

Alpha particle mutagenesis of human lymphoblastoid cell lines

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Abstract. Despite being derived from the same donor, the human lymphoblastoid cell lines WTK1 and TK6 have markedly different responses to low LET radiation. We originally observed that WTK1 was more resistant to the cytotoxic effects of X-irradiation, but significantly more sensitive to mutation induction at both the TK and HPRT loci. In an effort to better understand these properties, we have examined the effects of α -particles on these cells. Relative to TK6, WTK1 has enhanced survival and mutation after both X-ray and α -particle exposure. While the HPRT locus was significantly more mutable in WTK1 as a function of α -particle versus X-ray dose, the TK locus was only slightly more sensitive to α -particle mutagenesis. In addition, the slowly growing TK mutants that constitute the majority of X-ray-induced TK mutants of TK6 were recovered in lower proportions following α -particle exposures. This is consistent with the further finding that in both cell lines, loss of heterozygosity occurred in a smaller fraction of α -induced TK mutants than X-ray-induced mutants. These results are consistent with our previous model suggesting that WTK1 has an error-prone repair pathway that is either missing or deficient in TK6, and further suggest that this pathway may be involved in the processing of α -particle-induced damage.

1. Introduction

The threat of domestic exposures to radon in many homes and from drinking water has raised concern over the health effects of alpha particles. Radon and its daughters decay predominantly by emission of α -particles, and radon exposure has been linked to the causation of lung cancers (National Research Council 1988, Samet 1989), and leukaemias (Humphreys et al. 1985, Forman et al. 1987, Henshaw et al. 1990). There does not appear to be a simple relationship between linear energy transfer (LET) and biological endpoints (Goodhead et al. 1992), thereby necessitating the study of specific particles that may have relevance to human exposures and disease.

Equal doses of X-ray and alpha-particle radiation seem to produce similar initial yields of double

strand break (dsb) damage in cells (Frankenberg et al. 1981), but these breaks appear to have different biological effects (Jenner et al. 1992, Loucas and Geard 1994). The greater density of ionizations from high LET radiation is thought to result in more complex clustered damage within the DNA which is less likely to be repaired by the cells (Goodhead 1990). Studies of the extent of dsb rejoining following high LET irradiations show a trend toward increasing numbers of unrepaired breaks at higher LET (Cole et al. 1975, Weber and Flentje 1993).

Both rodent (Raju et al. 1991) and human (Chen et al. 1984) cells in culture have been shown to be more sensitive to killing by α -particles in comparison to low LET X-rays. Alpha-particles also induced higher levels of mutation at both the HPRT and TK loci (Thacker et al. 1982, Chen et al. 1984, Schwartz et al. 1991). The effects of cellular deficiencies in DNA repair have been explored in a few studies. Shadley et al. (1991) found the *xrs5* mutant of CHO to be more susceptible to both α -particle induced killing and HPRT mutant induction than CHO. However, a dsb repair deficient mutant of mouse lymphoma cells had a similar D_0 , but less mutation induction at the TK locus in response to α -particle irradiation (Evans et al. 1993a).

The human lymphoblastoid cell line TK6 has been used extensively in mutation studies. Many reports on the chemical and X-ray induction of mutations at the hypoxanthine-guanine phosphoribosyl transferase (HPRT) (Grososky and Little 1985, Amundson and Liber 1991), thymidine kinase (TK) (Liber and Thilly 1982, Liber et al. 1989, Amundson and Liber 1991), and other loci have contributed to the large body of data on these cells. Like TK6, WTK1 is a TK heterozygote derived from the same original WI-L2 isolate. Although closely related and similar in growth characteristics, WTK1 is considerably more resistant to the cytotoxic effects of X-rays. However, WTK1 is also more sensitive to the mutagenic effects of this agent both per unit dose and at equitoxic doses at both the TK and HPRT loci (Amundson et al. 1993). We have suggested that the different

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responses of these cell lines may result from a higher capacity of WTK1 to catalyze recombination as part of an error prone repair mechanism. This hypothesis is supported by molecular analysis of X-ray-induced TK mutants and the results of an *in vitro* plasmid recombination assay (Xia et al. 1994). The presence of a homozygous mutation in the p53 gene of WTK1 but not TK6 (Xia et al. 1995) may also affect radiation survival and mutation induction in these cell lines. TK6 is also deficient in double strand break repair (Evans et al. 1993b). More recently WTK1 has been shown to have a higher capacity than TK6 for rejoining X-ray-induced double-strand breaks (M. Story, personal communication, and H. Evans, personal communication).

