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Mutant frequencies and spectra depend on growth state and passage number in cells cultured from transgenic *lacZ*-plasmid reporter mice

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Abstract

Transgenic mice harboring the *lacZ* gene within a plasmid that can be recovered and amplified in *Escherichia coli*, to establish mutant frequencies and spectra, have provided crucial insights into the relationships between mutations, cancer and aging in vivo. Here, we use embryonic fibroblasts from transgenic *lacZ*-plasmid reporter mice to determine the relationship between cell proliferation in culture and mutations induced by ultraviolet (UV) light. A single dose of 2.5 J/m^2 of UVC to actively proliferating cells caused an approximately eight-fold increase in mutant frequency 24 h after irradiation. Identically treated quiescent cells showed a two-fold increase in mutant frequency. Thus, whereas proliferation facilitated the acquisition of mutations, it was not an absolute requirement. Characterization of the UV-induced mutations indicated that the lower mutant frequency in quiescent cells was due mainly to a reduction in point mutations; size-change mutations, indicative of translocations or deletions, were relatively unaffected by the growth state of the cells. To investigate long-term genomic stability after UVC-induced damage, we monitored the *lacZ* locus in irradiated cells passaged for many generations in culture. The results indicated the emergence of jackpot mutations of rapidly changing frequency, most likely reflecting the successive emergence and decline of dominant cell clones during long-term culture. These findings show that the *lacZ*-plasmid locus is a valid reporter for studying induced mutations in short-term cultures of both quiescent and proliferating fibroblasts. In long-term cultures, the locus is less suitable for studying induced mutations owing to the instability of the cell population.

1. Introduction

Ultraviolet (UV) light is the major cause of skin cancer and one of the most widely studied DNA damaging agents [1]. UV-irradiation is frequently used in cultured human and rodent cells as a model genotoxic agent to understand how DNA damage is processed and repaired [2]. There is an extensive literature on the types of DNA damage induced by UV, and the mechanisms by which that damage is repaired, in various cell types in culture and in vivo. Much less is known about

UV-induced mutations. To some extent, this is due to a lack of suitable assays for measuring mutant frequencies and spectra in mammalian cells, especially in cells that are not proliferating. It can be argued that quiescent cells in culture are a better model for cells in vivo because there are no tissues with a turnover rate comparable to that of most cell cultures, particularly cultured fibroblasts, the most common cell type used to study UV-induced DNA damage and repair. Most tissues in vivo, including the skin, contain a relatively small number of cells in S-phase or no proliferating cells at all. Moreover, stem cells, which are prime targets for carcinogenesis, exist mainly in a quiescent state [3].

With the advent of transgenic mouse models harboring reporter genes that can be recovered from their integrated state at one or more chromosomal sites and subsequently screened in *Escherichia coli* for mutations [4], it has become possible to study spontaneous and induced mutagenesis both in vivo and in culture [5]. One of these models, based on chromosomally integrated plasmids containing the *lacZ* reporter gene, made it possible to quantify and characterize a wide range of somatic mutations (including large genome rearrangements) at the neutral, non-expressed marker locus in various mouse organs and tissues [6], [7] and [8].

Recently, we established mouse embryonic fibroblast (MEF) cultures from *lacZ*-plasmid reporter mice carrying approximately 10 *lacZ*-plasmid copies per haploid genome on chromosomes 3 and 4. We measured spontaneous mutant frequencies and spectra in early passage cells proliferating in physiological (3%) or atmospheric (20%) oxygen concentrations. We found mutant frequencies significantly higher in cells cultured in 20% oxygen relative to cells in 3% oxygen, with G:C to T:A transversions, the signature mutations of 8-oxoguanine, being most frequent [9]. These results indicated that, at least for early passage proliferating cells, the integrated *lacZ*-plasmid reporter could be used to study the acquisition of mutations in cell cultures.

Cell proliferation is often considered important, if not essential, for the acquisition of mutations. That is, most mutations arise from errors made during replicative DNA synthesis, when the cells have very little time to repair damage accurately. Indeed, recent evidence suggests that proliferation is necessary for both repair and the acquisition of mutations when MEFs harboring the bacterial *lacI* gene in a lambda phage shuttle vector are treated with the powerful mutagen ethylnitrosourea (ENU) [10]. Here, we show that UVC by contrast induces mutations in quiescent *lacZ* reporter MEFs, albeit at a significantly lower frequency than in proliferating cells. As these UVC-damaged MEFs continued to proliferate during serial passage in culture, we observed large successive shifts in the mutant frequency and the predominance of specific mutations. These results suggest that clonal cell populations successively emerge and diminish long after the damage has occurred. Hence, *lacZ*-plasmid MEFs are useful for studying mutations induced by UVC, and possibly other mutagens, in short-term cultures, including quiescent cells. Moreover, they can be used to monitor the dynamics with which clonal genotypes emerge and decline subsequent to DNA damage, although this phenomenon interfered with the ability to monitor the frequency with which new mutations arise or are lost in the population.

