UV Radiation Induces Delayed Hyperrecombination Associated with Hypermutation in Human Cells†

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Ionizing radiation induces delayed genomic instability in human cells, including chromosomal abnormalities and hyperrecombination. Here, we investigate delayed genome instability of cells exposed to UV radiation. We examined homologous recombination-mediated reactivation of a green fluorescent protein (GFP) gene in p53-proficient human cells. We observed an ~5-fold enhancement of delayed hyperrecombination (DHR) among cells surviving a low dose of UV-C (5 J/m²), revealed as mixed GFP⁺/⁻ colonies. UV-B did not induce DHR at an equitoxic (75 J/m²) dose or a higher dose (150 J/m²). UV is known to induce delayed hypermutation associated with increased oxidative stress. We found that hypoxanthine phosphoribosyltransferase (HPRT) mutation frequencies were ~5-fold higher in strains derived from GFP⁺/⁻ (DHR) colonies than in strains in which recombination was directly induced by UV (GFP⁺ colonies). To determine whether hypermutation was directly caused by hyperrecombination, we analyzed hprt mutation spectra. Large-scale alterations reflecting large deletions and insertions were observed in 25% of GFP⁺ strains, and most mutants had a single change in HPRT. In striking contrast, all mutations arising in the hypermutable GFP⁺/⁻ strains were small (1- to 2-base) changes, including substitutions, deletions, and insertions (reminiscent of mutagenesis from oxidative damage), and the majority were compound, with an average of four hprt mutations per mutant. The absence of large hprt deletions in DHR strains indicates that DHR does not cause hypermutation. We propose that UV-induced DHR and hypermutation result from a common source, namely, increased oxidative stress. These two forms of delayed genome instability may collaborate in skin cancer initiation and progression.

UV radiation elicits many cellular stress responses attributed to its induction of reactive oxygen species (ROS) and its ability to directly damage DNA, predominantly forming cyclobutane pyrimidine dimers (CPDs) and pyrimidine-pyrimidone 6-4 photoproducts (12, 38, 68). UV also indirectly produces single-strand breaks and double-strand breaks (DSBs) during DNA replication (42). Because UV can produce strand breaks and other DNA lesions in several ways, it is a powerful mutagen and carcinogen. Solar UV-A (320 to 400 nm) and the higher-energy and shorter wavelength UV-B (290 to 320 nm) penetrate the Earth’s atmosphere, and chronic exposures have been linked to melanoma, the most fatal form of skin cancer (56). UV-C (100 to 290 nm) has the highest energy but is blocked by the ozone layer. However, UV-C exposures are possible from artificial sources, including germicidal lamps, arc welding equipment, and mercury arc lamps in older tanning beds. A significant fraction of UV damage is repaired by the nucleotide excision repair system, and patients with nucleotide excision repair defects show marked susceptibility to skin cancer (1, 27, 56). Human nonmelanoma skin cancer correlates with sun exposure (1) and is often associated with p53 mutations at dipyrimidines or longer pyrimidine tracts. Several mutational hotspots in p53 occur at 5’ CCG and 5’ TCG sequences; these are likely to contain 5-methylcytosine in the CG dinucleotide sequence, suggesting an increased potential for damage or decreased/less-accurate repair at these methylated sites (56).

UV induces mutations that can be detected soon after exposure. This direct mutagenesis results from errors during DNA repair or translesion synthesis. UV and other genotoxins, such as ethyl methane sulfonate, also induce mutations many generations after the initial exposure. These delayed mutations arise in a significant fraction (>10%) of exposed cells and reflect a hypermutation phenotype triggered by the original exposure (16, 18, 61, 62). In one study, delayed hypermutation was observed after exposure to UV-A, UV-B, and ionizing radiation (IR) (16). While there is a great deal of information about the spectrum of mutations directly induced by UV (56), only one study compared mutation spectra of early and delayed mutations, and this was limited to a multiplex PCR approach that distinguishes partial and total gene deletions from small-scale changes, such as single-base changes and small deletions/insertions (18). This analysis revealed that nearly 25% of delayed mutations were large deletions and...
IR induces DSBs as well as a complex array of direct or indirect DNA adducts, including base damage, single-strand breaks, and DNA-protein cross-links (69). IR can directly induce mutations (40), chromosomal aberrations (14), and homologous recombination (4, 10). IR also induces delayed mutations (16) as well as several other forms of delayed genomic instability, including chromosome aberrations, micronuclei, microsatellite instability, and low viability (47, 49, 50). It was recently shown that IR induces delayed hyperrecombination (DHR); this is a distinct type of genomic instability, as cells expressing the DHR phenotype do not show chromosomal instability or low viability (33). Thus, IR induces at least two mechanistically distinct types of delayed genomic instability.