The effects of high LET irradiation of TK6 have previously been examined by the incorporation of ^{125}I (Liber et al. 1983, Whaley et al. 1990), bombardment with ^{28}Si and ^{40}Ar ions (Kronenberg and Little 1989a), neutrons (Kronenberg and Little 1989b), radon (Bao et al. 1995, Chaudhry et al. 1996) and chelated ^{212}Bi in solution (Metting et al. 1992). Although no molecular analysis of TK mutants was presented by Metting et al. (1992) they reported that α -particle exposure from ^{212}Bi did not induce true slow growth mutants. This was of interest as our previous data indicated that the reduction in stable slow growth mutants seen in WTK1 was associated with the greater participation of recombination in mutagenesis in this cell line (Xia et al. 1994). WTK1 and TK6 give us a unique system for the comparison of the effects of DNA damaging agents on human cells with different capacities for recombination and dsb repair. We have irradiated these two cell lines with α -particles and compared the survival and induced mutation at the HPRT and TK loci with that induced by X-irradiation.

2. Materials and methods

2.1. Cell lines

The human lymphoblast cell lines used in this study were both derived from WI-L2, a nonclonal culture isolated from a human spleen by Levy et al. (1968). TK6 (ATCC CRL-8015) was derived from an unselected subclone of WI-L2 following treatment with ICR-191 and selection for TK heterozygosity (Skopek et al. 1978). WTK1, also a TK heterozygote, was selected from WI-L2-NS (ATCC CRL-8155), a different subclone of WI-L2 (Benjamin et al. 1991). The different responses of these cell lines to treatment with X-rays and

chemical mutagens have been described previously (Amundson et al. 1993).

Cells were maintained as exponentially growing cultures in RPMI 1640 medium supplemented with 10% horse serum (heat treated for 2 h at 56°C). Penicillin (100 U/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$) were added to the medium for some experiments. The cultures were incubated at 37°C in 5% CO_2 and 100% humidity and maintained at densities of $1\text{--}12 \times 10^5$ cells/ml.

2.2. Irradiations

Prior to the start of mutation experiments, CHAT (deoxycytidine, hypoxanthine, aminopterin, and thymidine) treatment of cultures was carried out as previously described (Liber and Thilly 1982). The alpha particle source used for these experiments has been described in detail elsewhere (Inkret et al. 1990), and consists of a thin layer of ^{238}Pu electrodeposited onto a stainless steel disc. The beam passes through an aluminium collimator, and exposure times are controlled with a photographic shutter. At the cell mylar interface the beam has a mean energy of 3.5 MeV and LET of $116 \text{keV} \mu\text{m}^{-1}$. The dose rate to the cells was approximately 0.037Gys^{-1} , with a fluence of $1840 \text{particles}/\text{mm}^2 \text{s}^{-1}$. The gamma component of the dose delivered by this source is less than 0.1% of the total dose (Eisen et al. 1991).

Lymphoblasts growing in suspension culture were pelleted by centrifugation and pipetted directly onto the $1.5 \mu\text{m}$ thick mylar-bottomed dishes especially constructed for use with this alpha source. The cells were then covered with a sterile glass coverslip to force them into a 'monolayer' on the mylar. Although the absolute 'monolayer' state of these cells cannot be stated unequivocally, microscopic examination indicated no appreciable stacking of cells above the mylar. A similar technique has been described for irradiating bone marrow cells (Lorimore et al. 1993).