2. Materials and methods

2.1. Cell isolation and culture

Mouse embryonic fibroblasts (MEFs) were isolated from day 13.5 embryos of pUR-288-*lacZ* transgenic mice, homozygous for the *lacZ* gene at either two loci (one on chromosome 3, another on chromosome 4; line 60) or one locus (on chromosome 11; line 30). Mutant frequencies at these three loci were identical [7]. We isolated MEFs as described previously [9]. Briefly, the placental membranes, amniotic sac, head and primordial blood organs were removed. The remaining carcass was rinsed with phosphate buffered saline (PBS) and minced in 2 ml PBS. Using a syringe and 18-gauge needle, the mixture was passed through a 100-μm strainer to remove large fragments, and placed in a 25-cm^2 flask containing DMEM (GIBCO), 10% (v/v) fetal bovine serum (FBS; GIBCO), 50 units/ml penicillin and 50 μg/ml streptomycin. At this stage, cells were cultured either under standard conditions, i.e., 20% oxygen, for studying UV-induced mutations in quiescent and proliferating cells, or 3% oxygen for long-term passaging. After 3 days, cells were transferred to a 75-cm^2 flask, cultured until 90% confluent and then transferred to 150-cm^2 flasks. These cells were considered passage 1 and population doubling (PD) 0. We used cells at passage 2 for short-term culture experiments.

2.2. Generation of proliferating and quiescent cells

MEFs were prepared from three individual embryos derived from line 30 mice and pooled to provide sufficient cells for the experiment. To generate proliferating cell populations, cells were seeded in six-well plates (100,000 cells/35-mm well) to measure [^{3}H]-thymidine incorporation or 10-cm dishes (600,000 cells) to measure mutant frequencies. Cells were cultured in 10% serum and the medium was replaced every 3–4 days for the duration of the experiment. To generate quiescent populations, cells were seeded as described for proliferating cells. After 24 h, the cells were washed three times with PBS and given DMEM containing 0.5% serum. The cells were maintained in this medium throughout the period of the experiment and used after 5 days. Visual inspection indicated no increase in cell number, which was confirmed by counting similarly treated cultures in six-well plates.

2.3. FACS analysis

Proliferating and quiescent cells were prepared as described above. A total of 2.5×10^5 cells were harvested by trypsinization and pelleted by centrifugation. The cells were resuspended in 400 μl of 0.1% Triton X-100 (Sigma) and 1% (v/v) RNase A (Sigma) in PBS, incubated for 10 min at room temperature, after which 400 μl of propidium iodide (100 μg/ml in PBS) was added. After vortexing briefly, the cells were incubated at 4 °C for 30 min and analysed by a fluorescence activated cell sorter (FACS; Becton Dickinson Immunocytometry Systems FACStar Plus) at the Institutional Flow Cytometry Core Facility of the University of Texas Health Science Center (San Antonio, TX, USA).

2.4. [^{3}H]-thymidine incorporation

Six hours prior to harvesting, 1 μCi [^{3}H]-TdR was added to each well of six-well plates containing proliferating or quiescent cell populations and incubation was continued at 37 °C for 6 h. The cells were washed twice with ice cold PBS and once with ice cold 5% trichloroacetic acid (TCA). After addition of 1 ml TCA, the cells were placed at 4 °C for 30 min, washed twice with PBS, and solubilized with

500 µl 0.5 M NaOH, 0.5% SDS. The solubilized cells were mixed with 10 ml scintillation cocktail plus 100 µl glacial acetic acid, and radioactivity was quantified using a scintillation counter (Beckman Instruments, CA).

2.5. Ultraviolet irradiation

Proliferating and quiescent cells were washed twice with PBS, covered with a thin layer of PBS and irradiated in lidless culture dishes using a germicidal lamp (254 nm, 15 W, General Electric, USA). Cells were then either harvested immediately and frozen for future analysis, or returned to culture. For proliferating cells, fresh medium was provided. For quiescent cells, the low-serum medium, which had been retained, was returned to the plates.

2.6. DNA isolation and mutation frequency determination

DNA was extracted from MEFs as described [9]. Briefly, cultured cells were trypsinized and collected by centrifugation at 4 °C. The cell pellet was washed with PBS and stored at −80 °C. Frozen pellets were suspended in lysis buffer (10 mM Tris–HCl, pH 8.0, 10 mM EDTA, 150 mM NaCl, 1% SDS, 0.5 mg/ml proteinase K, 120 µg/ml RNAse A) and incubated overnight at 50 °C while rotating. Genomic DNA was isolated as described using phenol:chloroform:isoamyl alcohol and chloroform extraction [9]. Mutant frequencies were determined as described [11]. Briefly, 20 µg of genomic DNA was digested with *Hin*dIII and linearized plasmids were recovered using magnetic beads pre-coated with *lacZ*/*lacI* fusion protein. After washing, plasmid DNA was eluted using isopropylthio-ß-galactoside (IPTG), circularized with T4 DNA ligase and electroporated into *E. coli* host cells. To determine the number of transformants recovered, 0.1% of the transformed bacterial cells were plated in agar containing 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal). The remaining transformants were plated in 0.3% phenyl-galactoside (p-gal) to select for cells harboring plasmids with *lacZ* mutations. Mutant frequencies were calculated as the number of mutant colonies divided by the number of recovered transformants. Each mutant frequency determination point was based on at least 300,000 recovered plasmids per culture.

