Hprr mutants in a transplantable murine tumour arise more frequently in vivo than in vitro

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Summary A model system was developed to allow investigation of the frequency at which clastogenic and or mutagenic events occur in situ in a transplantable murine fibrosarcoma tumour (MC1A-C1) compared with in vitro culture. The marker selected for detecting these events was the X-linked hprt (hypoxanthine–guanine phosphoribosyltransferase) gene. We found that the hprt gene in MC1A-C1 was not suitable for this purpose, most likely because multiple active copies were present. To circumvent the problem, HPRT+ [6-thioguanine (6-TG)-resistant] clones were isolated by inactivating all hprt genes with methylnitrosourea. Spontaneous revertants to hypoxanthine aminopterin thymidine resistance (HAT+) were isolated and found to be approximately 1000 times more sensitive than the parental tumour to induction of 6-TG-X mutants by cobalt-60 γ-rays. This sensitivity is expected for a heterozygous marker; these revertants may therefore possess only one functional hprt locus but two or more active X chromosomes. A clone with a stable hprt gene was identified and a neo gene was introduced. The resulting cell line (MN-11) could be grown as a subcutaneous tumour in syngeneic C57BL/6 animals. The frequency of mutations arising in vivo in the marker hprt gene could be estimated by culturing explanted tumour cells in the presence of 6-TG, using G418 selection to distinguish tumour from host cells. The frequency of mutants in MN-11 cells grown as tumours was found to be 3.4-fold higher than in tissue culture for an equivalent period of time. These data provide the first direct evidence for the existence of mutagenic factors in a tumour environment that might contribute to tumour progression.

Keywords: hprt gene; MC1A-C1 fibrosarcoma; transplantable tumour

It is well established that initiation and progression of cancer is associated with multiple genomic alterations, such as altered patterns of methylation, small-scale (intra-locus) mutations, or large-scale (multilocus) events. Multilocus clastogenic events such as recombinations, translocations, inversions or deletions observed at the chromosome level are a reflection of the increased genomic instability commonly observed in tumours (Yunis, 1983). The cause of this observed instability is only partially understood. Loeb (1991) has postulated that a mutation early in the development of cancer causes a mutator phenotype that is responsible for subsequent instability; recent reports of such a mutator gene in a subset of colorectal cancers support this possibility (Leach et al., 1993; Parsons et al., 1993). Mutations in genes affecting cell cycle checkpoints may also predispose cells to genomic instability (Livingstone et al., 1992; Weinert and Lydall, 1993).

As well as these endogenous mechanisms for genomic instability, we and others have postulated that exogenous factors presumed to be present in the tumour environment, such as reactive oxygen species and nitric oxide, may contribute to genotoxicity (Birnboim, 1983; Emerit and Cerutti, 1983; Heppner et al., 1984; Weitzman and Weitberg, 1985; Troll and Wiesner, 1985; Bennett et al., 1993). To test this hypothesis, we set out to develop a tumour model system in which loss of a marker gene (hprt) could be detected with high sensitivity. The hprt locus is widely used for the detection of mutagenic events (intra-locus and multilocus) in mammalian cells, including man (Vrielang et al., 1985; Fuscoe et al., 1986; Köberle and Speit, 1991; Nicklas et al., 1991; Cole and Skopek, 1994). Most rodent studies have been carried out using Chinese hamster cells (Stout and Caskey, 1985; Nastasi-Caló et al., 1989; Köberle and Speit, 1991; Schwartz et al., 1991) with relatively few studies in mouse cells (Evans et al., 1986; Vrielang et al., 1988; Morita et al., 1991). A reported limitation to the use of the hprt locus (which is X linked and essentially single copy) for studies of clastogenic events is that concomitant loss of neighbouring essential genes (multilocus lesions) will result in non-viable mutants, i.e. lowered sensitivity for detecting induced mutations. This explanation was proposed by Evans et al. (1986) who demonstrated that the apparent rate of mutation by a clastogenic agent (250 kVp X-rays) of a single-copy autosomal tk gene in mouse cell lines was approximately 100-fold higher than an X-linked hprt gene.

