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Genetics

Carcinogen-induced loss of heterozygosity at the *Aprt* locus in somatic cells of the mouse

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ABSTRACT **Genetic events leading to the loss of heterozygosity (LOH) have been shown to play a crucial role in the development of cancer. However, LOH events do not occur only in genetically unstable cancer cells but also have been detected in normal somatic cells of mouse and man. Mice, in which one of the alleles for adenine phosphoribosyltransferase (*Aprt*) has been disrupted by gene targeting, were used to investigate the potency of carcinogens to induce LOH *in vivo*. After 7,12-dimethyl-1,2-benz[*a*]anthracene (DMBA) exposure, a 3-fold stronger mutagenic response was detected at the autosomal *Aprt* gene than at the X chromosomal hypoxantine-guanine phosphoribosyltransferase (*Hprt)* gene in splenic T-lymphocytes. Allele-specific PCR analysis showed that the normal, nontargeted *Aprt* allele was lost in 70% of the DMBA-induced *Aprt* mutants. Fluorescence *in situ* hybridization analysis demonstrated that the targeted allele had become duplicated in almost all DMBA-induced mutants that displayed LOH at *Aprt*. These results indicate that the main mechanisms by which DMBA caused LOH were mitotic recombination or chromosome loss and duplication but not deletion. However, after treatment with the alkylating agent *N*-ethyl-*N*-nitrosourea, *Aprt* had a similar mutagenic response to *Hprt* while the majority (90%) of *N*-ethyl-*N*-nitrosourea-induced *Aprt* mutants had retained both alleles. Unexpectedly, irradiation with x-rays, which induce primarily large deletions, resulted in a significant increase of the mutant frequency at *Hprt* but not at *Aprt*. This *in vivo* study clearly indicates that, in normal somatic cells, carcinogen exposure can result in the induction of LOH events that are compatible with cell survival and may represent an initiating event in tumorigenesis.**

Tumorigenesis is an *in vivo* process that can be caused by exposure to various environmental factors, such as chemical or physical agents (1). Epidemiological studies have shown that exposure to tobacco smoke, aflatoxin B_1, and UV light causes an increase in the rate of lung, liver, and skin cancers, respectively, in humans. Mutational spectra analysis in these cancers have revealed characteristic fingerprints associated with exposure to benzo[a]pyrene, aflatoxin B_1, and UV light (2, 3). However, such strong relationships between genotoxic exposure, mutations, and cancer are not well defined for other human cancers. For example, the role of diet in the induction of colorectal cancer is still unclear because the most characteristic mutations found in *p53* and *APC* tumor suppressor genes are C to T transitions, probably caused by spontaneous deamination of 5-methylcytosine residues (4). However, if predominantly genetic alterations are induced that result in loss of the wild-type allele, a carcinogen-specific fingerprint will not be detectable. This type of genetic events has been shown to be a major cause for loss of heterozygosity (LOH) in sporadic tumors as well as hereditary tumors with a germ-line mutation in one allele of a tumor suppressor gene such as *Rb-1, WT-1*, or *p53* (5). Apart from intragenic mutations, the underlying mechanisms of LOH include chromosomal events such as mitotic recombination, mitotic nondisjunction, gene conversion, and deletion (6, 7).

Although it is not known whether genetic events causing LOH can initiate the neoplastic process, it is clear that LOH can take place in an early stage of tumorigenesis. For example, mutation of one allele of the *APC* tumor suppressor gene and consequent loss of the remaining allele are the first events in the formation of sporadic intestinal tumors (8). It already has been reported that LOH events can occur in normal somatic cells of mice and man that are, in contrast to tumor cells, genetically stable and have not undergone multiple genetic changes (9–12). However, little information is available on the relative contribution of the various mechanisms leading to LOH in normal diploid cells *in vivo* and what factors are involved. We and others have studied the mechanisms underlying LOH by using mice heterozygous for the autosomal adenine phosphoribosyltransferase *(Aprt*) gene on chromosome 8 (11, 13). These $Aprt^{+/-}$ mouse models have been generated by using a conventional gene targeting approach (13, 14) and allow the study of mutations occurring *in vivo* at both *Aprt* and *Hprt*. Recovery of mutants at the hemizygous X chromosomal *Hprt* locus is limited to intragenic mutations and deletions whereas at the autosomal *Aprt* locus both intragenic mutations and chromosomal changes can be monitored. In somatic cells from $Aprt^{+/-}$ mice or human individuals heterozygous for *APRT,* background mutant frequencies were found to be substantial higher at the *Aprt* locus than at the *Hprt* locus (9, 13). Molecular analysis of *Aprt*-deficient mutants showed that these differences were mainly caused by LOH events at the *Aprt* locus, i.e., mitotic recombination, which cannot be recovered at the *Hprt* locus (10–13).

