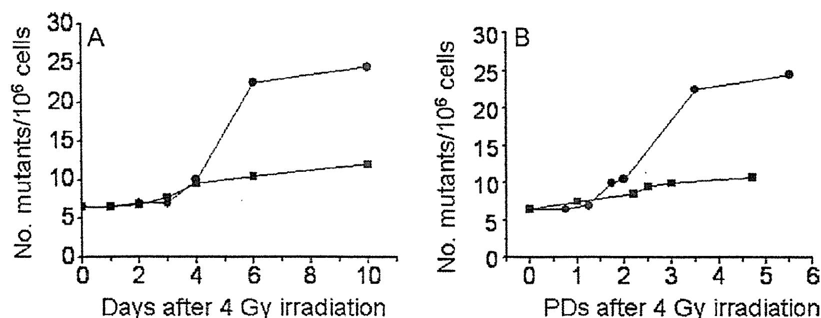

Fig. 3. Time course of mutant phenotype expression. Control and 4 Gy irradiated cells were serially cultured, and mutant frequencies were determined at the occasions of each split. ●, irradiated cells; ■, control cells. PDs: population doublings.

cells (10^7 cells) were irradiated and examined at each dose level since the radiation-related increase in the mutant frequency was expected to be much smaller. The results indicated a linear trend of dose response at a dose range of 50–400 mGy (Fig. 4C). Experiments at lower doses (below 50 mGy) were also conducted but the results were quite unstable.

3.4. Characterization of spontaneous and radiation-induced mutants

Mutants from irradiated or un-irradiated cultures were sorted using a JSAN cell sorter, and the mutant cells were grown individually in each well of 96-well microtiter plates to characterize the mutations at *HPRT* and *TetR* genes. Spontaneously arising mutants were obtained from large-scale untreated cultures of ~10^8 cells. Radiation-induced mutants were obtained from cultures of 6×10^6 cells irradiated with 4 Gy of X rays followed by a 6-day expression (the cell survival level following exposure to 4 Gy is ~20% by a colony assay in the parental cells). It was found that 10 spontaneous mutants were all normal with regard to the presence of 7 exons of *HPRT* gene and *TetR* gene (Fig. 2C left panel), and base sequencing study of four mutants showed that three bore base-change mutations and one had one-base deletion; namely, G to C mutation at base 260 (G260C), G283C, T231G, and deletion of one base at 649 (the numbers represent bases from the *TetR* start codon). All the point mutations caused amino acid substitution or premature stop. In contrast, among the mutants recovered from 4 Gy-irradiated cultures, only three out of seven retained the *HPRT* exons (Fig. 2C right panel, lanes 1, 2, and 4) and ultimately were found to carry base change mutations within the *TetR* gene (most likely originated from spontaneous events). The remaining four bore large-scale deletions encompassing 4–10 Mb including the entire *HPRT* locus as revealed by the results of multiplex PCR of *HPRT* exons (mutants 3, 5, 6, and 7) and genome walking with STS primers; specifically, mutants 3, 5, and 7 had deletions of ~4 Mb (and mutant 7 accompanied a complex rearrangement), and mutant 6 had a deletion of ~6 Mb.

3.5. Delayed effects of radiation

We examined whether the present system is able to detect radiation-induced genomic instability, which is expressed as an