We are unaware of any previous studies that have directly addressed the question of mutagenic events in a transplantable tumour in a syngeneic animal. To establish a suitable system, we started with a murine fibrosarcoma containing three X chromosomes (presumably three active hprt loci). The multiple hprt loci were inactivated by treatment with a mutagen, following which spontaneous revertants to HAT resistance were identified. These were screened for stable hprt expression and tumorigenicity. Selected clones demonstrated high sensitivity to mutation induction by ionising radiation, indicating that a single hprt locus had reverted and that cells were at least diploid for X-linked essential genes. The clones were used to compare the frequency of mutational events in cells growing as a subcutaneous tumour compared with the same cells grown in culture.

Materials and methods

Chemicals 6-Thioguanine (6-TG), aminopterin, and N-methyl-N-nitrosourea (MNU) were purchased from Sigma, St. Louis, MO, USA. Geneticin (G418) was from Gibco BRL, New York, NY, USA.

Derivation of MC1A and sublines

MC1A fibrosarcoma was originally isolated from a male C57BL/6 mouse that had been treated with methylcholanthrene (Kadhim and Rees, 1984; Kadhim et al., 1987). MC1A-C1 is a variant capable of in vitro growth that arose spontaneously when MC1A cells were maintained in culture for several weeks. The hprt gene(s) in MC1A-C1 cells were inactivated by treatment with MNU (125 μM) for 1 h, giving rise to mutants resistant to 6-TG. Tumorigenicity of 18
6-TG\(^8\) mutants were tested by selecting for clones that most readily formed subcutaneous tumours in syngeneic C57BL/6 female mice, 8–10 weeks of age (Charles River Laboratories). Of those tested, MC-TGRI7 was chosen. The final step was to reactivate one of what was believed to be two or more inactive copies of the hpgr gene. Approximately 5 \times 10^5 viable cells were cultured in HAT-medium (see below) and 54 spontaneously arising HAT-resistant (HAT\(^8\)) clones were selected. Most exhibited a HAT\(^8\), 6-TG-sensitive (6-TG\(^5\)) phenotype and were labelled as MC-TGSI7-1 to MC-TGSI7-54. These clones in turn were screened to identify which exhibited most stable HPTR expression (lowest spontaneous reversion to 6-TG-resistance); clone MC-TGSI7-51 was selected (see Results). MN-5, MN-11 and MN-12 are derivatives of MC-TGSI7-51 into which a neo gene in a retroviral vector was introduced to confer G418-resistance, allowing tumour cells to be readily distinguished from host cells. The vector expressing the neo gene was derived from a retrovirus which spontaneously lost expression of a human c-H-ras-1 oncogene driven by a Mu-MuLV LTR but retained a TN10 neomycin resistance gene driven by a SV40 early-region promoter (Bennett et al., 1994).

Cell culture conditions and challenge with inhibitors

Cells were grown in Dulbecco’s modified Eagle medium (DMEM) plus 10% fetal calf serum (Gibco BRL, Burlington, Ontario, Canada; non-selective medium) in a 5% carbon dioxide/95% air incubator at 37°C. Plating efficiency of all clones tested was about 50% under these conditions. HAT medium was non-selective medium supplemented with hypoxanthine, aminopterin and thymidine at concentrations of 1 \times 10^{-4} \text{M}, 4 \times 10^{-7} \text{M}, and 1.5 \times 10^{-5} \text{M}, respectively. 6-TG-medium was non-selective medium supplemented with 5 \times 10^{-3} \text{M} 6-thioguanine. Twice weekly, the culture medium was replaced with fresh non-selective medium containing the appropriate drug(s). 6-TG\(^8\) and HAT\(^8\) colonies were scored at either 9 days (for MN series cells) or 14 days (for MC series cells), because of differences in the growth rates of the two series of cells. When cells grown in HAT medium needed to be transferred to non-selective medium, they were first cultured in HT medium (1 \times 10^{-4} \text{M} hypoxanthine and 1.5 \times 10^{-5} \text{M} thymidine) for 2 days. G418 medium was non-selective medium supplemented with 500 \mu\text{g} \text{ml}^{-1} G418.

