Carcinogens induce genome-wide loss of heterozygosity in normal stem cells without persistent chromosomal instability

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Widespread losses of heterozygosity (LOH) in human cancer have been thought to result from chromosomal instability caused by mutations affecting DNA repair/genome maintenance. However, the origin of LOH in most tumors is unknown. The present study examined the ability of carcinogenic agents to induce LOH at 53 sites throughout the genome of normal diploid mouse ES cells. Brief exposures to nontoxic levels of methyl nitrosourea, diepoxybutane, mitomycin C, hydroxyurea, doxorubicin, and UV light stimulated LOH at all loci at frequencies ranging from $1 \times 10^{-3}$ to $8 \times 10^{-9}$ per cell (10–123 times higher than in untreated cells). This greatly exceeds the frequencies at which these agents have been reported to induce point mutations and is comparable to the rates of LOH observed in ES cells lacking the gene responsible for Bloom syndrome, an inherited DNA repair defect that results in genomic instability during the carcinogenic process. Finally, as a practical matter, chemically induced LOH is expected to enhance the recovery of homozygous recessive mutants from phenotype-based genetic screens in mammalian cells.

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Abbreviations: LOH, loss(es) of heterozygosity; MNU, methyl nitrosourea; HU, hydroxyurea.
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that can splice to the 3′ recombination appears to be the preferred mechanism of homozygous mutations induced by gene targeting (33). Mitotic higher concentrations of G418, a method first shown to select for from heterozygous cells simply by selecting for resistance to homozygous for GTR1.3-induced mutations can be selected the mouse genome. Previous studies have shown that cells /H11003

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Results and Discussion

Carcinogen-induced LOH. Carcinogen-induced LOH was measured in a panel of 53 mouse ES cell clones, each containing a neomycin resistance gene (Neo) inserted into a different cellular gene (Fig. 1) by the GTR1.3 gene trap retrovirus. Gene entrapment by GTR1.3 involves selection for inserted Neo sequences that can splice to the 3′ ends of cellular genes (Q.L., S.L.D., and H.E.R., unpublished work). The disrupted genes were identified by sequencing Neo-gene fusion transcripts and were localized on the mouse genome. Previous studies have shown that cells homozygous for GTR1.3-induced mutations can be selected from heterozygous cells simply by selecting for resistance to higher concentrations of G418, a method first shown to select for homozygous mutations induced by gene targeting (33). Mitotic recombination appears to be the preferred mechanism of spontaneous LOH involving Neo genes inserted in ES cells (34) and LOH involving other genes and cell types in vivo (35, 36). LOH doubles the number of Neo genes per cell and thus allows moderately resistant cells to acquire resistance to higher concentrations of G418. The frequencies of spontaneous LOH measured at the 53 different sites ranged from 1.3 × 10^{-3} to 1.2 × 10^{-4} (Table 2, which is published as supporting information on the PNAS web site), similar to those reported for other inserted neomycin resistance genes (33, 34) in ES cells, and for loci such as TK and APRT in other cell types (35, 36).

A variety of chemical agents were tested for their ability to enhance the frequencies at which mutant cells survive in 2.0 mg/ml G418. Methylnitrosourea (MNU) is an alkylating agent that produces a variety of monomethylated DNA adducts, hydroxyurea (HU) stalls DNA replication complexes, doxorubicin interferes with DNA synthesis, methotrexate is a competitive inhibitor of dihydrofolate reductase but is not genotoxic, diepoxybutane and mitomycin C induce interstrand DNA crosslinks, UV irradiation causes intra- and interstrand pyrimidine dimmers, and ethidium bromide intercalates between DNA strands to damage DNA (for additional information about these agents, see http://toxnet.nlm.nih.gov and http://listweb.swan.ac.uk/cmgt/index.htm). Treatment with 0.5 mM MNU or 0.25 mM HU dramatically increased the number of colonies surviving in high G418 (Fig. 2). The fold increase for MNU and HU ranged from 39–123 and 18–68, respectively (Table 2). The optimal concentrations of each agent to stimulate colony formation with minimal toxicity (<5% loss of cell viability) were determined in advance (Fig. 5, which is published as supporting information on the PNAS web site). Other genotoxic agents that have been reported to promote recombination doxorubicin (0.1 μM), diepoxybutane (100 ng/ml), mitomycin C (50 ng/ml), and UV light (5 J/m²) also enhanced colony formation by an average of 15-, 16-, 14-, and 10-fold. However, ethidium bromide (25 μg/ml) and methotrexate (50 μM) had no significant effect (Table 2 and data not shown). Each of these agents was tested two or more times on at least 25 entrapment lines.

