Determination of spontaneous loss of heterozygosity mutations in Aprt heterozygous mice

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ABSTRACT

A mouse model was generated to investigate loss of heterozygosity (LOH) events in somatic cells. The adenine phosphoribosyltransferase (Aprt) gene was disrupted in embryonic stem cells using a conventional gene targeting approach and subsequently Aprt heterozygous and homozygous mice were derived. Aprt homozygous deficient animals were viable though the mendelian inheritance pattern was skewed. On average these mice died at 6 months of age from severe renal failure. In T-lymphocytes of Aprt heterozygous mice the mean spontaneous mutant frequency at the Aprt locus was $8.7 \times 10^{-6}$ while the frequency was $0.8 \times 10^{-6}$ at the hypoxanthine phosphoribosyltransferase locus. In order to determine whether LOH events contribute to the high spontaneous mutant frequency at the Aprt locus, 140 Aprt mutant T-lymphocyte clones were expanded and analysed by allele-specific PCR. In 97 (69%) of these clones the wild-type allele had been lost. Nine of the mutant clones were characterized in more detail using dual-coloured fluorescence in situ hybridization analysis. Five out of six of the mutant clones which arose from an LOH event, based on the PCR assay, contained a duplication of the targeted allele. Therefore, mitotic recombination or chromosome loss followed by duplication of the remaining homologue appears to be the predominant mechanism for the in vivo generation of Aprt mutant T-lymphocytes.

INTRODUCTION

The inactivation of tumour suppressor genes has been recognized as a key event in the development of heritable cancers. In the germ-line of patients suffering from Retinoblastoma, Li-Fraumeni syndrome or Wilm’s tumour, one allele of a tumour suppressor gene (Rb, p53 and WT-1, respectively) is inactivated due to an intragenic mutation. The inactivation of the remaining wild-type allele in somatic cells of these patients involves events of a more complex nature such as multi-locus deletion, gene conversion, mitotic recombination and non-disjunction, resulting in loss of heterozygosity (LOH) (1–3). The importance of LOH events for the inactivation of tumour suppressor genes in sporadic tumour formation can be inferred from the observation that in sporadic tumours up to 50% of all chromosomes have undergone LOH events (4). It is, however, still unclear to what extent LOH events play an initiating role in cancer or are predominantly a consequence of the general genetic instability acquired during tumour development. The involvement of LOH events in the initiation of tumorigenesis, therefore, cannot be established in advanced sporadic tumours. Only in normal somatic cells will it be possible to determine the actual contribution of LOH events to spontaneous mutagenesis.

An autosomal endogenous gene suitable for detection of mutations including LOH events is the adenine phosphoribosyltransferase (Aprt) gene. This gene encodes an enzyme that is involved in the purine salvage pathway and catalyses the conversion of adenine into adenosine monophosphate (AMP). Aprt deficient cells can be selected in vitro on the basis of their resistance to toxic purine analogs such as 8-azaadenine (8-AA) or 2,6-diaminopurine (DAP).

Using a gene targeting approach in embryonic stem (ES) cells, mice were derived with only one functional Aprt allele. This mouse model can be used to investigate the influence of various environmental and genetic factors on the frequency and types of LOH mutations that are recovered. Similar Aprt knock-out mouse models have recently independently been generated by other investigators (5,6), who have extensively described the physiological consequences of Aprt deficiency in mice.

Events leading to loss of Aprt function in somatic cells of Aprt heterozygous mice can be detected and analysed in any cell type that can be propagated in vitro. T-lymphocytes isolated from the mouse spleen have been used successfully for the determination of spontaneous and induced mutations at the hypoxanthine phosphoribosyltransferase (Hprt) locus. Detection of mutagenic events at the functionally hemizygous Hprt gene is, however,
limited to point mutations and deletions, due to its location on the X-chromosome. The mouseAprt gene is located near the telomere on chromosome 8, providing a large target for proximal chromosomal events like mitotic recombination and translocations (7).

