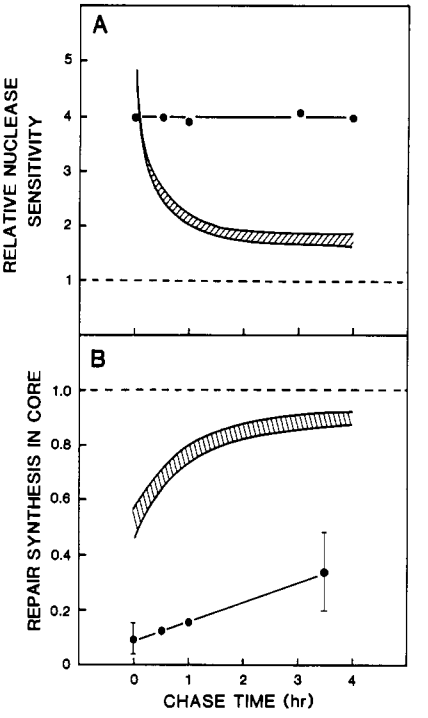

FIGURE 4: (A) Relative nuclease sensitivity of repair-incorporated nucleotides following short chase times. Confluent cells, prelabeled with [^{14}C]dThd and pretreated with 2 mM HU, were incubated with 100 μg/mL BLM, 2 mM HU, and [^{3}H]dThd for 30 min. Immediately after the 30-min incubation, the cells were either harvested or incubated for various times with unlabeled dThd in the presence of 2 mM HU. In each case, nuclei isolated from these cells were digested with staphylococcal nuclease and analyzed as previously described (Smerdon et al., 1978, 1979). The data represent the ratio of the fraction of repair-incorporated nucleotides that is nuclease sensitive and the fraction of bulk DNA that is nuclease sensitive and was determined from the initial slopes of normalized difference curves (e.g., Figure 3). (B) Repaired DNA associated with 146-bp nucleosome core DNA following short chase times. Cells were prelabeled and treated as in (A). Digestion of nuclei with staphylococcal nuclease was carried out until 20–25% of the ^{14}C-labeled DNA was rendered acid soluble. The DNA was isolated and electrophoresed on 2.8% agarose gels, and the ^{3}H and ^{14}C dpm profiles for the 146-bp (core) DNA band were determined as described previously [e.g., Smerdon (1983)]. Calculation of the ordinate values was as described in Smerdon (1983). Values for the 0.5- and 1-h chase times represent averages obtained from two different experiments, while the values shown for the 0 and 3.5-h chase times represent the mean ± 1 SD of the values obtained from three experiments. In each panel, the shaded region shows the range of values measured for the same cell strain irradiated with 254-nm UV radiation and labeled during repair with [^{3}H]dThd for 30 min (Smerdon et al., 1979).

for 30 min. This treatment was performed in the presence of 2 mM HU to suppress residual replicative synthesis to very low levels. (Treatment with HU during the repair labeling step was found to be necessary for these experiments since the amount of repair incorporation is significantly reduced from that observed following long patch repair.) The cells were either harvested immediately following the 30-min incubation or incubated in conditioned medium containing unlabeled dThd (chased) for different time periods. For each chase time, nuclei were isolated and digested with staphylococcal nuclease for varying times. Following different extents of digestion, the acid-soluble radioactivity was determined, and the rates of release of ^{3}H (repair label) and ^{14}C (bulk label) were analyzed by using a method described in detail previously (Smerdon et al., 1978, 1979). In using this method, the nuclease digestion data are represented as "normalized difference curves", which are plots of the difference between the fraction of ^{3}H dpm (repair label) and ^{14}C dpm (bulk DNA label) rendered acid soluble [f(^{3}H) − f(^{14}C)] versus the fraction of ^{14}C dpm rendered acid soluble [f(^{14}C)]. An example of such curves is shown in Figure 3. As can be seen, when cells are harvested immediately after the repair labeling period, or chased for 24 h in the presence of 2 mM HU, repair-incorporated nucleotides are released much more rapidly by staphylococcal nuclease than the bulk of the DNA in chromatin. However, when HU was omitted from the chase medium, the rates of release of the two labels were much closer (Figure 3).

From the initial slope (m_i) of these curves, one can determine the ratio of the fraction of repair-incorporated nucleotides that is nuclease sensitive (f_s) and the fraction of bulk DNA that is nuclease sensitive (ξ) using the following equation [see Smerdon and Lieberman (1978)]:

$$f_s/\xi = \text{relative nuclease sensitivity} = \frac{(m_i + 1)\sigma - 1}{\sigma - 1}$$

where σ is a correction of the data for nucleotides incorporated via replicative synthesis (Figure 2; Smerdon et al., 1978). The relative nuclease sensitivity should be 1.0 if the digestion rates are the same for both repair-incorporated nucleotides and bulk DNA, and >1.0 if the repaired DNA is digested more rapidly than the bulk DNA in chromatin. In Figure 4, we present data from an experiment where cells were subjected to short chase times (0–4 h). As can be seen, nucleotides inserted during repair of BLM damage were much more sensitive than bulk DNA immediately following the labeling period and the value of f_s/ξ was similar to that observed for long patch agents after the same labeling time (Figure 4A). In contrast to the repair of such agents (shaded area), however, BLM-induced repair patches remained nuclease sensitive following subsequent chase times in the presence of HU.