2.3. Mutants

Immediately after irradiation, cultures were plated for survival in 96-well microtiter plates at between 1 and 100 cells/well. Daily post-irradiation platings for mutant fraction determination were done to determine expression time for the two loci. Following α -particle irradiation, expression of mutant phenotypes was the same as previously determined following X-rays (3 days for

TK, 6–7 days for HPRT). After allowing the appropriate expression times, cells were plated in 1 $\mu\text{g}/\text{ml}$ 6-thioguanine to select for HPRT mutants, or 2.0 $\mu\text{g}/\text{ml}$ trifluorothymidine to select for TK mutants. Plates were incubated and scored for colony formation after 11 days, at which time fresh trifluorothymidine was added to the TK plates in order to score for late appearing colonies. Mutant fractions were calculated using the method of Furth et al. (1981).

TK mutants were cloned from independent cultures of TK6 exposed to 0.95, 1.14, or 1.52 Gy α -particles. TK mutants of WTK1 were cloned from cultures exposed to 1.14 or 1.52 Gy α -particles. These doses were chosen for the best recovery of induced mutants to minimize the proportion of spontaneous mutants analysed in each set, while still allowing a reasonable number of mutants to survive the inducing exposure. Approximately 91% of the WTK1 mutants and 83% of the TK6 mutants were induced by the α -particle exposure. In some cases more than one WTK1 mutant was kept from a single culture, because the large number of initial mutants surviving treatment (several thousands per culture) meant the probability of selecting sibling mutants would be extremely small (on the order of 1/2500). Independent spontaneous mutants were isolated from individual cultures started from 2500 cells. Phenotypes were confirmed by seeding mutants in 24 well plates in 2 ml medium with the appropriate concentrations of CHAT or TFT, and scoring for growth after several days. Doubling times were determined from daily cell counts of exponentially growing cultures.

2.4. Denaturing gradient gel electrophoresis

TK6 and WTK1 are TK heterozygotes due to single base insertions in different positions of the same TK allele in the two cell lines. Both cell lines have an inactivating frameshift mutation in exon 4, but their location at different positions (4866 bp in TK6 and 4851 bp in WTK1) allow us to distinguish both mutant alleles and the wildtype allele with a PCR/DGGE strategy. Genomic DNA was extracted from mutant and wildtype cells using a simple salting out method (Miller et al. 1988). Exon 4 fragments were then PCR amplified by two separate reactions that included a first round amplification with the following primers: 5'-GAACACTGAGCCTGCTTTGCA-3' (upstream) and 5'-CACTATGACAGGGAACTAGA-3' (downstream). This was followed by a nested PCR reaction using the upstream primer: 5'-TGGCTTT

CTCTTCCCAGGAAC-3' and a primer containing 20 bp homology to a downstream region of exon 4 plus 42 basepairs of an artificial high melting 'clamp': 5'-CGCCCGCCGCGCCCCGCGCCCGTC CCGCCGCCCGCCCGCGCCTCGTTCGATGCC TATGACA-3' (see Myers et al. 1985). The 50 μl PCR mixtures contained 2.75 mM MgCl_2 , 60 mM KCl, 15 mM Tris (pH 8.8), 2.5 μM deoxynucleotide triphosphates, 0.6 μM each primer, 0.02 units/ μl Taq polymerase (Cetus) with 5 μl diluted template DNA for round one amplifications, or 1 μl round one product for round two. One round of PCR amplification consisted of 32 cycles of 15 s melting at 94 $^\circ\text{C}$, 15 s annealing at 55 $^\circ\text{C}$, and 20 s synthesis at 72 $^\circ\text{C}$. These amplifications were carried out in a Perkin Elmer GeneAmp 9600 PCR System.

Fifteen μl of the second-round PCR products were mixed with loading buffer (0.25% bromophenol blue and 80% glycerol) heated to 95 $^\circ\text{C}$ for 10 min, then allowed to reanneal at room temperature. Samples were run for 8 h on a 12% polyacrylamide gel with a 0–60% denaturing gradient (100% denaturant is 7 M urea/40% formamide) at 150 V submerged in 60 $^\circ\text{C}$ TAE buffer (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA) as described previously (Okinaka et al. 1993). Gels were then soaked for 30 min in distilled water, stained with ethidium bromide, and photographed on a UV transilluminator with a Polaroid camera.

