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Inhaled benzene increases the frequency and length of *lacI* deletion mutations in lung tissues of mice

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This study investigated the frequency and pattern of mutations that arose in *lacI* transgenes in lung tissues of mice exposed to 300 p.p.m. of benzene for 6 h/day × 5 days/week for 12 weeks. The nucleotide sequence changes in 86 *lacI*$^-$ transgenes from lung tissues of eight benzene-exposed mice (BEM) and 78 spontaneous *lacI*$^-$ transgenes from lung tissues of eight unexposed control mice (UCM) were identified and compared. A total of 31% (27/86) of the *lacI* mutations in BEM are deletions compared with 9% (7/78) deletions in UCM. In BEM, 44% (12/27) of the deletions were longer than 10 bp, whereas only 14% (1/7) of the deletions in UCM exceeded 10 bp in length. Statistical tests supported the hypothesis that benzene exposure resulted in significant increases in both the frequency and length of deletions. Based on the *lacI* mutant frequency and fraction of unique mutations, lung tissues of BEM were estimated to have a 1.8-fold increase in *lacI* mutation frequency compared with lung tissues of UCM. The results presented in this paper demonstrate that inhaled benzene is a gene mutagen in lung tissues of mice.

Introduction

Benzene is a toxic compound that is classified as a carcinogen and has been the subject of numerous investigations because it is an ubiquitous environmental pollutant (for reviews, see 1,2). Although benzene is no longer widely used as a solvent, large amounts are produced for industrial synthesis of organic compounds and as an additive for fuels.

The human health effects of inhaled benzene depend on the concentration and time of exposure. At 25 000 p.p.m., benzene is lethal within minutes (3). Limited inhalation of 4000 p.p.m. can cause giddiness, euphoria, headache and nausea, whereas longer exposures at this level can lead to unconsciousness (3,4). It is generally thought that hematopoietic tissues are the most sensitive targets of benzene toxicity because chronic low level exposure correlates with the development of a variety of blood disorders in humans, which range from reductions in the concentration of peripheral blood cells to aplastic anemia, pancytopenia, acute myelogenous leukemia and its variants, and lymphoma (5–9). The cytopenic effects of benzene have also been observed in laboratory animals, and chronic exposure has been linked to increased frequencies of lymphoma, leukemia, lung tumors and other neoplasms (1,10–13).

Benzene is oxidized enzymatically in liver tissues and the phenolic products can be transformed in bone marrow to yield metabolites that are thought to play important roles in the hematotoxicity and carcinogenicity that are associated with exposure to benzene (for a review see 14). Evidence of the importance of cytochrome P450 2E1 (Cyp2E1) in benzene metabolism is provided by the finding that *cyp2e1*$^{-/-}$ mice have greatly reduced levels of benzene metabolites in their urine relative to those of a *cyp2e1*$^{+/+}$ strain (15).

Covalent adducts formed by reaction of benzene metabolites with nucleic acids and proteins have been proposed to play a direct role in hematotoxicity and tumorigenicity (16–18). In addition to adduct formation, benzene exposure increases oxidative DNA damage as indicated by an increase in the levels of 8-hydroxy-2′-deoxyguanosine in DNA of a human leukemia cell line and in the bone marrow of mice (19).

Epidemiological studies of humans exposed to benzene in the workplace have identified genetic damage in peripheral blood cells based on increased frequencies of chromosomal aberrations, micronuclei and sister chromatid exchange (20–25). Clastogenic effects of benzene have also been observed in animal studies (26–28) and cell culture (29,30). A long-standing and somewhat controversial question about the genotoxicity of benzene concerns its ability to cause mutations in genes. Although benzene is regarded as weakly mutagenic or non-mutagenic in most short term *in vitro* gene mutation assays (2), we found that inhalation of 300 p.p.m. of benzene for 6 h/day for 5 days/week for 12 weeks resulted in a statistically significant 1.7-fold increase in the average mutant frequency of *lacI* transgenes in lung tissues of mice (31).

