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Carcinogens Can Induce Homologous Recombination between Duplicated Chromosomal Sequences in Mouse L Cells

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The ability of a series of DNA-damaging agents to induce homologous intrachromosomal recombination between duplicated genes in the chromosome of mouse cells was investigated. The target cells were the thymidine kinase-deficient mouse L-cell strain 333M, which contains a single integrated copy of a plasmid with two herpes simplex virus thymidine kinase (H*tk*) genes, each containing an 8-base-pair *Xho*I linker inserted at a unique site. Expression of a functional H*tk* enzyme requires a productive recombinational event between the two nonfunctional genes. The spontaneous rate of recombination in this strain is 3 per 10^6 cells per generation. The agents tested represent physical carcinogens (UV and ionizing radiation), a simple alkylating agent (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine), an alkylating cross-linking agent (mitomycin C), and a reactive metabolite of a polycyclic aromatic hydrocarbon {(±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene [BPDE]}. The background frequency of tk^+ recombinants in the untreated population averaged 18 × 10^{-6} ± 5 × 10^{-6}. Ionizing radiation had little or no effect on recombination; exposure to mitomycin C, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, BPDE, or UV, at doses that lowered the survival to between 90 and 10% of the control, caused a dose-dependent increase in frequency of recombinants, reaching 50 × 10^{-6} to 100 × 10^{-6}. No tk^+ cells could be generated with a control cell line that contained only one mutant copy of the H*tk* gene. Molecular hybridization analysis showed that 85 to 90% of the tk^+ recombinants retained the H*tk* gene duplication, consistent with nonreciprocal transfer of wild-type genetic information, gene conversion. In the rest, only a single copy of the H*tk* gene remained, reflecting a single reciprocal exchange within a chromatid or a single unequal exchange between sister chromatids. Each recombinant tested contained an *Xho*I-resistant (wild-type) H*tk* gene.

Homologous mitotic recombination in somatic cells has been suggested as one mechanism in the multistepped process of tumorigenesis. For example, Cavanee et al. (5), using recombinant DNA technology, showed evidence in tumor cells from a retinoblastoma patient that mitotic recombination between genes on chromosomes 13 had led to homozygosity. Such recombination caused homozygosis of the mutant allele of the Rb-1 locus, allowing expression of the recessive phenotype in the patient's somatic tissues. Wasmuth and Vock Hall (40) provided genetic evidence for the occurrence of mitotic recombination between chromosomes 2 of Chinese hamster ovary cells in culture. In addition, the recent development of techniques for gene transfer and manipulation in mammalian cells has led to the development of a number of systems for detecting extrachromosomal (1–3, 6–8, 10–13, 16, 19, 24–28, 37, 38) and intrachromosomal (20–22, 25, 27, 32, 38a) homologous recombination in such cells, as well as targeted recombination between exogenous DNA and a chromosomal sequence (11, 18, 29, 30, 33–35).

Studies with lower eucaryotic organisms have demonstrated that DNA-damaging agents, such as UV (254 nm) and ionizing radiation, as well as simple alkylating agents, such as methyl methanesulfonate, can induce mitotic and meiotic allelic recombination and the frequency of recombination can depend on the DNA repair capacity of the cells. (See reference 14 for a review of these kinds of studies in yeasts.) However, less is known about the ability of such agents to induce homologous recombination in mammalian cells. One aim of the present studies was to determine whether carcinogens can induce homologous recombination between duplicated genes within the genome of mouse L cells. A second aim was to characterize the kinds of recombination events induced and draw inferences about possible mechanisms.

The system used involves a thymidine kinase-deficient mouse L-cell line, 333M, containing a single stably integrated copy of a plasmid carrying duplicated copies of the herpes simplex virus thymidine kinase gene (H*tk*), each inactivated by an 8-base-pair *Xho*I linker mutation at a unique site (22). Expression of a functional H*tk* enzyme requires a productive recombinational event between the two nonfunctional genes. The agents tested included two physical carcinogens (UV and cobalt-60), a simple alkylating agent (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine [MNNG]), an alkylating cross-linking agent (mitomycin C [MC]), and a polycyclic aromatic carcinogen {(±)-7β,8α-dihydroxy-9α, 10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene [BPDE]}. The results show that each agent, except cobalt-60, induced a dose-dependent increase in the frequency of tk^+ recombinants and that the majority of the recombination events represented gene conversions.

MATERIALS AND METHODS

Chemicals. MNNG (Pfaltz and Bauer, Flushing, N.Y.), BPDE (Chemical Carcinogenesis Program, National Cancer Institute, Bethesda, Md.), and MC (Sigma Chemical Co., St. Louis, Mo.) were stored at −20°C in a desiccator. Geneticin (G-418 sulfate) was purchased from GIBCO, Grand Island, N.Y., dimethyl sulfoxide was from Burdick and Jackson,

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Muskegon, Mich., and HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) was from Sigma.

Cells. The target cells were the mouse *tk* L-cell line 333M, which contains a single stably integrated copy of a plasmid carrying duplicated H*tk* gene sequences, each with an 8-base-pair *Xho*I linker insertion mutation at a different site (22). The *Xho*I linker insertion mutations map in the coding region of the H*tk* gene at positions 735 (mutant 26) and 1220 (mutant 8). The *neo* gene coding for Geneticin resistance is located between the two H*tk* genes. A second mouse L-cell line, used as a negative control, contains only one copy of the H*tk* gene with an *Xho*I 8-base-pair linker insertion mutation (mutant 26) (16).