The present study focuses on the role of carcinogens in inducing LOH in somatic cells *in vivo*. $Aprt^{+/-}$ mice were exposed to the model compounds 7,12-dimethyl-1,2-benz[a]anthracene (DMBA) and *N*-ethyl-*N*-nitrosourea (ENU) as well as to x-rays. The polycyclic aromatic hydrocarbon DMBA induces bulky DNA lesions, mainly at guanine and adenine residues, whereas the direct acting agent ENU ethylates *O*- and *N*-atoms of all four types of bases in the DNA, and x-rays induce mainly DNA strand breaks. All of these genotoxic agents induce high frequencies of *Hprt* mutants in

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Abbreviations: DMBA, 7,12-dimethyl-1,2-benz[*a*]anthracene; ENU, *N*-ethyl-*N*-nitrosourea; LOH, loss of heterozygosity; FISH, fluorescence *in situ* hybridization; 8-AA, 8-azaadenine; 8-AA^r, 8-AA-resistant.
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mouse or rat somatic cells *in vivo* and are potent rodent carcinogens (refs. 15–17 and A. D. Tates, personal communication). In the present study, mutations were detected at the *Aprt* and *Hprt* loci in splenic T-lymphocytes by using a mouse splenocyte clonal assay (18). The *Aprt* mutant splenic T-lymphocyte clones induced by DMBA, ENU, or x-rays were analyzed at the molecular level by using an allele-specific PCR analysis. Dual colored fluorescence *in situ* hybridization (FISH) analysis was performed to investigate which mechanism is primarily responsible for LOH at the *Aprt* locus.

MATERIALS AND METHODS

Mice. The generation of $Aprt^{+/-}$ mice in which one copy of the *Aprt* gene was inactivated by gene targeting with a neo construct in embryonic stem cells is described elsewhere (13). $Aprt^{+/-}$ mice used in these mutational studies were obtained by intercrossing either F1 (50% Ola129, 50% C57BL/6) heterozygotes or crossing F1 heterozygous mice with F1 homozygous deficient mice ($Aprt^{+/-} \times Aprt^{+/-}$ or $Aprt^{+/-} \times Aprt^{-/-}$). Genotypes were determined by allele-specific multiplex PCR analysis of DNA isolated from tail tips. DNA isolation was carried out by the salting-out technique as described (19).

In the PCR, 3 primers were used:

aprt 531, 5′-CCCCAGGTCCAGAAGACTAG-3′;

aprt 831, 5′-CACGCTAAACTCACGTCAATC-3′ and;

Ums 1, 5′-GGGTTTGATATGCGTGCACAG-3′.

The PCR reaction was performed in a mix containing 6.7 mM $MgCl_2$, 16.6 mM $(NH_4)_2SO_4$, 5 mM 2-mercaptoethanol, 6.8 mM EDTA, 67 mM Tris·HCl (pH 8.8), 10% dimethyl sulfoxide, 0.2 mM of each of the four deoxyribonucleotide triphosphates, 20 pmol of each of the PCR primers, and 1.5 units of Amplitaq polymerase (Perkin–Elmer) in a total volume of 50 μl. After an initial denaturation step at 93°C for 5 min, 35 cycles of 1 min at 94°C, 1 min at 55°C, and 3 min at 72°C were performed in a Thermal Cycler (Perkin–Elmer). The wild-type allele (aprt 531–aprt 831) was identified as a 300-bp PCR product, and the targeted allele (aprt 531–Ums 1) was identified as a 170-bp PCR product.