2.7. Mutant classification and characterization

Mutant colonies were transferred to 96-well-round bottom plates and cultured overnight at 37 °C, in 150 µl LB medium, 25 µg/ml kanamycin and 75 µg/ml ampicillin. One µl was directly plated onto X-gal plates to screen for galactose-insensitive host cells and this background was then subtracted [12]. One microliter was added to a PCR reaction mix and the DNA amplified as described [9]. The resulting PCR product was digested with *Ava*I and size separated on a 1% agarose gel to classify mutations as no-change (those exhibiting the wild-type restriction banding pattern) or size-change plasmids (those deviating from the wild type restriction pattern). The mutant spectra were adjusted for *Hin*dIII star-activity mutants as described [13]. For each sample, at least 48 mutants were characterized. One microliter of selected mutant glycerol stocks were grown overnight at 37 °C in 6 ml LB medium and used for plasmid mini preparation (QIAprep Spin Miniprep Kit, Qiagen). Sequence reactions of purified mutant plasmids were outsourced to Davis Sequencing (Davis, CA). The chromatograms were analyzed with Sequencher (Gene Codes, Ann Arbor, MI). The primers used for the sequence reactions have been described [8].

2.8. Statistical analyses

Student's *t*-test was performed in all cases using the statistical program JMP (SAS Institutes, Inc. Cary, NC). A *p* value of <0.05 was considered significant.

3. Results

3.1. Mutation induction in proliferating versus quiescent cells

Fig. 1 shows the dose response of UV-induced mutations at the *lacZ* reporter locus in early passage (PD2) proliferating MEFs cultured at 20% O_2. The mutant frequency was clearly above background levels at the lowest UV dose tested (2.5 J/m^2), and cell survival at this dose was approximately 90%, as expected [14] and [15]. We therefore used 2.5 J/m^2 to compare UV-induced mutations between proliferating and quiescent cells cultured in 20% O_2.

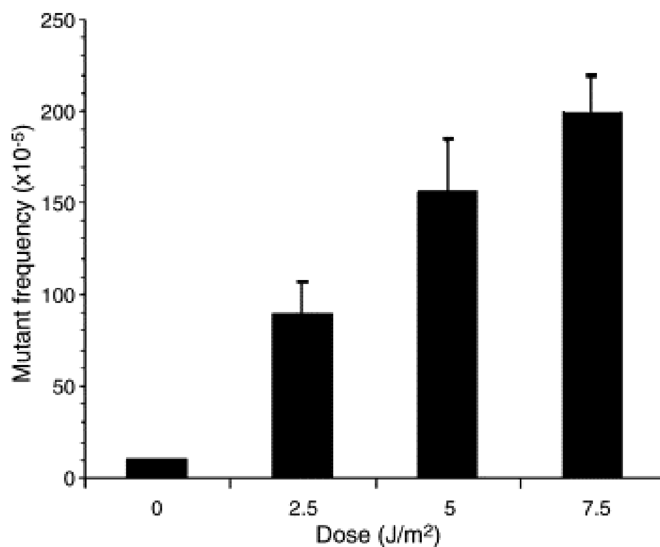

Fig. 1. Dose response curve. Mouse embryonic fibroblasts derived from transgenic *lacZ*60 mice were treated with various doses of UVC. Cells were harvested 24 h postirradiation and their mutant frequency (±S.D.) was determined.

First, we eliminated the possibility that *E. coli* might contribute to the mutant frequencies we measure after UV-irradiation. For this purpose, we irradiated *lacZ*-plasmid reporter MEFs with 2.5 J/m^2 UVC, then harvested the cells immediately thereafter, thereby preventing them from fixing mutations. If *E. coli* were to process the UV-induced lesions, thereby generating mutations in the recovered *lacZ* plasmids, we would expect to see an increase in mutant frequency after irradiation. Since this was not the case (Fig. 2; day 0), we conclude that *E. coli* does not contribute significantly to measurements of UV-induced mutations in MEFs. This observation confirms our previous results indicating that the p-gal selection system used to eliminate *lacZ*-positive transformants is only permissive for mutants with no more than 10% remaining beta-galactosidase activity [12]. If an adduct or mismatch were to be converted into a mutation by replication in *E. coli*, this would result in one wild type and one mutant copy of the plasmid in the same host cell. Assuming that both copies have an equal chance of being replicated within the host (it is a multicopy plasmid), and that all copies are transcribed with equal efficiency, the resulting mosaic of plasmids would have at

least 50% remaining beta-galactosidase activity compared to wild type. Hence, these cells will be eliminated by the selection system.