To complement these studies, we have also examined the level of repair-incorporated nucleotides in nucleosome core DNA isolated from cells treated as above. Staphylococcal nuclease digestion products obtained from these cells were separated by agarose gel electrophoresis and the ^{3}H and ^{14}C profiles were determined for the core DNA band (146 bp) in each case. The data were then analyzed to give the relative

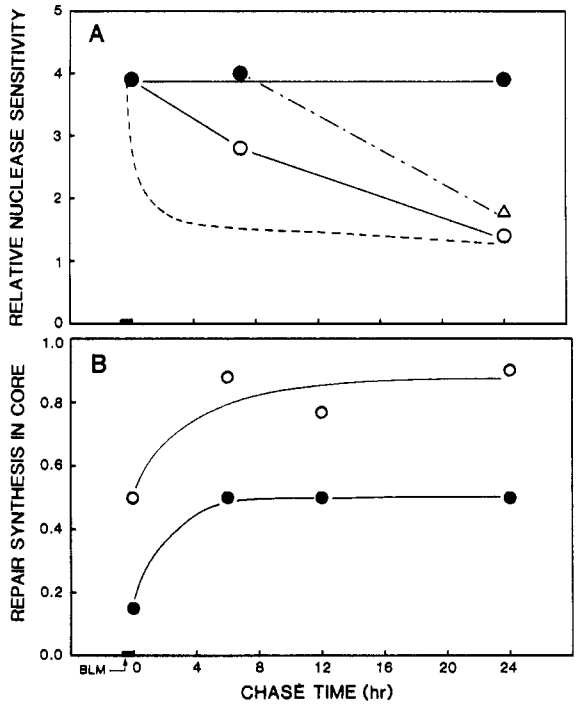

FIGURE 5: (A) Relative nuclease sensitivity of repair-incorporated nucleotides following long chase times. Confluent cells were treated as in Figure 4. Immediately after BLM treatment and labeling, the cells were incubated with unlabeled dThd in the presence (●) or absence (○) of 2 mM HU. A third set of cells was incubated for 7 h in the presence of HU and an additional 17 h in the absence of HU (△). In each case, nuclei isolated from these cells were digested with staphylococcal nuclease and analyzed as in Figure 4A. The dashed line represents a fit to the data obtained for cells irradiated with 12 J/m² UV light and labeled during repair for 30 min (Smerdon et al., 1979). (B) Repair synthesis associated with nucleosome core DNA following long chase times. Confluent cells, treated as in (A), were incubated with unlabeled dThd for the times shown in the presence (○) or absence (●) of 2 mM HU. Nuclei digestion and data analysis was performed as described in Figure 4B.

amount of repair-incorporated nucleotides in the core regions (Smerdon, 1983). If the repair-incorporated nucleotides are distributed randomly between nucleosome core and linker regions, the relative amount of repair synthesis in core DNA will be 1.0 (Sidik & Smerdon, 1984). As shown in Figure 4B, immediately following the 30-min labeling pulse, core DNA is almost devoid of repair-incorporated nucleotides. Furthermore, the level of repair patches remains low following subsequent chase times (up to 4 h), although the values gradually increase (in contrast to no change in the relative nuclease sensitivity). These results are also in marked contrast to repair induced by UV radiation and UV mimetic chemicals (shaded area).

Following longer chase times, we found that the relative nuclease sensitivity depends on the presence of HU in the chase medium. When the repair label was chased in the presence of HU, repair patches remained nuclease sensitive for 24 h (Figure 5A). In fact, this high level of nuclease sensitivity persists for at least 72 h after repair labeling (data not shown). If HU was omitted from the chase medium, we observed that these patches become increasingly nuclease resistant with time. However, this loss in nuclease sensitivity occurred at a much slower rate than observed for repair of UV photoproducts (Figure 5A, dashed line). Furthermore, if HU was present in the chase medium for 7 h and then omitted during a subsequent 17-h chase, the repair-incorporated nucleotides were slightly more sensitive than if HU was absent for the entire 24-h period (Figure 5A, open triangle). This latter result indicates that the "blockage of rearrangement" by HU is reversible even after periods of sustained exposure.

Table III: Percentage of Nuclei Undergoing Replicative Synthesis at Various Times after Treatment with or without BLM

labeling time (h)	treatment[a] +BLM +HU (%)	+BLM −HU (%)	−BLM +HU (%)	−BLM −HU (%)
1	0.55 ± 0.40[b]	0.63 ± 0.34	0.77 ± 0.40	0.91 ± 0.38
3	0.68 ± 0.24	0.69 ± 0.30	0.77 ± 0.31	0.90 ± 0.30
8	0.90 ● 0.28	0.86 ± 0.48	1.0 ± 0.26	0.90 ± 0.38
24	0.90 ± 0.30	0.70 ± 0.30	3.1 ± 1.0	5.8 ± 1.3
48	0.75 ± 0.25	0.75 ± 0.34	3.9 ± 1.2	5.8 ± 2.1

[a] Cells were either exposed or not exposed to 2 mM HU for 45 min and then to 100 μg/mL BLM for 30 min, prior to incubation in media containing [³H]dThd with or without 2 mM HU. Control cells, not receiving BLM, were treated in the same way. Cells were fixed, developed for autoradiography, and viewed under a phase-contrast microscope. [b] Values represent the mean ± 1 SD of the percent of cells labeled by replicative synthesis in 20 or more fields in each case.