Although HR is often characterized as an accurate DNA repair pathway, unregulated HR is associated with genome instability. Thus, the hyperrecombination phenotype of cells with defects in RecQ helicases (e.g., human BLM and Saccharomyces cerevisiae Sgs1) is associated with genome instability and cancer predisposition in humans and mice (51, 32, 51). The most conservative HR outcome is gene conversion without an associated crossover, but this still results in localized loss of heterozygosity. HR events associated with crossovers can have more-serious genetic consequences, including large-scale loss of heterozygosity extending from the crossover point to the telomere as well as deletions, inversions, and translocations (53).

Given that IR and UV radiation each induce multiple forms of DNA damage and delayed genomic instability, we have tested the hypothesis that UV induces DHR and that DHR is associated with hypermutation. We exposed human colorectal carcinoma cells carrying a green fluorescent protein (GFP)-based HR substrate to various doses of UV-B or UV-C. In this system, direct induction of HR produces homogeneous GFP colonies, whereas DHR produces mixed GFP+/− colonies. We show that UV-C, but not UV-B, induces DHR in up to 15% of surviving cells. Interestingly, mutation frequencies in DHR strains, measured at the HPRT locus, were ~5-fold higher than in non-DHR cells (GFP+) isolated from the same UV-irradiated population that gave rise to DHR cells. The mutation spectrum indicated that hypermutation is not caused by HR but showed evidence of arising from oxidative DNA damage. Oxidative damage also stimulates HR, suggesting a model in which UV-induced DHR and hypermutation are independent forms of delayed genome instability resulting from a common stressor, increased oxidative DNA damage.

**Materials and Methods**

Cell culture and measurement of cell survival after UV exposure. A derivative of the RKO human colorectal carcinoma cell line, RK306, carrying a single, integrated copy of an EGFP (hereafter GFP) direct repeat HR substrate was described previously (33). Cells were grown in monolayer cultures in Dulbecco’s modified Eagle’s medium supplemented with 10% bovine serum, 100 U of penicillin/ml, 100 µg of streptomycin/ml, and 0.2% sodium bicarbonate at 37°C in a 5% CO2 atmosphere. Prior to UV irradiation, cells were seeded into 10-cm (diameter) dishes to yield ~100 colonies and allowed to adhere for at least 3 h. Cells were rinsed twice with phosphate-buffered saline (PBS) and resuspended in PBS during UV exposures. Dishes were uncovered and exposed to UV-C irradiation using the germicidal UV lamp in a tissue culture hood and to UV-B using a UV-B lamp (UV Products, Upland, CA). Doses of UV-B and UV-C were determined with a UVX radiometer (UV Products). Emission spectra of the light sources were obtained using a calibrated Optronics 742 scanning spectroradiometer (Optronic Laboratories, Orlando, FL). After exposure, fresh growth medium was added and cultures were incubated for 10 to 14 days. To measure cell survival after UV treatment, colonies were stained with crystal violet in methanol, and those with at least 50 cells were scored.

**Delayed hyperrecombination assay.** To measure DHR, 10- to 14-day-old colonies treated as described above were washed twice with PBS. Green fluorescent cells were detected with an inverted Nikon TE2000 microscope powered by an argon laser (488 nm), and images were captured using LaserSharp 2000 confocal software using a fluorescein isothiocyanate filter (Bio-Rad, Hercules, CA). Differential interference contrast images were captured with a transmission filter. For each determination, a total of 90 colonies were analyzed in three dishes. Colonies were scored as GFP+, GFP−, or mixed GFP+/−. GFP+/− colonies with >4 GFP+ cells in an otherwise GFP− colony were scored as DHR, and colonies with <4 GFP+ cells in an otherwise GFP− colony were scored as delayed mutation.