Scoring of 6-TG-resistant colonies

To measure spontaneous or induced drug-resistant colonies, 1 \times 10^5 cells were plated per 10 cm tissue culture dish. Following incubation in 6-TG-Medium (MN series, 8–9 days; MC series, 14 days), dishes were washed in PBS (calcium-, magnesium-free phosphate buffered saline), colonies fixed in methanol for 20 min, rinsed with PBS and stained with Wright’s stain for 5 min. Excess stain was removed by gentle washing with running water. Up to about 300 colonies (> 50 cells per colony) per 10 cm dish could readily be scored under 2.5 \times magnification. All results are expressed as mutants per 1 \times 10^6 clonable cells, i.e. corrected for the plating efficiency, which ranged from 40–50%.

In vitro growth rate determination

The \textit{in vitro} growth rate of several MC-TGS and MN clones was established by plating a known number of cells (1 \times 10^5 per 6 cm dish) and measuring the increase in cell number with a Coulter Counter. Growth was followed daily for up to 7 days or until confluence. Cell doubling times were estimated from the logarithmic phase of the growth curve.

Cell irradiation

Cells (in non-selective medium) were irradiated with cobalt-60 \(\gamma\)-rays (dose rate 1.8–1.4 Gy min\(^{-1}\); Theratron 780, Atomic Energy of Canada). After 7 days’ expression time with subculturing as needed, 1 \times 10^5 viable cells (as determined by trypan blue exclusion) were replated and challenged with 6-TG medium. Unirradiated (control) cells were treated similarly, without irradiation.

\textbf{Establishing conditions of in vivo mutation frequency}

When tumours reached approximately 1 cm in size (14–21 days after subcutaneous injection in 0.1 ml of PBS of 1 \times 10^5 MC-TGS cells or 12–17 days after injection of 5 \times 10^5 MN-11 cells), animals were euthanised by carbon dioxide narcosis and cervical dislocation. Tumours were removed under aseptic conditions and cell suspensions were prepared by mechanical disruption. Cells were incubated for 2–4 days in non-selective medium to allow cell attachment and to remove debris. Approximately 1 \times 10^5 viable (i.e. trypan blue-excluding) cells per 10 cm dish were replated in 6-TG medium for scoring of 6-TG\(^8\) mutants. Cells were also plated in non-selective medium for determination of plating efficiency. For MN-11, the percentage of total cells which were G418 resistant was determined by incubating a fraction of the cells for 9 days in G418 medium; typically, 80–90% of total cells were G418 resistant. The number of 6-TG\(^8\) mutants was corrected for plating efficiency and for G418 resistance. All animal procedures were carried out in accordance with guidelines of the Canadian Council on Animal Care and the Animals for Research Act RSO-1990

\textbf{Karyotype analysis}

Metaphase cells were prepared by addition of 0.1 \mu\text{g} \text{ml}^{-1} colchicin 4 h before harvest. Cells were allowed to swell in 0.075 M potassium chloride for 15–20 min, then fixed in three changes of methanol–acetic acid (3:1, v/v) and dropped onto microscope slides. Identification of X chromosomal material in these cells was accomplished by hybridisation with a biotinylated X chromosome-specific composite DNA probe (Brenerman et al., 1994). Hybridisation and detection of bound probe was accomplished with two layers of avidin–FITC as described (Brenerman et al., 1994). Representative cells were photographed with Kodak Ektachrome 400 film on a Zeiss Axiohot photo microscope.