Chemicals and Exposure. Both male and female $Aprt^{+/-}$ mice were used that were 8–12 weeks of age at the time of treatment. ENU (Pfaltz and Bauer) was purified as described (20), and, shortly before use, it was dissolved in Sørensen's phosphate buffer (pH 6.0). ENU was administered i.p. at single doses up to 100 mg/kg. DMBA (Sigma) was dissolved in tricaprilyn (Fluka) and was administered i.p. at single doses up to 60 mg/kg. For x-rays, $Aprt^{+/-}$ mice received a whole body exposure up to 3 Gy. The number of untreated control animals per experiment varied between two and nine mice. For the mutagen-treatments, 4–10 mice per dose group were used. Seven weeks after treatment, mice were killed, and spleens were isolated.

Isolation and Culturing of Splenic T-Lymphocytes. *Culture medium.* Priming and cloning of T-lymphocytes was done in RPMI culture medium 1640 as described by Tates *et al.* (18) with some minor modifications. The serum-free medium DCCM-1 was replaced by AIM-V (Life Technologies, Grand Island, NY), and the antibiotics consisted of 100 units/ml penicillin and 100 μg/ml streptomycin sulfate.

Isolation and priming of T-lymphocytes. Mouse T-lymphocytes were isolated from the spleen by rubbing the spleen through a sterile 70-μm nylon mesh (Falcon Cell Strainer, 2350) and subsequently were frozen in RPMI medium 1640 supplemented with 10% dimethyl sulfoxide and 40% fetal bovine serum by using a Cryomed freezing apparatus (Forma Scientific, Marietta, OH). When required, frozen cells were thawed at 37°C and immediately were stored on ice. Subsequently, 10 ml of RPMI medium 1640 supplemented with 40% fetal bovine serum was added slowly to the cells. Priming of the cells was performed in 15 ml of culture medium supplemented with 4 μg/ml Con A (Pharmacia) for 44 hr at 37°C, 5% CO_2.

Selection of 6-thioguanine- and 8-azaadenine-resistant mutants. Stimulated T-lymphocytes were cultured and selected for *Hprt* deficiency in the presence of lethally irradiated feeder cells as described by Tates *et al.* (18). Similar conditions were used to select *Aprt*-deficient mutants except that 50 μg/ml 8-azaadenine (8-AA; Sigma) was used as a selecting agent. The Sp2/0 feeder cells were mouse lymphoblastoid cells irradiated with 30 Gy of x-rays. After incubation for 6–8 days at 37°C, 5% CO_2, plates were scored for colony growth by using an inverted microscope. Cloning efficiencies and mutant frequencies were calculated as described (18).

Molecular Analysis of *Aprt*-Deficient Mutants. 8-AA-resistant ($8\text{-}AA^r$) clones were selected and diluted 1:3 in culture medium containing 8-AA (50 μg/ml). After 3 days of culturing, crude cell lysates were made (21). Cells were incubated for 1 hr at 55°C in 100 μl of Nonidet-lysis buffer (50 mM KCl/10 mM Tris·HCl, pH 8.3/2.5 mM $MgCl_2$/0.1 mg/ml gelatin/0.45% Nonidet P40/0.45% Tween 20) with 60 μg/ml proteinase K. Proteinase K was heat inactivated for 10 min at 95°C. A portion (10 μl) of this crude cell lysate was used in an allele-specific multiplex PCR in a total volume of 50 μl, as described for the tail tips.

FISH. Dilution and culturing of clones was performed as described for molecular analysis of *Aprt* mutants, followed by a second 1:2 dilution. After another 3 days of culturing, colcemid (0.1 μg/ml) was added to the cells 2 hr before harvesting. The cells were fixed by the conventional methanol-acetic acid procedure after a hypotonic shock with 0.075 M KCl at 4°C. Cell suspensions were dropped on clean slides at room temperature.

Probes used for FISH analysis were (*i*) a 20-kilobase genomic *Sal*I fragment containing the mouse *Aprt* gene in pGEMEX-2 (APRT) and (*ii*) a 2-kilobase PGKneopA fragment in a pSPORT-1 vector (NEO).