The level of repair-incorporated nucleotides in isolated core DNA increased during these longer chase periods (Figure 5B). Surprisingly, this was the case whether or not HU was present in the chase medium. However, in the presence of HU, the amount of repair-incorporated nucleotides associated with core DNA increased to only about 60% of the value obtained when HU was absent from the chase medium (Figure 5B).

It was shown previously that the formation of nucleosomes in newly repaired regions of DNA occurs after the ligation step is completed (Hunting et al., 1985; Smerdon, 1986). Thus, a slow rate of ligation during BLM-induced repair could yield a slow rate of nucleosome rearrangement at these sites. Therefore, we examined the levels of ligation of BLM-induced repair patches in cells that were chased in the presence and absence of HU (as above). The DNA isolated from these cells was digested with *E. coli* exonuclease III, as described previously (Smerdon, 1986). Unligated repair patches contain nicks or gaps and are rapidly digested to acid-soluble nucleotides, whereas ligated repair patches and prelabeled DNA are digested much slower. The fraction of unligated repair patches was determined from the fraction of total ³H label that was *rapidly* rendered acid soluble (Smerdon, 1986). As shown in Figure 6A (solid symbols), the rate of ligation was the same whether the cells were chased in the presence or absence of HU, and ligation was almost complete after 6 h. Thus, during the repair of BLM-induced strand breaks, repair patches are ligated to completion in the presence of HU and the sustained nuclease sensitivity of these regions (Figure 5) is not due to incomplete ligation.

Another possible explanation for the difference observed in Figure 5 relates to the blockage of replicative synthesis by HU [e.g., Collins and Johnson (1981)]. If a significant number of cells in the population were able to undergo replication during the long chase periods in the absence of HU, then the repair patches could be randomized as replication forks pass through these sites. This process could cause the slow rearrangement observed in Figure 5A. To test this hypothesis, cells were analyzed by autoradiography (Cleaver & Thomas, 1981). The fraction of cells undergoing replicative synthesis (i.e., with many silver grains over the nucleus) was determined for different incubation times with and without HU present and the results are shown in Table III. After BLM treatment, the number of cells undergoing replicative synthesis remained essentially constant (<1%) over a 48-h period, and HU had little affect on the fraction of cells labeled. On the other hand, in the control cells (not treated with BLM), we observed a small increase in the number of nuclei undergoing replicative

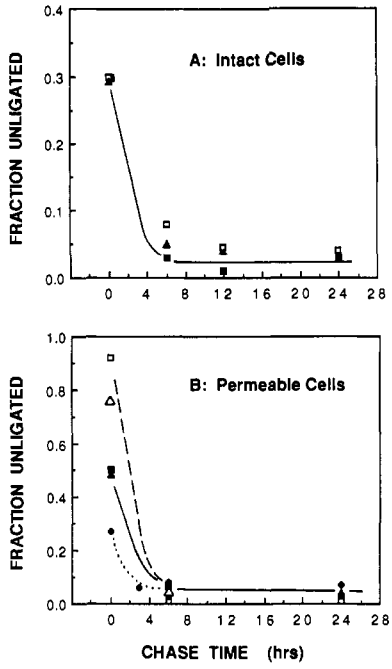

FIGURE 6: Fraction of unligated repair patches remaining following different chase times in intact (A) and permeabilized (B) cells. (A) Confluent cells were double labeled as described in Figure 4, except that some cells were labeled in the presence of 65 μM APC (□) instead of HU. Following the ^3H labeling period, the cells were either harvested immediately or incubated with unlabeled dThd in the presence (▲) or absence (■) or 2 mM HU, or in the presence of 65 μM APC (□), for the times shown. DNA isolated from these cells was digested with *E. coli* exonuclease III, as previously described (Smerdon, 1986). The fraction of unligated repair patches was determined from the fraction of total ^3H that was *rapidly* rendered acid soluble. These values were corrected for the (slow) digestion of ligated repair patches by subtraction of the fraction of ^{14}C rendered acid soluble during the same times (Smerdon, 1986). (B) Confluent cells, prelabeled with [^{14}C]dThd, were permeabilized by incubation with 80 μg/mL LPC (in PBS buffer) for 2 min at 0 °C. The cells were then incubated with "repair mix salts" (Materials and Methods) containing 1 μg/mL (◆), 5 μg/mL (▲, △), or 25 μg/mL (■, □) BLM and 10 μCi/mL [^3H]dThd for 30 min at 37 °C. Following the labeling period, cells were incubated for the times shown in unlabeled dThd (chased) and the DNA digested with exonuclease III as in (A). As in (A), some of these cells were exposed to 65 μM APC during the labeling and chase periods (open symbols).