\textbf{Results}

\textbf{Karyotype analysis of MC1A derivatives}

MC1A was originally derived from a tumour arising in a male mouse injected with a carcinoembryonic antigen methylcholangiome (Kadhim and Rees, 1984; Kadhim et al., 1987). It was anticipated that it would be aneuploid but the number of X chromosomes was uncertain. Since it had proven to be rather difficult to obtain HPRT\(^-\) (6-TG\(^5\)) mutants of MC1A-C1 by MNU treatment or cobalt-60 \(\gamma\)-rays, this suggested that more than one functional copy of the X-linked hpgr gene was present. Karyotype analyses, including the use of new X-specific hybridisation probes (Brenerman et al., 1995), was carried out (Table I, Figure 1). All lines were found to be hypotetraploid with about three X chromosomes plus other regions detected by the fluorescent probe, consistent with the notion of multiple copies of the hpgr gene in MC1A.

\textbf{Establishing conditions for growth in 6-TG to minimise metabolic cooperation}

Mutant cells lacking hpgr (normally resistant to 6-TG) can still be killed by ‘metabolic cooperation’, i.e. passive acquisition of 6-TG nucleotide through gap junctions from neighbouring cells that contain active enzyme (Trosko and Chang, 1984). A reconstruction experiment was carried out to determine the cell density at which this effect could be minimised for our cell lines. One hundred 6-TG\(^8\) MC-TGRI7 cells were seeded onto 10 cm tissue culture dishes with increasing numbers of 6-TG\(^5\) MC-TGSI7-51 cells. The cells were grown in 6-TG for 14 days, following which 6-TG\(^8\) colonies were...
Table 1 Karyotype of MCIA-C1 and its derivatives

| Cell line            | Total number of chromosomes | Number of X chromosomes | Number of other painted chromosomes |
|----------------------|-----------------------------|-------------------------|-------------------------------------|
| MCIA-C1 (n = 19)     | 71.3 ± 14.3                 | 2.8 ± 0.6               | 3.4 ± 1.4                           |
| MC-TGR17 (n = 39)    | 65.6 ± 5.0                  | 2.8 ± 0.6               | 3.2 ± 0.8                           |
| MC-TGS17-51 (n = 20) | 93.5 ± 11.1**               | 3.9 ± 0.9**             | 7.0 ± 0.1**                         |

Results represent means ± standard deviations. The differences in number of total chromosomes and X chromosomes are both statistically significant when MC-TGS17-51 was compared with the other two strains (**P < 0.001, ANOVA, Bonferroni post test). Other details given in Materials and methods.

Figure 1 Detection of X chromosome-specific sequences by fluorescence in situ hybridisation in MC-TGR cells. Details as described in Materials and methods. The large arrows locate multiple X-chromosomes. Small arrows point to examples of interstitial and centromeric X-specific sequences.

The recovery of 6-TG<sup>8</sup> colonies was unchanged in the presence of up to 1 x 10<sup>7</sup> 6-TG<sup>9</sup> cells per 10 cm dish. Above this cell number, there was a clear decrease in recovery of 6-TG<sup>8</sup> colonies such that, at 1 x 10<sup>9</sup> cells, very few colonies were recovered (data not shown). All experiments were therefore conducted at 1 x 10<sup>9</sup> cells per 10 cm dish.

Spontaneous mutation frequency and growth rates of different HAT<sup>8</sup> clones