Although most studies of benzene genotoxicity have focused on hematopoietic tissues or blood cells, we decided to investigate lung tissues because the major point of entry of benzene polluted air into the body is through the lungs. In this report we have investigated the nucleotide sequence changes and estimated the mutation frequency in *lacI* transgenes collected previously from lung tissues of benzene-exposed mice and unexposed control mice (31).

Materials and methods

Benzene exposure

The inhalation chambers and conditions for benzene exposure have been previously described in detail (31). Briefly, 6-week-old male Big Blue C57BL/6 mice [λ LIZα: C57BL/6(LIZα)] obtained from Stratagene (La Jolla, CA) were quarantined for 2 weeks and observed for anomalous behavior. Mice were randomly distributed into test and control groups. Mice exposed to benzene received whole body exposures. Eight mice were exposed to a target dose of 300 p.p.m. of analytical grade, thiophene free benzene (CAS# 71-43-2) vapor for 6 h/day×5 days/week for 12 weeks and eight control animals were exposed to filtered conditioned air in a duplicate chamber during the same time period. After the 12 week exposure was completed, the mice were exposed to filtered conditioned air for 2 weeks and then killed by cervical dislocation.

Abbreviations: BEM, benzene exposed mice; DMM, dislocation mutagenesis model; SSM, strand slippage model; UCM, unexposed control mice; X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

Nucleotide sequence determination

A total of 634 phenotypically LacI$^-$ λ LIZα phage were collected while measuring the *lacI* mutant frequency in lung tissues of benzene-exposed mice (BEM) and unexposed control mice (UCM) (31). Of the total, 97 such LacI$^-$ mutants recovered from eight BEM were chosen randomly for nucleotide sequence analysis. Of these, 13 mutants were selected from one mouse and 12 mutants were selected from each of seven other mice. Eighty-three LacI$^-$ mutants recovered from the 8 UCM were chosen randomly for nucleotide sequence analysis. Eleven mutants were selected from each of three mice and 10 mutants each were selected from the five remaining mice.

DNA fragments for determining the nucleotide sequence of mutant *lacI* genes were generated using a thermocycling reaction protocol with template DNA derived from phage λ LIZα plaques. Phage of a single plaque were suspended in 0.5 ml of SM buffer (100 mM NaCl, 8 mM $MgS0_4$, 50 mM Tris pH 7.5, 0.01% gelatin) that contained 60 µl of $CHCl_3$, and ~10 µl of each phage preparation was streaked onto the surface of an NZ amine soft agarose plate overlay that contained *Escherichia coli* host strain SCS-8 [*recA1*, *endA1*, *mcrA*, Δ(*mcrBC-hsdRMS-mrr*), Δ(*argF-lac*)U169, ϕ80d*lacZ*ΔM15, Tn*10* (*tet*^{r})] (32) and X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside). For each mutant that was analyzed, a single-well isolated blue plaque was picked from the agarose overlay with a sterile wooden applicator stick, suspended in 30 µl of TE (10 mM Tris, 0.1 mM EDTA, pH 8.0), and boiled for 5 min to release phage DNA. Thermocycling DNA amplification reactions (100 µl final volume) were prepared by combining 3 µl of the released phage DNA, 1 U of exo$^-$ Vent DNA polymerase (New England Biolabs, Beverly, MA), 10 µl of 10× Vent polymerase buffer [100 mM KCl, 200 mM Tris–HCl pH 8.8, 100 mM $(NH_4)_2SO_4$, 20 mM $MgSO_4$, 1% (v/v) Triton X-100] and 2 µl 100 mM $MgSO_4$. In addition, dATP, dCTP, dGTP and dTTP were added at final concentrations of 200 µM each, and oligodeoxyribonucleotide primers designed to amplify *lacI* (upstream primer: 5′-GTATTACCGCC-ATGCATACTAG-3′, downstream primer: 5′-CGTAATCATGGTCATAGC-TGTT-3′) were added at a final concentration of 1 µM each. The thermocycling reaction started with a 2 min incubation at 97°C, followed by 30 reaction cycles of 97°C for 1 min, 53°C for 45 s and 72°C for 1 min 20 s. Finally, the reaction was incubated at 72°C for 10 min.