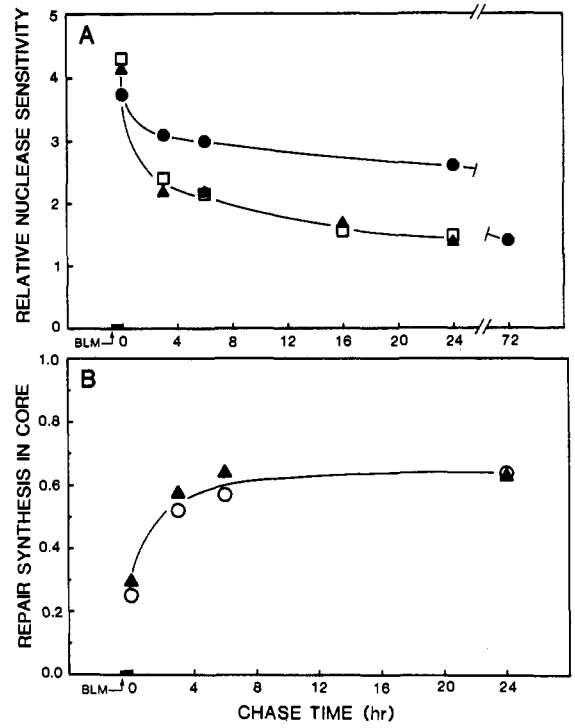

FIGURE 7: (A) Relative nuclease sensitivity of repair-incorporated nucleotides as a function of chase time for permeabilized cells treated with BLM. Confluent cells, prelabeled with [^{14}C]dThd, were permeabilized as in Figure 6B. The cells were then incubated in "repair mix salts" containing 1 μg/mL (●), 5 μg/mL (▲), or 25 μg/mL (□) BLM and 10 μCi/mL [^3H]dThd for 30 min at 37 °C. Cells were then incubated in unlabeled dThd for the times shown. Digestion of the nuclei with staphylococcal nuclease and data analysis were performed as described in Figure 4A. (B) Repair-incorporated nucleotides in nucleosome core DNA as a function of different chase times in permeabilized cells. Cells were treated as described in (A) and the amount of repair synthesis in isolated 146-bp core DNA was determined for cells exposed to 1 μg/mL (○) or 5 μg/mL (▲) BLM. Data were analyzed as described in Figure 4B.

synthesis during a 48-h period and, as expected, replicative synthesis was reduced in the presence of HU. However, even in the absence of HU, only about 6% of the cells entered S-phase during the 48-h period (Table III). Furthermore, in intact cells, BLM-induced damage and repair occurs in clusters of 10–250 cells (Sidik & Smerdon, 1990). Therefore, if the slow rearrangement observed in Figure 5 is caused by replicative synthesis, cells undergoing replicative synthesis should also be in clusters. This, however, was not observed (data not shown). Thus, the slow rearrangement observed in the absence of HU (Figure 5) does not appear to result from an increased number of cells undergoing replicative synthesis during the chase period.

Nucleosome Rearrangement in Permeabilized Cells. We have also examined the nuclease digestion characteristics of repair-incorporated nucleotides in permeabilized cells exposed to BLM. As discussed earlier, pretreatment of confluent AG1518 cells with 80 μg/mL LPC yields significant levels of DNA damage and repair in the majority of the cells following low doses of BLM (Sidik & Smerdon, 1990). For these experiments, cells were prelabeled with [^{14}C]dThd, permeabilized with LPC, and incubated with repair mix containing [^3H]dThd and various concentrations of BLM for 30 min. [Labeled dThd was used because the majority of cells reseal their membranes within 15 min (Lorenz et al., 1988) and a larger portion of the cell population could be labeled.] Following the labeling period, cells were subjected to varying chase periods, nuclei were isolated and digested with staphylococcal nuclease, and the acid-soluble radioactivity was analyzed as before. The relative nuclease sensitivity of the inserted repair patches is shown in Figure 7A. When cells were damaged with either 5 μg/mL or 25 μg/mL BLM, the repair patches were initially sensitive to nuclease and, during subsequent chase times, these regions became increasingly nuclease resistant. Furthermore, the decrease in relative nuclease sensitivity with time was more similar to that observed during repair of UV-induced damage than observed in intact cells treated with BLM [Figure 5; see also Lorenz et al. (1988)]. However, when only 1 μg/mL BLM was used the decrease in relative nuclease sensitivity was much slower, and repair patches remained more nuclease sensitive for an extended period of time (Figure 7A). Therefore, following this lower dose of BLM, it appears that a smaller fraction of the total repair-incorporated label is associated with the rapid structural rearrangement observed for higher BLM doses.