Culture conditions. The cells were cultured in Eagle minimal essential medium supplemented with 0.2 mM L-aspartic acid, 0.2 mM L-serine, 1 mM sodium pyruvate, 15% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) (culture medium) in a humidified atmosphere of 5% CO_2–95% air at 37°C. To select tk^+ cells, this culture medium was supplemented with cytidine (2×10^{-5} M), hypoxanthine (10^{-6} M), aminopterin (2×10^{-6} M), and thymidine (3×10^{-5} M) (CHAT medium).

Assay of recombination induced by chemical carcinogens. Populations of exponentially growing cells were detached with 0.25% trypsin, suspended in culture medium, pooled, counted with an electronic cell counter, and plated into a series of 100-mm-diameter dishes at a density of 10^4 cells per cm^2 (5×10^5 cells per dish), unless otherwise designated. The number of dishes per determination was adjusted for the expected cell survival so as to have at least 2×10^6 surviving target cells after treatment, e.g., 20 dishes for an expected survival of 20%. An extra set of cells to be used for assaying cytotoxicity was included with each dose and with the untreated control. The target cells were allowed 6 h to attach and elongate before being exposed to the carcinogen. Just before treatment, the medium was replaced with culture medium lacking serum. In the case of MNNG, this serum-free medium was buffered to pH 7.3 with HEPES (15 mM). The test chemicals were dissolved just prior to use and delivered into the dishes by micropipette. The solvent for MC was distilled H_2O, that for MNNG was anhydrous dimethyl sulfoxide, and that for BPDE was anhydrous acetone. After 1-h exposure to the test agent or to the solvent control, the medium containing the test chemical was removed and the cells were rinsed with phosphate-buffered saline and refed with culture medium containing serum. In the case of MC, the treated cells were rinsed three times.

The cells in one dish for each dose were immediately plated at cloning densities to assay cytotoxicity (see below). The rest were allowed an expression period of ~20 h before selection for tk^+ recombinants was begun, using CHAT medium. Selection medium was renewed every 2 to 3 days for 12 to 14 days. When macroscopic CHAT-resistant colonies developed, representative colonies were isolated for further analysis (see below), and the rest of the clones were stained with crystal violet to determine their frequency. The frequency observed in the treated population was corrected for the number of viable target cells, as determined from the accompanying cytotoxicity studies. The frequency of induced recombination was determined by subtracting the background frequency observed in the control dishes accompanying each experiment.

Assay for recombination induced by UV radiation. The methods used to assay recombination induced by UV radiation were as described above for chemical treatment except that cells to be assayed for survival (cytotoxicity) were plated at cloning densities at the time the rest were plated at 5×10^5 cells per dish. After 6 h, the medium was removed and the cells were rinsed with phosphate-buffered saline and irradiated with a Mineralight UVSL-54 germicidal lamp as described previously (23). The incident dose was 0.5 J/m^2 per s as determined with an IL570 radiometer (International Light, Newburyport, Mass.). After irradiation, the cells were fed with fresh culture medium and allowed to undergo cell replication, and the recombinants were selected after 18 to 20 h.

Assay for recombination induced by ionizing radiation. The methods used to assay recombination induced by ionizing radiation were the same as for the chemicals except the target cells were suspended in culture medium at 5×10^5 cells per ml in plastic conical centrifuge tubes, placed on ice, and irradiated with a U.S. Nuclear ^{60}Co variable flux sealed source irradiator (exposure rate, 2.74 Gy/min), as described previously (39). Following irradiation, 5×10^5 cells were plated into each of a series of 100-mm-diameter dishes, enough to have at least 2×10^6 surviving target cells per dose. Cells were also diluted appropriately and plated at cloning densities to assay cytotoxicity (see below).

Cytotoxicity assay. The cytotoxic effect of the test agents was determined from the colony-forming ability of the treated cells compared with that of the solvent-treated control cells. Except for those receiving UV radiation, the cells were treated at the same density as for recombination and then plated at cloning densities (i.e., densities adjusted to obtain 50 to 100 microscopic colonies per dish; 75 to 750 cells per dish, 6 to 10 dishes per determination) and allowed 10 to 12 days to form macroscopic colonies. The cloning efficiency of the treated cells divided by that of the untreated cells was used to estimate the fraction of surviving cells.

Assay for Geneticin resistance. CHAT-resistant colonies were isolated by using sterile filter paper moistened with 0.25% trypsin, transferred into 23-mm-diameter wells (six-well plates) containing culture medium, and allowed to attach. Twenty-four hours later, the medium was changed to CHAT medium and the cells were allowed to grow. When the well was almost full, the cells were trypsinized and transferred to a 25-cm^2 flask in CHAT medium to be expanded and used for DNA analysis. At the same time, a sample was plated into each of two 8-cm^2 wells (12-well plates) and the cells were allowed to attach. After 24 h, Geneticin (400 μg/ml) was added to one well of each set. The medium in each well was renewed every 2 to 3 days, and the growth of the cells was monitored microscopically after 7 to 8 days.

DNA blotting and hybridization. Cells taken from individual isolated tk^+ colonies were expanded, and the DNA was prepared as previously described (21). Restriction enzymes were purchased from New England BioLabs, Inc., Beverly, Mass., and digestions were carried out under the supplier's recommended conditions. Digested DNAs (8 μg) were electrophoresed on 0.8% agarose (Sigma) gels, transferred to nitrocellulose, and hybridized with a ^{32}P-labeled probe (21). The probe consisted of a 2.4-kilobase-pair *Bam*HI fragment containing the H*tk* gene.