The amplified DNA products were purified using PCR SELECT-III spin chromatography columns (5 Prime → 3 Prime Inc., Boulder, CO) and used as templates in dideoxynucleotide sequencing reactions (33) using Cyclist DNA sequencing kits (Stratagene, La Jolla, CA) and [α-^{32}P]dATP as the radiolabel. A set of seven synthetic oligodeoxyribonucleotides were used as primers in reactions to determine the complete nucleotide sequence of one DNA strand of *lacI* for each mutant. The sequence change identified for each mutant was confirmed by determining the nucleotide sequence of the complementary DNA strand at the position of each identified mutation.

Statistical analysis

The statistical significance of the differences in frequency and length of deletions between the BEM and UCM was evaluated using three different two-sample tests. If p_1 specifies the proportion of deletions in the nucleotide sequence for a benzene-exposed mouse and p_2 the proportion of deletions in an unexposed control mouse, the evidence of $p_1 > p_2$ would indicate that the increase in deletion mutations was caused by benzene exposure.

The observations in the benzene group are Y_{ij}, $i = 1$–8; $j = 1$–n_i, where $Y_{ij} = 1$ means that there are deletions in the jth mutant obtained from the ith mouse and $Y_{ij} = 0$ means that there are no deletions. Similarly, the observations in the control group are Z_{ij}, $i = 1$–8; $j = 1$–m_i. The proportion of deletions p_k ($k = 1$ or 2) in a particular mouse is random, meaning that it varies from mouse to mouse; F denotes the distribution function (df) of p_1 and G the df of p_2. Although p_1 is a random variable, for a particular benzene-exposed mouse, for example, the ith mouse, the proportion p_1 of *lacI* deletion mutations is a fixed number between 0 and 1, and an observation Y_{ij} has a Bernoulli distribution with mean P_1. This leads to

$$P\{Y_{ij} = 1\} = E\{Y_{ij}\} = E\{E\{Y_{ij}|p_1\}\} = E\{p_1\} = \mu_1 \quad (1)$$

where μ_1 is the average proportion of deletions among the BEM and is the mean of F. Thus, observations Y_{ij} have an identical Bernoulli distribution with mean μ_1. By a similar argument, we know that observations Z_{ij} in UCM have an identical Bernoulli distribution with mean μ_2, where μ_2 is the average proportion of deletions among UCM and is the mean of G.

To compare the distributions F of BEM and G of UCM, the following hypothesis test was conducted based on observations Y_{ij}, $i = 1$–8; $j = 1$–n_i, and Z_{ij}, $i = 1$–8; $j = 1$–m_i.

$$H_0\text{: } F = G \text{ versus } H_1\text{: } F > G \quad (2)$$

Note that $p_1 > p_2$ is investigated by this test in terms of its statistical description F > G. A two-sample permutation test (34) was used to test equation 2, and the test statistic was given by

$$\hat{\theta} = \hat{\theta}_1 - \hat{\theta}_2 \quad (3)$$

where

$$N_1 = \sum_{i=1}^{8} n_i = [\text{total number of observations in BEM}]$$

$$N_2 = \sum_{i=1}^{8} m_i = [\text{total number of observations in UCM}]$$

$$\hat{\theta}_1 = \frac{1}{N_1} \sum_{i=1}^{8} \sum_{j=1}^{n_i} Y_{ij} \text{ and } \hat{\theta}_2 = \frac{1}{N_2} \sum_{i=1}^{8} \sum_{j=1}^{m_i} Z_{ij}. \quad (4)$$