Once again, the rate of ligation of nascent repair patches was measured to determine if these patches were completed in the permeabilized cells. As can be seen in Figure 6B (solid symbols), the rate of ligation was similar for the two higher BLM concentrations used in Figure 7A and somewhat faster for 1 μg/mL BLM. However, in each case, over 90% of the

repair patches were ligated by 6 h. Therefore, as observed for intact cells, the slow decrease in relative nuclease sensitivity observed after 1 μg/mL BLM (Figure 7A) was not due to a decrease in the rate of ligation of repair patches in these cells.

The level of the repair-incorporated nucleotides in nucleosome core DNA, isolated from permeable cells that were labeled and chased as described above, is shown in Figure 7B. Cells were damaged with either 5 μg/mL or 1 μg/mL BLM. As expected, in either case, repair-incorporated nucleotides were significantly lower in core DNA immediately following the labeling period. Furthermore, as with intact cells treated with HU, the amount of repair-incorporated nucleotides in core DNA increased with time even after 1 μg/mL BLM, where the relative nuclease sensitivity changed much more slowly (Figure 7A). However, even after a 24-h chase period, repair synthesis was not randomly distributed in nucleosomes and remained higher in "noncore" (e.g., linker) regions of the genome (Figure 7B).

Nucleosome Rearrangement in Cells Treated with Aphidicolin. Since it is possible that the slow rearrangement we observe after low levels of BLM damage is due (at least in part) to the minor fraction of repair synthesis thought to occur by polymerase δ (DiGiuseppe & Dresler, 1989; Table II), we also examined the maturation of repair patches in both intact and permeabilized cells treated with 65 μM APC. As shown in Figure 8A, under these conditions, the newly repaired DNA in intact cells remained nuclease sensitive for at least 24 h. Also, the presence of APC in the *chase medium* did not appear to affect the relative nuclease sensitivity during this time period. Furthermore, as observed for HU-treated intact cells, APC had little affect on the ligation of repair patches, which was almost complete by 6 h (Figure 6A, open symbols).

We also examined the affect of APC on nucleosome rearrangement in permeabilized cells exposed to 5 and 25 μg/mL BLM. As shown in Figure 8B, maturation of repair patches in permeabilized cells exposed to 65 μM APC is associated with rapid structural rearrangements at a rate and extent similar to those incorporated in the absence of APC. Furthermore, in this case, the presence of APC during repair synthesis appears to retard the rate of ligation at these repair sites (Figure 6B). Immediately following the 30-min labeling period, the fraction of unligated repair patches is almost twice that observed in the absence of APC. However, even with this reduction in the rate of ligation, almost all of the repair patches are ligated after a 6-h chase period (Figure 6B).

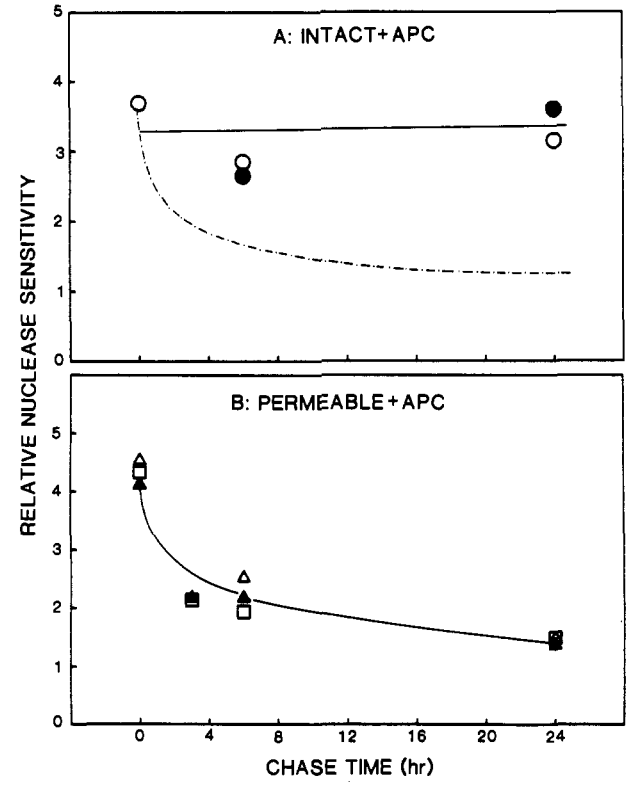

FIGURE 8: Relative nuclease sensitivity of repair-incorporated nucleotides in intact (A) and permeabilized cells (B) exposed to BLM and APC. (A) Confluent cells, prelabeled with [^{14}C]dThd and pretreated with 65 μM APC, were incubated with 100 μg/mL BLM, 65 μM APC, and 10 μCi/mL [^{3}H]dThd for 30 min at 37 °C. Immediately after BLM treatment and labeling, the cells were incubated with 50 μM unlabeled dThd either in the presence (○) or absence (●) of APC. In each case, nuclei were isolated and digested with staphylococcal nuclease and the data analyzed as in Figure 4A. The dot–dashed line (···) represents a fit to the data obtained for cells irradiated with 12 J/m^2 UV light and labeled during repair for 30 min (Smerdon et al., 1979). (B) Confluent cells, prelabeled with [^{14}C]dThd, were pretreated with (△, □) or without (▲) 65 μM APC. Cells were then permeabilized as in Figure 6B and incubated with "repair mix salts" (Materials and Methods) containing 5 μg/mL (△, ▲) or 25 μg/mL (□) BLM and 20 μCi/mL [^{3}H]dThd for 30 min at 37 °C. Following the labeling period, cells were incubated for the times shown in 50 μM unlabeled dThd, and 65 μM APC in the case of APC treated cells, prior to nuclei isolation and digestion with staphylococcal nuclease (as in Figure 4).