The probes were labeled with either biotin-16-dUTP or digoxigenin-11-dUTP (Boehringer Mannheim) in a standard nick translation procedure. Dual-colored FISH was carried out by using avidin fluorescein isothiocyanate and Texas Red as described (22) with minor modifications. In the case of $APRT^{bio}/NEO^{dig}$ hybridization, 250 ng of $APRT^{bio}$ was preannealed with 10 μg of mouse Cot-1 DNA for 20–30 min, after which 250 ng of NEO^{dig} was added. The $APRT^{dig}/NEO^{bio}$ hybridization was performed without preannealing, and the hybridization mix contained 250 ng of each of the probes and 10 μg of mouse-Cot-1 DNA. Pretreatment of the slides, hybridization, washing, and immunochemical detection was performed as described (23). All metaphases on a slide were evaluated. The dual-colored spots (yellow) at chromosome 8 were checked with separate microscope filters for the individual colors (red and green) to ascertain colocalization of the two probes.

RESULTS

Carcinogen-Induced *Aprt* and *Hprt* Mutant Frequencies. Young adult $Aprt^{+/-}$ mice were exposed to different exposure levels of DMBA, ENU, or x-rays. Seven weeks after treatment, both *Aprt* and *Hprt* mutant frequencies were determined in the same splenic T-lymphocyte cell population of each animal. The mean cloning efficiency (cloning efficiency ± SEM) per dose group varied between 9.5 (± 0.4%) and 20.5% (± 6.6%). After DMBA-treatment, a dose-dependent, significant increase in mutant frequency was seen at both the *Aprt* and *Hprt* genes (Fig. 1*A*). At equal dose levels, however, DMBA was 3- to 4-fold more mutagenic at the *Aprt* locus than at the *Hprt* locus. ENU also induced $8\text{-}AA^r$ and 6-thioguanine-resistant mutants in a dose-dependent manner, the induction curves for mutant frequencies having the same kinetics (Fig. 1*B*). Exposure to different doses of x-rays resulted in a clear increase in

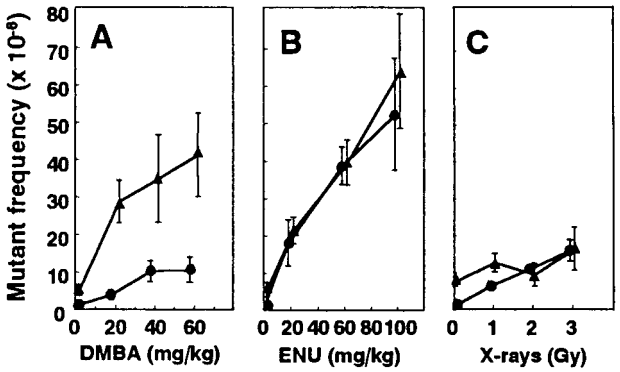

FIG. 1. Mutant frequencies (± SEM) in splenic T-lymphocytes of carcinogen-exposed *Aprt*$^{+/-}$ mice. Mutant frequencies were determined at two endogenous loci, i.e., *Aprt* (closed triangles) and *Hprt* (closed circles) after exposure to either DMBA (*A*), ENU (*B*), or x-rays (*C*).

the *Hprt* mutant frequency, which is linear with dose (Fig. 1*C*). Surprisingly, mutant frequencies at *Aprt* were not significantly different between x-irradiated and control animals, although there appeared to be a slight increase in *Aprt* mutant frequency at the highest dose. However, the high background mutant frequencies at *Aprt* versus *Hprt* ($7.4 \pm 1.2 \times 10^{-6}$ vs. $0.6 \pm 0.4 \times 10^{-6}$ in this experiment) made it more difficult to detect small increases in mutant frequency at *Aprt* than at *Hprt*. No differences in mutant frequencies were found between males and females for all three treatments.

These results indicate (*i*) that both the *Aprt* and *Hprt* loci appear to be similarly sensitive targets to the types of mutations induced by ENU, (*ii*) that DMBA induces a class of mutations at the *Aprt* locus that is not detectable at the *Hprt* locus, and (*iii*) that the hemizygous *Hprt* locus is a better target for the recovery of x-ray-induced genetic alterations than is the heterozygous *Aprt* locus.