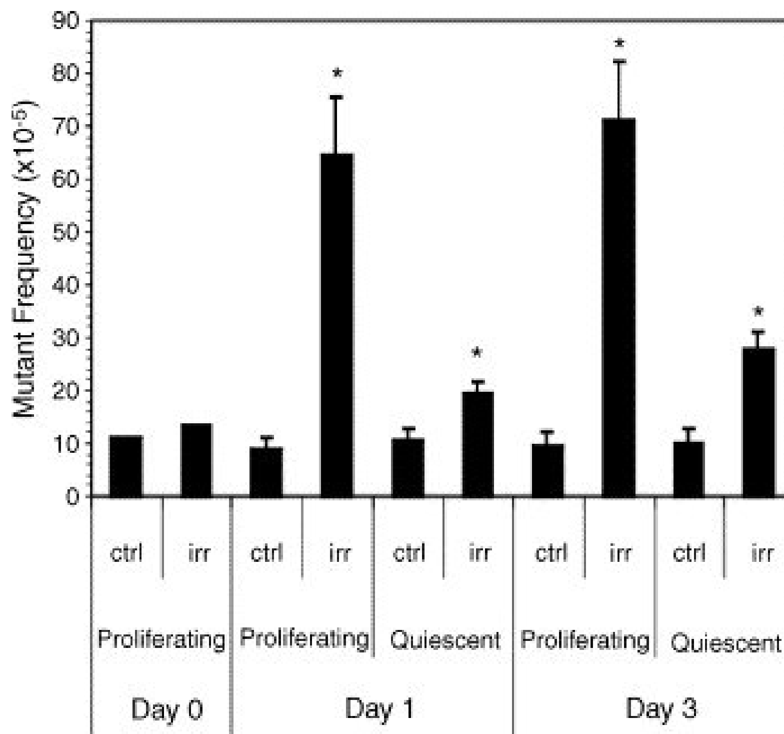

Fig. 2. Average *lacZ*-mutant frequencies (±S.D.) of proliferating and quiescent MEFs harvested 0, 1 and 3 days after treatment with an acute dose of 2.5 J/m^2 UVC radiation. Values at day 0 indicate the lack of mutations occurring as a result of artifacts due to fixation of mutations by *E. coli*. At day 1 both proliferating (p = 0.0101) and quiescent (p = 0.0077) cells displayed a significant increase in mutant frequency. Similarly, at day 3 there was a significant elevation of mutations following UV treatment in both the proliferating (p = 0.0083) and quiescent (p = 0.0014) cell populations.

To determine whether and to what extent UV-induced mutations depended on cell proliferation, we irradiated early passage proliferating or quiescent cells with 2.5 J/m^2 UVC. Unirradiated proliferating or quiescent cells served as controls. We harvested the cells at various intervals after irradiation and measured the mutant frequency. Within 24 h after irradiation, proliferating cells accumulated eight-fold more mutations than unirradiated proliferating control cells (Fig. 2). Two days later, i.e., at 3 days after irradiation, the mutant frequency was essentially identical to the frequencies observed at day 1, in both control and irradiated cells. Thus, *lacZ*-plasmid reporter MEFs acquired mutations at the expected rate and stability.

Interestingly, quiescent cells also accumulated mutations within 24 h after irradiation, albeit only 1.8-fold more than controls (unirradiated quiescent cells) (Fig. 2). Two days later, the mutant frequency was the same in unirradiated, quiescent control cells, but was slightly increased to 2.5-fold over controls in the irradiated, quiescent cells (Fig. 2). In the absence of UV irradiation, proliferation state per se had no effect on the mutant frequency (Fig. 2; compare proliferating and quiescent controls).

The UV-induced elevation of mutation frequency in quiescent cells was not associated with a significant amount of replicative DNA synthesis, since S-phase cells were virtually absent from these populations. This was demonstrated by flow cytometric analysis in three independent replicate experiments; a typical example is shown in Fig. 3. This result indicates that replicative DNA synthesis is not essential for the fixation of UVC-induced mutations. Therefore, such mutations might result from errors during DNA repair synthesis. However, some remaining replicative DNA synthesis in the quiescent cell cultures cannot be ruled out. To assess the possible contribution of DNA synthesis per se to the generation of mutations in UV-irradiated quiescent cells, we measured tritiated thymidine incorporation by cultures irradiated in parallel with the cultures used for plasmid recovery. As expected, proliferating cultures incorporated high levels of [^{3}H]-thymidine during a 6-h pulse (Fig. 4), consistent with a relatively large fraction of the population undergoing DNA synthesis. Compared to unirradiated controls, UV reduced [^{3}H]-thymidine incorporation by proliferating cells at 3 days after irradiation. Quiescent cultures incorporated much less [^{3}H]-thymidine than proliferating cultures, consistent with the majority of cells having withdrawn from the cell cycle. Nonetheless, both control and irradiated quiescent cultures incorporated low levels of the radiolabel, possibly due to DNA repair synthesis and some remaining replicative synthesis not readily detectable by flow cytometric analysis (compare Fig. 3 with Fig. 4).