Discussion

We have performed a comprehensive study of the nuclease digestion characteristics of repair patches inserted into chromatin of intact and permeabilized human diploid fibroblasts exposed to bleomycin (BLM). From our past experience with different DNA damaging agents (Smerdon et al., 1978; Sidik & Smerdon, 1984, 1987), we incorporated several features in these studies that allow less equivocal evaluation of the data. First, the nuclease digestion data for intact cells were corrected for the contribution of (residual) replicative synthesis taking place during repair (Smerdon et al., 1979; Smerdon, 1983), since the level of short patch repair synthesis is low. Somewhat surprisingly, during the first 6 h following treatment with 100 μg/mL BLM, the level of replicative synthesis was similar to that of untreated cells (Figure 2). However, at later times, the BLM-induced lesions inhibited replicative synthesis. These observations were supported by studies using whole-cell autoradiography (Table III). Second, *both* the relative nuclease sensitivity of newly repaired DNA (using staphylococcal nuclease as a probe) and the levels of repair-incorporated nucleotides in isolated nucleosome core DNA were determined. In the case of long patch repair, these two methods examine different features of the same structural transition(s) [see Smerdon (1989)]. However, since different types of repair may yield differences in the "coupling" of these two parameters (see below), it is imperative that both methods be employed when comparing conformational states of newly repaired regions following treatment with untested DNA damaging agents.

BLM Induces Short Patch Repair in Human Cells. Our results are in general agreement with DiGiuseppe and Dresler (1989), who reported that repair of BLM-induced DNA damage in human diploid fibroblasts consists of both long patch and short patch components. We obtained average repair patch sizes of six to nine bases for permeabilized cells exposed to three different doses of BLM (1, 5, and 25 μg/mL; Table I) and <20% of the repair incorporation (after 5 μg/mL BLM) was inhibited by 65 μM aphidicolin (APC). However, DiGiuseppe and Dresler (1989) observed an average patch size of ~20 bases when permeabilized cells were damaged with 5 μg/mL BLM on ice prior to labeling with [^{32}P]dGTP during repair. Patches of four bases were observed by these authors

when 200 μg/mL BLM and [^{32}P]dCTP were used (DiGiuseppe & Dresler, 1989). As demonstrated by these authors, the different nucleotide triphosphates used to label BLM-induced repair patches select for different repair components. Furthermore, the differences in cell permeabilization and how the cells were treated with BLM (i.e., on ice versus 37 °C) could also introduce variations between the two studies. For example, exposure of permeabilized cells to 200 μg/mL BLM at 37 °C would result in extensive degradation of genomic DNA in our system, with a significant fraction of strand breaks being <200 bases apart (Sidik & Smerdon, 1990).

Given that the average size of short and long repair patches is 4 and 20 bases, respectively (see above), we estimate that (in the absence of APC) between 69% and 88% of the labeled, BLM-induced repair synthesis in our study is of the short patch type (i.e., for average patch sizes of six to nine bases). These values agree well with our observation that 82% of the labeled repair synthesis is resistant to 65 μM APC (Table II). These considerations indicate that our findings (discussed below) are indeed representative of bona fide short patch repair synthesis.

Maturation of Short Patch Repair in Intact Cells. When intact cells are treated with 100 μg/mL BLM, repair patches are initially quite sensitive to staphylococcal nuclease digestion and the degree of sensitivity is similar to that of repair synthesis induced by long patch agents (Figures 4 and 5). Furthermore, there is almost *no* nascent repair synthesis associated with isolated nucleosome core DNA. This latter result differs from previous observations with long patch repair, where a significant fraction of the repair-incorporated nucleotides are associated with core DNA immediately after the labeling period [see Smerdon (1989)]. Also unlike the situation with long patch repair, BLM-induced repair patches lose their enhanced nuclease sensitivity much more slowly and the rate of loss is dependent on the presence of hydroxyurea (HU) in the chase medium. Indeed, in the presence of 2 μM HU, the newly inserted repair patches remain nuclease sensitive for at least 72 h and only about 50% of the repair-incorporated nucleotides become associated with isolated core DNA (Figure 5). However, if HU is not included in the chase medium, the newly repaired DNA gradually becomes more nuclease resistant, although at a rate much slower than that observed during long patch repair (Smerdon & Lieberman, 1978, 1980; Tlsty & Lieberman, 1978; Oleson et al., 1979; Zolan et al., 1982; Sidik & Smerdon, 1984, 1987). Thus, our results indicate that in intact cells exposed to 100 μg/mL BLM, the resulting short patch repair is not associated with the rapid phase of nucleosome rearrangement observed during long patch repair. As stated in the introduction, this phase is thought to be associated with the rapid formation of native nucleosome structures at repair sites (Smerdon, 1989).