**Analysis of HPRT mutations.** GFP+, GFP−, and GFP+/− colonies were expanded, and hypoxanthine phosphoribosyltransferase (HPRT) mutation frequencies were measured as described by Glaab et al. (29). Briefly, preexisting hprrt mutants were eliminated from expanded colonies by culturing in HAT selection medium (Dulbecco’s modified Eagle’s medium with 10% bovine growth serum, 100 µM hypoxanthine, 0.4 µM aminopterin, 16 µM thymidine; Sigma) for at least 14 days. Cells were then seeded into 10-cm dishes at 5 × 104 cells per dish and incubated in fresh growth medium for another 14 days to express the hprrt mutation phenotype before being plated at 5 × 103 per dish in selective growth medium containing 30 µM 6-thioguanine (6-TG; Sigma). Cell viability was measured by plating 1,000 cells per dish in nonselective medium, calculated as the ratio of colonies formed to cells plated (plating efficiency). After 14 days, 6-TG-resistant colonies were chosen for expansion and subsequent analysis of mutation spectra; the remaining colonies were stained and counted as described above. Mutation frequencies were calculated as the number of 6-TG-resistant colonies divided by the number of viable cells plated in 6-TG medium. Because hprrt mutants were eliminated from initial populations and initial populations were large, mutation frequencies are not strongly affected by “jackpots” and are therefore valid measures of spontaneous events. Mutations in expanded 6-TG-resistant colonies were analyzed by reverse-transcriptase PCR (RT-PCR) essentially as described by Denault et al. (20). Briefly, total RNA was extracted from 6-TG-resistant cells using the RNasy mini kit (QIAGEN, Valencia, CA). cDNA was produced by reverse transcription using 2 µg RNA in 20-µl reaction mixtures with 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl2, 10 mM dithiothreitol, 10 mM each of four deoxynucleoside triphosphates, 0.1 µg/µl bovine serum albumin, 2 pmol primer VM2 (5′-GATAATTTTCTGGCAGTGT), 40 units/µl RNase inhibitor (Applied Biosystems, Foster City, CA), 250 nM-400, 200 units/µl Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA). Reaction mixtures were incubated at 37°C for 1 h and stopped by heating to 70°C for 15 min. Two microliters of each cDNA reaction mixture was added to a 50-µl PCR mixture with 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 2.5 mM MgCl2, 400 µM each of deoxynucleoside triphosphate, 0.2 pmol primer VM1 (5′-CTGTTTCGGCAACCGGCTTC), 0.2 pmol primer VM2, and 2.5 units Taq Gold polymerase (PerkinElmer, Wellesley, MA). Reaction mixtures were incubated for 35 cycles comprising 1 min at 94°C, 1 min at 50°C, and 2 min at 72°C. A second round of PCR with nested primers was performed by using 1-µl aliquots of the initial PCR in a 50-µl PCR mixture as described above, except that 0.5 ng/µl each of primers VM3 (5′-CCTGAGCAGTCGAGCCGGGC) and VM4 (5′-CAATGAGCCTACGGCATGGT) was used. PCR products were analyzed by agarose gel electrophoresis. PCR products were then sequenced using primers UNC3 (5′-GCGCCTCCGTATGGCGAC) and UNC2 (5′-GTTGTTTATTCCTCTAGGC) and identified using the ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

**Detection of ROS.** Microscopic and fluorescence-activated cell sorter analyses of intracellular ROS were performed as described by Shi et al. (59). Briefly, cells were seeded into 10-cm dishes with or without coverslips and grown to 75% confluence. Cells were incubated with 2 µM dihydroethidium (DHE; Sigma) at 37°C for 1 h. Positive controls included 150 µM sodium dichromate dihydrate (Sigma), an oxidizing agent. Cells on coverslips were washed twice with PBS and fixed in 3% formaldehyde (Sigma) for 30 min, rinsed with PBS, mounted onto slides, and analyzed using a Zeiss Axioskop 10 microscope with Texas Red filter and Picture Frame software. Cells in dishes without coverslips were trypsinized, fixed in 70% ethanol for 30 min, suspended in PBS, and analyzed with a Becton Dickinson FACScan and CellQuest Pro 5.2 software. Gated excitation of DHE-mediated forward scatter was detected at 585 ± 21 nm (mean ± standard deviation), and side scatter was used to detect all cells.
RESULTS