RESULTS

Optimization of assay conditions for recombination. Before examining the effect of various DNA-damaging agents on the frequency of homologous recombination, we carried out experiments to optimize the system. For example, we ex-

TABLE 1. Example of data used to determine frequency of carcinogen-induced recombination

Agent	Dose	Cloning efficiency	Survival (% of control)	Target cells (10^{-6})	Viable cells (10^{-6})	tk^+ colonies observed	tk^+ per 10^6 viable cells	Induced frequency (10^6)
MC (μg/ml)	0	0.73	100	2.0	2.0	26	13.0	
	0.5	0.68	93	2.0	1.86	45	24.2	11.2
	1.0	0.60	82	2.5	2.05	68	33.2	20.2
	1.5	0.52	71	3.0	2.05	78	36.2	23.2
	2.0	0.43	59	3.5	2.06	66	31.6	18.6
BPDE (μM)	0	0.72	100	2.0	2.0	39	19.5	
	0.15	0.42	58	3.5	2.03	75	36.9	17.4
	0.20	0.33	45	5.0	2.25	85	38.1	18.6
	0.25	0.145	20	10.0	2.0	123	60.3	40.8
MNNG (μM)	0	0.786	100	2.0	2.0	37	18.5	
	4.0	0.46	59	3.0	1.77	46	26.0	7.5
	5.5	0.30	38	5.0	1.9	63	33.2	14.7
	7.0	0.17	21.6	7.5	1.62	92	56.8	38.3
UV (J/m^2)	0	0.36	100	2.0	2.0	21	10.5	
	6.0	0.24	67	3.0	2.0	32	16	5.5
	8.0	0.087	24	15.0	3.6	87	24.5	14.0
	10.0	0.058	16	14.0	2.25	55	24.5	14.0

amined the effect of cell density at the beginning of selection on the frequency of CHAT-resistant colonies. Cells were treated for 1 h with 0.2 μM BPDE, a concentration previously determined to reduce the survival to ~40% and to induce recombination. They were then plated into a series of dishes at densities ranging from 0.5×10^4 to 2×10^4 cells per cm^2 and selected for CHAT resistance 18 h later as described above. The results indicated that the frequency of tk^+ cells in the untreated population was the same at each cell density, but that of the BPDE-treated population was slightly lower for the cells plated at $2 \times 10^4/cm^2$ than for those plated at the lower densities (data not shown). Therefore, a density of $10^4/cm^2$ was used for subsequent studies.

The length of time required for recombination to occur and be expressed, i.e., for cells with wild-type H*tk* gene to be able to survive CHAT selection, was also examined. The results showed that selection could be started as early as 12 h post-treatment or as late as 48 h without a significant effect on the frequency of CHAT-resistant colonies (data not shown). Therefore, an expression period of 18 to 20 h was used for convenience. Experiments designed to see if it was necessary to continue selection with CHAT medium for the entire period of colony formation to ensure that only the tk^+ recombinants formed colonies indicated that continuous selection was optimal (data not shown).

The spontaneous rate of homologous recombination in the 333M cell line is 3 per 10^6 per cell generation (22). Therefore, whenever the untreated population was found to have accumulated a high frequency of tk^+ cells, i.e., $>30 \times 10^{-6}$, the background frequency was lowered by diluting out the tk^+ cells. A series of 30 flasks was seeded with 10^3 cells per flask, and the cells were grown to large populations ($>2 \times 10^6$ cells). A portion of each population was assayed for the background frequency of tk^+ cells and the rest was frozen for future use. Only those populations with a low background were then used for assaying induced recombination.

Reconstruction studies were also carried out to determine the cloning efficiency of the cells plated at $10^4/cm^2$ and allowed to grow for 6 h before carcinogen treatment and for 20 h post-treatment before CHAT medium selection. CHAT-resistant tk^+ recombinant cells were seeded into dishes alone or on top of a lawn of 333M cells at 10^4 cells per cm^2. A third set of dishes was seeded with 333M cells at 10^4 cells per cm^2, but with no CHAT-resistant tk^+ recombinant cells added. One set of each series was exposed to 6 J of UV per m^2 and selected with CHAT medium according to the protocol described. The frequency of CHAT-resistant colonies in these three series of dishes indicated that the cloning efficiency of the cells was 100% (data not shown). Therefore, in subsequent experiments the frequency of tk^+ recombinants induced by carcinogens was calculated directly from the observed number of CHAT-resistant colonies per viable target cells. An example for each of the active agents is shown in Table 1.

Determining the doses to be used for recombination studies. Before testing the ability of each carcinogen to induce homologous recombination, we determined the concentration needed to lower the survival of the target cells to between 80 and 10% of the untreated control. The survival data from these preliminary cytotoxicity experiments (two to four experiments per agent) have been included in the top panels of Fig. 1 and 2, along with the survival data obtained in the subsequent series of experiments carried out to measure induction of homologous recombination. For the latter experiments, concentrations expected to yield survivals between 80 and 20% of the control population were used.

Induction of recombination with BPDE and MNNG. The ability of BPDE to decrease the survival of the target 333M cells and to induce recombination between the two mutant H*tk* genes was assayed in six experiments with various concentrations. An untreated control population accompanied each experiment. Similarly, the ability of MNNG to cause cell killing and recombination was assayed in six experiments. The data from each of these experiments are shown in Fig. 1. Both agents increased the frequency of recombination significantly above the background frequencies. For example, the highest concentration of BPDE tested, 0.25 μM, gave an observed frequency of 60.3 recombinants per 10^6 viable cells, which was three times higher than the background frequency of 19.5×10^{-6} (Table 1). This was plotted in Fig. 1 as an induced frequency of 40.8×10^{-6}. Similarly, the highest dose of MNNG gave an observed frequency of 95 recombinants per 10^6 viable cells in an experiment with a background frequency of 19×10^{-6},

giving an induced frequency of 76 × 10^{-6}. To compare the results of one experiment with another, the individual background frequencies were subtracted and the induced frequencies were plotted in Fig. 1.