At present, it is not clear why HU interferes with the slow structural rearrangements that occur following repair synthesis. It is important to note that this is not observed following long patch repair, although some retardation in the rapid rearrangement phase does occur (Smerdon, 1983). We have shown that this cannot be due to decreased ligation of the repair patches since ligation is essentially complete in 6 h and follows the same time course in the presence or absence of the drug (Figure 6). Furthermore, the slow rearrangement observed in the absence of HU is not due to accumulation of randomized repair patches resulting from passage of replication forks (Table III). Other factors that may contribute to the slow loss of nuclease sensitivity in the absence of HU include (1) higher order folding of the chromatin structure during maturation of repair sites [see Smerdon (1989)] or (2) "long-range" rearrangements (e.g., nucleosome sliding) induced by the residual long patch repair component (see below). Indeed, it is well-known that HU is an inhibitor of ribonucleotide reductase and, in its presence, there is a decrease of deoxynucleotide triphosphates (Adams & Lindsay, 1967; Collins et al., 1977). Therefore, it is possible that BLM-induced repair synthesis is more sensitive to reduced precursor pools than long patch repair synthesis, particularly because they appear to involve different DNA polymerases (Miller & Chinault, 1982; Seki et al., 1982; Dresler & Lieberman, 1983; DiGiuseppe & Dresler, 1989).

Maturation of Short Patch Repair in Permeable Cells. The nuclease digestion characteristics were also examined in permeabilized cells to assess the maturation rate of repair patches inserted after higher levels of DNA damage by BLM. We report elsewhere that when cells are reversibly permeabilized with lysophosphatidylcholine (LPC), the number of cells undergoing measurable repair synthesis increases from ~5% in intact cells treated with 100 μg/mL BLM to 86% following a dose of only 5 μg/mL BLM (Sidik & Smerdon, 1990). Therefore, in this case, measurable short patch repair takes place in a much larger fraction of the cells and with much higher levels of DNA strand breaks. Indeed, a major fraction of the DNA is in fragments <1000 bases (or five nucleosomes) in length (Sidik & Smerdon, 1990). Furthermore, Lorenz et al. (1988) showed that in LPC-permeabilized cells dNTP pools are very low and not able to support replicative synthesis. Therefore, these experiments were performed in the absence of inhibitors such as HU.

The structural rearrangements of BLM-induced repair patches were dependent on the concentration of BLM. At high BLM concentrations (5 and 25 μg/mL), there was a more rapid loss of nuclease sensitivity than observed for intact cells (compare Figures 5 and 7). However, when the BLM concentration was reduced to 1 μg/mL, the loss in nuclease sensitivity occurred at a slower rate and was more like the time course of intact cells (in the absence of HU). These observations may reflect the fact that most DNA strand breaks occur in regions of chromatin that are most accessible to BLM. These regions include nucleosome linker DNA (Kuo & Hsu, 1978; Sidik & Smerdon, 1900), at least some transcriptionally active genes (Kuo, 1981; Villeponteau & Martinson, 1987), and, perhaps, other open regions in chromatin (Ljungman, 1989). Thus, at low levels of DNA damage by BLM (i.e., in intact cells or permeabilized cells treated with ≤1 μg/mL BLM), strand breaks may be located almost exclusively in nucleosome linkers of open chromatin regions. This feature, plus the fact that BLM is (primarily) a short patch agent, may reduce the extent of chromatin unfolding required to allow access to repair enzymes (Smerdon, 1989). When the concentration of BLM is increased, however, strand breaks also occur in linker regions of less accessible chromatin and (to a small extent) within core DNA [see scans of Sidik and Smerdon (1990)]. At this level of damage, short patch repair enzymes may require unfolding of nucleosomal structure to allow access to many of the lesions.

Uncoupling of Nuclease Sensitivity and Association with Nucleosome Cores. Under certain conditions, in both intact and permeabilized cells, the appearance of repair-incorporated nucleotides in isolated core DNA during the chase period was more rapid than the rate of loss of nuclease sensitivity of these regions (Figures 4, 5, and 7). In the case of intact cells, there was a reassociation of ~50% of the repair-incorporated label with isolated core DNA and no change in the nuclease sensitivity when HU was present in the chase medium (Figure

5). With permeable cells, there was no difference in the rate of association of repair patches with core DNA when cells were treated with 1 and 5 μg/mL BLM, even though after 1 μg/mL BLM the loss of nuclease sensitivity was clearly slower than after 5 μg/mL (Figure 7). A possible explanation for this "uncoupling" is that the two methods of analysis examine chromatin populations that differ in sensitivity to nucleases. Analysis of the relative nuclease sensitivity examines nucleotides that are released during the first ~10% of digestion of bulk DNA with staphylococcal nuclease (i.e., ~10% of the bulk DNA is rendered acid soluble; Figure 3). Therefore, in this case, one examines the regions in chromatin that are very accessible to nuclease digestion. On the other hand, analysis of the appearance of repair patches in isolated core DNA examines nucleosomes remaining after 20–25% of the bulk DNA is digested to acid solubility. These nucleosomes include regions in chromatin that are more resistant to nucleases and are from more compact, higher order structures. Thus, it is possible that the preferential attack of open chromatin regions by BLM, along with the fact that BLM elicits short patch repair, gives rise to a heterogeneity in repair patch distribution, which causes the uncoupling we observe. However, even after rearrangement is complete in the permeable cells, the core DNA population is clearly underrepresented in BLM-induced repair patches (Figure 7). This observation may also reflect the preference of BLM for linker DNA, even in highly condensed chromatin.