In this report, we describe the generation of ourAprt knock-out mouse model using homologous recombination in ES cells. The resulting Aprt heterozygous ES cells and mice were used to determine spontaneous mutant frequencies at both theHprtandAprt loci. Furthermore, using dual-coloured fluorescence in situ hybridization (FISH), we have analysed if detected LOH events were the result of deletion, mitotic recombination or chromosome loss and duplication.

**MATERIALS AND METHODS**

**Generation of the targeting vector**

The construct for disruption of theAprt gene in mouse ES cells was designed in such a way that part of the promoter region and theATGstart codon were deleted. An 800 bp promoter fragment and a 2100 bp coding fragment were amplified from genomic DNA isolated from ES cells using primer combinations 85, 86 and 58, 32, respectively.

These fragments were random labelled (Boehringer Mannheim) and used as probes to screen a CCE (129Sv/Ev) EMBL3 genomic library (provided by G. Weeda) by standard plaque hybridization methods (8). A 20 kb Sall fragment, an internal 12 kb EcoRI fragment and an internal 11.5 kb EcoRV–Sall fragment were isolated from EMBL clone 6.1.2 and subcloned into pGEMEX-2 (Promega).

The construct pAprtneo was generated by subcloning a blunted 3.5 kb EcoRI–BamHI fragment into the bluntedClaI site of pTKNeoUMS (a gift of M. Gassmann) (9). This vector contains a UMS sequence that terminates transcripts and both a neomycin resistance cassette (PGKneoA) and aHerpes simplex virus thymidine kinase cassette (HSV-TK). The long arm of homology was introduced into theNotI–SacI sites as an 8 kb NotI–SacI fragment of the pGEMEX-2 EcoRV–SacI subclone. The uniqueSacI restriction recognition site was used for linearization of the targeting vector. A schematic representation of the resulting targeting vector is depicted in Figure 1A.

**Cell culture and gene targeting**

The E14 ES cell subline IB10 (kindly provided by E. Robanus-Maandag, Dutch Cancer Institute, Amsterdam) was cultured as described (10) on a feeder cell layer of X-ray (40 Gy) irradiated mouse embryonic fibroblasts (MEFs) in DMEM high glucose supplemented with 10% FCS (ES qualified), 1× non-essential amino acids, 50 μM β-mercaptoethanol, 500 U LIF/ml (ESGRO), 50 IU penicillin, 50 μg/ml streptomycin (all obtained from Life Technologies, Breda, The Netherlands) and 3.33 μM nucleosides (Sigma, St Louis, MO). Cells were electroporated with pAprtneo, using 2×107 cells and 25 μg of the linearized targeting vector DNA. Cells were washed once in 10 mM HEPES buffered PBS (pH 7.0) and electroporated in 0.8 ml of this buffer. The BioRad gene pulser was set at 400 V, 250 μF with a resulting time constant of 6.8.

After electroporation, cells were divided over five 100 mm dishes containing G418-resistant lethally irradiated (40 Gy) MEF feeder layers. Twenty-four hours after electroporation, cells were incubated with G418 (Geneticin 200 μg/ml active weight) and 48 h after electroporation gancyclovir (Cymovene, Mijdrecht, The Netherlands) was added to the medium to a final concentration of 2 μM. Selection was continued for 9 days before colonies were isolated and expanded in 24-well plates containing MEF feeders.

**Southern blot analysis of selected clones and mouse tails**

Cells or tails were lysed in buffer containing 10 mM Tris–HCl pH 8.0, 150 mM NaCl, 10 mM EDTA, 1% SDS, 100 μg/ml