Sensitivity of recombinants to the test agents. If background tk^+ recombinants preexisting in the target population were more resistant than *tk* cells to the cytotoxic effects of the test agents, this would cause an apparent increase in frequency of such recombinants. We tested and showed that this was not the case. Cells from two tk^+ colonies from plates receiving the highest dose of BPDE and two taken from plates receiving the highest dose of MNNG were isolated and tested for their sensitivity to killing by a series of doses of BPDE and MNNG. The results indicated that these tk^+ recombinants were just as sensitive as the original parent target population of 333M cells (data not shown).

Dependence on presence of duplicated H*tk* genes. As a negative control for recombination, the ability of BPDE and MNNG to induce CHAT-resistant cells in a population of mouse L cells containing only a single mutant copy of the H*tk* gene was investigated. The target cells for these studies were derived by using plasmid pIAT-2, which carries only one mutant H*tk* gene (22). As expected, even when the cells were treated with doses of BPDE and of MNNG that lowered their survival to between 50 and 30%, no tk^+ CHAT-resistant cells were found out of 8 × 10^6 viable cells assayed (data not shown). Letsou and Liskay (16) also

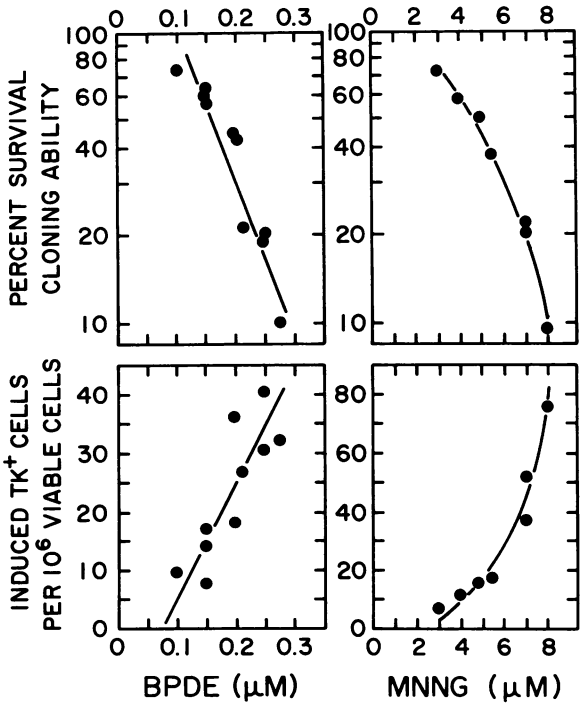

FIG. 1. Cell killing and induction of recombination as a function of the concentration of BPDE and MNNG. Cells plated at 10^4 cells per cm^2 into 100-mm-diameter dishes were treated for 1 h with BPDE or MNNG. Immediately following treatment, one set of cells was plated at cloning densities to determine survival. The rest were allowed a ~20-h expression period and then assayed for a functional *tk* gene by being selected for resistance to CHAT medium. The background frequencies in the populations ranged from 16.3 × 10^{-6} to 19.5 × 10^{-6}, with an average of 17.7 × 10^{-6}. These have been subtracted to give the induced frequencies. The BPDE line was fitted by least-squares regression; the MNNG curve was best fit.

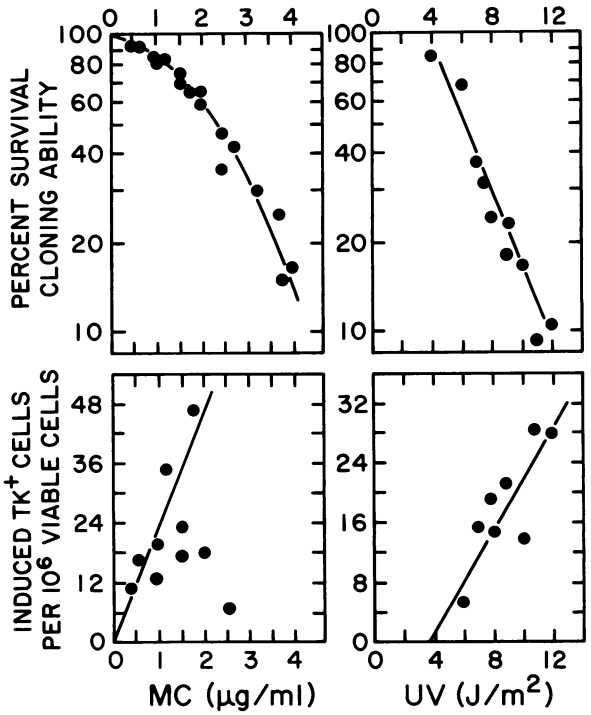

FIG. 2. Cell killing and induction of recombination as a function of the concentration of MC or dose of UV radiation. The background frequencies for the MC experiments were 8.5 × 10^{-6}, 13 × 10^{-6}, and 29 × 10^{-6}; those for the UV experiments were 10.5 × 10^{-6}, 25 × 10^{-6}, and 26 × 10^{-6}. These backgrounds have been subtracted to give the induced frequencies. The UV line was fitted by least-squares regression, using all data. The MC line was fitted by least-squares regression for the concentrations <1.5 μg/ml.

reported no spontaneous tk^+ cells out of 3 × 10^8 cells assayed.