Molecular Analysis of x-ray-, DMBA-, and ENU-Induced Mutants at the *Aprt* Locus. An allele-specific PCR-analysis was performed to detect the extent of loss of the normal, nontargeted *Aprt* allele in isolated mutants obtained from mice exposed to DMBA (40 and 60 mg/kg), ENU (60 and 100 mg/kg), or x-rays (2 and 3 Gy) (Fig. 2). After DMBA treatment, 70% of the mutant clones had lost the normal *Aprt* allele (64 of 92 mutants; Table 1). However, 70% of the DMBA-induced *Hprt* mutant clones still produced *Hprt* mRNA as determined by reverse transcription–PCR (data not shown). This finding suggests that deletions play at most a minor role in DMBA mutagenesis at *Hprt*. Among the ENU-induced mutants, only 13 of 125 *Aprt* mutants had lost the normal *Aprt* allele whereas x-rays induced loss of the normal *Aprt* locus in 30 of 43 mutants (70%) analyzed (Table 1). It should be noted that spontaneous mutants may contribute significantly to this latter set of mutants because x-rays caused only a small increase of the *Aprt* mutant frequency above the background level and 69% of the spontaneous *Aprt* mutants lack the normal *Aprt* allele (Table 1).

FISH Analysis of Mutant Clones. The underlying mechanisms by which LOH events arise include deletion, nondisjunction with or without chromosome duplication, mitotic recombination, and gene conversion. To investigate which mechanism is responsible for LOH at the *Aprt* locus in mutagen-induced 8-AA^r clones, a dual-colored FISH analysis was performed by using an APRT- and a NEO-specific probe. In *Aprt*$^{+/-}$ cells, the APRT-specific probe will hybridize to both chromosomes 8 because it recognizes the normal and targeted *Aprt* allele whereas the NEO-probe will hybridize solely to chromosome 8 harboring the targeted *Aprt* allele. Therefore, in metaphase spreads of nonmutated cells, the chromosome 8 homologue containing the targeted *Aprt* gene will have a dual-colored signal at both chromatids whereas the other homologue of chromosome 8 will contain a single-colored signal at both chromatids. The efficiency of the FISH analysis was determined by using *Aprt*$^{+/-}$ cells. In 10–22% of the metaphases, both chromatids of the targeted chromosome 8 contained a dual-colored spot (Table 2), which is the expected efficiency of FISH with small probes (24) such as the 2-kilobase NEO-probe used in these experiments.

Table 1. Allele-specific PCR analysis of *Aprt* mutant clones

	Total no. of clones	No. of clones lacking the nontargeted *Aprt* allele	LOH*, %
Background†	140	97	69
DMBA	92	64	70
ENU	125	13	10
x-rays	43	30	70

*Loss of heterozygosity.
†Ref. 13.

In all clones tested, the majority of the metaphases (85–90%) were diploid. To determine whether the targeted *Aprt* allele had become duplicated in mutant clones, the ratio of the number of dual-colored spots at both chromosomes 8 (two, three, or four) divided by the number of dual-colored spots at one chromosome 8 (one or two) of the individual mutants was calculated. In six of seven DMBA-induced 8-AA^r clones that showed loss of the nontargeted *Aprt* allele by PCR (I.1, I.3, I.4, II.1, III.1, and III.2; Table 2), this ratio was significantly different ($P < 0.0001$) from the ratio for the combined group of DMBA- and ENU-induced mutants that had retained the nontargeted allele (Table 2). This result indicates that, in these six mutants, both chromosomes 8 contain the neo-insert (Fig. 3). Apparently, DMBA-induced LOH at the *Aprt* locus in *Aprt*$^{+/-}$ splenic T-lymphocytes frequently is accompanied by duplication of the targeted allele.

In all nine ENU-induced 8-AA^r clones, no LOH of the *Aprt* locus was detected by using allele-specific PCR. These mutants contained almost exclusively dual-colored signals at the chromosome 8 harboring the targeted allele and single-colored signals at the other chromosome 8 (Table 2). However, occasionally, because of relatively high background signals of the NEO-probe, some metaphases were detected that contained dual-colored spots at both chromosomes 8 (Table 2).