UV-C induces delayed hyperrecombination. IR induces multiple forms of delayed genomic instability, and UV induces delayed hypermutation (16, 18, 33, 48, 61, 62). To investigate whether UV induces DHR, we used RKO36 cells, which carry a single-copy GFP direct repeat HR substrate (Fig. 1A). RKO36, like its parent cell line RKO, expresses wild-type p53, and has a near-diploid karyotype and normal responses to radiation exposure, including stabilization of p53 and p21 activation (33). We chose the RKO cell model because it has previously been characterized in delayed genomic instability assays (33) and is more relevant to human cancer than rodent cell models. RKO36 populations have both GFP$^-$ and GFP$^+$ cells that produce uniform GFP$^-$ and GFP$^+$ colonies, respectively, and it was previously established that GFP$^+$ cells arise from HR between GFP repeats (33). UV-induced DNA damage can stimulate HR, converting GFP$^-$ cells to GFP$^+$, and it can induce mutations that convert GFP$^+$ cells to GFP$^-$. These direct UV effects also produce uniform GFP$^-$ and GFP$^+$ colonies, although both types of events are expected to be rare because of the small size of the GFP target. However, if UV induces genomic instability at later times, this can give rise to mixed GFP$^{+/-}$ colonies, reflecting DHR (GFP$^-$→GFP$^{+/-}$, predominantly GFP$^+$) or delayed mutation (GFP$^+→$GFP$^{+/-}$, predominantly GFP$^-$). In this study, we used the GFP assay to score DHR and a more sensitive hprt mutation assay to score delayed hypermutation (Fig. 1B).

As expected, at equivalent doses, UV-C resulted in greater cytotoxicity than the lower-energy UV-B (Fig. 2). To determine whether UV-B induces DHR, we tested two doses that gave 0.5 to 5% survival (75 and 150 J/m$^2$). Neither UV-B dose increased the percentage of GFP$^{+/-}$ colonies above background levels (Fig. 3). Thus, even with relatively high cytotoxicity, acute UV-B exposure did not induce DHR. In contrast, we observed...
a modest increase in DHR with a 2.5-J/m² dose of UV-C and a marked increase at 5 J/m². Note that 20 to 50% of cells survived these UV-C doses and, at the higher dose, nearly 15% of surviving cells showed DHR. At these relatively low doses of UV-C, there was no significant increase in directly induced HR (GFP⁺) (data not shown), consistent with our previous report (21). The induction of DHR with UV-C, but not UV-B, was also apparent in two subsequent experiments (Fig. 3).

**Delayed hyperrecombination is associated with hypermutation but not reduced cell viability.** IR induces at least two types of delayed genomic instability, one showing chromosomal instability coupled with low cell viability and a second showing DHR and normal viability (33). To determine whether the DHR induced by UV-C was associated with the previously described delayed hypermutation phenotype (16, 18, 61, 62), we expanded nine colonies each from unexposed cells (GFP⁺), cells in which HR had directly been induced by UV-C (GFP⁺), and cells showing DHR (GFP⁺/+); the last two sets arose from cells that survived an initial dose of 5 J/m². We then measured mutation frequencies at the HPRT locus in each strain. After the initial colony expansion, the cultures were cleansed of hprt mutants by growth in medium with HAT and then expanded in nonselective medium to allow new hprt mutants to arise before selection in medium with 6-TG (Fig. 1B). The mutation frequency of unexposed control cells averaged ~10⁻⁴, consistent with previous reports (23, 41). The average mutation frequency of the GFP⁺ isolates was severalfold higher, but there was some overlap among individual GFP⁺ (exposed) and GFP⁺ (unexposed) strains. In contrast, all GFP⁺/+ strains showed strong hypermutation phenotypes, with mutation frequencies averaging >100-fold higher than GFP⁺ strains and ~13-fold higher than GFP⁺/+ strains (Fig. 4A). In addition, plating efficiencies of GFP⁺ and GFP⁺/+ strains were similar, and neither was lower than that of GFP⁺ strains (Fig. 4B). These results indicate that UV-C induces DHR that is associated with hypermutation and that these forms of genetic instability do not reduce cell viability.