Induction of recombination by MC and UV radiation. The ability of the cross-linking agent MC and of UV radiation to induce homologous recombination was also investigated. Three experiments were carried out with MC, and three were done with UV. The data from all of these experiments are shown in Fig. 2. UV radiation gave a dose-dependent increase in recombinants. Similarly, low concentrations of MC (survival, 70% or above) induced a dose-dependent increase in frequency of recombination. But at higher concentrations, i.e., those yielding survival below 50%, the frequency decreased below that induced by the lower concentrations (Fig. 2). It should be noted that the size of the cells exposed to the higher concentrations of MC had increased dramatically by the time CHAT selection was begun. In effect, this enlargement increased the cell density, and this may have affected recovery of CHAT-resistant colonies.

Lack of induction of homologous recombination by ionizing radiation. The ability of ionizing radiation (cobalt-60) to increase the frequency of tk^+ cells was also investigated, using doses that lowered the survival of the population to between 90 and 10% of the control. No evidence of recombination above the background frequencies could be detected in the target population.

Comparing the agents for recombination efficiency. Since the biological effects of radiation and chemicals cannot be compared on the basis of applied concentration, these carcinogens are often compared on the basis of equal cytotox-

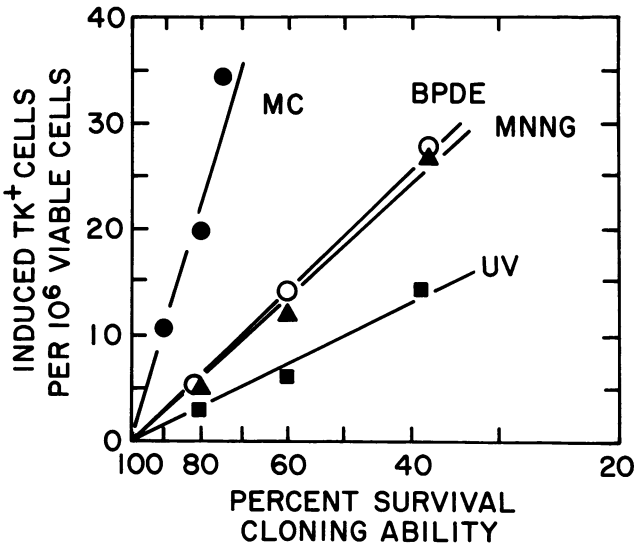

FIG. 3. Frequency of homologous recombination induced by the test agents as a function of the extent of cell killing induced by each agent. Data were extrapolated from the respective survival and recombination curves shown in Fig. 1 and 2.

icity. For example, the term "mutagenic efficiency" is used to refer to the frequency of mutations induced per mean lethal event. To compare the four active test agents for their ability to induce recombination, we plotted the frequency of recombination induced by various doses of each agent as a function of the corresponding degree of cell killing (Fig. 3). Analyzed in this way, the data indicate that MC was the most efficient agent for inducing homologous recombination and UV was the least efficient.

Types of recombination events induced by these agents. To facilitate analysis of the kinds of recombination events which occur, the plasmid used to transfect the mouse L cells and prepare the 333M cell line contains the *neo* gene coding for Geneticin resistance located between the two H*tk* genes (22). If the recombination event involves a single reciprocal exchange within a chromatid, or a single unequal exchange between chromatids, only a single wild-type copy of the H*tk* gene will be present and the *neo* gene will be lost. In contrast, if the event consists of a nonreciprocal transfer of wild-type information, gene conversion, the H*tk* gene duplication with the *neo* gene will be retained in the recombinant (see diagram in Fig. 4). To determine what kind of recombination event was involved, 13 to 22 CHAT-resistant colonies were isolated from each of the populations receiving a high dose of MC, BPDE, MNNG, or UV and tested for resistance to Geneticin, as well as for continued ability to grow in CHAT medium. The results showed that 85 to 90% of the recombinants from each group retained the *neo* gene and represent gene conversions.

DNA from seven of the Geneticin-resistant recombinants and one of the Geneticin-sensitive colonies was analyzed by Southern blot hybridization, using the H*tk* gene as a probe (bottom, Fig. 4). DNA in each of the odd-numbered lanes was digested with *Hin*dIII and *Bam*HI to release the H*tk* genes as individual bands (2.4 or 1.8 kilobases). The DNA in each of the even-numbered lanes was digested with *Hin*dIII, *Bam*HI, and *Xho*I to test for the presence of the *Xho*I site in the H*tk* genes. Mutant H*tk* genes are sensitive to *Xho*I digestion, whereas wild-type H*tk* genes are not. Except for the Geneticin-sensitive recombinant used to prepare the DNA shown in lanes 7 and 8, each of the recombinants contained two H*tk* genes, one wild type and one still retaining an *Xho*I-sensitive band. They represent products of gene conversion events. The two recombinants used to prepare DNA for lanes 1 and 2 and lanes 5 and 6 contained a wild-type H*tk* gene of 2.4 kilobases. The other four gene conversion recombinants analyzed in Fig. 4 contained a wild-type H*tk* gene of 1.8 kilobases. The presence of a single *Xho*I-resistant band in lane 8 indicates that the recombinant used for lanes 7 and 8 was generated by a single reciprocal exchange within a chromatid or a single unequal exchange between sister chromatids. These results with Southern blot analysis confirmed that the Geneticin-resistant recombinants retained the H*tk* gene duplication, with one H*tk* gene being wild type, i.e., lacking an *Xho*I restriction site. The Geneticin-sensitive recombinants analyzed contained only a single (wild-type) H*tk* gene.

