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THE PKZ1 RECOMBINATION MUTATION ASSAY: A SENSITIVE ASSAY FOR LOW DOSE STUDIES

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□ The majority of mutation studies are performed at high doses of DNA damaging agents due to the insensitivity of most mutation assays. Extrapolation using a linear no-threshold (LNT) dose response model is then used to estimate the extent of possible DNA damage at lower doses. There is increasing evidence to suggest that the LNT model may not be correct at low doses of at least some DNA damaging agents. The pKZ1 *in vivo* and *in vitro* recombination assays have proven to be very sensitive for detection of changes in chromosomal inversion in lymphoid tissue in response to low doses of DNA damaging agents. Non-linear dose response curves for chromosomal inversion as an end-point have been identified at low doses of DNA damaging agents using this assay. Here, we review the inversion results obtained to date with the pKZ1 assays and discuss their suitability for low dose studies.

Keywords: chromosomal inversion, recombination, low dose, non-linear dose response

INTRODUCTION

Nearly all laboratory studies of mutagens and carcinogens involve high levels of exposure, whereas the importance of mutagens and carcinogens for human populations involves the exposure of relatively large numbers of individuals to relatively low doses of the specific agent. However, the majority of mutation assays are only capable of detecting mutations at high doses of agents. Therefore, at present, safe levels of environmental and occupational agents are determined largely by extrapolation of data obtained using high doses of agents rather than from true measurements. Extrapolation generally uses the linear no-threshold (LNT) model, which is based on the conventional paradigm that an agent which produces a harmful effect at a high dose will produce a similarly harmful effect at low doses. There is a need for assays that enable study of doses of agents which are relevant to environmental exposure. If some or all mutagens are anti-mutagenic at low doses then current concepts of low dose population exposure may need substantial revision. Hazard assessment for toxicological agents would need to be changed to accommodate this new paradigm. Furthermore, understanding the mechanism

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of such an anti-mutagenic effect could ultimately lead to new insights into situations such as cancer and aging in which mutagenesis is important.

There are several lines of evidence that suggest that the conventional paradigm might not always be correct. The term hormesis was invoked to describe the phenomenon whereby small doses of irradiation might actually be advantageous in terms of increasing life-span (reviewed in Upton, 2001). The proposed mechanism behind this phenomenon has involved an adaptive response whereby low doses of radiation could, for example, stimulate the expression of repair enzymes, which repair not only the X-radiation induced mutations, but also other mutations in the cell, thus reducing the overall mutation frequency (a J-curve response). This type of response could equally be hypothesised for any low level of genotoxic agent in the environment. All organisms including humans have evolved in the presence of low doses of genotoxic agents and have evolved mechanisms to cope with them. These mechanisms are likely to have commonalities at the level of maintenance of DNA integrity. Calabrese and Baldwin (2001a) have defined experimental criteria necessary for studying low dose responses. These criteria include the need to use assay systems which are very sensitive *in vitro* and *in vivo* with the same mutation end-point over a wide dose range, and have an endogenous mutation frequency which is relatively high in order to see reductions in frequency below endogenous frequency**.** Most mutation assays and studies of low dose effects have been unable to fulfil many, let alone all of these criteria.

Our laboratory has recently made several observations that suggest that the conventional paradigm of LNT-dose response is incorrect for at least some agents. Somatic intrachromosomal recombination, which leads to chromosomal deletions and inversions, is an important mutational mechanism in cancer. We have been studying DNA damaging agents using the pKZ1 transgenic mouse as a mutation assay with somatic intrachromosomal inversion as the end-point. Here we review the results obtained at low doses of DNA damaging agents using the pKZ1 inversion assays and discuss their suitability for low dose mutation studies.

THE PKZ1 TRANSGENIC MOUSE

The pKZ1 mouse was originally developed by Matsuoka *et al.* (1991) to study somatic DNA recombination in the brain. The pKZ1 transgenic founder was an F_2 C57BL/6J X DBA2 mouse and the subsequent transgenic progeny were backcrossed to C57BL/6J mice. The pKZ1 transgene is described in detail in Matsuoka *et al.* (1991). Briefly, the transgene is comprised of an *E. coli* β-galactosidase gene (*LacZ)* which is in inverse orientation with respect to a chicken β-actin promoter. The transgene is flanked by the mouse immunoglobulin gene recombination signal sequences $J_{\kappa 5}$ and $V_{\kappa 21C}$. The pKZ1 mice are outbred to C57BL/6J mice resulting in 50:50 transgenic:non-transgenic progeny (n = 1164, p = 0.42,

Chi Square). Transgenic animals are identified using DNA from mouse tail clippings and an *E. coli lacZ*-specific polymerase chain reaction (PCR) which has previously been described in detail (Bej *et al.* 1990; Sykes *et al.* 1998). When an inversion occurs in the transgene, facilitated by the recombination signal sequences, the *lacZ* gene is brought into the correct juxtaposition with respect to the β-actin promoter and the *lacZ* gene can now be expressed (Fig 1).