**Frequent compound point mutations in hypermutable DHR cells.** Given the association between DHR and hypermutation (Fig. 4A), we tested whether the increased mutations in hprt were arising by a recombinational mechanism. Mutations that inactivate HPRT can range from single-base changes to deletions of an exon or the entire gene. We expected that HR-mediated mutations would result in large-scale changes, such as exon deletions, resulting from recombination between repeated elements in introns (28). RT-PCR was used for initial analysis of hprt mutations arising spontaneously from GFP⁺, GFP⁺, and GFP⁺/+ isolates after UV-C exposure and control GFP⁻ isolates from unexposed cells. As shown in Fig. 5A, 4 of 15 hprt mutants derived from non-DHR strains (UV-C-exposed GFP⁺ and GFP⁺ cells and unexposed GFP⁺) showed large-scale alterations. In contrast, no large-scale changes were observed among 18 hprt mutations arising spontaneously from GFP⁺/+ isolates (P = 0.033; Fisher’s exact test). The lack of large-scale changes in hprt mutants from GFP⁺/+ cells indicates that hypermutation is not a result of hyperrecombination.

We characterized a subset of these hprt mutants by sequencing the RT-PCR products. The results are summarized in Fig. 5B, Table 1, and Tables S1 and S2 in the supplemental material. Among 12 hprt mutants arising from non-DHR cells, 8 had 1- and 2-base changes and 4 had large deletions of 3.7 and 8.1 kbp. Two of the mutants had an identical 1-bp insertion and therefore may not have arisen independently. Three of the large deletions began at the same nucleotide near the beginning of exon 4 (A323) but differed in other respects. Two had identical 3,723-bp deletions, but one had an additional 84-bp insertion, and the third had a 3,726-bp deletion with an associated 80-bp insertion. The large insertions included 60- and 56-bp sequences, respectively, identical or nearly identical to the terminal 21 bp of HPRT exon 9 and 36 to 39 bp of contiguous downstream sequence. The remaining 24 bp of these insertions were 83% identical to each other but unrelated to any known sequence (by BLAST analysis). These fragments were inserted 20 bp downstream of the exon 9 TAA stop codon. Thus, the duplicated DNA inserted into itself (Fig. 5B). The fourth large deletion (8,189 bp) occurred at a distinct site and resulted in complete loss of exon 6 and partial loss of exons 5 and 7. In summary, spontaneous hprt mutations from non-
DHR cells resulted primarily from single mutational events, comprising single-base substitutions/deletions/insertions, and larger-scale deletion/insertion events whose structures suggest that they arose by error-prone DSB repair (see Discussion). None of the large deletions resulted in precise loss of one or more exons and therefore do not reflect point mutations in mRNA splice acceptor/donor sequences.

As expected from the agarose gel analysis of the RT-PCR products (Fig. 5A), sequence analysis of seven hprt mutants from DHR (GFP⁺/H11001/H11002) cells revealed only point mutations. All of these mutations arose independently. Interestingly, five of seven were compound mutants with two or more changes in the HPRT coding sequence; three mutants had 5 to 11 point mutations. Most of these mutations were single-base substitutions, deletions, or insertions. The largest changes were a tandem 2-bp substitution and a tandem 2-bp deletion (Fig. 5B) (see Table S2 in the supplemental material). This mutation spectrum, with a majority of mutants having compound point mutations, is significantly different from the non-DHR spectrum ($P = 0.045$, Fisher’s exact test). The mutation spectra of these mutations arose independently. Interestingly, five of seven were compound mutants with two or more changes in the HPRT coding sequence; three mutants had 5 to 11 point mutations. Most of these mutations were single-base substitutions, deletions, or insertions. The largest changes were a tandem 2-bp substitution and a tandem 2-bp deletion (Fig. 5B) (see Table S2 in the supplemental material). This mutation spectrum, with a majority of mutants having compound point mutations, is significantly different from the non-DHR spectrum ($P = 0.045$, Fisher’s exact test). The mutation spectra