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**Figure 1.** (A) Schematic representation of the targeting construct. Five boxes represent the exons of theAprt gene. Restriction sites (BamHI; C, ClaI; E, EcoRI; EV, EcoRV; H, HindIII; N, NotI; SII, SacI) and PCR primers are depicted. (B) Southern blot analysis of ES cell DNA digested byHindIII. The homologous recombinant ES cell clones were identified using probe A generating a 4.5 and a 4.0 kb band representing the wild-type and targeted allele. (C) Southern blot analysis of tail DNA using probe B resulting in a 12 kb wild-typeEcoRI fragment and a band of 4 kb representing the recombinated allele.
proteinase K at 37–55°C. The DNA lysates were phenol extracted, precipitated with isopropanol and the DNA was washed once in 70% ethanol. Approximately 5 µg DNA was digested with HindIII, electrophoresed and Southern blots were generated using Hybond-N+ membrane according to the alkaline blotting procedure (Amersham, Roosendaal, The Netherlands). The filters were analysed using random primer labelled probes (Boehringer Mannheim). Targeting of ES clones was determined using the 500 bp EcoRI–HindIII fragment adjacent to the 5′ arm as a probe. Hybridization of HindIII digested DNA with this probe resulted in a 4.5 kb band for the wild-type allele and a 4 kb band for the targeted allele (Fig. 1B). Blots of EcoRI digests were probed with the 2100 bp coding region PCR fragment. Additional integrations of the targeting vector were checked using the 2.0 kb PGKneoA fragment (results not shown). EcoRI tailblots were analysed using the 800 bp Aprt promoter fragment (Fig. 1C).

Allele specific multiplex PCR analysis

A 300 bp PCR product specific for the wild-type allele was amplified using primers 531 and UMS1 (531: 5′-CCCCAGGTCCAGAGACACTAG-3′, UMS1: 5′-CAGCTGAACATCACTGCAATC-3′). The mutant allele was amplified with primers 531 and UMS1 generating a 170 bp product (UMS1: 5′-GGGTTTTGATATGCGTGCAAGTAGC-3′). The multiplex PCR with these three primers was performed in a total volume of 50 µl in a mix containing 6.7 mM MgCl₂, 16.6 mM (NH₄)₂SO₄, 5 mM 2-mercaptoethanol, 6.8 mM EDTA (pH 8.0), 67 mM Tris–HCl (pH 8.8), 10% dimethyl sulphoxide (DMSO), 0.2 mM of each of four deoxyribonucleotide triphosphates, 20 pmol of each primer and 1.5 U AmpliTaq polymerase (Perkin Elmer). After an initial denaturation step at 93°C for 5 min, 35 cycles of 1 min at 94°C, 1 min at 55°C, 3 min at 72°C were performed in a Thermal Cycler (Perkin Elmer).

Cell lines and cell culture

The Aprt heterozygous ES cell line IB10.25 was cultured on lethally irradiated (40 Gy) MEFs. When plated on 0.1% gelatin (Sigma, St Louis, MO) coated culture dishes medium without nucleosides was supplemented with 50% BRL cell conditioned medium (10), hereafter referred to as BRL complete medium. Analysis of spontaneous Aprt and Hprt mutant frequencies was performed as follows. Five gelatin coated 100 mm dishes were seeded with 2 × 10⁶ cells each for the selection of spontaneous mutants using 50 µg/ml 8-AA and 5 µg/ml 6-thioguanine (6-TG), respectively. Simultaneously, five gelatin coated 100 mm dishes were seeded with 500 cells each to determine the plating efficiency. Cells from a few 8-AA resistant colonies were isolated for the determination of APRT enzyme activity.

Derivation of Aprt+/− and −/− mice

Three diploid ES cell clones were selected for injection of C57Bl/6 blastocysts (11). Male chimaeras were mated with C57Bl/6 females for germ line transmission. Resulting agouti offspring was analysed by Southern blot for the presence of the mutant allele. One chimaera derived of the IB10.25 line gave germ line transmission. The Aprt heterozygous offspring was interbred for the analysis of the phenotype of Aprt homozygous deficient mice. Standard genotyping of mice was performed using an allele specific multiplex PCR assay on tail DNA isolated by the salting-out procedure described by Miller et al. (12).