DISCUSSION

To gain information on the nature of adducts that are capable of inducing LOH *in vivo*, *Aprt*$^{+/-}$ mice were exposed to three mutagens causing different types of DNA-alterations, i.e., bulky lesions, alkylation damage, and DNA breaks. Exposure of *Aprt*$^{+/-}$ mice to the potent rodent carcinogen DMBA (17), a model-agent for polycyclic aromatic hydrocarbons, resulted in a 3- to 4-fold increase in the induction of *Aprt* mutant clones compared with the induction of *Hprt* mutant clones, at the same exposure level. Dual-colored FISH analysis revealed that six of seven DMBA-induced *Aprt* mutants showing LOH had lost the normal, nontargeted *Aprt* allele whereas the targeted *Aprt* allele had become duplicated. DMBA-induced mutations at the *Hprt* locus consisted presumably of intragenic mutations, as was found in rats in which the majority of DMBA-induced *Hprt* mutants carried bp substitutions (16). ENU treatment of *Aprt*$^{+/-}$ mice gave rise to comparable mutant frequencies at the *Aprt* gene and the *Hprt* gene by using the same dose. The fraction of *Aprt* mutants that showed LOH by allele-specific PCR was small (10%). Mutational spectra analysis at the *Hprt* gene in skin fibroblasts or T-lymphocytes of rats, mice, and monkeys exposed *in vivo* to ENU have shown that the predominant types of mutation were transversions at A:T base pairs probably caused by *O*-ethylated thymines mispairing with thymine or cytosine during DNA replication (15, 25, 26).

Table 2. Dual-colored FISH analysis of 2 *Aprt*$^{+/-}$ clones and 20 *Aprt* mutant clones

	Clone	Total no. of metaphases	Hybridization patterns on chromosomes 8						LOH by PCR§
Aprt$^{+/-}$*	1	30	17	10	3	0	0	0	
	2	100	46	32	22	0	0	0	
DMBA†	I.1	51	19	16	8	3	5	0	Y*
	I.2	15	12	1	2	0	0	0	N
	I.3	51	7	4	25	3	1	11	Y*
	I.4	96	36	34	11	13	1	1	Y*
	I.5	24	9	9	5	1	1	0	Y
	II.1	176	74	47	31	19	2	3	Y*
	II.2	59	23	19	16	1	0	0	N
	II.3	46	36	6	4	0	0	0	N
	III.1	84	24	14	25	4	13	4	Y*
	III.2	101	3	13	37	14	18	16	Y*
	III.3	28	17	6	4	0	1	0	N
ENU‡	IV.1	71	35	21	14	0	0	1	N
	IV.2	61	34	12	12	0	3	0	N
	IV.3	19	15	4	0	0	0	0	N
	IV.4	37	21	10	5	0	1	0	N
	IV.5	53	5	22	23	2	1	0	N
	V.1	61	28	7	22	3	2	1	N
	V.2	105	61	36	8	0	0	0	N
	V.3	166	106	49	11	0	0	0	N
	V.4	74	21	28	21	3	1	0	N

Nonselected *Aprt*$^{+/-}$ clones* were isolated from cloning efficiency plates. 8-AA^r mutant clones were derived from five different animals (I, II, III, IV, V) treated with either 40 mg/kg DMBA† or 100 mg/kg ENU‡. ⋔ represents a metaphase chromosome 8; ● represents simultaneous staining of the APRT- and NEO-probes. All clones also were analyzed by allele-specific PCR and were checked for loss of heterozygosity§. The ratio of the number of dual-colored spots at both chromosomes 8 (2, 3, or 4 spots) divided by the number of dual-colored spots at one chromosome 8 (1 or 2 spots) was used to determine whether duplication of the targeted *Aprt* allele had occurred in the mutant clones showing LOH by PCR. In the clones marked with an asterisk, a statistically significant difference (χ^2 test, $P < 0.0001$) was found in this ratio compared to the ratio for the combined group of DMBA- and ENU-induced mutants without LOH by PCR, indicating that, in the marked clones, the targeted *Aprt* allele had become duplicated.

The relative potency of DMBA and ENU to induce chromosomal type of LOH events as shown here is in good agreement with literature data. DMBA-induced mouse skin carcinomas had a wide spectrum of alterations at chromosome 7, including trisomy, mitotic recombination, deletion, and gene duplication whereas the alkylating agent *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine-induced tumors only rarely exhibited chromosomal alterations (27). Also, DMBA-induced mouse mammary tumors showed LOH on chromosomes 4 (in 25% of the analyzed tumors), 8 (in 20%), and 11 (in 30%) (28). Moreover, ENU treatment has been shown to cause more frequently intragenic mutations than recombinational events at the Chinchilla locus by using the spot test in mice (29).