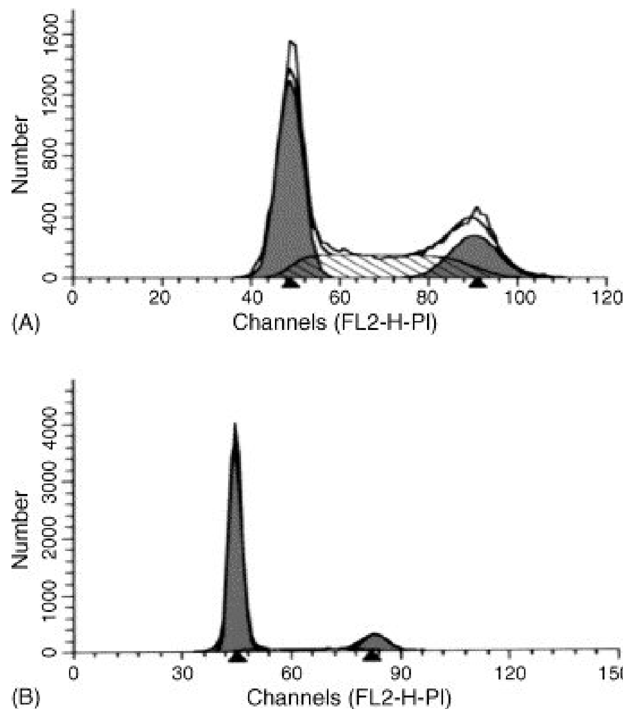

Fig. 3. Flow cytometric analysis of (A) proliferating and (B) quiescent MEFs. The small peak at G2 in the quiescent cells likely corresponds to tetraploid cells, which start to arise in MEFs early upon culture (J. Campisi, unpublished results).

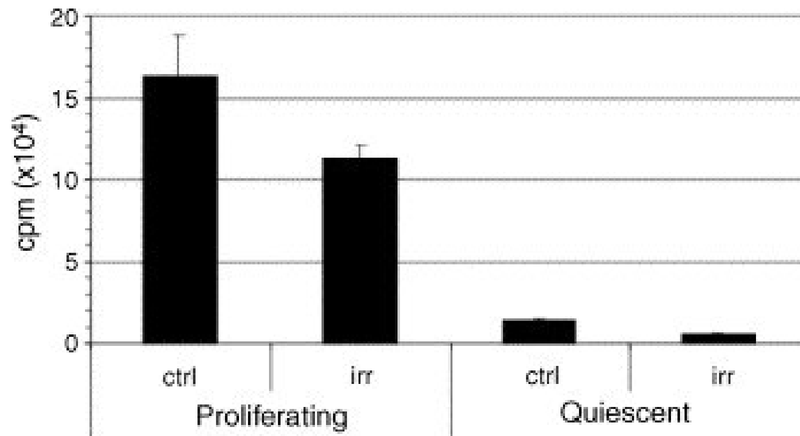

Fig. 4. [^{3}H]-thymidine incorporation in proliferating and quiescent cells harvested 3 days after treatment with 2.5 J/m^2 UVC radiation. Background levels of thymidine incorporation were subtracted.

To deduce the fraction of UV-induced mutations truly independent of DNA synthesis, we normalized mutant frequencies 3 days after irradiation for [^{3}H]-thymidine incorporation. This was done individually for replicate experiments by dividing the mutation frequency by the [^{3}H]-thymidine labeling index, as determined from the parallel cultures. If in both cultures the UV-induced mutations were dependent on replicative DNA synthesis, normalization should yield similar mutant frequencies for proliferating and quiescent cultures. However, the significantly higher normalized mutant frequencies in the quiescent population indicates that these extra mutations are independent of DNA synthesis (Fig. 5).

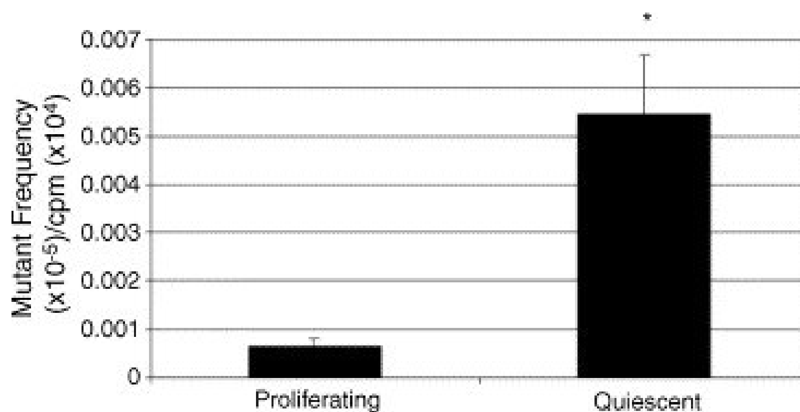

Fig. 5. Mutant frequencies at 3 days after treatment with 2.5 J/m^2 UVC radiation normalized by [^{3}H]-thymidine incorporation. A significantly higher frequency of mutations ($p = 0.0024$) was observed in the quiescent cells as compared to proliferating cells.