Measurements of APRT enzyme activity

APRT enzyme activity was measured in ES cells and red blood cells by a semiquantitative assay. Blood from the tails of mice was collected in heparinized tubes. After centrifugation, the cell pellet was washed three times in 0.85% saline. Red blood cells and ES cells were lysed in 50 mM Tris (pH 7.6), 0.2% Triton X-100. Cell lysates were pelleted and the clear supernatant was mixed in several dilutions with assay mix containing 5 mM MgCl₂, 30 mM Tris–HCl pH 7.5, 1.25 mM M-5-phosphorylribosyl-1-pyrophosphate, 2.7 nM adenine and 0.3 nM [14C]-labelled adenine (13). After incubation, various amounts of the reaction mixture were spotted on DE81 paper. Non-converted [14C]-adenine was removed by washing the DE81 paper four times in Tris–HCl pH 8.0, followed by scintillation counting of the amount of [14C]AMP. Protein concentration was estimated using the BioRad protein assay. Enzyme activity was determined as the amount of [14C]adenine converted to [14C]AMP/µg of protein.

Histopathology

Tissues of wild-type, Aprt heterozygous and Aprt deficient mice were fixed in 4% neutral buffered formalin, dehydrated through a series of alcohol gradients and embedded in paraffin. Sections of the kidneys of wild-type, heterozygous and mutant mice were mounted onto slides and compared after hematoxylin and eosin or periodic acid–Shift (PAS) staining.

T-lymphocyte clonal assay

The clonal assay was essentially performed as described by Yates et al., (14) for the analysis of mutations of the Hprt gene. Aprt mutant cells were selected using 50 µg/ml 8-AA. The culture medium for priming and cloning of mouse T-lymphocytes consisted of RPMI 1640 with 25 mM HEPES, 2 g/l NaHCO₃ and 2 mM L-glutamine supplemented with 30% AIM-V (Gibco, Life Technologies), 10% LAK supernatant (15), 15% heat inactivated fetal bovine serum (HyClone, Logan, UT), 50 µM 2-mercapto-ethanol (Merck, Darmstadt, Germany), 1 mM sodium pyruvate, 4 mM L-glutamaxes and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin sulphate). Cells were cultured at 37°C in a 5% CO₂ atmosphere. Mouse T-lymphocytes were isolated from the spleen using a 70 µM Falcon Cell Strainer. No Ficoll density gradient was performed before priming or freezing of the cells. The cell suspension was primed for 44 h in 15 ml culture medium supplemented with 4 µg/ml Canconavalin A (Pharmacia). Prior to proliferation of the T-lymphocytes in vitro, stimulated T-cells were plated for determination of cloning efficiency and Aprt or Hprt mutant selection as described (14).

Analysis of spontaneous 8-AA⁻ T-lymphocyte mutants

To estimate the frequency of LOH, 8-AA⁻ T-lymphocyte clones were expanded in culture medium supplemented with 50 µg/ml 8-AA. Each clone was expanded to three wells of a 96-well plate to reduce the number of dead wild-type T-lymphocytes and feeder cells present in the population. After 3 days culturing, cells were resuspended in medium and pelleted in Eppendorf tubes. The cells were washed once in PBS before crude cell lysates were made (16). Cells were incubated for 1 h at 55°C in 100 µl nonidet-p-lys buffer, consisting of 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 2.5 mM MgCl₂, 0.1 mg/ml gelatin, 0.45% NP-40, 0.45% Tween 20 and 60 µg/ml proteinase K. After heat inactivation of
proteinase K for 10 min at 95°C. 10 µl of each lysate was used in an allele specific multiplex PCR assay as described above.

FISH

Spontaneous mutant T-lymphocyte clones were expanded to three wells of a 96-well dish followed by another expansion to a total of six wells in culture medium containing 50 µg/ml 8-AA. Three days later cells were treated for 2 h with colcemid (0.1 µg/ml) and subsequently harvested. After a hypotonic shock in cold 0.075 M KCl buffer (4°C), cells were fixed by conventional 70% methanol/30% acetic acid procedure and chromosome spreads were prepared. Two probes were used for FISH analysis. The 20 kb Sall genomic Aprt fragment in pGEMEX-2 and a pSPORT-1 vector containing a 2 kb PGKneoUMS fragment, subcloned from pTKNeoUMS, were labelled with either biotin-16-dUTP or digoxigenin-11-dUTP (Boehringer Mannheim) in a standard nick-translation procedure. Dual-coloured FISH using avidin FITC and Texas Red was performed with minor modifications according to Hagemeijer et al. (17). The Aprt<sup>dig</sup>/PGKneoUMS<sup>bio</sup> hybridization was performed in a mixture containing 250 ng of each probe and 10 µg mouse Cot-I DNA. In the case of Aprt<sup>bio</sup>/PGKneoUMS<sup>dig</sup> hybridization, 250 ng of Aprt<sup>bio</sup> was pre-annealed with 10 µg mouse Cot-I DNA for 20–30 min after which 250 ng of PGKneoUMS<sup>dig</sup> was added. Pretreatment of slides, hybridization, washing and immunochromical detection were performed as previously described (18).