ANALYSIS OF INVERSIONS IN PKZ1 TISSUES

The *E. coli* β-galactosidase protein (β-gal) can be detected in frozen mouse tissue sections using the chromogenic substrate X-gal (5-bromo-4-chloro-3-indolyl-β-galactopyranoside) which results in a blue stain. The blue staining cells can be counted under the light microscope and an inversion frequency is obtained by calculating the number of blue staining cells divided by the total number of cells counted.

The method for tissue preparation, X-gal staining and calculation of inversion frequency are described in detail in Sykes *et al.* (1998) and colour photographs of positive staining cells have been published previously (Sykes *et al.* 1998; Hooker *et al.* 2004a). Scoring of blue staining cells is time consuming and requires considerable expertise, suggesting that analysis might be subject to operator bias. The mean endogenous inversion frequency observed in spleen can differ between operators but the overall spread of data observed within operators is similar (data are presented for three operators in Fig 2). For the pooled data in Fig 2, the 95% confidence interval for the mean inversion frequenciy in pKZ1 animals is

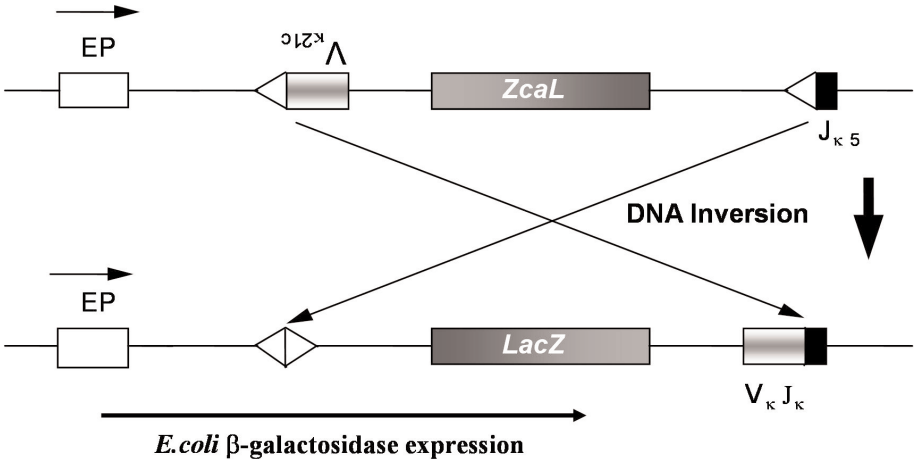

FIGURE 1: The pKZ1 transgenic mouse construct. *LacZ*, *E. coli* β-galactosidase gene; EP, chicken β-actin enhancer/promoter complex; $V_{\kappa 21C}$ and $J_{\kappa 5}$, mouse immunoglobulin recombination signal sequences (adapted from Hooker *et al.* 2004b).

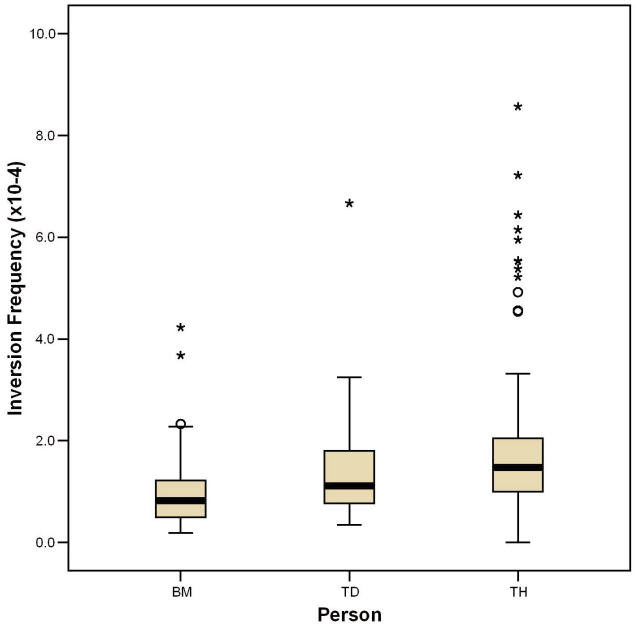

FIGURE 2: Boxplot comparison of operators (BM, TD & TH) showing the median ± interquartile range for inversion frequencies in spleen of pKZ1 untreated animals. The black bar indicates the median inversion frequency and the box represents the interquartile range (middle 50% of values). The error bar indicates the next quartile range (25%) of inversion frequencies. The open circles and asterisks indicate outliers and extreme outliers respectively. For BM (n = 40), TD (n = 30), TH (n = 194).