<table>
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<tr>
<th>Source</th>
<th>No. of mutants</th>
<th>No. of mutations</th>
<th>No. (%) of mutants with compound point mutations</th>
<th>No. (%) of mutations with indicated base substitution</th>
<th>No. (%) of mutations with indicated frameshift</th>
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<td></td>
<td></td>
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<td>Transition</td>
<td>Transversion</td>
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<tr>
<td>GFP⁺⁺</td>
<td>12</td>
<td>18</td>
<td>2 (17)</td>
<td>4 (22)</td>
<td>0</td>
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<tr>
<td>GFP⁺⁻</td>
<td>7</td>
<td>28</td>
<td>5 (71)</td>
<td>3 (11)</td>
<td>9 (32)</td>
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showed similar fractions of +1 and −1 frameshifts. However, transversions comprised a third of the 28 DHR mutations but none of the 18 non-DHR mutations (P = 0.0074; Fisher’s exact test).

Transversion mutations are commonly induced by oxidative DNA damage (3, 25, 34, 35, 39). To determine whether DHR-associated hypermutation resulted from persistent oxidative stress, we examined the oxidation states of UV-C-irradiated DHR and non-DHR cells by using the fluorescent probe DHE (59). Microscopic analysis revealed no difference in oxidation state between DHR and non-DHR cells (Fig. 6), nor was a difference apparent by fluorescence-activated cell sorter analysis (data not shown). While these results indicate that persistent oxidative stress is not the underlying cause of UV-C-induced DHR/hypermutation, they do not rule out the possibility that these phenotypes result from time-limited “bursts” of oxidative stress (see Discussion).

Although the hprt mutants arose in progeny of cells exposed to UV-C, it is important to note that all mutations arose more than 5 weeks after UV-C exposure. Moreover, mutants arose after cells were treated with HAT medium to eliminate preexisting mutants (Fig. 1B). Thus, the mutations in both DHR and non-DHR cells arose spontaneously and were not due to residual UV-C damage. This conclusion is further supported by the observation that neither mutation spectrum showed a bias toward pyrimidine-rich sequences near mutation sites (see Tables S1 and S2 in the supplemental material). We also note that among mutants from DHR cells showing multiple mutations, there were a few examples of clustered mutations, but most were widely distributed along the HPRT coding sequence (Fig. 5B).

DISCUSSION

Genome instability can result in a wide variety of changes, including point mutations, short (di- or trinucleotide) repeat expansion or contraction, larger (gene-sized) repeat duplication, deletion, or inversion, and chromosome-scale changes, such as translocations and aneuploidy. In some cases, instability can be traced to defects in specific DNA repair pathways, e.g., microsatellite instability associated with defective mismatch repair (6). The “mutator phenotypes” associated with these defects have in several cases directly been linked to cancer predisposition in mouse models or humans (8, 27, 36, 65, 67). Genotoxins, including IR, UV, and DNA-reactive chemicals, directly induce DNA damage that can result in both large- and small-scale mutational changes. Less well understood, but potentially just as important for tumor initiation and perhaps more important for tumor progression, are delayed effects of radiation, including hypermutation and chromosomal instability. Huang et al. (33) recently defined DHR as a new form of delayed genetic instability induced by IR that is mechanistically distinct from delayed chromosomal instability. Thus, these phenotypes were mutually exclusive, and chromosomal instability, but not DHR, was associated with low cell viability. Here, we show that UV-C radiation also induces DHR that similarly has no effect on cell viability. IR-induced DHR is observed at doses that cause minimal cell killing and does not show a classical dose-response curve (33). UV-C, on the other hand, induces DHR at a dose giving significant cytotoxicity (~20% survival) but not at a lower dose giving 50% survival (Fig. 2 and 3), suggesting a more typical dose response. Nonetheless, the significant cell survival and the high frequency of DHR induction, which approached 15% of cells surviving 5 J/m² UV-C, suggest that DHR is not due to inactivation of one or a few specific target genes but is more likely an epigenetic effect with a target perhaps as large as the nucleus or the entire cell, as suggested for IR-induced DHR (33). DHR is not induced by acute doses of UV-B (Fig. 3B), but Dahle et al. (18) showed that UV-B does induce delayed hypermutation. Although this might suggest distinct mechanisms for induction of hypermutation by UV-B and UV-C, these results may reflect differences in study design. We measured hprt mutagenesis after screening for DHR, 40 days after UV exposure (Fig. 1B), whereas Dahle et al. (18) detected hprt hypermutation 10 days after UV exposure. In addition, the lack of a DHR response with acute UV-B exposure does not preclude the possibility of DHR induction by chronic UV-B exposure from sunlight. The UV-B and UV-C doses used in the present study required short exposures (several seconds), so repair occurring during the exposure cannot account for the difference in DHR induction. UV-C has higher energy than UV-B, and the more-acute damaging effects of UV-C may explain this difference.