RESULTS

Targeted disruption of the Aprt gene in mouse ES cells

To obtain functional inactivation of the Aprt gene in mouse cells, a lambda clone containing a 20 kb Sall insert including the promoter region and coding sequence of the Aprt gene was isolated from a CCE (129Sv/Ev) genomic library. A targeting vector containing the neomycin and thymidine kinase genes for ‘positive and negative’ selection was constructed (Fig. 1A). The vector was linearized at the II site and transfected into IB10 ES cells by electro- transfection

| Table 1. Analysis of mendelian inheritance patterns and body weight of mice born from Aprt<sup>+/–</sup> × Aprt<sup>+/–</sup> crosses |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Aprt genotype | Animals weaned | Average body weight (s.e.m.) |
|                |                 | <sup>+</sup> | <sup>+</sup> | <sup>–</sup> | <sup>–</sup> | <sup>–</sup> | <sup>–</sup> |
| +/+             | 69              | 17.3%         | 58            | 14.6%          | 22.9           | 100%           | 30.3           | (2.8)          | 100%           |
| +/–             | 102             | 25.7%         | 113           | 28.5%          | 21.8           | (1.4)          | 95%            | 27.5           | (3.5)          | 91%            |
| –/–             | 34              | 8.6%          | 21            | 5.3%           | 18.8           | (2.2)          | 82%            | 20.5           | (2.9)          | 68%            |

The number and relative proportion (%) of offspring are given per genotype and sex. Body weight was determined of 41 mice (26 <sup>+</sup> and 15 <sup>–</sup> mice) between 4 and 11 weeks of age. Body weight measurements were performed for large litters only and which had the expected distribution of the three genotypes. Mean body weight, standard error of the mean (s.e.m.) and the proportion (%) to wild-type offspring are presented per genotype and sex.

Demonstration of the Aprt null phenotype

To test whether the insertion of a neomycin cassette into one of the two alleles of the Aprt gene indeed abolished its expression, allowing selection of 8-AA<sup>–</sup> clones mutated at the remaining wild-type allele, several spontaneous 8-AA resistant (8-AA<sup>+</sup>) ES clones were selected from Aprt<sup>+/–</sup> cells. Crude cell extracts were prepared from the parental cell line, four heterozygous ES clones and five 8-AA<sup>–</sup> clones, for determination of the Aprt enzyme activity. The Aprt enzyme activity in Aprt<sup>+/–</sup> and 8-AA<sup>+</sup> ES cells was, respectively, 39–65% and 0–7% of wild-type cells, indicating that the gene targeting event at Aprt indeed resulted in loss of Aprt function in ES cells.

Generation of Aprt<sup>+/–</sup> and Aprt<sup>–/–</sup> mice

Diploid ES cells of clone IB10.25 were used to construct male chimerae that were mated with C57Bl/6 females. Germ-line transmission of the targeted allele was obtained and subsequent brother–sister matings of Aprt heterozygous mice gave rise to Aprt<sup>–/–</sup> offspring. Blood from tails of mice of all three genotypes was analysed for Aprt enzyme activity. The residual Aprt activity in Aprt<sup>+/–</sup> and Aprt<sup>–/–</sup> mice was 62 and 1%, respectively, again indicating that no functional Aprt mRNA was produced from the targeted Aprt allele.