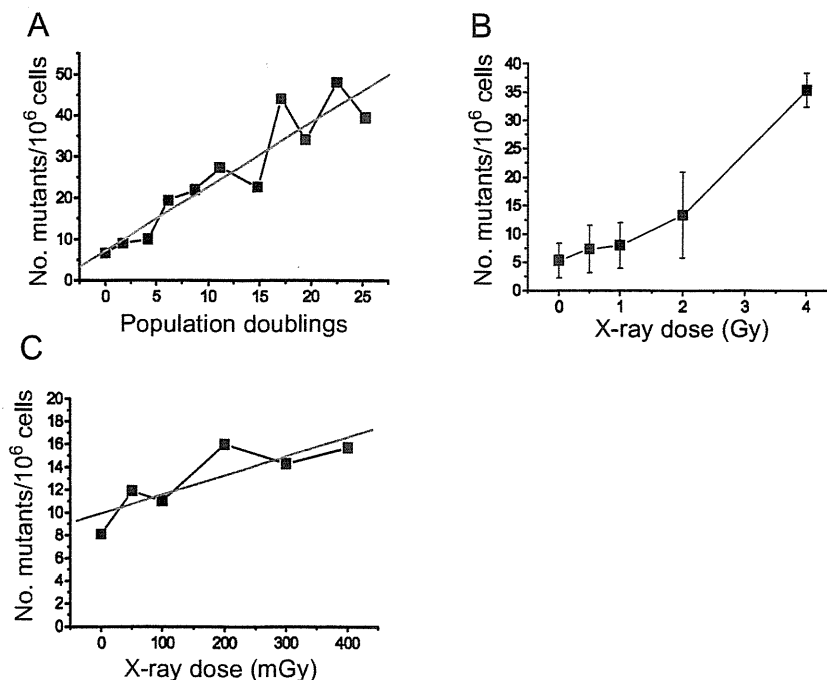

Fig. 4. Kinetics for the accumulation of spontaneous mutations, and dose response of radiation mutagenesis. (A) Mutant frequency at the time of each subculture was plotted against the number of population doublings. (B) X-ray dose response. Triplicate measurements were conducted at each dose level. (C) Radiation dose response at doses below 400 mGy.

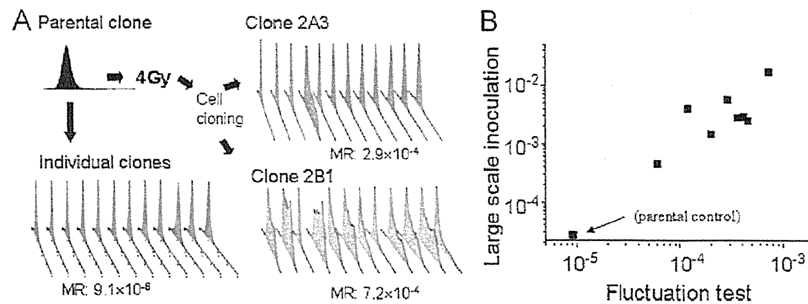

Fig. 5. Isolation of clones showing genomic instability following 4 Gy irradiation. (A) Cells were X-irradiated and 400 colonies were isolated in each well of 96-well microtiter plates. Candidate clones indicating genomic instability were screened under fluorescent microscopy. Spontaneous mutation rate was estimated either by "large-scale inoculation and short-term culture" method for the mass cultures of each clone or by the classic fluctuation test following further isolation of 12 colonies from each clone. In both cases, mutant frequency was measured by flow cytometry. The histograms shown for control cells and two clones 2A3 and 2B1, isolated after 4 Gy irradiation, indicate the frequency distribution of cells with different levels of fluorescence intensity. MR stands for the estimated mutation rate. (B) Comparison of the two mutation rates thus estimated.

enhanced mutation rate following radiation exposure. Three million cells were irradiated with 4 Gy of X rays, cultured for 6 days, and surviving cells were plated at low densities to isolate individual colonies. About 400 colonies were randomly isolated and cells from each clone were propagated in each well of 96-well plates until they reached confluence ($\sim 2 \times 10^4$/well). Then each well was examined with a fluorescent microscope to screen wells that contained more than 10 green cells as candidate clones exhibiting genomic instability. Given the spontaneous mutation rate is 2.8×10^{-6}/cell generation in the parental cells (#15-2 clone), the probability for a clonal cell population to yield 10 or more mutants among 2×10^4 cells is quite low.

In the initial screening, 22 candidate clones were isolated and mutation rates were measured using the "large-scale inoculation and short-term culture" method. Almost all the clones exhibited enhanced mutation rate except for four; namely, three had mutation rates lower than the parental ones and one was unable to be measured because of slow growth. Mutation rates showed a large variation from a relatively modest to extremely high levels; 6.0×10^{-5} to 2.0×10^{-2}/cell division. To compare the results with those by the classical fluctuation test, cells from typical candidate clones exhibiting modest to high mutation rates were inoculated at low densities and more than 10 colonies were randomly isolated and propagated to $\sim 2 \times 10^6$ cells, followed by flow-cytometric analyses to determine the mutant frequency. Whereas the fluctuation test of parental unirradiated #15-2 cells showed exactly the same mutant distribution profile in individual cell propagations, those of modestly unstable (2A3; 4×10^{-3}/cell division) and highly unstable clones (2B1; 2×10^{-2}/cell division) apparently produced abundant mutants when individual cells constituting the clone were propagated (Fig. 5A). Classical calculation (formula 2) gave rise to a mutation rate for parental cells of 9.1×10^{-6}, while 2A3 and 2B1 gave much higher rates, 2.9×10^{-4} and 7.2×10^{-4}/cell division, respectively. The results of the "large-scale inoculation and short-term culture" method were plotted against the results of fluctuation tests, which indicated a fairly good correlation between the two calculations (Fig. 5B). However, the large-scale inoculation method consistently showed higher rates than those obtained by the classic fluctuation tests, which could perhaps be partly due to a decreased colony-forming efficiency in highly unstable cells in the latter.