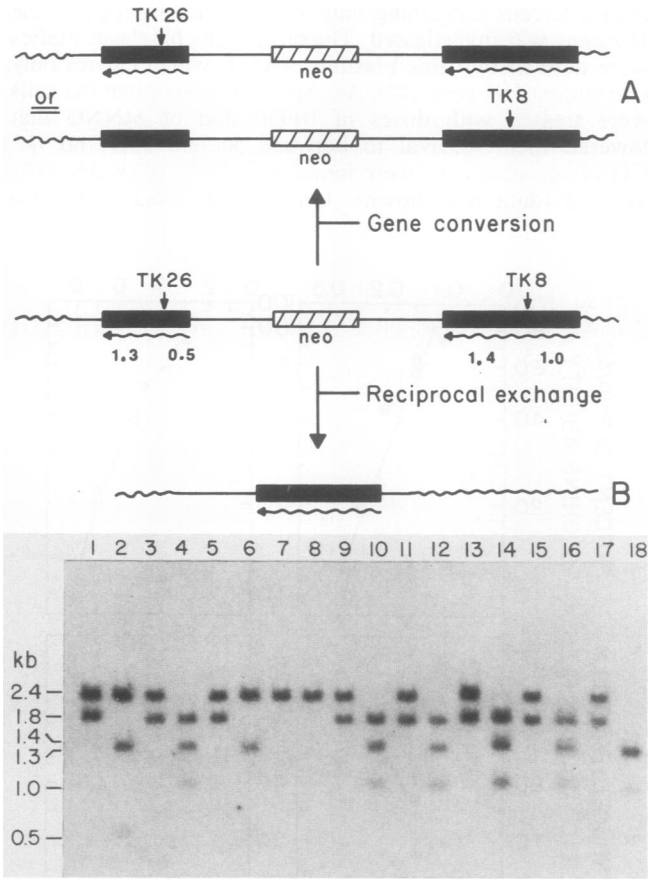

FIG. 4. Types of recombination events induced by test agents. (Top) Diagram of kinds of recombination products generated by gene conversion or reciprocal exchange from the parental strain with its duplicated mutant H*tk* genes and the neomycin gene (*neo*) in the intervening sequence. (Bottom) Southern blotting analysis of DNA from eight recombinant clones of CHAT-resistant cell strains (lanes 1 to 16) compared with their parental cell strain 333M (lanes 17 and 18). The cells were analyzed with a probe specific for H*tk* sequences. DNA in each of the odd-numbered lanes was digested with *Hin*dIII and *Bam*HI to release the H*tk* genes as individual bands (2.4 or 1.8 kilobases [kb]). The DNA in each of the even-numbered lanes was digested with *Hin*dIII, *Bam*HI, and *Xho*I to test for the presence of the *Xho*I site. Mutant H*tk* genes are sensitive to *Xho*I digestion, whereas wild-type H*tk* genes are not. (See text for interpretation.)

DISCUSSION

Our first aim was to determine whether selected carcinogens can induce homologous recombination between genes integrated within the genome of mouse L cells. The data indicate that UV, MC, BPDE, and MNNG caused a dose-dependent increase in the frequency of recombination, but that ionizing radiation did not. At the low doses which allowed cell survival above 60%, MC was the most efficient agent; i.e., it induced the highest frequency of recombinants per mean lethal event. However, at doses that caused greater cell killing, the frequency decreased so that this agent appeared to be less efficient in inducing recombination than the other three active carcinogens.

That UV induced recombination is consistent with reports by Dasgupta and Summers (9) that UV irradiation of human cells infected with multiple copies of defective herpes simplex virus stimulated productive recombination. Unlike ours, their assay involved extrachromosomal recombination. Van Duin et al. (36), using an assay not closely related to ours, showed that UV irradiation of mammalian host cells prior to transfection of a plasmid or irradiation of the plasmid itself increased the frequency of DNA-mediated stable transformation to drug resistance.

There have been no previous reports of homologous recombination induced by BPDE or MNNG, but the increase in recombination frequency we observed with MC supports the MC-induced 3.3-fold increase above background reported by Lin and Sternberg (20), who used a closely related assay involving intrachromosomal recombination in mouse L cells. We had expected ionizing radiation to induce homologous recombination in our assay because it stimulates mitotic recombination in yeasts (14) and causes sister chromatid exchanges in mammalian cells (15). Furthermore, the introduction of double-strand breaks into plasmids by using restriction endonucleases increases the frequency of extrachromosomal recombination between genes transfected into mammalian cells on plasmids (2–4, 10, 13, 17, 28, 31). However, there is evidence that intrachromosomal homologous recombination differs from extrachromosomal recombination (38a).

A second aim of our study was to characterize the kinds of recombination events induced by the four carcinogens and draw inferences about possible mechanisms. The 10 to 20 recombinant tk^+ clones for each agent that we examined for resistance to Geneticin were taken from dishes receiving the highest doses to lessen the possibility of choosing a background recombinant. The drug resistance results showed that the majority (80 to 90%) of the tk^+ cells analyzed retained the *neo* gene located on the DNA sequence between the two H*tk* genes. The presence of one wild-type gene and one mutant gene in such cells was confirmed in a representative sample of these recombinants by molecular analysis, suggesting that they most likely resulted from gene conversion events. The remaining 10 to 20% of the events most likely involved single reciprocal exchanges. This is the same ratio of gene conversion to reciprocal exchanges observed for spontaneous recombination in this cell line (22), indicating that the carcinogen-induced damage or process(es) which causes the observed increase leads to both types of events. Because many studies in fungi and recent studies on intrachromosomal recombination in mouse L cells (using inverted repeats [R. Bollag and R. M. Liskay, manuscript in preparation]) suggest a mechanistic association between gene conversions and reciprocal exchanges, the concomitant increases induced by the carcinogens are not surprising. To get more information on the mechanisms involved, it will be interesting to detect DNA-damaging agents that discriminate between the two kinds of events.