Whole body exposure of *Aprt*$^{+/-}$ mice to a dose of 1, 2, or 3 Gy of x-rays resulted in a significant mutation induction at the *Hprt* locus but surprisingly not at the *Aprt* locus. The predominant type of mutations induced by x-rays have been shown to be large multilocus deletions (30, 31). The recovery

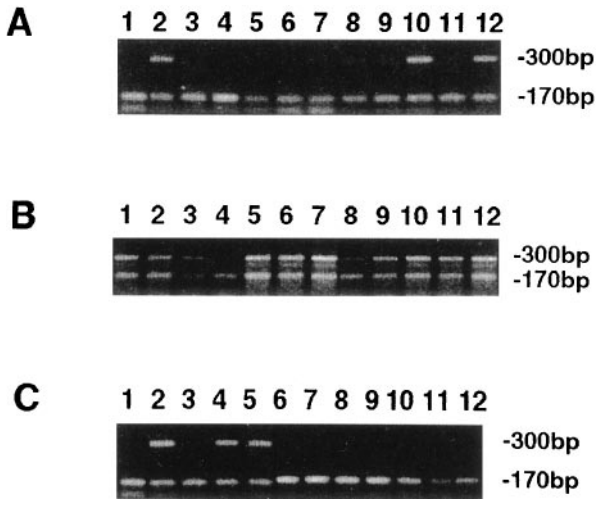

FIG. 2. Allele-specific PCR analysis for the detection of loss of the wild-type *Aprt* allele in *Aprt* mutant clones. Loss of heterozygosity in *A*, DMBA-induced 8-AA^r clones; in *B*, ENU-induced 8-AA^r clones; in *C*, x-ray-induced 8-AA^r clones. All lanes represent different mutant clones. The normal allele is shown as a 300-bp fragment; the targeted allele is shown as a 170-bp fragment.

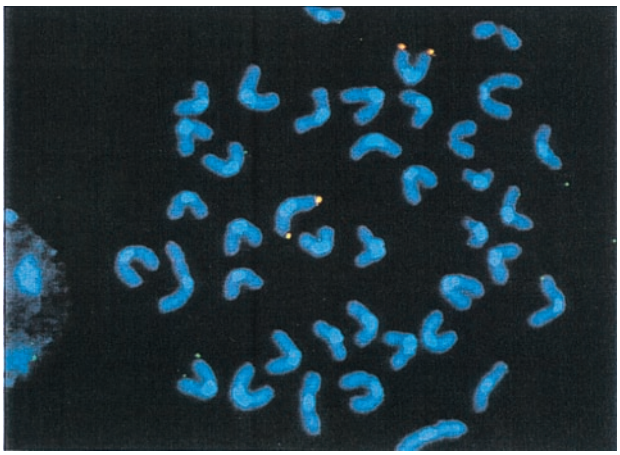

FIG. 3. Dual-colored FISH-analysis of a DMBA-induced *Aprt* mutant clone. Duplication of the targeted *Aprt* allele can be recognized because both chromosomes 8 are hybridized with the APRT- (red) and NEO-specific (green) probe. Colocalization of these two probes gives rise to a red/yellow spot at the telomeric end of chromosome 8.