Analysis of the mutant phenotype in Aprt<sup>+/–</sup> mice

No phenotypic abnormalities were detected in Aprt<sup>+/–</sup> mice. Matings of Aprt heterozygotes resulted in offspring of all three genotypes but the Mendelian inheritance pattern was skewed. Only 5 and 8% male and female homozygous deficient animals respectively were weaned compared to 12.5% expected for each sex. Within these litters some of the pups were clearly smaller in size. The small body size invariably correlated with the Aprt<sup>–/–</sup> genotype of these mice. At 4–11 weeks of age the average body weight of Aprt<sup>–/–</sup> offspring was 75% of their wild-type and heterozygous littermates (Table 1), which might be the result of early onset of the kidney defects observed in the Aprt deficient mice (5,6). The severity of the kidney pathology varied between individual mice, but correlated well with the health status. Severely affected mice had a low body weight, dehydrated appearance and a hunched posture and died prematurely. The life span of Aprt<sup>–/–</sup> mice ranged from 6 weeks to 12 months with a mean age of death of 6 months. Relatively healthy Aprt<sup>–/–</sup> mice were fertile, but fertility dropped with deteriorating kidney function.
T-lymphocytes isolated from conditions for 8-AA selection of conditions described by Tates et al. (14) we defined selection conditions for 8-AA selection of Aprt mutant lymphocytes in population of T-lymphocytes isolated from Aprt heterozygous mice. For this purpose, splenic T-lymphocytes were isolated from Aprt–/+ mice. Concentrations up to 75 µg/ml of the selecting agent 8-AA did not reduce the cloning efficiencies of Aprt mutant T-lymphocytes. Cloning efficiencies of Aprt mutant cells were furthermore not affected by the presence of wild-type or heterozygous T-lymphocytes at a density of 2 × 10^6 cells/well in a 96-well plate.

To determine the spontaneous mutant frequency in T-lymphocytes, 61 Aprt+/− mice were sacrificed at 15 weeks of age and splenocytes were isolated. Primed lymphocytes were plated for 6-TG or 8-AA selection to determine spontaneous Hprt and Aprt mutant frequencies, respectively. In 12 animals, enough primed lymphocytes were available to select for spontaneous mutant frequencies at both loci within the same animal. The average mutant frequency was 8.7 × 10^−6 for Aprt and 0.8 × 10^−6 for Hprt. This 10-fold difference in mutant frequency between Aprt and Hprt indicates that mutagenic events which cannot be scored at Hprt, such as mitotic recombination, may be the predominant cause for mutation at Aprt. (Fig. 2)

**FIGURE 3.** Allele-specific PCR analysis of Aprt mutant T-lymphocyte clones. The normal allele is shown as a 300 bp fragment and the targeted allele as a 170 bp fragment. The picture of the ethidiumbromide stained 2% agarose gel is representative for the analysis of 140 mutant clones.

**LOH as determined by allele-specific PCR and FISH**

To investigate whether the non-targeted Aprt allele was still present in the 8-AA+ clones, DNA was analysed by a multiplex allele-specific PCR. Mutant T-lymphocyte clones were subcultured twice to reduce the possibility of contamination by DNA of dead wild-type T-lymphocytes and feeder cells in the crude cell lysates. Of 140 spontaneous mutants, 97 (69%) had lost the wild-type Aprt allele, indicative of LOH (Fig. 3). To confirm the PCR results, nine clones were analysed by in situ hybridization. The presence of the non-targeted and the targeted Aprt alleles was determined by dual-coloured FISH. A 20 kb genomic Aprt fragment was used to visualize the location of the Aprt gene on both chromosomes 8, while the targeted Aprt allele was specifically stained using a 2 kb PGKneoUMS containing probe. Specific hybridizing signal by the PGKneoUMS probe, co-localizing with the Aprt signals, could however only be observed in a fraction of the metaphase spreads because of the small size of the probe. If in a diploid cell PGKneoUMS signals could be detected on both chromosomes 8 in >10% of the scored metaphases, this mutant was considered to have a duplication of the targeted Aprt allele. Five out of six spontaneous Aprt mutants which had shown LOH determined by allele-specific PCR contained a duplication of the targeted Aprt allele (Table 2; Fig. 4). These results indicate that LOH at the Aprt gene in mouse T-lymphocytes is predominantly caused by events such as mitotic recombination, gene conversion or chromosome loss and endo-reduplication.