3. Results

Survival dose-response curves (Figure 1)

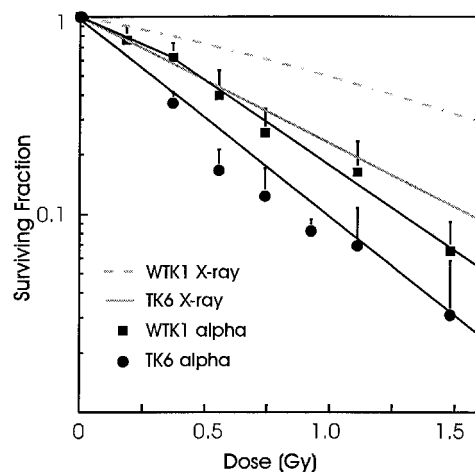


Figure 1. Survival of TK6 and WTK1 cells following exposure to α -particles. Each point is the average of 3–9 independent experiments and error bars are standard errors of the means. For comparison, X-ray data are shown with dashed lines only, from Amundson et al. (1993).

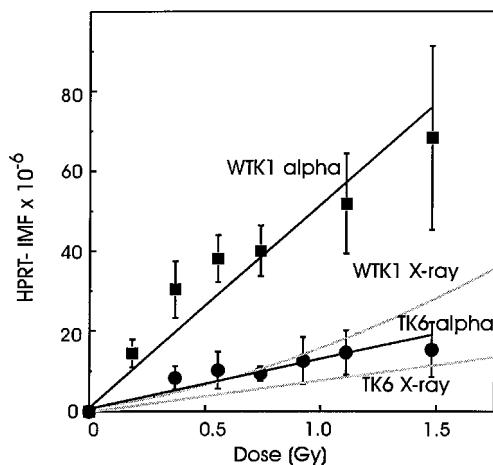


Figure 2. Mutation induction at the HPRT locus in TK6 and WTK1 cells by α -particles. Each point is the average of 3–7 independent experiments and error bars are standard errors of the means. The lines for α -particle-induced mutation were fitted by linear regression. X-ray data from Amundson et al. (1993) are shown as dashed lines only. The background mutant fractions averaged 2.6×10^{-6} in TK6 and 6.1×10^{-6} in WTK1, and have been subtracted.

demonstrated that alpha particles were slightly more cytotoxic than X-rays in both TK6 and WTK1. The RBE (relative biological efficiency) for killing by α -particles was approximately 1.6 in WTK1 and 1.8 in TK6, based on a comparison of

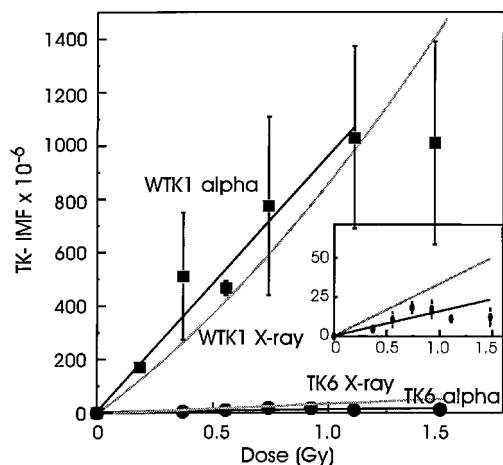


Figure 3. Mutation induction at the TK locus in TK6 and WTK1 cells by α -particles. Each point is the average of 3–7 independent experiments and error bars are standard errors of the means. The lines for α -particle-induced mutation were fitted by linear regression. X-ray data from Amundson et al. (1993) are shown as dashed lines only for comparison. The background mutant fractions averaged 2.9×10^{-6} in TK6 and 103×10^{-6} in WTK1, and have been subtracted. The insert shows the TK6 data on an enlarged vertical scale.