3.2. Mutational spectra in proliferating and quiescent cells

To further investigate the types of mutations induced by UV in quiescent and proliferating cells, we analyzed the *lacZ* mutants recovered from cells 3 days after irradiation. Plasmids that show no size change after restriction digestion (no-change mutations) are generally point mutations, while plasmids showing a size-change after digestion are generally rearrangements with one breakpoint in the

lacZ gene and the other elsewhere in the mouse genome [8]. As expected, most of the UV-induced mutations were point mutations (Fig. 6). However, some size-change mutations were also observed. Interestingly, such mutations were considerably more prevalent in the quiescent cells (34.5%) compared to proliferating cells (11.8%) (Fig. 6). At the UV dose used for these experiments, the induction of size-change mutations was significant in quiescent cells, but not significant in proliferating cells. At higher doses, UV did induce a significant increase in size-change mutations in proliferating cells (data not shown).

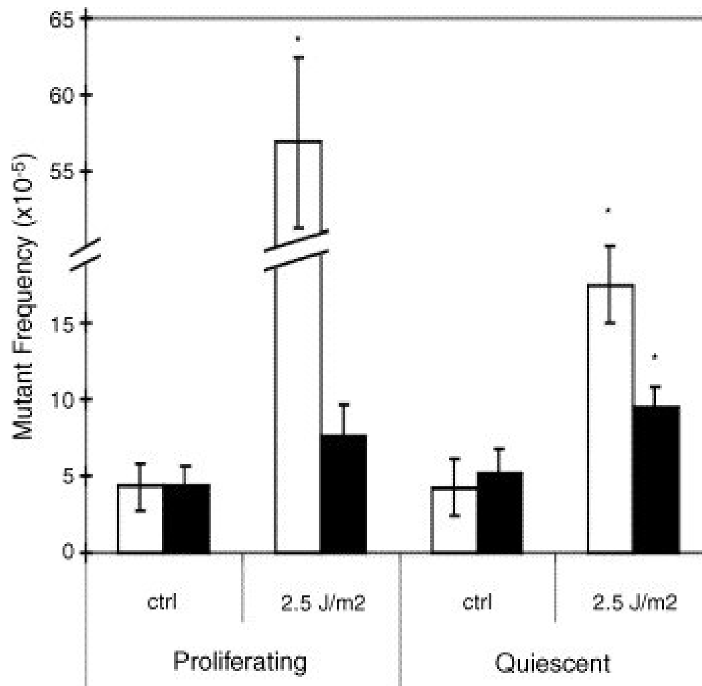

Fig. 6. Frequencies (±S.D.) of no-change mutations and size-change mutations in MEFs irradiated with 2.5 J/m^2 UVC radiation at 3 days posttreatment. White bars show the frequency of no-change mutations (point mutations, small insertions or small deletions) and the black bars show the frequency of size-change mutations (genomic rearrangements). We observed a significant (p = 0.0044) induction of no-change mutations in the proliferating cells and a significant induction of both no-change (p = 0.0034) and size-change mutations (p = 0.0054) in the quiescent cell populations 3 days following UV treatment.

To investigate the nature of the point mutations in the two cell populations, we sequenced about 20 plasmids that showed no size change after digestion. The mutations were mainly G:C to A:T basepair substitutions, with essentially no difference whether the plasmids were recovered from quiescent or proliferating cell populations (Fig. 7).

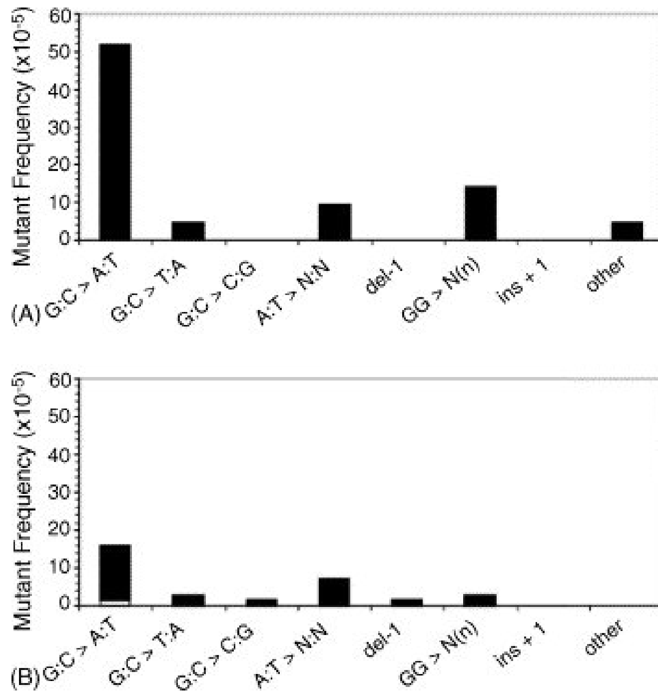

Fig. 7. Point mutational spectra of (A) proliferating and (B) quiescent cells, 24 h after treatment with 2.5 J/m^2 UVC radiation. The white areas in the G:C to A:T bars indicate the fraction of these mutations that occurred at CpG sites.