Genotypic analysis of five different mutants confirmed that 100% of colonies that survived high G418 selection after carcinogen treatment had undergone LOH (Fig. 3) compared to 85% of spontaneously resistant colonies. Thus, colony formation in 2.0 mg/ml G418 provided a direct measure of carcinogen-induced LOH at each entrapment locus. The overall extent of LOH induced by a single exposure to nontoxic levels of either MNU or HU was remarkably high (Table 2), in some cases exceeding 1% of the genome.

LOH Is a Transient Response to Carcinogens. Two types of experiments were performed to assess whether frequencies of LOH were transiently or stably elevated after carcinogen exposure. First, cells were treated with MNU and HU as before, and the percentages of cells having undergone LOH were determined by selection in 2.0 mg/ml G418 at various times thereafter. Over 90% of the total LOH was induced within 24 h of MNU and HU exposure, and only minimal additional LOH occurred subsequently (Fig. 6, which is published as supporting information on the PNAS web site). Second, we asked whether LOH frequencies at a second locus were elevated in cells having undergone LOH at the entrapment locus. For this, a herpes simplex virus thymidine kinase (TK) gene was introduced into cells containing an entrapment allele of the Hesx1 gene, and frequencies of TK gene loss were measured by selection in gancyclovir. These studies used the cell line containing the TK gene (C8TK1) and derivatives of C8TK1 that had undergone LOH at the entrapment locus

Fig. 1. Distribution of entrapment mutations in the murine genome. Stars represent the locations of GTR1.3 retroviral vector inserts in 53 clones on murine chromosomes 1–15 and 17–19. The centromere for each chromosome is positioned at the top of the ideogram.

Fig. 2. Limited carcinogen exposure enhances the survival of mutant ES cells in media containing 2.0 mg/ml G418. ES cells heterozygous for an entrapment mutation in Xrc5β were selected in high G418 directly (A) or after treatment for 4 h with 0.5 mM MNU (B), 0.25 mM HU (C), or 100 ng/ml diepoxybutane (D). After 12 days in selection, colonies were washed with PBS and stained with crystal violet.
induced spontaneously (C8TK1sN) or after treatment with either MNU (C8TKmN) or HU (C8TKhN). As shown in Table 1, the frequencies of spontaneous TK gene loss were similar in all cells regardless of whether carcinogens had been used previously to induce LOH at the entrapment locus. Moreover, the stability of the TK gene after carcinogen treatment was largely unaffected by prior selection for LOH involving the entrapment locus. Similar results were also obtained with a second TK-containing line (C8TK2; Table 3, which is published as supporting information on the PNAS web site). Together, these experiments indicate that carcinogen-induced LOH results from an acute response rather than from a stably altered cellular phenotype.

**Effect of Chromosome Position on Carcinogen-Induced LOH.** The frequency of spontaneous colony formation in high G418 was previously reported to increase with increasing distance from the centromere (Q.L., S.L.D., and H.E.R., unpublished work), consistent with previous studies suggesting that mitotic recombination plays a significant role in spontaneous LOH (34). Similar chromosome position effects were also observed (Fig. 4) after treatment with HU but not MNU (for example, $R^2$ values for loci on chromosome 4 were 0.65 and 0.11 after HU and MNU treatment, respectively), suggesting that mechanisms other than mitotic recombination were responsible for most of the MNU-induced LOH, consistent with studies in mouse lymphocytes (37). However, the ES cells used in the present study were derived from inbred mice and are naturally homozygous at all loci and thus cannot be used to distinguish among the possible mechanisms for generating LOH.