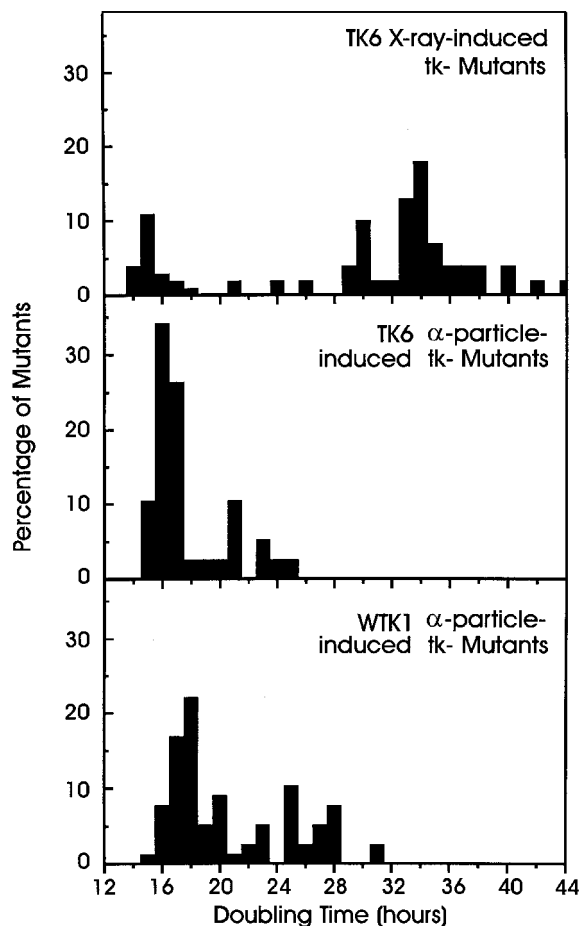


Figure 4. Distributions of doubling times of TK-mutants (X-ray data from Amundson et al. (1993)). As roughly equal numbers of early and late mutants were analysed in each category, percentages have been weighted to reflect the frequency of each class of mutant in the total mutant fractions. Doubling times were determined for 54 α -induced mutants of WTK1 and 52 α -induced mutants of TK6.

D_0 values. WTK1 ($D_0 \approx 0.57$ Gy) also displayed enhanced survival compared to that observed for TK6 ($D_0 \approx 0.37$ Gy) following α -particle irradiation.

Alpha particles induced four- to fivefold more HPRT mutants per Gy in WTK1 than did X-rays (Figure 2). On the other hand, in TK6 there did not appear to be a statistically significant difference between the dose response curves for the induction of HPRT mutants by α -particles compared with X-rays ($0.01 > p > 0.001$). The consequence of these observations is that the HPRT locus is approximately fourfold more sensitive to mutation induction in WTK1 than in TK6 following exposure to α -particles.

Dose-response comparisons between X-ray- and α -particle-induced mutations at the autosomal TK locus are illustrated in Figure 3. WTK1

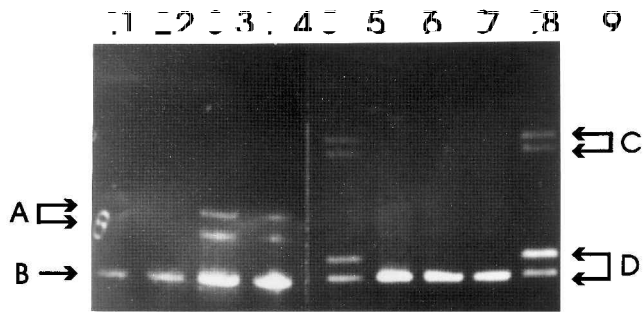


Figure 5. Representative DGGE gel showing wildtype and LOH patterns in exon 4 of both TK6 and WTK1. Lanes 1–3 TK6 mutants, lane 4 TK6, lanes 5–8 WTK1 mutants, lane 9 WTK1. The letters A and C mark the heteroduplex bands, while B and D mark the homoduplexes. When heterozygosity has been lost, no heteroduplex bands can form, and the one remaining allele forms a single homoduplex band.

is considerably more sensitive (> 50-fold) to mutation induction by α -particles than is TK6. The difference in response between the two cell lines is enhanced by the fact that TK6 is approximately threefold less sensitive to α -particle-induced mutation versus that induced by X-rays at the TK locus.

Previous studies have indicated that the majority of X-ray and spontaneous TK mutants of TK6 have a stable slow growth rate in culture. Although 80% of TK mutants of WTK1 also appear only after 18 days of incubation, less than half of these exhibit a stable slow growth phenotype (Amundson et al. 1993). Doubling time distributions of TK mutants (Figure 4) suggest that there is a striking difference between the kinds of mutants induced by X-rays versus those induced by α -particles. Slowly-growing mutants are not a major component of α -particle-induced TK mutants recoverable by this protocol in either TK6 or WTK1.