1.45 to 1.75 x 10^{-4} (n = 264), and approximately 5% of animals exhibit unusually high inversion frequencies. These high inversion frequencies may be the result of localised clonal expansion of a cell which has undergone an inversion within the haemopoietic system .

Intra-animal variation was determined by analysing the inversion frequencies of fifteen untreated pKZ1 animals, scored on at least two occasions on new frozen sections, and separated by a time period ranging from 3 weeks to 8 months. There was no statistical difference in the inversion frequency between replicate counting of inversions within an animal (p= 0.24, Exact Friedman Test) indicating that differences in time of freezing, preparation and sectioning did not significantly affect the observed inversion frequency.

The expression of the *E. coli* β-gal is differentiated from the endogenous mammalian β-gal by ensuring that staining is performed at pH 7.4. (MacGregor *et al.* 1987). Non-transgenic and treated non-transgenic animals are always included in the experimental design to account for any non-specific staining due to mammalian β-galactosidase. Non-specific staining in untreated non-transgenic animals is rare. The mean frequency of non-specific staining observed to date in spleen of 58 untreated non-

transgenic animals is $1.22 \pm 0.73 \times 10^{-6}$. Non-specific staining is increased after administration of very high doses of DNA damaging agents ranging from $8.9 \pm 4.6 \times 10^{-5}$ after whole body exposure to 2 Gy X-radiation to $1.3 \pm 0.3 \times 10^{-4}$ after IP injection of 50 mg/kg etoposide. Non-specific staining observed in treated non-transgenic animals is always subtracted from the staining observed in treated pKZ1 animals.

ENDOGENOUS INVERSION FREQUENCY IN PKZ1 TISSUES

Spleen was chosen as the first tissue for mutational analysis as spleen is a relatively homogeneous tissue for ease of scoring of blue staining cells in a total number of cells in any one microscope field. The mean endogenous frequency (±SE) observed in pKZ1 spleen is $1.61 \pm 0.08 \times 10^{-4}$ ($n = 264$) and the frequencies ranged from $0 - 8.57 \times 10^{-4}$. The endogenous inversion frequency in spleen in pKZ1 animals follows a log normal distribution.

The endogenous inversion frequency in spleen in pKZ1 mice increases two-fold with age from $1.54 \pm 0.24 \times 10^{-4}$ in 4-month old mice to $3.12 \pm 0.67 \times 10^{-4}$ in 22 month old mice (Sykes *et al.* 1998). There is no difference in endogenous inversion frequency in spleen between 4-month old pKZ1 males ($1.5 \pm 0.1 \times 10^{-4}$, $n = 64$) and females ($1.4 \pm 0.09 \times 10^{-4}$, $n = 53$) ($p = 0.51$, Mann-Whitney U test). After correction for transgene copy number (pKZ1 mice have 15 copies, Matsuoka *et al.* 1991), the endogenous inversion frequency for 4-month old mice equates to 1×10^{-5}/copy. This is 50-fold higher that that observed in spleen for point mutations in 8-10 week-old Muta™Mouse (Myhr, 1991) and 25-fold higher than 6-8 week BigBlue® mice (Köhler *et al.* 1991) when corrected for copy number. Comparison with *in vivo* results in pKZ1 transgenic spleen indicate that the endogenous inversion frequency observed in this study was approximately 15-fold less than that observed by Dempsey *et al.* (1993) for spontaneous H-2 mutants via mitotic recombination and 7 fold higher than that observed for HPRT mutations (Dempsey and Morley, 1986; Jones *et al.* 1985).

Inversions in the pKZ1 transgene have been identified in other tissues in pKZ1 mice. Matsuoka *et al.* (1991) demonstrated that inversions occurred in the brain, spleen, marrow and to a lesser extent in thymus by histochemistry. Inversions have also been identified in pKZ1 prostate luminal epithelial or basal cells (Hooker *et al.* 2004a). Studies on different stages of foetal and neonatal development in the brain of pKZ1 mice revealed that *E. coli* β-gal activity was widespread in the central nervous system and was not random, suggesting that inversion occurs early in development of the pKZ1 mouse brain. Matsuoka *et al.* (1991) also designed polymerase chain reaction (PCR) primers for detection of inversion events in the pKZ1 transgene at the DNA level, and sequenced PCR products from spleen and brain to identify the molecular DNA breakpoints at the inversions. They demonstrated that the inversion breakpoints in spleen