**DISCUSSION**

Genetic events which lead to LOH play an important role in the development of tumours as they may convert a heterozygous configuration of a mutated tumour-suppressor gene into homozygosity. LOH can be brought about by various mechanisms, such as deletion, mitotic recombination, gene conversion and chromosome loss. Here, we report the development of Aprt heterozygous mice, which can be used to study the cellular processes that lead to loss of heterozygosity in normal somatic cells in vivo.
As has been described by Engle et al. (5) and Redhead et al. (6), the loss of one allele of the Aprt gene has no phenotypic effects while loss of both copies results in viable but clearly affected animals. In the purine-salvage pathway the APRT enzyme plays a role in the conversion of adenine into AMP. In the absence of the APRT enzyme, adenine will be hydroxylated to DHA which easily precipitates and forms renal stones. Ultimately, these DHA precipitates will destroy the kidney tissue resulting in renal failure and premature death (19). Aprt homozygous deficient mice displayed the renal pathology previously described for two other Aprt deficient mouse models (5,6). In a hybrid (C57Bl/6 × 129/Ola) background Aprt deficient mice had a reduced body weight (75%) compared to their wild-type and heterozygous littermates. On average, Aprt deficient mice died at 6 months of age with a considerable variation consistent with the severity of the kidney pathology. Engle et al. (5) and Redhead et al. (6) have already indicated that Aprt deficient mice were more often lost before weaning than their heterozygous and normal littermates, skewing the mendelian inheritance pattern. In our hands, only 50% of the homozygous deficient mice expected, based on mendelian inheritance, reached weaning age. Male offspring appeared to be more affected than females, both in the proportion being weaned and in the reduction of body weight. A similar sex-difference in the severity of the renal pathology of adult being weaned and in the reduction of body weight. A similar appeared to be more affected than females, both in the proportion

Table 2. Dual-coloured FISH analysis of spontaneous 8-AA⁴ T-lymphocyte clones

<table>
<thead>
<tr>
<th>Mutant</th>
<th>No. interpretable metaphases</th>
<th>No. metaphases with duplication (%)</th>
<th>LOH by PCR</th>
<th>Y/N</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.1</td>
<td>37</td>
<td>9 (24)</td>
<td>Y</td>
<td>Y/N</td>
</tr>
<tr>
<td>I.2</td>
<td>10</td>
<td>4 (40)</td>
<td>Y</td>
<td>Y/N</td>
</tr>
<tr>
<td>II.1</td>
<td>9</td>
<td>– (0)</td>
<td>N</td>
<td>Y/N</td>
</tr>
<tr>
<td>II.2</td>
<td>42</td>
<td>3 (7)</td>
<td>N</td>
<td>Y/N</td>
</tr>
<tr>
<td>II.3</td>
<td>55</td>
<td>9 (20)</td>
<td>Y</td>
<td>Y/N</td>
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<td>II.4</td>
<td>23</td>
<td>1 (4)</td>
<td>N</td>
<td>Y/N</td>
</tr>
<tr>
<td>II.5</td>
<td>15</td>
<td>– (0)</td>
<td>Y</td>
<td>Y/N</td>
</tr>
<tr>
<td>II.6</td>
<td>24</td>
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<td>Y/N</td>
</tr>
<tr>
<td>II.7</td>
<td>26</td>
<td>7 (27)</td>
<td>Y</td>
<td>Y/N</td>
</tr>
</tbody>
</table>

Dual-coloured FISH analysis of nine independent spontaneous 8-AA⁴ clones derived from two different animals (I, II). If >10% of the interpretable metaphases contained two chromosomes 8 with dual-coloured signals; this mutant was scored as having a duplication of the targeted allele. All clones were also analysed by allele-specific PCR and checked for heterozygosity.