of x-ray-induced mutants, therefore, is expected to be better for a heterozygous locus than for a hemizygous locus because concomitant deletion of a nearby essential gene will lead to cell death in the latter case. Indeed, in various mammalian cell lines, a large difference in mutant frequency after x-irradiation was reported between autosomal loci in a heterozygous versus hemizygous configuration (32, 33). In cultured mouse embryonal carcinoma cells, x-ray-induced *Aprt* mutants contained large chromosomal events at chromosome 8 that were predominantly interstitial deletions (34, 35). However, in this *in vivo* study, large deletions appear to be tolerated better at the hemizygous *Hprt* gene than at the heterozygous *Aprt* gene. The reasons for this discrepancy in sensitivity of the mouse *Aprt* locus to x-rays are unclear. Genetic events might be induced that are viable in transformed embryonal carcinoma cells but are lethal to normal somatic cells *in vivo*. Possibly, because of the relatively telomeric location of the mouse *Aprt* gene, its loss is accompanied by loss of the telomere, resulting in a chromosome instability and reduced cell viability. Alternatively, an essential gene may be located close to the mouse *Aprt* gene of which a double gene dosage is necessary for cell survival, so that deletion of this gene together with *Aprt* will result in a cell lethal phenotype. Recently, such a candidate gene, thought to be essential for cell survival, was identified just downstream of the *Aprt* gene (36). However, hamster and human cells hemizygous for this gene seem to have normal growth characteristics. The weak mutagenic response to x-rays does not only indicate that large deletions are recovered poorly at the *Aprt* locus but also that LOH events of the type induced by DMBA do not occur after ionizing radiation.

Several cellular mechanisms can result in loss of the normal *Aprt* allele. The finding that, in most of the DMBA-induced *Aprt* mutants, the targeted *Aprt* allele had become duplicated indicates that the main mechanisms causing LOH at the *Aprt* gene were mitotic recombination or chromosome loss and duplication but not deletion. In contrast, alkylation damage induced by ENU gave rise predominantly to intragenic mutations at *Aprt* whereas exposure to x-rays resulted in a marginal induction of *Aprt* mutants. Among spontaneous human and mouse *Aprt* mutants *in vivo*, mitotic recombination seems to be the major pathway leading to LOH (10–13). *In vitro* studies in mammalian cell lines also showed, for both the autosomal *APRT* and *TK* genes, that mitotic recombination is the main mechanism leading to LOH in spontaneous and induced mutants (37, 39). Molecular analysis of small-colony mouse lymphoma *Tk* mutants revealed that, because of somatic recombination, the majority of the mutants had lost the Tk^+ allele whereas the Tk^- allele had become duplicated (40). Additionally, chromosome loss and duplication appeared to be a major mechanism for LOH *in vitro*, as determined at the human leukocyte antigen gene in 4 different human lymphoblastoid cell lines (41).

Which aspect of DNA lesions trigger the induction of recombinational events? Studies using duplicated mutant reporter genes have shown that DNA damage-induced intrachromosomal recombination is increased in the absence of nucleotide excision repair, suggesting that single-stranded nicks induced by this repair pathway are not a major cause for induction of recombinational events (42). More likely, blocked DNA replication forks might be the initiating events leading to recombination, as has been suggested for the induction of sister chromatid exchanges (43). Bulky DNA adducts such as those caused by DMBA probably represent a much stronger block for DNA replication than ethylated bases, which are bypassed more readily during DNA replication. If so, the relative potency of a mutagen to induce intragenic versus chromosomal mutations will depend on the ability of DNA replication to bypass the induced mutagenic adducts.

From epidemiological studies, it is known that human cancer rates are influenced by environmental factors, suggesting that environmental mutagens contribute to the induction of somatic mutations during lifetime (1). Carcinogenic polycylic aromatic hydrocarbons have been identified in many foods, including broiled, barbecued, and smoked meat or fish (44). However, there is often no clear relationship between specific carcinogenic components in the diet and cancer (4), and no characteristic mutational fingerprint can be found in colorectal tumors. The results obtained with DMBA-exposed $Aprt^{+/-}$ mice indicate that environmental carcinogens such as polycyclic aromatic hydrocarbons can induce chromosomal types of changes that lead to LOH in normal somatic cells without interfering with cell viability and that may contribute to the initiation of cancer. No mutational fingerprint will appear if a carcinogen predominantly induces chromosomal type of LOH events, providing an explanation for the absence of a carcinogen-specific fingerprint in colorectal cancer.

In the present study, we show that some carcinogens can cause LOH of a housekeeping gene in normal somatic cells *in vivo,* similar to the alterations causing allelic loss of tumor suppressor genes during cancer development. The mechanisms involved in the generation of LOH now can be analyzed carefully by using $Aprt^{+/-}$ mice crossed with transgenic mouse models defective in pathways that guard genome integrity. These studies should provide more insight into the role of LOH events in early stages of tumorigenesis.

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