**Gene Entrapment in Studies of Genome-Wide LOH.** Entrapment ES cell clones provide an important *in vitro* model to study spon-
taneous and chemically induced LOH. ES cells are representative of self-renewing stem cells that serve as the precursors to cancer (38), and their use in mutagenesis studies is potentially important, because stem cells may possess specialized mechanisms to suppress mutations as a defense against oncogenic transformation (25–29). The clones are genotypically normal, as assessed by their ability to produce germine chimeras (10 of 10 clones tested) and normal offspring and thus lack coincidental mutations that might affect genome maintenance. Libraries of entrapment clones characterized for mouse genome mutagenesis provide large numbers of genetic markers that allow LOH frequencies to be measured at many sites in the genome. Rates of spontaneous LOH observed in ES cells are similar to those reported in a variety of other mammalian cell types (39, 40). Moreover, the influence of chromosome position indicates that the rates of spontaneous and HU-induced LOH do not primarily reflect localized effects of the integrated gene trap vector.

The use of entrapment ES cells also permits direct comparisons between the effects of chemical carcinogens and specific DNA repair defects such as the Bloom’s syndrome mutation. Given the ease of creating defined mutations that can be transferred back and forth between ES cells and mice, ES cells provide an ideal system to compare the effects of different mutations on spontaneous and carcinogen-induced LOH in a normal and potentially isogenic cellular background. It will be important to test whether endogenous or exogenous carcinogens contribute to genome-wide changes associated with defects in genome maintenance. For example, mice expressing reduced levels of Bub1B, a protein involved in mitotic spindle checkpoint control, form tumors only after carcinogen exposure (41). It should also be possible to assess how specific DNA repair/genome maintenance pathways influence the types of recombination events induced by different genotoxic agents (37).

The GTR1.3 vector has features that allow the selection of homozygous mutant cells except in cases where gene entrapment disrupts genes required for cell growth or viability. The present study suggests that MNU and HU can be used to enhance the recovery of clones homozygous for recessive mutations during phenotype-based genetic screens in mammalian cells (31, 32, 42). The vector may also allow mutagenesis screens to be carried out in a greater variety of cell backgrounds.

LOH as a Somatic Mutation: Implications for the Carcinogenicity of Mutagens Like MNU. Our results extend previous studies in which mutagens such as MNU have been reported to induce LOH (39, 43–48). However, these studies used nonmammalian systems or tumor-derived cell lines or were limited to only one or two loci. The present study provides a genome-wide analysis of carcinogen-induced LOH and an analysis involving normal diploid stem cells. As with most laboratory assessments of carcinogen risk, it is not possible to extrapolate from the concentrations of carcinogen used experimentally to the levels of exposure in human populations that typically occur over several decades. However, carcinogen concentrations were minimally toxic and were similar to those commonly used to induce tumors in animals.

LOH contributes to carcinogenesis by altering the dosage of genetically and epigenetically modified genes (22), including recessive cancer genes (tumor suppressors) of which >60 have been characterized (23). The ability of MNU and other agents used in the present study to induce point mutations is well established. These agents are also clastogens, as assessed by their ability to induce chromosome aberrations and sister-chromatid exchanges (http://toxnet.nlm.nih.gov). Our results indicate that the induction of LOH by a variety of mutagens occurs in normal stem cells at frequencies 2–4 orders of magnitude higher on a per-gene basis than the reported induction of point mutations. This could contribute to the notion that chromosome alterations such as LOH appear to have a greater impact on tumor cell genomes than the accumulation of point mutations (7, 14–16).

Frequencies of carcinogen-induced LOH were higher in some cases than the reported rates of LOH observed in ES cells homozygous for a mutation in the Bloom syndrome gene (Blm; refs. 30 and 31), an induced DNA repair defect that results in greatly increased risk of cancer. Higher LOH frequencies were observed, even allowing for differences in plating efficiencies of entrapment clones (10–50%; data not shown), as compared to the Blm-deficient ES cells (30%; ref. 31). In short, the carcinogens tested produced the appearance of chromosomal instability in normal stem cells in the absence of a genetically determined mutator phenotype. Of course, the Blm mutation causes a persistent state of chromosome instability, whereas the rates of carcinogen-induced LOH are elevated only transiently following carcinogen exposure. Although it is not clear how certain mutations affecting DNA repair induce LOH, it would appear that certain types of adducted DNA and/or stalled replication complexes can promote LOH regardless of whether they are caused directly by genotoxic agents or indirectly by genetic attenuation of DNA repair pathways. Just as the carcinogenicity of the Blm mutation has been attributed to high rates of LOH, the carcinogenicity of a variety of mutagens may result as much from their ability to induce LOH as from their ability to induce point mutations.