3.3. UV-induced mutation frequency and spectra after long-term passaging

To determine whether and how *lacZ* mutant frequencies and spectra change during long-term culture, we monitored MEFs during extended passaging in culture after a single dose of UV. We previously showed that standard culture conditions, which include atmospheric (20%) oxygen, caused a population doubling-dependent increase in mutant frequencies in serially passaged MEFs; this increase did not occur when MEFs were passaged at a physiological oxygen tension (3%) [9]. To eliminate scoring increases in mutant frequencies due to the stress of culture in 20% oxygen, we performed this experiment at 3% oxygen. We established and cultured MEFs from *lacZ* line 60 animals in 3% O_2 and irradiated the cells with 5 J/m^2 UVC. We then continually passaged the cells every 3–4 days and measured mutant frequencies at selected population doubling (PD) levels. Fig. 8A and B shows the cumulated increase in cell number of two independent MEF populations prior and subsequent to UV irradiation. The arrows indicate the PD at which the cultures were irradiated. Immediately after irradiation, both populations showed a temporary decline in growth lasting approximately 1 week. Thereafter, both cultures recovered and resumed their pre-irradiation growth rate.

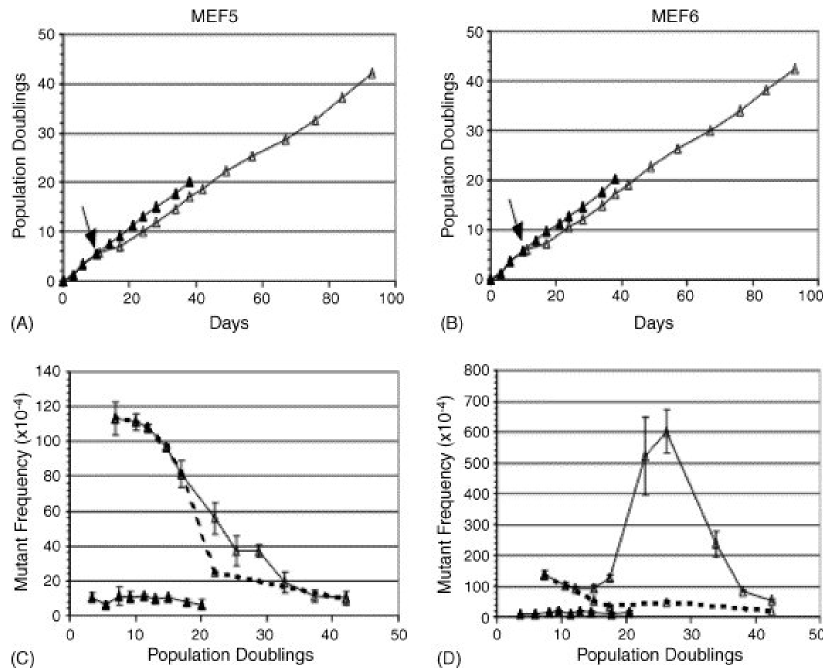

Fig. 8. (A and B) growth of MEFs from two independent embryo cultures (MEF 5 and MEF 6) at 3% oxygen both prior to and after treatment with 5 J/m^2 UVC. Arrows indicate the point at which the UV treatment was applied. (C and D) *lacZ* mutation frequencies (±S.D.) of irradiated MEF cultures as a function of population doubling level.

In one culture the mutant frequency gradually declined and approached that of unirradiated cells (solid line; Fig. 8C). The other cell population initially followed the same trend, decreasing in mutant frequency for a short period of time. Thereafter, however, the culture showed a significant increase in mutant frequency, followed by an equally substantial decline (solid line; Fig. 8D). Characterization of these mutants by restriction analysis revealed that the increase in mutant frequency was due to a "jackpot" mutation, that is, a mutation that arose and subsequently dominated the population as the mutant cells propagated. This mutation was of the size-change class and comprised the majority of the mutations at PD 27. After correction for the presence of the jackpot mutation (subtracting its contribution to the overall mutation frequency), it was apparent that the mutant frequency in the rest of the population actually declined, similar to the behavior of the other MEF culture (compare dashed lines in Fig. 8C and D).

The behavior of the jackpot mutation most likely indicates a striking example of a single cell lineage transiently dominating the population. Indeed, sequence analysis of the point mutants in MEF 5 (Fig. 8C) identified several other jackpot mutations, in this case of the no-change class (data not shown). The dashed line in this figure indicates the corrected mutant frequency after subtracting the jackpot mutations. These results indicate extensive shifts in cell lineages during serial passage of MEFs, even at 3% oxygen. Since the *lacZ*-plasmid reporter is neutral, *lacZ* mutant (and non-mutant) cells with a growth advantage can dominate the population, at least transiently. Under these conditions, the *lacZ* plasmid no longer faithfully reports the mutant frequency of the remainder of the MEF populations. Instead, it now serves as a marker for shifts in the clonal composition of the population.

4. Discussion

We previously reported an age-related accumulation of mutations in several mouse tissues. The greatest increase occurred in tissues that contained a relatively high fraction of proliferating cells, such as the small intestine [8]. There was almost no increase in the brain, which has a large proportion of postmitotic cells [7]. These findings were in keeping with the generally accepted idea that proliferation is required, or at least greatly facilitates, the induction of mutations. However, the accepted idea does not explain the age-related accumulation of mutations in organs comprised largely of postmitotic cells, such as the heart, and the relatively slow increase in mutations in proliferative organs, such as spleen and testis [8]. Of course, many factors likely contribute to mutation accumulation in vivo. However, MEFs harboring the *lacZ* plasmid reporter offer the opportunity to directly study the influence of cell proliferation on mutation induction.