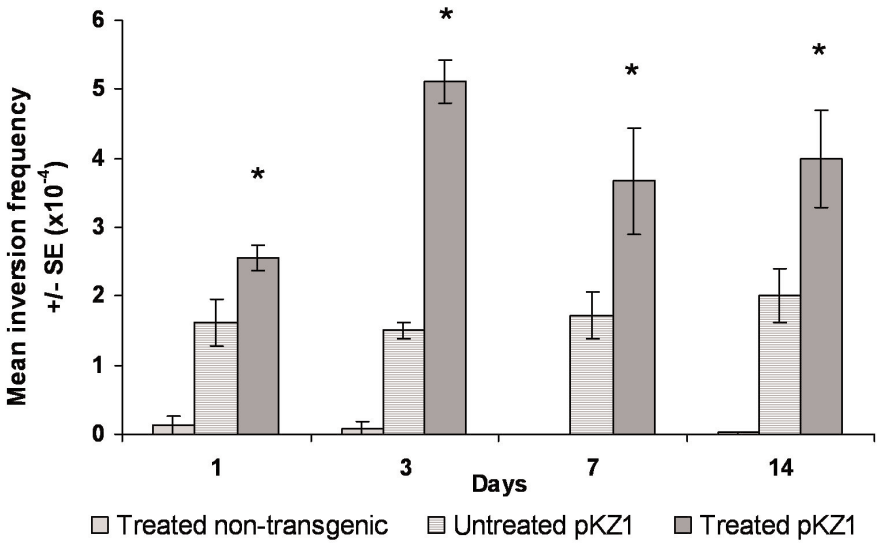

FIGURE 3: Mean inversion frequency (± SE) in spleen of 4-month old pKZ1 mice observed at 1, 3, 7 or 14 days after a single IP injection of 1 mg/kg cyclophosphamide. $n = 5$ for all treatment groups in each experiment. (*) Significant increase in inversion events between (untreated pKZ1 – untreated non-transgenic) and (treated pKZ1 – treated non-transgenic) ($p < 0.05$, two-tailed unpaired Mann-Whitney U-test).

involved the heptamer-nonamer sequences found in immunoglobulin gene rearrangements (Sakano *et al.* 1979) suggesting that the RAG1/RAG2 complex may be involved in resolution of the inversion event. Studies in other tissues by Southern analysis of inversion PCR products revealed inversion events at the molecular level in spleen, thymus and liver, however no inversion events were detected in tail or kidney tissue (Matsuoka *et al.* 1991). We utilised the same PCR primers in a semi-quantitative limiting dilution PCR analysis on pKZ1 spleen DNA and demonstrated that the frequency of inversion events closely approximated that observed by histochemistry of frozen spleen sections (Sykes *et al.* 1998).

THE PKZ1 ASSAY AS AN IN VIVO MUTATION ASSAY

Three- to four-month old animals were chosen for all subsequent studies as this age represented young but mature animals. A time course study was performed for exposure to 1 mg/kg cyclophosphamide to determine the time at which maximal induction of inversions was observed. Spleen was analysed from groups of animals either at 1, 3, 7 or 14 days after cyclophosphamide treatment. The results are presented in Fig 3. A statistically significant induction in inversions was observed at all

time-points of analysis. The fold induction rose from 1.6-fold ($p = 0.048$) at day 1 to 3-fold ($p = 0.001$) at day 3 and then decreased to 2.1- ($p = 0.016$) and 2-fold ($p = 0.028$) at days 7 and 14 respectively (two-tailed Mann-Whitney U test). Day 3 post-treatment was chosen for all subsequent experiments and it was expected that most DNA repair would have been completed by this time. Time-course experiments *in vivo* have not been performed for other DNA damaging agents. Initial experiments performed to determine if a variety of DNA damaging agents would induce inversions involved intraperitoneal injection of single high doses of DNA damaging agents, or whole body X-radiation, with subsequent analysis of spleen tissue for chromosomal inversions in the pKZ1 transgene. An induction in inversions was observed 3 days after treatment with a range of different DNA damaging agents; cyclophosphamide, mitomycin C, etoposide, X-radiation and methylene chloride (Sykes *et al.* 1998; 1999). All of these agents are mutagens in a number of mutation assays as well as known carcinogens. Cyclophosphamide is an alkylating agent which can result in guanine adducts in the DNA, mitomycin C is an alkalating cross-linking agent, etoposide is a topoisomerase II inhibitor, and X-radiation causes results in DNA strand breaks and free radicals which can damage DNA. Methylene chloride was chosen for study because it is known to be carcinogenic in animals and causes intrachromosomal recombination but not interchromosomal recombination in yeast (Scheistl, 1993). The induction of inversions in response to all of these agents suggested that chromosomal inversion was a common mutational response to different DNA damaging agents with different mechanisms of genotoxic action.