4. Discussion

The present assay system can detect a wide range of mutational events covering from base change mutations to megabase deletions, and does not require extensive processes and time necessary for colony formation of mutant cells (i.e., 1–2 weeks depending on the cells). That is a great advantage over the conventional mutation assays that require huge numbers of culture dishes as well as specific culture conditions. Thus the straightforward, flow cytometric analyses are promising future applications for general use. The only requirement is a 6-day post-irradiation subculture before determining the mutant frequency. Thus, the overall time for one set of experiments can be reduced from 2 to 3 weeks to one. It is unfortunate that the 6-day subculture (i.e., expression time) is still necessary, which is the same as that of the classic assay at the endogenous *HPRT* gene. It would be beneficial if the expression time could also be shortened by, say one half or 3–4 days. That is especially attractive for screening a large number of environmental chemicals for mutagenicity [25]. In that regard, dilution of the TetR proteins is the rate-limiting process and not the accumulation of GFP proteins, since only an overnight culture in the presence of tetracycline is sufficient for the full expression of GFP-positive phenotype (Fig. 2A) (tetracycline binds to the TetR protein and removes them from the TetO sequences by allosteric inhibition). Thus, to shorten the expression time, strategies to make the TetR proteins undergo faster turnover are needed; for example, genetic modification of the TetR protein to be more labile while maximally maintaining the binding capability to the TetO sequences.

In the present study, we used HT1080 cells (derived from a fibrosarcoma) as they show a stable karyotype near diploid ($2n = 46$), which we thought is best suited for gene-targeting experiments (knock-in experiments). Only a few of human cell lines, like HT1080 and HCT116, etc., are suited for gene targeting, and above all, HT1080 has been utilized for mutation assay in several studies.

However, this cell line turned out to have a spontaneous mutation rate somewhat higher than that in normal cells; namely, it was estimated as 12×10^{-6}/cell division in HT1080 cells (resistance to 6TG) [18] while the rates for normal cells are, for example, $0.45–1.8 \times 10^{-6}$/cell generation in human skin fibroblasts (resistance to 8-azaguanine) [26] or $0.055–0.24 \times 10^{-6}$ [27], or $5–10 \times 10^{-6}$ [28] in human blood lymphocytes (resistance to 6TG), although the published data varies extensively. We felt that the elevated level of spontaneous mutation rate of HT1080 cells could be related to the characteristics of its stable karyotype; namely, chromosomal instability (CIN) and mismatch-repair deficiency leading to microsatellite instability (MIN) are often mutually exclusive (but not necessarily so) in colorectal cancers, and HT1080 cells are at least non-CIN and normal regarding the p53 gene status [29]. However, we did not find any indication that the cells are MIN; namely, the spectrum of base-change mutations at the *TetR* gene did not indicate any clustering at microsatellite or repeat sequences. In any event, to maintain the spontaneous mutation rate low in the control culture, it would probably be beneficial, for example, to use cells of

non-tumor origin and expressing *hTERT* gene so that natural aging processes may be avoided.

It has long been known that radiation-induced mutation rates varied considerably among genes. For example, among the 7 loci examined by Russell et al., *s* gene mutated much more frequently than other genes and the difference was about 20-fold when compared with the frequency at *a* gene, which was the lowest [30]. Since a positive correlation has been seen between the spontaneous and radiation-induced mutation rates in mouse gene loci, it was once imagined that the difference could be partly due to physical gene size; that is, the larger the gene size, the higher both spontaneous and radiation-induced mutation rates would be. However, following genome sequencing studies, gene size turned out not to matter any longer; for example, *s* and *a* genes consist of 7 and 4 exons and span about 29 kb and 37 kb, respectively. Alternatively, since radiation-induced mutations often accompany large deletions, the presence of vital gene(s) near the marker gene may also be a factor to be considered, while only a limited set of information is thus far available (some papers by Russell's group). Other factors, such as different chromatin conformation, can be part of the reason, but there is practically no relevant information available with regard to radiation mutagenesis. To pursue the issue, *TetR* gene can be inserted at any locus in the genome of cultured cells as the reporter of mutations provided that the locus is stably expressed under the normal culture conditions.

Finally, if the present system is realized in an *in vivo* mouse system, it would become possible to obtain three sets of data simultaneously; namely, quantitative data necessary for evaluation of mutagenicity *in vivo*, tissue dependence of the activity, and carcinogenicity, which may help better understanding the role of somatic mutations in carcinogenesis. There are two major hurdles to be cleared, however; one is identifying the effective promoter that may work on different tissues, and the other is choosing the sites for single integrations of each of *TetR* and *TetO/FGP* genes into the genome.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mrgentox.2010.12.010

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