We used denaturing gradient gel electrophoresis (DGGE) of PCR (polymerase chain reaction) products to distinguish the active and inactive TK alleles and to screen mutants for the loss of heterozygosity (LOH). TK6 and WTK1 have inactivating mutations in exon 4 of one allele of the TK gene. The exon 4 alleles can be amplified with the same primer sets, and the mutant and wildtype alleles of both cell lines can be resolved as heteroduplex molecules under the same DGGE conditions. Figure 5 illustrates that the exons 4 of TK6 and WTK1 produce distinct DGGE patterns that allow us to distinguish the heterozygous status of each mutant. Although the homoduplex bands of TK6 did not resolve clearly, the mutant/wildtype and wildtype/mutant heteroduplexes of both wildtype cell lines could be distinguished easily. Mutants

that had retained the active allele displayed the wildtype pattern of both homoduplex bands and two heteroduplex bands. The loss of both heteroduplexes and the wildtype homoduplex band can be seen clearly in the LOH mutants (Figure 5). The majority of all mutants had lost heterozygosity at the TK locus. In both cell lines, α -particles induced a lower overall proportion of recoverable LOH mutants than did X-rays, and approximately the same proportion as seen among the spontaneous clones (Table 1).

4. Discussion

We have irradiated human lymphoblastoid cells with α -particles using mylar bottomed dishes and a well defined ^{238}Pu source. Our irradiation protocol was similar to one previously established (Lorimore et al. 1993). Both TK6 and WTK1 were more sensitive to killing by α -particles compared with X-rays. However, the increased cytotoxicity of α -particles was not as great as that reported for human fibroblasts (Chen et al. 1984). This result may be related to the heightened sensitivity of lymphoid cells to X-rays compared with cells from other tissues (Hendry 1985, Cronkite et al. 1987).

TK6 cells were clearly more sensitive to killing by α -particles than were WTK1 cells. WTK1 has a homozygous mutation in the p53 gene not present in TK6 and this has been associated with a delay in X-ray-induced apoptosis (Xia et al. 1995). Although apoptosis did reach the same levels in both cell lines, the different kinetics may contribute to the apparently higher survival in WTK1 following both X- and α -irradiation. In addition, TK6 has been shown to be DNA double strand break (dsb) rejoining deficient (Evans et al. 1993), whilst WTK1 has normal levels of dsb rejoining (H. Evans, personal communication, M. Story, personal communication). Results of a plasmid based recombination assay also indicate WTK1 is more proficient at both homologous and nonhomologous recombination than TK6 (Xia et al. 1994).

Xrs-5, a double strand break repair deficient line, has also been reported to have lower survival than its parent CHO-K1 following exposure to ^{212}Bi (Shadley et al. 1991) and ^{238}Pu α -particles (Thacker and Stretch 1985). Unlike TK6 and WTK1, the responses of four repair deficient xrs rodent cell lines were not found to differ between α -particle and X-ray exposures (Thacker and Stretch 1985). On the other hand, Evans et al. (1993) reported very similar dose response curves after ^{222}Rn α -particle exposure of two mouse

Table 1. Per cent of mutants that had lost heterozygosity at the TK locus.

	TK6	WTK1
Spontaneous	72% (46)	78% (46)
Induced: α -particle	71% (41)	92% (54)
X-ray	84%*	100%†

As roughly equal numbers of early and late mutants were analysed in each category, percentages have been weighted to reflect the frequency of each class of mutant in the total mutant fractions. The number of each type of mutant analysed in this study is given in parenthesis.

*Data from Amundson and Liber (1991).

†Data from Amundson et al. (1993).

L5178Y cell lines with different repair capacities and different X-ray sensitivities (Alexander and Mikulski 1961). These results suggest that some repair of α -particle damage is possible, but the mechanisms involved may differ from those of X-ray damage repair.