THE PKZ1 IN VIVO ASSAY IS EXTREMELY SENSITIVE IN SPLEEN TISSUE

When lower doses of DNA damaging agents were studied, it was found that the pKZ1 recombination mutagenesis assay was extremely sensitive compared with other *in vivo* mutation assays. For cyclophosphamide, an induction of inversions was observed at doses that were 4 orders of magnitude lower (Sykes *et al.* 1998) than doses which had previously been shown to induce point mutations in the BigBlue® mutagenesis assay (Köhler *et al.* 1991). For etoposide, a change in inversion frequency was observed at doses that were an order of magnitude lower than had been studied in other assays. However, not only was the assay more sensitive, the dose response followed a J-curve (Fig 4A) (Hooker *et al.* 2002) with low doses of etoposide causing a reduction below endogenous inversion frequency suggesting that low doses of etoposide may be antimutagenic. Etoposide is a known mutagen causing point mutations, small and large deletions, insertions and illigitimate recombination in mammalian cells (Berger *et al.* 1991; Han *et al.* 1993; Maraschin *et al.* 1990; and Ferguson and Baguley, 1996). There have been no reports in other muta-

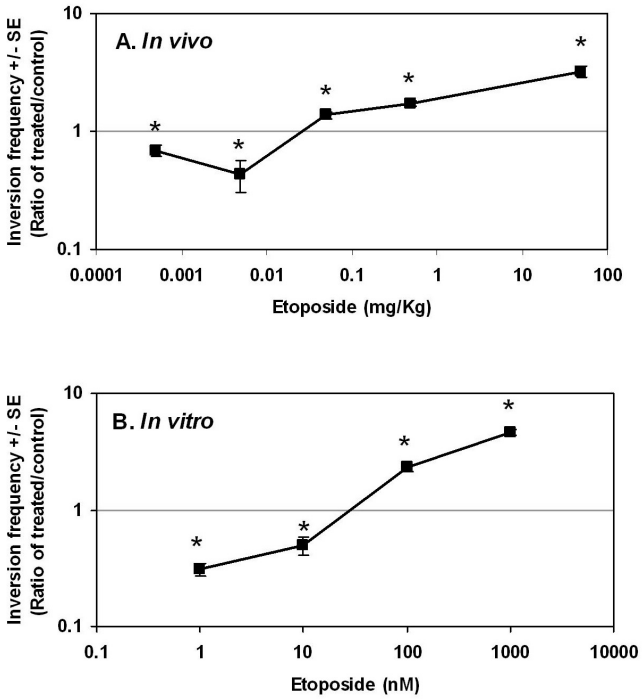

FIGURE 4: Inversion frequency ± SE (ratio of treated/control) A) *in vivo* in pKZ1 spleen three days after a single intraperitoneal injection with etoposide and B) *in vitro* in a pKZ1 cell line three days after exposure to etoposide for 6 hours. $n = 5$, *statistically significant ($p < 0.05$, two tailed T-test) (Hooker *et al.* 2002).

tion assays showing a reduction below endogenous frequency in response to low dose etoposide exposure. A significant reduction below endogenous inversion frequency was also detected in pKZ1 spleen after 25 days of 4 W/kg, 900 MHz radiofrequency exposure (Sykes *et al.* 2001). Whether 900 Mhz radiofrequency can cause genotoxicity in other mutation assays is controversial as is a possible mode of genotoxicity (reviewed in Brusick *et al.* 1998). In the case of acute whole body X-radiation, changes in inversion frequency were observed at doses that were at least three orders of magnitude lower than other investigators had reported for other mutation end-points, and in this case the dose response was even more complex than a J-curve. We have termed this response an S-curve response (Fig 5) (Hooker *et al.* 2004b). These results suggested that ultra-low doses of X-radiation were more mutagenic than low doses and that low doses may be anti-mutagenic. An induction of inversions was observed in pKZ1 mice with single acute whole body X-radiation exposures of ultra-low doses of 5 and 10 μGy. At these doses bystander effects must be playing a role as the number of cells sustaining damage directly