In equation 4, $\hat{\theta}_1$ and $\hat{\theta}_2$ are the sample proportions of deletions in the BEM and UCM, respectively, and they are also the estimators for the means of F and G (μ_1 and μ_2), respectively. The P-value of this test is estimated using Algorithm 15.1 of Efron and Tibshirani (34), using 10 000 permutation vectors for the calculation. The bootstrap test for equation 2 is conducted using Algorithm 16.1 of Efron and Tibshirani (34), of which the test statistic is given by equation 3. Alternatively, a bootstrap t-test was conducted for

$$H_0\text{: } \mu_1 = \mu_2 \text{ versus } H_1\text{: } m_1 > m_2 \quad (5)$$

using algorithm 16.2 of Efron and Tibshirani (34), of which the test statistic is given by

$$t = \frac{(\hat{\theta}_1 - \hat{\theta}_2)}{\sqrt{\frac{\hat{\sigma}_1^2}{N_1} + \frac{\hat{\sigma}_1^2}{N_2}}} \quad (6)$$

where

$$\hat{\sigma}_1^2 = \frac{N_1}{(N_1 - 1)} \hat{\theta}_1(1 - \hat{\theta}_1) \text{ and } \hat{\sigma}_2^2 = \frac{N_2}{(N_2 - 1)} \hat{\theta}_2(1 - \hat{\theta}_2).$$

The permutation test, Bootstrap test and Bootstrap t-test described above were also used to evaluate the statistical significance of the differences in length of deletions between the BEM and UCM. For this analysis, p_{l1} denotes the proportion of deletions with length ⩾10 bp for a particular benzene exposed mouse and p_{l2} represents the proportion of deletions with length ⩾10 bp in a particular unexposed control mouse. The evidence of $p_{l1} > p_{l2}$ would indicate that the increase in the length of deletions is caused by exposure to benzene. For this study we denote F_l as the df of p_{l1}, and G_l the distribution of p_{l2}; μ_{l1} and μ_{l2} represent the means of F_l and G_l respectively.

Results

The types of mutations that arose in lung tissues of male Big Blue™ transgenic mice after inhalation of benzene at a level of 300 p.p.m. for 6 h/day×5 days/week for 12 weeks were investigated by nucleotide sequence analysis. Big Blue™ transgenic mice carry ~40 tandemly integrated copies of the *lacI*$^+$ phage λ LIZα (32). Phage λ LIZα transgenes were recovered *in vitro* by packaging of genomic DNA from mouse lung tissues, and screened for their LacI phenotypes using a colorimetric plaque assay. Phage that formed blue plaques when propagated in *E.coli* host strain SCS-8 on NZ amine agar plates supplemented with X-Gal were presumed to carry *lacI* mutations, and phage that formed clear plaques were presumed to carry wild-type copies of *lacI* (31). A total of 634 independent phage clones that expressed the blue plaque phenotype were collected from lung tissues of eight BEM and eight UCM. The nucleotide sequences of 97 presumptive *lacI*