The frequency of spontaneous mutants constitutes a background which affects the sensitivity of detection of induced mutants. To obtain cell lines with the lowest background possible, we screened 54 independently arising MC-TGS (HAT<sup>8</sup>, 6-TG<sup>9</sup>) clones to identify those with a low rate of spontaneous loss of the HPRT function. These clones were first grown in HAT medium (to eliminate any accumulated 6-TG<sup>9</sup> spontaneous mutants) and then grown in non-selective medium for up to 28 days, following which 6-TG<sup>8</sup> colonies were scored. Three clones were selected and analysed in greater detail for rate of spontaneous loss of HPRT function. At times up to 28 days following removal of HAT, clones were challenged with 6-TG (Figure 2a). In all cases, there was an increase in the number of 6-TG<sup>8</sup> colonies seen as a function of time in non-selective medium. Clone MC-TGS17-51 appeared to be the most stable. Its spontaneous mutation frequency was estimated to be 7.1 x 10<sup>-5</sup> ± 0.2 x 10<sup>-8</sup> per day in one set of experiments (Figure 2a) and 8.2 x 10<sup>-6</sup> ± 0.8 x 10<sup>-8</sup> per day in a second set of experiments (Figure 2b). The equivalent rate for the two other clones (17-1 and 17-25) ranged from 10 to 16 x 10<sup>-6</sup> per day. The growth rates of the three MC-TGS clones were not detectably different from parental MCIA-C1; doubling times were about 20 h (data not shown). Three G418-resistant subclones derived from MC-TGS17-51, namely, MN-5, MN-11 and MN-12, were also tested (Figure 2b). The spontaneous mutation frequency of MN-11 was estimated to be 5.1 x 10<sup>-6</sup> ± 0.2 x 10<sup>-6</sup> per day. All three MN clones had a significantly shorter doubling time (15-16 h) than the parental clone (data not shown). When corrected for differences in doubling times, the rate of mutation was estimated to be 6.4 x 10<sup>-6</sup> cells per generation for MC-TGS17-51 and 3.3 x 10<sup>-6</sup> cells per generation for MN-11. The average growth rate of a pool of five spontaneously arising 6-TG<sup>8</sup> mutant clones was not detectably different from parental MN-11 cells, as assessed by a mixing experiment in which 6-TG<sup>8</sup> mutants were grown in the presence of MN-11 cells (data not shown).

Radiation-induced 6-TG<sup>8</sup> mutants

Our objective was to develop a cell line which could detect loss of hprt gene function with high sensitivity, that is, have a high ratio of induced to spontaneous mutations. We screened three MC-TGS17 clones to assess their sensitivity to mutation induction by cobalt-60 γ-radiation. Within experimental error, the number of induced mutations was similar (data not shown). MC-TGS17-51 was chosen because of its stability at the hprt locus. MC-TGS17-51, a G418<sup>8</sup> derivative (MN-11) and parental MC1A-C1 cells were compared in terms of sensitivity to induction of 6-TG<sup>8</sup> mutants by cobalt-60 γ-rays (Figure 3). Dose-dependent increases in the number of 6-TG<sup>8</sup> colonies were seen in the case of MC-TGS17-51 and MN-11, while virtually no 6-TG<sup>8</sup> colonies were observed in the parental MC1A-C1 cells. In fact, no 6-TG<sup>8</sup> colonies were detected in three independent experiments involving a total of 3.4 x 10<sup>8</sup> unirradiated MC1A-C1 cells and 1.5 x 10<sup>7</sup> irradiated (0.5-5 Gy) cells. The proposed explanation is that multiple functional copies of the X-linked hprt gene are present in MC1A-C1 cells (Table 1 and Figure 1). The induced levels of 6-TG<sup>8</sup> colonies in MC-TGS17-51 and MN-11 were 451 and 300 per 10<sup>6</sup> viable cells per Gy respectively. At 5 Gy, we estimate that the induction of 6-TG<sup>8</sup> colonies in the derived clones was at least 1000-fold greater than in the
Figure 2  Spontaneous generation of 6-TG<sup>A</sup> mutants. (a) Three different MC-TGS clones were grown in non-selective medium for the indicated periods of time, then challenged with 6-TG as described in Materials and methods. Error bars represent the s.e.m. of the average 3–9 replicate plates. (b) MN subclones, compared with MC-TGS17-51. Note difference in scale for parts (a) and (b). Error bars represent the s.e.m. of the average of 3–6 experiments, each involving 8–10 replicate plates.