Although our analysis of UV-induced DHR focused on a single cell type derived from a human tumor, it is likely to be a general effect because the various forms of delayed genomic instability have been observed in a wide variety of cell types, in animal models, and after exposures to many types of genotoxic agents (13, 16, 22, 24, 30, 52, 60, 64). In addition, the RKO-derived cells we studied express functional p53 (33), so DHR is not restricted to p53-defective cells (i.e., the majority of tumor cells). UV-induced delayed hypermutation has been observed in several studies (16, 18, 61, 62), but only one included an analysis of mutation spectra, and this was limited to agarose gel analysis of PCR-amplified hprt exons (18). In that study, delayed mutations after UV-B exposure showed a higher fraction of large-scale deletions than directly induced mutations; presumably, the directly induced mutations would show a typical “UV-signature,” with a high fraction occurring at dipyrimidines and longer pyrimidine runs (7, 66). In the present study, we did not measure directly induced mutations but focused instead on spontaneous mutations arising in DHR or non-DHR cells nearly 6 weeks after UV-C exposure and found that all DHR strains were hypermutable. However, if the enhanced mutability of DHR cells was a direct consequence of HR (i.e.,
between Alu repeats in hprt introns), one would expect the DHR mutation spectrum to show a significant fraction of exon deletions (28). Instead, all hprt mutations arising in DHR strains were point mutations, defined here as 1-bp or tandem 2-bp substitutions, deletions, and insertions. Thus, while DHR and hypermutation are associated, the extra mutations are not directly caused by HR. Instead, DHR and hypermutation probably result from a common upstream event or cellular state (see below).

Large-scale deletions and insertions, as well as point mutations, were observed in non-DHR cells, consistent with the known spectrum of spontaneous hprt mutations (11). One difference between DHR and non-DHR mutation spectra was the absence of large deletions in the DHR mutants. A study of delayed hprt mutations after IR in Chinese hamster ovary cells showed similar results, with large deletions predominant among directly induced mutants but much less frequent among delayed mutants from IR-treated cells and spontaneous mutants from untreated cells (44). An even more striking difference in the DHR and non-DHR mutation spectra was the much larger fraction of compound point mutations arising in DHR strains. Although compound mutations were observed in non-DHR cells, three of five were associated with kilobase pair deletions and included large insertions or “sequence captures.” These are reminiscent of products formed during DSB repair by nonhomologous end joining (2, 43, 45) and may therefore reflect single mutational events, albeit with complex outcomes. That three of the four large deletion mutations were related, but not identical, is further evidence that they reflect different outcomes initiated by a single DSB repair event.

If we consider only point mutations (predominant in both spectra), the frequency of mutants with compound point mutations was fourfold higher in DHR cells than in non-DHR cells (Table 1). In addition, hprt mutations from DHR cells had an average of 4 mutations, and three harbored 5, 6, and 11 mutations, whereas non-DHR cells had at most 2 point mutations. Although the high frequency of compound point mutations in DHR cells is consistent with their hypermutation phenotype, questions arise as to their origin. Specifically, are compound mutations a consequence of linked mutational events, or does each mutation (or cluster of mutations) arise independently? Assuming that these mutations result from misrepair and/or error-prone translesion synthesis at sites of DNA damage, we can envision three different scenarios for compound mutation formation. In the first, DNA damage levels are low, but repair (or translesion) synthesis by an error-prone DNA polymerase initiates at a lesion and produces errors through base misincorporation or base skipping/ addition over a long distance (Fig. 7A). However, this is not consistent with the low processivity of repair and translesion DNA polymerases (57). In the second scenario, DNA damage levels are also low, but individual mutations arise sequentially in different cell cycles (Fig. 7B). In this case, one would expect a significant fraction of shared “founder” mutations, but this was not observed. For these reasons, we favor a third model in which DNA damage levels are high in hypermutable cells, with multiple mutations arising independently during a single cell cycle due to multiple misrepair/translesion synthesis events (Fig. 7C). This model is also consistent with the widely dispersed mutations in each compound mutant. Spontaneous compound mutations are quite rare; thus, it is surprising that they appear so frequently in DHR cells. These cells show normal viability (Fig. 4B), suggesting that the genome as a whole is not susceptible to an extreme mutagenic load. Perhaps these cells are suffering from localized “bursts” of damage.