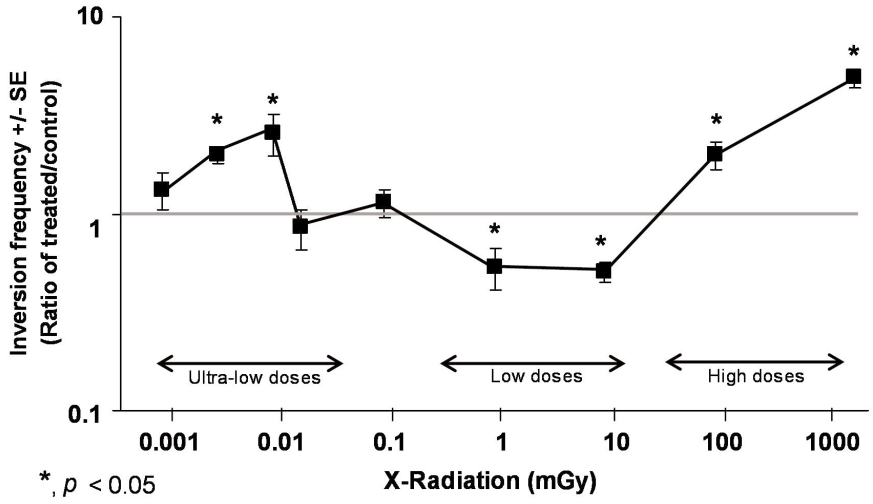

FIGURE 5: Inversion frequency ± SE (ratio of treated/control) in pKZ1 spleen three days after X-irradiation. Inversions were induced in pKZ1 spleen at ultra-low and at high doses of radiation exposure. Intermediate (low) doses of radiation caused a decrease below endogenous inversion frequency. * Statistically significiant (p<0.05, two-tailed T-test) (Hooker *et al.* 2004b)

from the radiation must only constitute a small fraction of the cells present (Azzam *et al.* 2003; Hall and Hei, 2003; Morgan, 2003; Mothersill and Seymour, 2003). Bystander effects may also be playing a role in response to very low doses of chemical DNA damaging agents. The results with low doses of etoposide and X-radiation do not conform to a LNT dose response model. Based on the same dose-response patterns that have been emerging through our studies, it is possible that had we continued to study lower doses of the other agents above, we might well have observed a similar dose-response pattern to that shown in Fig 5.

PKZ1 IN VITRO ASSAY

In vivo mutation assays are time-consuming and expensive, and studying mechanism *in vivo* can be difficult. We therefore developed a pKZ1 hybridoma cell line by fusing pKZ1 spleen cells with a myeloma cell line and then selected clones which contained the pKZ1 transgene (Hooker *et al.* 2002). One cell line (A11) which was chosen for subsequent study has an endogenous inversion frequency of 1×10^{-3} which is approximately 10-fold higher than the endogenous inversion frequency in spleen. The higher frequency may be the result of genomic instability in a vigorously growing immortal cell line or of increased recombination associated with rapid replication. The endogenous inversion frequency in A11 was studied over a 4 month period in culture (31 passages) and was

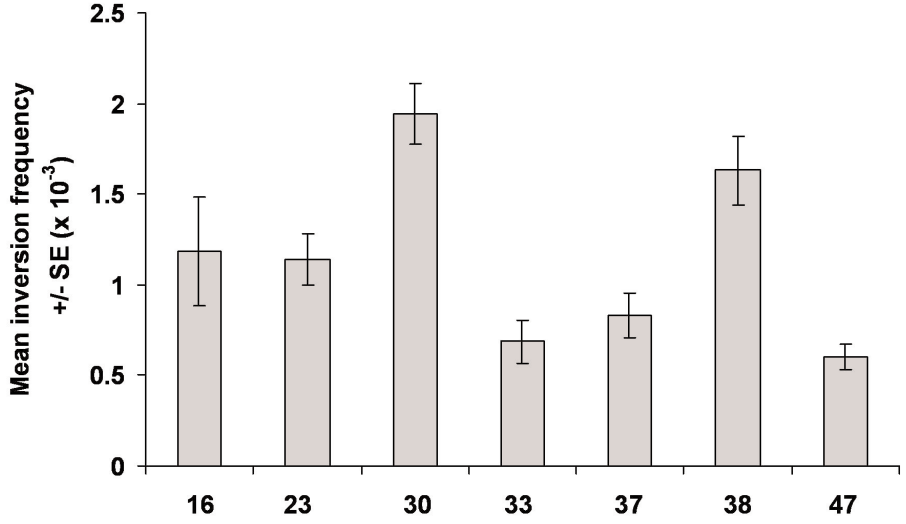

FIGURE 6: Endogenous inversion frequency in pKZ1 hybridoma A11 cells (mean ± SE). Endogenous inversion frequency was analysed by histochemistry over a period of approximately 4 months for cells in continuous culture. $n = 5$ for all groups except for passage 16 where $n = 2$.