![Graph showing spontaneous generation of 6-TG<sup>A</sup> mutants](image)

Figure 3 Generation of 6-TG<sup>A</sup> mutants in MC1A-C1 and various derivatives by cobalt-60 γ-rays. ■, MC-TGS17-51; ▲, MN-11; ■, parental MC1A-C1. Error bars represent the s.e.m. of the average of 3–5 experiments, each involving 3–10 replicate plates. Other details as described in Materials and methods.

![Graph showing generation of 6-TG<sup>A</sup> mutants](image)

MC1A-C1 parental line. Sensitivity to cell killing by ionising radiation was similar for the three lines, D<sub>20</sub> = 3.5 Gy for MC-TGS17-51 and MN-11 and 2.8 Gy for MC1A-C1 (data not shown).

**Detection of HPRT<sup>-</sup> mutants arising in tumours**

The primary use of the experimental model was to determine whether the frequency of HPRT<sup>-</sup> mutants is altered as a result of *in vivo* growth in subcutaneous tumours. Before injection, three MC-TGS17 clones with different spontaneous mutation frequencies *in vitro* were cultured in HAT-medium to remove pre-existing 6-TG<sup>A</sup> mutants. Once tumours reached about 1 cm in size, they were collected and cells were plated in the presence of 6-TG to detect mutants that had arisen during tumour growth. The mutation frequency of these cells and cells grown *in vitro* under non-selective conditions for an equivalent period of time is shown in Figure 4. There was a trend towards higher mutation frequency in cells derived from tumours as compared with cultured cells. Cells derived from MC-TGS17-36 tumours had a statistically significantly higher mutation frequency than cells from MC-TGS17-51 tumours. To explore in more detail the relationship between *in vitro* and *in vivo* mutation frequencies, MN-11 cells, which have a low background of spontaneous mutation and a selectable marker to allow distinguishing tumour from host cells, were used (Figure 5). The mutation frequencies of cells from 16 tumours (12–17 days of *in vivo* growth) and 12 independent cultures were compared. The differences in frequencies were highly significant (*P* < 0.0001). The *in vivo* frequency was 3.4 times higher than the *in vitro* frequency. Tumour data were also examined as a function of time to reach 1 cm diameter; no statistically significant differences were detected (Figure 5, inset).

**Discussion**

Genomic instability is a commonly observed feature of tumours (Nowell, 1982, 1991; Bringuier et al., 1993; Nielsen et al., 1993). Factors *intrinsic* to tumour cells (such as mutated forms of p53) may predispose these cells to further genomic instability (Weinert and Lydall, 1993). A second (not mutually exclusive) possibility is that instability is due to *extrinsic* factors in the tumour environment, such as reactive oxygen species or nitric oxide generated by phagocytic cells. To explore the latter hypothesis, we have developed a new *in vitro/in vivo* model system. A mouse tumour known to be infiltrated with macrophages and granulocytes, and which is readily transplantable in syngeneic animals, was chosen as a starting point (Kadhim and Rees, 1984; Kadhim et al., 1987). A tissue culture line was established from the tumour, verified to be tumorigenic, and then genetically altered to
allow more sensitive detection of mutagenic events. We chose the hprt gene as a surrogate for measuring cancer-related genetic changes since loss of function of this gene can be readily scored. A potential disadvantage of X-linked genes, such as hprt, is that mutations resulting in multilocus deletions (e.g., those caused by ionising radiation) may be non-viable if neighbouring essential genes are also lost. This biases the assay against such events (Evans et al., 1986; Bradley et al., 1988; Sankaranarayanan, 1991; Schwartz et al., 1991; Zhou et al., 1993). To overcome this problem, many workers have introduced an exogenous marker gene (Ashman and Davidson, 1985; Ashman, 1989; Ikehata et al., 1989; Tindall and Stankowski, Jr. 1989; Kimura et al., 1993; Lichter and Kaligis et al., 1993). Our difficulty in obtaining HPRT<sup>−</sup> mutants of MC1A-C1 and the subsequent detection of three copies of the X chromosome suggested a different strategy. Since it appeared that MC1A-C1 had more than one active X chromosome and more than one active hprt gene, we chose to create a hprt heterozygote, as has been carried out at the tk and aprt loci (Liber and Thilly, 1982; Sebastio et al., 1985). This was done by screening for revertants to HAT<sup>+</sup>. Clone MC-TGS17-51 and its derivatives were all very similar with respect to stability of hprt (Figure 2b), with spontaneous mutant frequencies estimated to be <7.0 x 10<sup>−4</sup> mutants per generation, corrected for plating efficiency. There was a very large gain in sensitivity (>1000-fold) for detection of radiation-induced hprt mutations, compared with the parental line that is presumed to express multiple copies of hprt. The frequency of hprt mutations induced by cobalt-60 γ-rays in the cell lines we describe (300–450 x 10<sup>−4</sup> per Gy) is within the range of that reported for other heterozygous marker genes (tk, aprt and gpt) in mouse and Chinese hamster lines (Evans et al., 1986; Bradley et al., 1988; Ikehata et al., 1989; Schwartz et al., 1991).