Compared with X-rays, α -particles induced only a slight increase of mutations/unit dose at the HPRT locus in TK6 cells ($p < 0.01$), but a highly significant increase ($p \ll 0.001$) in WTK1 at the same locus. The results are consistent with those of radon exposure of TK6 (Bao et al. 1995), but contrast with a ^{212}Bi study that reported an RBE of 3.8 for the HPRT locus in TK6 (Metting et al. 1992). Although mutation induction at HPRT varies widely, in most cell lines α -particles are significantly more mutagenic than X-rays (e.g. Thacker et al. 1982, Shadley et al. 1991). Many factors, including cellular repair status and tissue of origin, are likely to contribute to the variations observed.

In contrast with the response at the HPRT locus, the TK locus in WTK1 was not more sensitive to mutation induction by α -particle irradiation compared to X-irradiation (Figure 3, $p \ll 0.001$). The mutant yield was so reduced that there was no difference between the α -induced TK and HPRT mutant fractions in TK6 ($p > 0.2$). This is in marked contrast to the significantly different response of these two loci to X-rays ($p \ll 0.001$). Metting et al. (1992) also found lower induction of TK mutants in TK6 with ^{212}Bi than with X-rays, while Chaudhry et al. (1996) reported higher induction of TK mutants in TK6 by radon compared with X-rays.

The apparent differences in sensitivity to mutation between the TK locus and the HPRT locus following exposure to low LET radiation is due largely to the recovery of a class of stable slowly growing TK mutants. We previously demonstrated that 80% of X-ray-induced TK mutants of TK6

were 'late-appearing' (not visible until day 18) and had stable doubling times > 20 h (Liber et al. 1989, Amundson and Liber 1991). In contrast, although nearly half of the α -particle induced TK mutants from both TK6 and WTK1 were 'late-appearing', measurement of doubling times of mutants revealed that neither cell line produces a clear class of stable slow-growth mutants such as that observed among X-ray induced mutants of TK6 (Figure 4). This is consistent with results of ^{212}Bi irradiation of TK6 where approximately 2/3 of the TK mutants appeared late on the plates, but none of the mutants tested had doubling times greater than 20 h (Metting et al. 1992). More recently, Chaudhry et al. (1996) reported an elevation of TK mutants in TK6 following radon exposure in comparison with X-rays. These results were due to the recovery of a new class of very slowly growing mutants detected on soft agar plates. The relationship between this class of slow-growth mutants and those identified by the standard microtiter plate assay is not yet clear.

Previous studies with both rodent and human cells have associated slow growth TK mutants with large scale loss of heterozygosity (LOH) (Hozier et al. 1981, Moore et al. 1985, Little et al. 1987, Amundson and Liber 1991). The 'very slow' mutants of Chaudhry et al. (1996) were also associated with larger scale LOH. Our relative lack of stable slow growth mutants may reflect a reduced capacity of these cells to repair very large or complex lesions under the growth conditions used.

The reduced proportions of slow-growth TK mutants recovered from our α -particle exposures indicated a possible lower frequency of LOH among these mutants. Screening TK mutants for LOH revealed that in contrast to the 100% LOH induced by X-rays, both spontaneous and α -particle-induced mutants of WTK1 include smaller scale events not resulting in LOH (Table 1). The proportion of spontaneous WTK1 mutants with LOH is similar to the proportion of spontaneous LOH in TK6 mutants. Among α -particle-induced mutants, however, considerably more WTK1 mutants had LOH at the TK locus compared with TK6 mutants.

Alpha-irradiation resulted in fewer recoverable LOH mutants in both cell lines than did X-rays. These results are consistent with the observed decrease in stable slow growth TK mutants. Alpha-particles have previously been shown to induce point mutations in plasmid shuttle vectors (Jaberboansari et al. 1991, Lutze et al. 1993) and mammalian cells (Jostes et al. 1994, Bao et al.

1995, Chaudhry et al. 1996). It is likely that a subset of α -particle-induced damage is too extensive to be repaired as recoverable mutants under our plating conditions, thus biasing mutant recovery in this system slightly toward point mutations. This effect is more pronounced in the repair deficient TK6 line than in WTK1, possibly implicating DNA repair pathways requiring recombination in the processing of α -particle-induced damage.

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