The “heavy damage” model can also account for the association of DHR and hypermutation. Besides cyclobutane pyrimidine dimers and 6-4 photoprotections, UV also induces oxidative DNA damage (37), and increased oxidative stress was observed in cells displaying UV-induced hypermutation (15). Oxidative stress creates a wide variety of DNA lesions that give rise primarily to point mutations (5), mirroring the mutation spectrum observed in the hypermutable DHR cells. For example, oxidative damage (from IR, UV, or chemical agents) induces base substitutions and single-base frameshifts and, notably, a preponderance of transversions (3, 25, 34, 35, 39), as observed with DHR cells (Table 1). This spectrum of oxidative damage-induced mutations is seen in cell and mouse models, and enhanced in the absence of the oxidative damage repair enzyme OGG1 and MTH1, is suppressed by antioxidants, and has been linked to tumorigenesis (3, 25, 34, 35, 39, 46, 58). The idea that UV-induced hypermutation results from oxidative DNA damage is further supported by the absence of a UV mutagenesis signature in the DHR/hypermutable cells (see Table S2 in the supplemental material). Importantly, oxidative DNA damage also stimulates HR (70). Together, these results indicate that DHR and delayed hypermutation reflect distinct phenotypic outcomes of a common initial state, namely, increased oxidative stress in a subpopulation of UV-exposed cells (Fig. 8). It is not yet known whether UV-A stimulates DHR. UV-A directly induces oxidative damage (37) and should stimulate HR and mutation directly but may not trigger delayed events as does UV-C. Future experiments will test whether UV-C-induced DHR and hypermutation can be prevented by pretreatment with antioxidants, as shown for delayed hypermutation induced by UV-A and UV-B (17). If UV-induced DHR results from increased oxidative stress, mitochondria may emerge as a key target in this process. Mitochondria are a major source of cellular ROS, and a signaling pathway
linking mitochondrial ROS to nuclear gene expression was recently defined (63). It is tempting to speculate that dysregulation of this or other systems that control mitochondrial ROS production or detoxification, perhaps as a result of UV-induced damage in mitochondrial or nuclear DNA, could underlie the DHR/hypermutation phenotype described here. Alternatively, transient ROS “bursts” might result from loss of integrity of individual mitochondria. It is interesting that mitochondrial DNA shows greater induction of pyrimidine (6–4) pyrimidone photoproducts by UV-C and less-efficient repair of these and cyclobutane pyrimidine dimer lesions than nuclear DNA (55). In summary, the absence of a global increase in oxidative stress in DHR cells, the high frequency of compound mutations, and normal cell viability are consistent with damage occurring in transient bursts.

DHR and hypermutation each destabilize the genome, but these processes (as well as the underlying oxidative stress) do not reduce cell viability and thus may play important roles in the initiation and progression of skin cancer. In particular, there are strong connections between oxidative DNA damage and melanoma. Melanomas from sun-exposed sites (but not unexposed sites) show high rates of a specific BRAF transversion mutation (T1799→A), producing a V600→E amino acid substitution that activates the kinase domain and is sufficient to transform NIH 3T3 cells (previously thought to be V599→E) (9, 19). This T→A transition cannot arise from UV photoproducts but is more consistent with oxidative DNA damage. A strong oxidative damage signature was also seen in N-ras mutations in congenital melanocytic nevi, which have a high probability of transformation into malignant melanoma. More than half of the identified N-ras mutations were in codon 61, and 90% of these resulted from a C→A transversion, a signature oxidative damage mutation. This N-ras mutation is also prevalent in familial melanoma associated with CDKN2 mutations (26, 54). The association of hypermutation and DHR in UV-exposed cells suggests that these forms of genomic instability may collaborate in the initiation and progression of melanoma and other skin cancers.

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