Table I. Position and nature of *lacI* mutations in the BEM

Mutant[a]	Base no.[b]	Mutation	Mutant[a]	Base no.[b]	Mutation
20-3	42	G:C→A:T[c]	24–5	[d]	254 bp deletion
20-5	56	G:C→A:T[c]	24-20	56	G:C→A:T[c]
20-14	95	G:C→A:T[c]	24-7	180	G:C→A:T[c]
20-2	188	G:C→A:T	24-19	[d]	1 bp deletion
20-11	242 and 467	complex[f]	24-35	329	G:C→A:T[c]
20-12	[d]	13 bp deletion	24-6	381	G:C→C:G
20-28	269	G:C→A:T[c]	24-8	[d]	4 bp deletion
20-13	308	G:C→A:T[c]	24-10	1001	A:T→T:A
20-23	[d]	34 bp deletion	24-31	1206[e]	G:C→A:T[c]
20-25	[d]	4 bp deletion			
			25-1	[d]	433 bp deletion
21-3	[d]	1 bp deletion	25-8	95	G:C→A:T[c]
21-6	[d]	complex[g]	25-2	169	G:C→C:G
21-1	381	G:C→A:T[c]	25-12	180	G:C→A:T[c]
21-9	381	G:C→A:T[c]	25-6	[d]	16 bp deletion
21-11	381	G:C→A:T[c]	25-9	329	G:C→A:T[c]
21-17	381	G:C→A:T[c]	25-13	329	G:C→A:T[c]
21-8	[d]	119 bp deletion	25-7	[d]	4 bp deletion
21-4	[d]	1 bp deletion	25-4	792	G:C→A:T[c]
21-10	[d]	1 bp deletion	25-5	[d]	7 bp deletion
21-12	[d]	18 bp deletion	25-3	1205[e]	G:C→T:A
21-7	[d]	1 bp deletion			
			26-2	84, 85	complex[h]
22-16	56	G:C→A:T[c]	26-13	[d]	1 bp deletion
22-17	92	G:C→A:T[c]	26-11	131	G:C→A:T[c]
22-5	93	G:C→A:T[c]	26-1	180	G:C→A:T[c]
22-4	180	G:C→T:A	26-7	188	G:C→A:T
22-1	329	G:C→A:T[c]	26-16	189	A:T→T:A
22-9	[d]	4 bp deletion	26-5	213	A:T→T:A
22-15	[d]	34 bp deletion	26-4	267	A:T→G:C
22-14	719	G:C→A:T	26-8	369	A:T→C:G
22-34	792	G:C→A:T[c]	26-15	777	G:C→A:T[c]
22-36	792	G:C→A:T[c]	26-6	1005	G:C→C:G
22-7	[d]	1 bp deletion			
			27-4	[d]	57 bp deletion
23-5	[d]	199 bp deletion	27-22	[d]	13 bp deletion
23-8	[d]	1 bp deletion	27-3	56	G:C→A:T[c]
23-18	197	G:C→A:T	27-18	56	G:C→A:T[c]
23-1	221	G:C→T:A	27-2	131	G:C→A:T[c]
23-9	329	G:C→A:T[c]	27-1	237	G:C→T:A
23-15	381	G:C→A:T[c]	27-7	329	G:C→A:T[c]
23-16	381	G:C→A:T[c]	27-19	381	G:C→A:T[c]
23-4	[d]	1 bp deletion	27-6	[d]	44 bp deletion
23-2	[d]	5 bp insert	27-5	774	A:T→T:A
23-14	701	G:C→C:G	27-20	1205[e]	G:C→A:T[c]
23-17	714	G:C→T:A			
23-6	[d]	1 bp deletion			

[a]20-3, for example, refers to particular mutant isolated from mouse 20.
[b]Indicates the position of point mutations relative to +1, which is the *lac1* transcription start site (35).
[c]The G:C→A:T mutation occurs in a 5′-CpG-3′ sequence.
[d]The sequence context of each insertion and deletion is shown in Table V.
[e]These mutations are located outside of the *lac1* open-reading frame.
[f]This complex mutation consists of G:C→A:T and A:T→G:C at base pairs 242 and 467, respectively.
[g]This complex mutation consists of a 34 bp deletion and a base substitution at either the 5′ or the 3′ deletion endpoint.
[h]This complex mutation consists of a GG:CC→TT:AA at base pairs 84 and 85.

mutants from the BEM were determined, and the pattern of spontaneous mutations in *lacI* was examined by determining the nucleotide sequences of 83 presumptive *lacI* mutants recovered from lung tissues of eight UCM. The nucleotide sequence change in each mutant is shown in Tables I and II. The classes of mutations and their relative frequencies are summarized in Table III.

Some λ LIZα phage formed blue plaques but did not carry a mutation in the lacI structural gene

Although the vast majority of phage that formed blue plaques carry *lacI* mutations, ~14% (14/97) of the blue plaque phage from the BEM, and 6% (5/83) from the UCM carried no apparent mutation in the *lacI* promoter or in the region between the promoter and the end of the *lacI* structural gene. The finding of phage that form blue plaques, even though they carry a wild-type copy of *lacI*, suggests that certain mutations outside of the *lacI* structural gene in λ LIZα phage can block expression of LacI repressor activity. Consistent with this prediction, three of the blue plaque phage were each found to carry a mutation located downstream of the *lacI* stop codon at either bp 1205 or 1206 (Table I); however, it is unclear if these downstream