Aprt in ES cells is indeed subjected to higher spontaneous mutation than Tk. Sixty-one Aprt heterozygous mice were used to determine spontaneous Aprt and Hprt mutant frequencies in T-lymphocytes isolated from the spleen. The mean mutant frequency at the heterozygous Aprt locus (8.7 × 10⁻⁵) was ∼10-fold higher than at the hemizygous Hprt locus (0.8 × 10⁻⁶). Allele-specific PCR analysis of 140 Aprt mutant T-lymphocytes revealed that the normal Aprt allele was lost in 97 mutants (69%). This result indicates that cellular mechanisms leading to LOH play a major role in spontaneous mutagenesis of somatic cells of the mouse. Dual-coloured FISH analysis showed that in five out of six mutants, with LOH as determined by allele-specific PCR, the targeted Aprt allele had become duplicated. LOH at the Aprt gene in mouse T-lymphocytes can thus be attributed to events such as mitotic recombination, gene conversion or chromosome loss and endo-reduplication. Stambrook and co-workers (7) have recently determinedAprt mutant frequencies in skin fibroblasts of Aprt⁺/− mice. The mean mutant frequency at which Aprt mutant clones arose was 1.7 × 10⁻⁴. Molecular analysis of these colonies showed that most mutants had lost the wild-type allele. Testing for LOH at simple sequence length polymorphism (SSLP) markers flanking Aprt indicated that mitotic recombination was the predominant mechanism leading to LOH in vivo.

These results are in good agreement with reported Aprt and Hprt mutation data in humans. In peripheral blood T-lymphocytes from heterozygous individuals with characterized germ-line Aprt mutations, mutant frequencies for Aprt were found to be 10–30-fold higher than for Hprt (23). Approximately 80% of the Aprt-deficient T-cells exhibited loss of the normal Aprt allele (24) and of linked polymorphic markers (25), indicating that LOH events were the main cause for spontaneous mutation. Interestingly, a 7-fold lower Aprt mutant T-cell frequency was observed in heterozygous individuals carrying a gross DNA change at the Aprt locus mutated in the germ-line. This relatively low frequency coincided with the absence of the class of LOH mutations (26). Presumably, the expression of a gene essential for cell survival is closely linked to the Aprt gene and has been
damaged by the gross germ-line mutation. Loss of the normal Aprt allele in somatic cells through a chromosomal LOH event would thus simultaneously abolish expression of the linked essential gene, resulting in cell death. The recovery of this class of Aprt mutants in these individuals is therefore not possible. Evidence for the presence of an essential gene flanking the Aprt gene has been obtained from studies using hemizygous hamster cells (27,28).

The TK6 lymphoblastoid cell line has been used to determine the spontaneous frequency of LOH events in vitro at the Tk, HLA-A or the Aprt locus (29–32). Of the mutants analysed, 71, 84 and 78%, respectively, displayed LOH, which was due to chromosome loss, deletion, translocation or mitotic recombination. In vivo, the fraction of mutations resulting from extended LOH events in human T-lymphocytes was only 30% at the HLA-A locus (33) and was shown to be highly donor-dependent (33) (A.G.de Nooij-van Dalen, personal communication). Virtually all LOH events in vivo at both the HLA-A and APRT loci could be attributed to mitotic recombination (7,25,26,33). The difference in the contribution of LOH events in vivo spontaneous mutagenesis between the HLA-A locus and the APRT gene (30 versus 84%) may be explained by a lower tolerance for extended LOH events at the HLA-A locus in T-lymphocytes. This implies that the possibility to recover the various genetic alterations which lead to LOH in vivo will vary between different chromosomes, depending, for instance, on the presence of recessive lethal mutations or imprinted genes.

Inbred mouse strains are an excellent tool to study the cellular processes involved in the induction of LOH, since recessive lethal mutations have been selected against in the breeding process. The virtual absence of lethal mutations allows maximal recovery of the various events leading to LOH. Aprt+/- mice provide a unique opportunity to investigate the mechanisms by which LOH arises and the role carcinogens play in inducing LOH in normal somatic cells. Furthermore, the availability of transgenic mouse models with created specific defects in pathways that guard genome integrity allow careful dissection of the contribution of these pathways in the generation of LOH.

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