demonstrated to be relatively stable, fluctuating around a mean of 1.13 ± 0.12 × 10^{-3} (Fig 6). The A11 line has been used to study low doses of etoposide *in vitro* (Fig 4b) (Hooker *et al.* 2002). The pKZ1 A11 cell line responded in the same dose dependent manner in response to etoposide as was observed *in vivo* in that a J-curve response was observed. The lowest etoposide dose studied was 10-fold lower than had previously been reported to modulate a mutation end-point. There was no difference observed in proliferation at the lower doses compared with vehicle control indicating that the reduction in inversion frequency below endogenous inversion frequency was not largely proliferation dependent. The demonstration that a J-curve dose response was observed for etoposide both *in vivo* and *in vitro* confirmed that the pKZ1 assay is measuring a mutagenic response that is fundamental to the cell regardless of whether it is in immortalised cells in culture or cells present in spleen tissue in a live animal (Fig 4).

WHY ARE SIMILAR NON-LINEAR DOSE RESPONSE CURVES NOT OBSERVED IN OTHER MUTATION ASSAYS?

Non-linear dose response curves have been identified for a number of different biological end-points (reviewed in Calabrese and Baldwin, 2001b) but there is limited data demonstrating non-linear dose-response curves in mutation assays.

By contrast with most mutation assays, the pKZ1 assay has a very high endogenous inversion frequency in spleen tissue of approximately 1.5 ×

10^{-4} and 1 x 10^{-3} in the A11 cell line. This high frequency makes it feasible to observe a reduction below endogenous inversion frequency. Assays which have endogenous mutation frequencies of 1×10^{-6} would require enormous numbers of cells to be studied to observe significant reductions below the endogenous mutation frequency. This may be the reason why the non-linear dose response is rarely observed in other mutation assays. The results of the S-curve response for X-radiation suggest that investigators may have the opportunity to identify subsequent increases in mutation frequency with other mutation assays but may not have looked at doses that are several orders of magnitude lower than the doses at which no changes were observed previously in such assays. One μGy acute radiation dose is the lowest dose studied in the pKZ1 mice to date. Future experiments studying doses below 1 μGy still need to be performed to determine whether 1 μGy is the threshold radiation dose below which no changes in inversion frequency occur. Further low dose experiments also need to be performed to determine the threshold dose for the other chemical DNA damaging agents previously studied in pKZ1 animals.

WHY IS THE PKZ1 ASSAY SO SENSITIVE?

Effectively, the pKZ1 assay measures the ability of a cell to invert the *lacZ* transgene. In order for this to occur the chromatin must be unwound to enable a stem and loop structure to occur, and then that structure needs to be recognised by proteins which resolve the stem and loop structure into an inversion. The proteins involved are likely to be those involved in non-homologous end-joining (NHEJ) recombination as the molecular break-point identified in spleen tissue bears the heptamer-nonamer signature sequence of immunoglobulin gene rearrangements which occur via a NHEJ mechanism (Sykes *et al.* 1998; Hooker *et al.* 2004a). However, inversion events with this same signature sequence have been identified in pKZ1 tissues that do not express the RAG1/RAG2 complex known to be essential for immunoglobulin gene rearrangement (Matsuoka *et al.* 1991; Hooker *et al.* 2004a). pKZ1 mice have been crossed with RAG1 knock-out mice to make pKZ1/RAG1 knock-out mice. The progeny still underwent inversions in lymphocytes but the inversions did not involve the recombination signal sequences and only short regions of homology were involved (H. Sakano and F. Nagawa, personal communication). These results indicate that more ubiquitous enzymes involved in NHEJ-type processes may be involved. Regardless of the exact NHEJ enzymes involved, we hypothesise that the inversions in the pKZ1 gene act as a surrogate measure of NHEJ activity in a cell. Therefore an inversion detected in the pKZ1 transgene will not necessarily be due to repair of damage in the transgene DNA sequence. Instead, inversions can result from NHEJ activity as a response to DNA damage that has occurred anywhere in the genomic DNA of that cell or even in neighbouring cells

where bystander effects are involved. This hypothesis would account for the exquisite sensitivity of the pKZ1 assay. The actual damage to the DNA incurred by direct interaction with a DNA damaging agent may be linear but the repair response may not be. It is the responses of the cell (at least at low doses) to the damage that will ultimately be important for the subsequent mutational load and survival of the cell, rather than the direct amount of DNA damage at these low doses.