Table II. Position and nature of spontaneous *lacI* mutations in the UCM

Mutant[a]	Base no.[b]	Mutation	Mutant[a]	Base no.[b]	Mutation
1-8	56	G:C→A:T[c]	5-10	56	G:C→A:T[c]
1-15	56	G:C→A:T[c]	5-19	56	G:C→A:T[c]
1-24	81	A:T→G:C	5-11	93	G:C→A:T[c]
1-11	[d]	1 bp deletion	5-20	129	G:C→A:T[c]
1-6	178	G:C→T:A	5-3	269	G:C→A:T[c]
1-3	180	G:C→A:T[c]	5-16	270	G:C→A:T[c]
1-7	329	G:C→A:T[c]	5-49	329	G:C→A:T[c]
1-12	329	G:C→A:T[c]	5-8	369	A:T→C:G
1-23	329	G:C→A:T[c]	5-12	702	G:C→A:T
1-16	381	G:C→A:T[c]	5-7	[d]	1 bp deletion
2-1	42	G:C→A:T[c]	6-3	42	G:C→A:T[c]
2-6	93	G:C→A:T[c]	6-11	42	G:C→A:T[c]
2-3	180	G:C→A:T[c]	6-16	42	G:C→A:T[c]
2-10	270	G:C→A:T[c]	6-14	56	G:C→A:T[c]
2-15	270	G:C→A:T[c]	6-29	[d]	1 bp deletion
2-9	329	G:C→A:T[c]	6-2	179	G:C→A:T[c]
2-22	329	G:C→A:T[c]	6-19	179	G:C→A:T[c]
2-2	381	G:C→A:T[c]	6-20	180	G:C→A:T[c]
2-4	864	G:C→T:A	6-1	197	G:C→A:T
2-8	[d]	1 bp insert	6-17	530	G:C→A:T[c]
3-6	39	A:T→T:A	7-16	42	G:C→A:T[c]
3-14	41	A:T→C:G	7-22	80	G:C→A:T
3-27	41	A:T→C:G	7-2	155	G:C→C:G
3-30	180	G:C→A:T[c]	7-9	180	G:C→A:T[c]
3-2	269	G:C→A:T[c]	7-6	191	G:C→A:T
3-4	270	G:C→A:T[c]	7-11	269	G:C→A:T[c]
3-1	381	G:C→A:T[c]	7-10	329	G:C→A:T[c]
3-3	[d]	2 bp deletion	7-14	329	G:C→A:T[c]
3-13	792	G:C→A:T[c]	7-8	381	G:C→A:T[c]
4-5	−16	A:T→G:C	8-15	104	G:C→A:T
4-4	93	G:C→A:T[c]	8-20	[d]	2 bp deletion
4-13	131	G:C→A:T[c]	8-12	188	G:C→A:T
4-24	179	G:C→A:T[c]	8-11	198	G:C→A:T[c]
4-25	206	G:C→A:T	8-3	381	G:C→A:T[c]
4-8	369	A:T→C:G	8-14	381	G:C→A:T[c]
4-11	381	G:C→A:T[c]	8-22	381	G:C→A:T[c]
4-6	537	G:C→A:T	8-7	[d]	67 bp deletion
4-17	631	G:C→A:T	8-16	[c]	4 bp insert
4-12	769, 770	complex[e]	8-18	[c]	1 bp deletion

[a]Mutants are named as in Table I.
[b]Bases are numbered as in Table I.
[c]The G:C→A:T mutation occurs in a 5′-CpG-3′ sequence.
[d]The sequence context of each insertion and deletion is shown in Table V.
[e]This complex mutation consists of an AG:TC→TA:AT at base pairs 769 and 770.

mutations are responsible for the blue plaque phenotype of these phage.