Chromatin Rearrangements in the Absence of Repair Synthesis by Polymerases α and δ. Maturation of repair patches inserted primarily by polymerase β was measured in cells exposed to 65 μM APC, a potent inhibitor of polymerases α and δ (Fry & Loeb, 1986). Repair patches induced by BLM in the presence of APC were ligated within 6 h in both intact and permeabilized cells, although this rate was slowed in permeabilized cells treated with APC (Figure 6). In the case of intact cells, repair patches remained nuclease sensitive even after 24 h (Figure 8A). On the other hand, repair of high levels of BLM-induced damage in permeabilized cells lost the enhanced nuclease sensitivity at a rate similar to cells not exposed to APC (Figure 8B). In contrast to these results, the ligation and chromatin maturation of repair patches inserted following exposure to 12 J/m^2 UV light or 2 mM methyl methanesulfonate occur at a much slower rate than for those incorporated in the absence of APC (unpublished results). These latter results (persumably) reflect the inhibition of repair patch completion by polymerase α and/or δ and emphasize the sequential events taking place during repair patch maturation (i.e., ligation precedes association with nucleosome structures) (Hunting et al., 1985; Smerdon, 1986).

The above observations suggest the following: (1) The slow rate of nucleosome rearrangement observed in intact cells, not treated with HU, may be caused by residual repair (or replicative) synthesis via polymerases α and/or δ. (2) At higher levels of BLM damage (i.e., in permeabilized cells) repair involving polymerase β can induce rapid nucleosome rearrangement similar to that observed following long patch repair. These results support the notion (given above) that short patch repair occurring in open regions of chromatin via polymerase β does not require the (presumed) "unfolding/refolding" of nucleosomes associated with the fast phase of nucleosome rearrangement (Smerdon, 1989).

Concluding Remarks. To date, nucleosome rearrangement has been observed following repair of all long patch agents studied, as well as small alkylating agents, regardless of whether adducts are formed randomly in nucleosomes or preferentially in linker DNA [reviewed by Smerdon (1989)]. These observations may only apply to repair of DNA lesions in compact regions of chromatin and/or repair synthesis via the long patch mode. Our results with BLM (at low damage frequency) indicate that the reduced amount of DNA processing associated with short patch repair of linker-specific lesions does not require the same degree of nucleosome rearrangement required for long patch repair. Indeed, the short patches inserted should be confined primarily to the linker regions, whereas long repair patches may extend beyond the linker into the core region. Furthermore, nucleosome unfolding may be required for *any* repair synthesis involving polymerase α or δ. The much smaller polymerase β enzyme (Fry & Loeb, 1986) may not require this unfolding at all lesions. Thus, this study suggests for the first time that the different (patch size) components of the excision repair system have different requirements for rearrangements of chromatin structure during excision repair, and the extent of rearrangement during short patch repair depends on the location of the DNA lesions within chromatin.

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Effect of Central-Residue Replacements on the Helical Stability of a Monomeric Peptide†

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ABSTRACT: The peptide acetylYEAAAKEARAKEAAAKAamide exhibits the dichroic features characteristic of a monomeric helix/coil transition in aqueous solution. Nineteen variants of this peptide each containing a different residue at position 9 were prepared by solid-phase peptide synthesis and purified by reversed-phase chromatography. The thermal dependence of the far-ultraviolet dichroic spectrum of each of these peptides except that containing proline is characteristic for an α-helix/coil transition. The relative stability of the helical forms of these peptides does not correlate with the preference of the variable amino acid to occupy a middle position in a protein helix. It is likely that the specific interactions of the variable residue with its local environment obscure any inherent preference of the residue to reside in an α-helix.

Baldwin and his associates (Marqusee & Baldwin, 1987; Shoemaker et al., 1987) have demonstrated that peptides containing fewer than 20 residues can be designed to exhibit a significant population of helical residues in aqueous solution. In this paper, we have altered one of their designed peptides, acetylAEAAAKEAAAKEAAAKAamide, to investigate the effect of residue replacement on the population of helical residues. The alanine residue at position 1 was replaced with a tyrosine to increase the precision and rapidity of the measurement of peptide concentration. The central alanine residue was selected for replacement by each of the other 19 residues to minimally perturb both the helix dipole and the three potential salt bridges in the designed peptide. Accordingly, each peptide considered in this paper has the sequence acetyl-YEAAAKEAXAKEAAAKAamide and will be designated by the residue at position X with the one-letter code. The effect of each central-residue replacement on the population of helical residues was measured by circular dichroism, and the results are compared with the preference of each residue

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