LOH as Somatic Mutation: Implications Regarding the Origins of LOH in Human Cancer. Extensive LOH in cancer cells is widely assumed to result from chromosomal instability; however, this conclusion is almost always based on the prevalence of LOH rather than on actual rate measurements (6). The present study showed that extensive LOH is induced in normal stem cells as an acute response to nontoxic levels of various carcinogens. We hypothesize that much of the LOH observed in nonhereditary cancers could result from prior exposure to genotoxic agents rather than from a state of genomic instability during the carcinogenic process. This hypothesis is consistent with the fact that >80% of cancers are caused by carcinogens present in the environment or produced by cellular metabolism (3–5), explains the apparent absence of mutations in genes required for DNA repair/genome maintenance in most cancers (6, 15, 16), and may account for the high levels LOH reported in several types of noncancerous lesions (18–21).

In summary, the present study describes a mechanism capable of generating high levels of LOH in the absence of a genetically activated genomic instability phenotype. Intrinsically low mutation rates and apoptosis in self-renewing stem cells have been proposed as mechanisms to suppress carcinogenesis (25–29). Similarly, the efficient use of sequences from homologous chromosomes to repair DNA damage and/or resolve stalled replication complexes could function to prevent coding sequence mutations. However, the process causes extensive LOH, with the likely consequence of unmasking recessive mutations in tumor suppressor genes.

Materials and Methods

Cell Culture. The AC1 ES cell line was derived from 3.5-day blastocysts from 129esJ mice. AC1 cells were infected with the GTR1.3 poly(A) gene trap vector, and entrapment clones were isolated in 300 μg/ml G418. GTR1.3 inserts a neomycin phosphotransferase gene (Neo) expressed from the constitutive Pol2 gene promoter. Selection for neomycin (G418) resistance generates cell clones in which the Neo gene splices to 3’ exons of cellular genes (Q.L., S.L.D., and H.E.R., unpublished work). Genes disrupted in the entrapment clones were identified by sequencing cellular sequences appended to Neo fusion transcripts. ES cells were maintained at 37°C in DMEM supple-
mented with 15% FBS, nonessential amino acids, l-glutamine, 2-mercaptoethanol, and lymphocyte inhibitory factor.

Colony Selection and Chemical Treatment of Cells. Serially diluted cells were plated onto 150-mm plates containing drug-free media and allowed to attach overnight. Unattached cells were removed, and media containing the indicated concentrations of MNU, HU, ethidium bromide, doxorubicin, methotrexate, diepoxybutane, or mitomycin C were put onto cells for 4 h (or cells were exposed to UV light in the absence of media and allowed to recover in drug-free media for 4 h). Cells were then rinsed twice with drug-free media, and selection media containing 0.0, 0.3, or 2.0 mg/ml G418 were put onto cells. After 12 days of selection, the number of colonies surviving was counted, and the frequency of colony formation was determined by dividing the number of colonies obtained from 2.0 mg/ml G418 selection to that obtained from parallel experiments with 0.3 mg/ml G418 selection. 

TK gene loss was assessed after selection in media containing 2 μg/ml gancyclovir.

Genotypic Analysis of LOH. Genotypic analysis was performed by Southern blotting and PCR. Southern blot analysis was performed on 5 μg of genomic DNA that had been digested with a restriction enzyme and resolved on 0.9% agarose gels. Southern blot hybridization was performed by using DNA probes obtained by PCR amplification of genomic DNA adjacent to the site of retroviral vector insertion. PCR analysis was performed on 200 ng of genomic DNA with three primers. The first primer was in the sense orientation and was specific for genomic DNA 5’ to the site of retroviral vector insertion. Two additional primers were added that were in the antisense orientation; one was specific for sequence 3’ of the retroviral vector insertion and the other specific for the LTR portion of the retroviral vector insertion. Using these three primers, PCR amplification of genomic DNA yielded a smaller DNA fragment when the entrapment vector was present and a larger DNA fragment when the entrapment vector was absent.

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