We used UV radiation to induce mutations at the *lacZ* locus in proliferating or quiescent MEFs. Our results generally support the notion that replication errors during cell proliferation are the main source of mutagenesis, at least in this cell type after UV irradiation. Nevertheless, we also found that UV induced a significant number of mutations in quiescent MEF populations, albeit at a four-fold lower level than in proliferating cells. The significantly higher mutant frequency in quiescent cells compared to proliferative cells after normalization for replicative DNA synthesis, strongly suggests that at least a fraction of these mutations is independent of DNA synthesis.

Using MEFs harboring *lacI* bacteriophage lambda mutational reporters, Bielas and Heddle recently compared the ability of ENU or UV to induce mutations in proliferating and quiescent cells [15]. In apparent contrast to our findings, they found that quiescent cells do not accumulate mutations [10] and [15]. Several differences in study design may explain this ostensible discrepancy. First, Bielas and Heddle used much lower UV doses—0.125 J/m^2 compared to 2.5 J/m^2 used in our study, while inducing about the same number of mutations in proliferative cells as we do over the same time period. This may be a matter of differences in UV dosimetry, since at such low doses we cannot induce any mutations. Second, Bielas and Heddle used serum-free medium to induce cells into a quiescent state. In our hands, serum-free medium caused significant cell death within 4 days. This lethality is undoubtedly why standard protocols for arresting the proliferation of cultured MEFs use several days incubation in 0.5% serum [16], [17] and [18].

In addition, there are important differences in the mutation reporter locus used by Bielas and Heddle and us. Our *lacZ* plasmid reporter is sensitive to both no-change and size-change mutations. In contrast, the *lacI* bacteriophage lambda reporter used by Bielas and Heddle cannot detect large rearrangements due to the minimum vector size required for efficient packaging of bacteriophage lambda vectors. We found that a significant number of the mutations induced in quiescent cells were size-change mutations, the majority of which are large rearrangements [8]. Size-change mutations are generally toxic because each event can affect a large region of the genome. It is possible that actively proliferating cell populations select more strongly against such events than quiescent cells. Of note, genome rearrangements do not depend on cell replication and may result from erroneous repair of DNA double strand breaks or cross-links [19]. While UV induces mainly cyclobutane pyrimidine dimers and (6–4) photoproducts, some DNA-protein crosslinks and DNA strand breaks are known to occur [2].

Size-change mutations could be solely responsible for the UV-induced mutations that appeared to be independent of DNA synthesis in quiescent cells. However,

there is also evidence that point mutations can be independent of DNA synthesis, for example, as a result of error-prone short-patch synthesis across DNA single-strand breaks, similar to the mechanism proposed to underlie somatic hypermutation in B lymphocytes [20]. Interestingly, using an *E. coli* cell-free assay, Cohen-Fix and Livneh demonstrated the existence of a replication-independent UV mutagenesis pathway, which depended on excision repair [21]. Moreover, the mutation spectrum was similar to that associated with the replication-dependent UV mutagenesis pathway in *E. coli*, consisting mainly of G:C to A:T transition mutations, similar to our findings. Such mutations could result from closely opposed UV lesions, the removal of one of which would result in a short excision gap. Filling in such a gap would result in a mutation due to misincorporation opposite the other UV lesion. However, global genome nucleotide excision repair is deficient in rodent cells, at least for repairing pyrimidine dimers [22]. Therefore, pyrimidine dimers in quiescent MEFs could become subject to mismatch repair, which may be error prone in non-replicating cells [23].

Murine cells undergo the successive growth stages of proliferation, senescence, immortalization and eventual oncogenic transformation under standard culture conditions. However, when cultured under physiological oxygen concentrations, murine cells do not senesce, but rather proliferate indefinitely, consistent with their expression of telomerase [24]. We therefore assessed the *lacZ*-plasmid reporter during extensive passaging in physiological oxygen. To our surprise, we observed a striking variation following an acute dose of UV and extensive passage in one of two cultures. These changes in mutant frequency were due to the prevalence and subsequent decline of jackpot mutations. This finding indicates that there is extensive clonal selection of cell lineages characterized by a specific *lacZ* mutation during serial passage of MEFs, even at 3% oxygen. Thus, the *lacZ*-plasmid reporter showed that successive clonal lineages emerge during long-term culture of MEFs, despite physiological oxygen concentrations. These results also indicate that the relationship between lacZ-plasmid reporter mutations and the mutant frequency in an entire cell population as a whole needs to be verified by sequencing to rule out the possibility of clonal dominance. An obvious important question to be answered by future studies is whether clonal dominance occurs in non-neoplastic or pre-neoplastic mouse tissues in vivo or in human cells and tissues.

In conclusion, our results demonstrate the usefulness of short-term cultures of *lacZ*-plasmid-containing MEFs for studying the mechanisms by which a variety of agents and genetic modifications alter genome maintenance in mouse cells.

Acknowledgement

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