It is well known that MC, MNNG, and UV induce sister chromatid exchanges (15). Our data show that they also induce intrachromosomal homologous recombination between closely linked genes. Although the assay described here cannot distinguish between sister chromatid recombination and intrachromatid events, there is evidence that the former events, i.e., unequal sister chromatid exchanges, do occur (28). In fact, based on a comparison of the rates of recovery of the products of reciprocal exchanges for direct repeats versus inverted repeats (Bollag and Liskay, in preparation), it is very likely that sister chromatid interactions occur in our system as frequently as intrachromatid interactions.

In conclusion, we have shown that mouse L cells containing closely linked gene duplications in their genome are very useful for detecting agents that stimulate recombination of chromosomal sequences in a mammalian system. We are currently integrating the plasmid with its duplicated H*tk* genes into human cells, which differ significantly in DNA repair capacities. Further studies should provide insight into the relationship between the processes involved in DNA repair, mutation induction, sister chromatid exchanges, and homologous recombination. Our system could also prove useful in identifying classes of carcinogens not detected by more standard assays involving mutagenesis.

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LITERATURE CITED

1. **Bandyopadhyay, P. K., S. Watanabe, and H. M. Temin.** 1984. Recombination of transfected DNAs in vertebrate cells in culture. Proc. Natl. Acad. Sci. USA **81**:3476–3480.
2. **Brenner, D. A., S. Kato, R. A. Anderson, A. C. Smigocki, and R. D. Camerini-Otero.** 1984. The recombination and integration of DNAs introduced in mouse L cells. Cold Spring Harbor Symp. Quant. Biol. **49**:151–160.
3. **Brenner, D. A., A. C. Smigocki, and R. D. Camerini-Otero.** 1985. Effect of insertions, deletions, and double-strand breaks on homologous recombination in mouse L cells. Mol. Cell. Biol. **5**:684–691.
4. **Brenner, D. A., A. C. Smigocki, and R. D. Camerini-Otero.** 1986. Double-strand gap repair results in homologous recombination in mouse L cells. Proc. Natl. Acad. Sci. USA **83**: 1762–1766.
5. **Cavanee, W. K., T. P. Dryja, R. A. Phillips, W. F. Benedict, R. Godbout, B. L. Gallie, A. L. Morphree, L. C. Strong, and R. L. White.** 1983. Expression of recessive alleles by chromosomal mechanisms in retinoblastoma. Nature (London) **305**:779–784.
6. **Chakrabarti, S., S. Joffe, and M. M. Seidman.** 1985. Recombination and deletion of sequences in shuttle vector plasmids in mammalian cells. Mol. Cell. Biol. **5**:2265–2271.
7. **Chakrabarti, S., and M. M. Seidman.** 1986. Intramolecular recombination between transfected repeated sequences in mammalian cells is nonconservative. Mol. Cell. Biol. **6**:2520–2526.
8. **Cox, R., W. K. Masson, P. G. Debenham, and M. B. T. Webb.** 1984. The use of recombinant DNA plasmids for the determination of DNA-repair and recombination in cultured mammalian cells. Br. J. Cancer **49**:67–72.
9. **Dasgupta, U. B., and W. C. Summers.** 1980. Genetic recombination of *Herpes simplex* virus, the role of the host cell and