Our tumour model was developed to study factors in the tumour environment that cause mutagenic or clastogenic events. Clone MN-11, which exhibited the lowest in vitro rate of spontaneous gene loss, was used to test whether the in vitro environment was 'mutagenic', that is, whether an increase in mutation frequency could be detected compared with in vitro growth conditions. A highly statistically significant 3.4-fold increase in mutant frequency was observed. It is difficult to make precise estimates of the growth rate of cells in vivo, since tumours contain a mixture of growing, non-growing and dying cells. Thus, estimates of in vitro mutation frequency (mutants per total number of viable cells) not mutation rate (mutants per cell generation) are presented. It is worth noting that the doubling time of MN-11 cells in vitro is very rapid (16 h); hence, it is improbable that the observed increase in mutation frequency can be explained solely by a greater number of cell divisions in vitro compared with in vitro. A large variance in mutation frequency among different tumours was seen, compared with the small variance observed in replicates of a single tumour cell suspension. This suggests a Luria–Delbrück type of fluctuation (Kendal and Frost, 1988). To the best of our knowledge, these data are the first to directly demonstrate that factors in the tumour environment may cause mutations at the hprt locus in a murine syngenic tumour model.

Most solid tumours, including human tumours, are infiltrated with inflammatory cells, and it is well known that a variety of inflammatory conditions such as ulcerative colitis predispose to malignancy (Templeton, 1975; Camisa, 1984; Yamada and Grisham, 1991; Babbs, 1992; Frenkel, 1992;
Levin, 1992). While recent attention has been paid to endogenous changes in tumour cells such as p53 and possibly p16 mutations that can predispose cells to genomic instability (Livingstone et al., 1992; Tlsty et al., 1992; Kamb et al., 1994), the new experimental system we have described will allow us to study whether exogenous factors in the tumour environment such as pH, oxygen radicals, nitric oxide, and others that may vary with the number of infiltrating inflammatory cells and the necrotic state of the tumour (Yamashina et al., 1986; Dobrowsky et al., 1991; Mareel et al., 1991; Bennett et al., 1993; Ohshima and Bartsch, 1994; Rosin et al., 1994.a,b) are also important in genomic instability.

**Abbreviations**

aprt, adenine phosphoribosyltransferase gene; gpr, bacterial xanthine guanine phosphoribosyltransferase gene; HAT, hypoxanthine-aominopterin and thymidine; HAT\(^\#\), cells resistant to HAT; Hprt, hypoxanthine–guanine phosphoribosyltransferase gene; HPRT\(^-\), cells lacking functional hprt (seuro. bacterial neomycin gene, pgk, phosphoglycerate kinase; 6-TG, 6-thioguanine; 6-TG\(^-\), cells resistant to 6-TG; tk, thymidine kinase gene.

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