THE PKZ1 IN VIVO ASSAY CAN BE USED TO STUDY THE EFFECT OF CANCER-ASSOCIATED GENES ON INVERSION FREQUENCY

Chromosomal inversions are common mutations in cancer and we hypothesised that genes frequently altered in cancer would play a role in formation of chromosomal inversions. It has been difficult to study the effect of cancer-associated genes on chromosomal changes prior to tumour formation *in vivo* because of a lack of appropriate test systems. We utilised the pKZ1 assay to test this hypothesis by breeding pKZ1 mice to three tumour model strains, Eμ-*myc* (Corcoran *et al.* 1985), *Msh-2* knockout (de Wind *et al.* 1995) and transgenic adenocarcinoma mouse prostate (TRAMP) (Greenberg *et al.* 1995), to produce double-transgenic animals (Hooker *et al.* 2004a). The three tumour models studied involved overexpression of the *c-myc* proto-oncogene (resulting in lymphoma), loss of *Msh-2* (resulting in lymphoma) and expression of the SV40 T antigen (resulting in prostate tumours) respectively. The frequency of chromosomal inversions was affected in the case of all three cancer associated genes studied. An increase in inversions was demonstrated in spleen of animals over-expressing *c-myc* and a decrease in inversions was observed in spleen of animals lacking *Msh-2* and in luminal epithelial or basal cells of prostatic glands of animals expressing the SV40 T antigen. We hypothesise that the increase and decrease in inversions in the presence of cancer associated genes results from inappropriate NHEJ activity. These data suggest that the pKZ1 model may provide a powerful tool for studying the effect of cancer associated genes on chromosomal aberrations in the early stages of carcinogenesis as well as being a useful model for studying low dose effects.

ADVANTAGES AND DISADVANTAGES OF THE PKZ1 ASSAYS FOR STUDYING LOW DOSES OF DNA DAMAGING AGENTS

The pKZ1 assay has many advantages for the study of low doses of DNA damaging agents, as it is very sensitive and the high endogenous inversion frequency enables detection of decreases below endogenous inversion frequency. The model also exhibits modulation of inversion frequency over a very wide range of dose. The magnitude of the response to any one dose is not large, but the dose response is still clearly identified

and may represent the limit of variation of NHEJ activity. The J-curve response has been identified both *in vivo* and *in vitro* which suggests that a fundamental mechanism is involved in modulation of inversion events whether in an artificial biological situation *in vitro* or in the metabolic and physiologically relevant biological system of a complete mammal. Direct histochemical analysis of tissues can enable identification of the types of cells which are undergoing inversions in response to DNA damaging agents. This is a major advantage compared with *in vivo* mutagenesis assays which require isolation of DNA from the tissues and subsequent analysis in a second vector system, such as BigBlue® (Köhler *et al.* 1991), Muta™Mouse (Myhr, 1991) and the deletion model of Gossen *et al.* (1995). The COR3 mouse model by Murti *et al.* (1994) and the mouse pink-eye model by Brilliant *et al.* (1991) enable the study of mutations at the cellular level, but are restricted to the study of testis, or patches of eye and coat colour respectively. The main disadvantage of the pKZ1 assays is that screening tissue sections and cell lines for blue staining is labour intensive and the endpoint is visual scoring. It takes considerable time for staff to become competent at screening as the blue stain forms a small defined dot in cells which have undergone inversions. These small dots can be missed in the screening if the histochemical staining is suboptimal. False positives can also be scored if there is significant non-specific background staining. In order to ensure that these are not confounding problems in scoring, all tissues from any one experiments are always cut and stained on the same day and all slides are screened blind, whereby the slides are coded by another individual in the laboratory and the code is not broken until all the slides in an experiment have been screened. Both treated and untreated non-transgenic animal controls are also always included to control for X-gal staining and any effects that treatment may have on X-gal staining. Attempts are presently in progress to develop a quantitative real-time polymerase chain reaction to detect the inversion event in lymphoid tissue at the DNA level. Such an assay would remove subjective bias and allow larger numbers of cells to be screened rapidly. However, different tissues do exhibit different inversion break-points (unpublished data) and therefore only a subset of inversions will be scored at any one time in a real-time PCR. However, a number of different PCR's to the different break-points could be designed and compared quantitatively.

SUMMARY

The pKZ1 mutation assays fit the majority of the criteria set by Calabrese and Baldwin (2001a) for low dose studies. The assays are sensitive over a wide dose range and have the capacity to detect non-linear dose responses due to their high endogenous frequencies. Our results to

date using the pKZ1 *in vivo* and *in vitro* assays suggest that the LNT dose-response model may not hold for all DNA damaging agents. Inversions in pKZ1 spleen have been studied almost exclusively to date, but there is the potential to study a wide range of pKZ1 tissues both at the cellular and the molecular level. The pKZ1 *in vivo* assay has the potential to be used to study the effect of cancer genes in the early stages of carcinogenesis and may be useful for the study of low doses of DNA damaging agents in the presence of aberrant cancer gene expression.

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