UV-irradiation of the virus. Mol. Gen. Genet. **178**:617–623.
10. **de Saint Vincent, B. R., and G. M. Wahl.** 1983. Homologous recombination in mammalian cells mediates formation of a functional gene from two overlapping gene fragments. Proc. Natl. Acad. Sci. USA **80**:2002–2006.
11. **Folger, K., K. Thomas, and M. R. Capecchi.** 1984. Analysis of homologous recombination in cultured mammalian cells. Cold Spring Harbor Symp. Quant. Biol. **49**:123–138.
12. **Kucherlapati, R. S., D. Ayares, A. Hanneken, K. Noonan, S. Rauth, J. M. Spencer, L. Wallace, and P. D. Moore.** 1984. Homologous recombination in monkey cells and human cell-free extracts. Cold Spring Harbor Symp. Quant. Biol. **49**: 191–197.
13. **Kucherlapati, R. S., E. M. Eves, K.-Y. Song, B. S. Morse, and O. Smithies.** 1984. Homologous recombination between plasmids in mammalian cells can be enhanced by treatment of input DNA. Proc. Natl. Acad. Sci. USA **81**:3153–3157.
14. **Kunz, B. A., and R. H. Haynes.** 1981. Phenomenology and genetic control of mitotic recombination in yeast. Annu. Rev. Genet. **15**:57–89.
15. **Latt, S. A.** 1981. Sister chromatid exchange information. Annu. Rev. Genet. **15**:11–55.
16. **Letsou, A., and R. M. Liskay.** 1986. Intrachromosomal recombination in mammalian cells, p. 383–409. *In* R. Kucherlapati (ed.), Gene transfer. Plenum Publishing Corp., New York.
17. **Lin, F.-L., K. Sperle, and N. Sternberg.** 1984. Model for homologous recombination during transfer of DNA into mouse L cells: role for DNA ends in the recombination process. Mol. Cell. Biol. **4**:1020–1034.
18. **Lin, F.-L., K. Sperle, and N. Sternberg.** 1985. Recombination of mouse L cells between DNA introduced into cells and homologous chromosomal sequences. Proc. Natl. Acad. Sci. USA **82**: 1391–1395.
19. **Lin, F.-L. M., K. M. Sperle, and N. L. Sternberg.** 1987. Extrachromosomal recombination in mammalian cells as studied with single- and double-stranded DNA substrates. Mol. Cell. Biol. **7**:129–140.
20. **Lin, F.-L., and N. Sternberg.** 1984. Homologous recombination between overlapping thymidine kinase gene fragments stably inserted into a mouse cell genome. Mol. Cell. Biol. **4**:852–861.
21. **Liskay, R. M., and J. L. Stachelek.** 1983. Evidence for intrachromosomal gene conversion in cultured mammalian cells. Cell **35**:157.
22. **Liskay, R. M., J. L. Stachelek, and A. Letsou.** 1984. Homologous recombination between repeated chromosomal sequences in mouse cells. Cold Spring Harbor Symp. Quant. Biol. **49**: 183–189.
23. **Patton, J. D., L. A. Rowan, A. L. Mendrala, J. N. Howell, V. M. Maher, and J. J. McCormick.** 1984. Xeroderma pigmentosum (XP) fibroblasts including cells from XP variants are abnormally sensitive to the mutagenic and cytotoxic action of broad spectrum simulated sunlight. Photochem. Photobiol. **39**:37–42.
24. **Rubnitz, J., and S. Subramani.** 1985. Rapid assay for extrachromosomal homologous recombination in monkey cells. Mol. Cell. Biol. **5**:529–537.
25. **Rubnitz, J., and S. Subramani.** 1986. Extrachromosomal and chromosomal gene conversion in mammalian cells. Mol. Cell. Biol. **6**:1608–1614.
26. **Shapira, G., J. L. Stachelek, A. Letsou, L. K. Soodak, and R. M. Liskay.** 1983. Novel use of synthetic oligonucleotide insertion mutants for the study of homologous recombination in mammalian cells. Proc. Natl. Acad. Sci. USA **80**:1827–1831.
27. **Small, A., and G. Scanagos.** 1983. Recombination during gene transfer into mouse cells can restore the function of deleted genes. Science **219**:174–176.
28. **Smith, A. J. H., and P. Berg.** 1984. Homologous recombination between defective *neo* genes in mouse 3T6 cells. Cold Spring Harbor Symp. Quant. Biol. **49**:171–181.
29. **Smithies, O., R. G. Gregg, S. S. Boggs, M. A. Koralewski, and R. S. Kucherlapati.** 1985. Insertion of DNA sequences into the human chromosomal β-globin locus by homologous recombination. Nature (London) **317**:230–234.
30. **Smithies, O., M. A. Koralewski, K.-Y. Song, and R. S. Kucherlapati.** 1984. Homologous recombination with DNA introduced into mammalian cells. Cold Spring Harbor Symp. Quant. Biol. **49**:161–170.
31. **Song, K.-Y., L. Chekurk, S. Rauth, S. Ehrlich, and R. Kucherlapati.** 1985. Effect of double-strand breaks on homologous recombination in mammalian cells and extracts. Mol. Cell. Biol. **5**:3331–3336.
32. **Stringer, J. R., R. M. Kuhn, J. L. Newman, and J. C. Meade.** 1985. Unequal homologous recombination between tandemly arranged sequences stably incorporated into cultured rat cells. Mol. Cell. Biol. **5**:2613–2622.
33. **Subramani, S.** 1986. Rescue of chromosomal T-antigen sequences onto extrachromosomally replicating, defective simian virus 40 DNA by homologous recombination. Mol. Cell. Biol. **6**:1320–1325.
34. **Thomas, K. R., and M. R. Capecchi.** 1986. Introduction of homologous DNA sequences into mammalian cells induces mutations in the cognate gene. Nature (London) **324**:34–37.
35. **Thomas, K. R., K. R. Folger, and M. R. Capecchi.** 1986. High frequency targeting of genes to specific sites in the mammalian genoma. Cell **44**:419–428.
36. **van Duin, M., A. Westerveld, and J. H. J. Hoeijmakers.** 1985. UV stimulation of DNA-mediated transformation of human cells. Mol. Cell. Biol. **5**:734–741.
37. **Volkert, F. C., and C. S. H. Young.** 1983. The genetic analysis of recombination using adenovirus overlapping terminal DNA fragments. Virology **125**:175–193.
38. **Wake, C. T., and J. H. Wilson.** 1979. Simian virus 40 recombinants are produced at high frequency during infection with genetically mixed oligomeric DNA. Proc. Natl. Acad. Sci. USA **6**:2876–2880.

38a. **Waldman, A. S., and R. M. Liskay.** 1987. Differential effects of base pair mismatch on intrachromosomal versus extrachromosomal recombination in mouse L cells. Proc. Natl. Acad. Sci. USA **84**:5340–5344.

39. **Wang, Y., W. C. Parks, J. C. Wigle, V. M. Maher, and J. J. McCormick.** 1986. Fibroblasts from patients with inherited predisposition to retinoblastoma exhibit normal sensitivity to the mutagenic effects of ionizing radiation. Mutat. Res. **175**: 107–114.
40. **Wasmuth, J. J., and L. Vock Hall.** 1984. Genetic demonstration of mitotic recombination in cultured Chinese hamster cell hybrids. Cell **36**:696–707.