Benzene exposure increased the lacI mutation frequency in lung tissues

The data in Tables I and II show that at least 92% (79/86) of the *lacI* mutations from the BEM resulted from independent mutational events because they were either different from each other or were found in different animals. Using the same argument, at least 83% (65/78) of the mutations in the UCM arose as independent mutational events. The *lacI* mutant frequency of the BEM and UCM was determined in a previous study by dividing the number of LacI$^-$ transgenes by the total number of *lacI* transgenes recovered (31). The mutant frequency would be equal to the mutation frequency if each *lacI* mutant resulted from an independent mutational event. Based on the fraction of independent mutants, and the published *lacI* mutant frequencies (31), the *lacI* mutation frequencies in lung tissues of BEM and UCM were calculated to be $(9.7 \pm 1.3)\times10^{-5}$ and $(5.3 \pm 1.3)\times10^{-5}$, respectively. These findings suggest that the benzene exposure resulted in a 1.8-fold increase in the *lacI* mutation frequency in lung tissues, and this increase was judged to be statistically significant based on the calculated *P*-value of 9.5×10^{-6} using Student's two-tailed *t*-test. The 1.8-fold increase in mutation frequency that resulted from benzene exposure suggests that ~38 of the 86 *lacI* mutants recovered from lung tissues of BEM (Table III) were induced by benzene.

Exposure to benzene resulted in a statistically significant increase in both the frequency and length of deletion mutations

Although the number of mutations analyzed in this work was relatively small, some differences between the pattern of mutations in BEM and UCM are apparent. The most striking difference in mutational pattern is the higher frequency of deletions in BEM: 31% (27/86) compared with 9% (7/78) in UCM (Table III).

The size distribution of deletion mutations also differed

Table III. Frequency and spectrum of *lacI* mutations

		BEM		UCM	
		No.	%	No.	%
Transitions					
	G:C→A:T[a]	40	47	58	74
	A:T→G:C	1	1	2	3
Transversions					
	A:T→T:A	4	5	1	1
	G:C→T:A	5	6	2	3
	G:C→C:G	4	5	1	1
	A:T→C:G	1	1	4	5
Deletion mutations					
	1 bp	10	12	4	5
	2–10 bp	5	6	2	3
	11–25 bp	4	5	0	0
	26–50 bp	3	3	0	0
	51–100 bp	1	1	1	1
	>100 bp	4	5	0	0
Insertion mutations		1	1	2	3
Complex mutations		3	3	1	1
Total mutations		86	100	78	100

[a]90% (36/40) of G:C→A:T mutants in BEM and 84% (49/58) of G:C→A:T mutants in UCM occur at 5′-CpG dinucleotides.

Table IV. Two-sample statistical tests on deletions

Statistical test[a]	Test statistic	Estimated *P*-value
Permutation test H_0: $F = G$	0.2242	0.0005
Bootstrap test H_0: $F = G$	0.2242	0.0001
Bootstrap-t test H_0: $\mu_1 = \mu_2$	3.7395	0.0002
Permutation test H_0: $F_l = G_l$	0.1267	0.0021
Bootstrap test H_0: $F_l = G_l$	0.1267	0.0013
Bootstrap-t test H_0: $\mu_{l1} = \mu_{l2}$	3.1910	0.0001

[a]The statistical tests are described in detail in Materials and methods.

between BEM and UCM. In BEM, 44% (12/27) of the deletions were longer than 10 bp whereas only 14% (1/7) of the deletions in UCM were longer than 10 bp (Table III). These data suggest that exposure to benzene increased both the frequency and length of deletion mutations relative to those found in UCM.

The statistical significance of the differences in frequency and length of deletions between BEM and UCM was evaluated using three different two-sample tests that make few assumptions with regard to the distribution of data (Table IV). The small *P*-values calculated for each of the three statistical tests support the hypothesis that exposure to benzene was responsible for the increase in both the length and frequency of deletions (Table IV).

The 95% of bootstrap-*t* confidence intervals for the average proportion of deletions were calculated to be 0.2258, 0.04183 for BEM and 0.0393, 0.1654 for UCM (34). The finding that the 95% confidence intervals are disjoint provides further evidence that benzene exposure was responsible for the increase in frequency of deletions in lung tissues of mice.

Distribution and nucleotide sequence features of lacI deletion mutations

Except for mutation 23-5, the deletions of BEM are all predicted to cause reading-frame shifts that result in synthesis of truncated *lacI* proteins. Mutation 23-5 resulted in a LacI$^-$ phenotype because it disrupted the promoter of *lacI* and

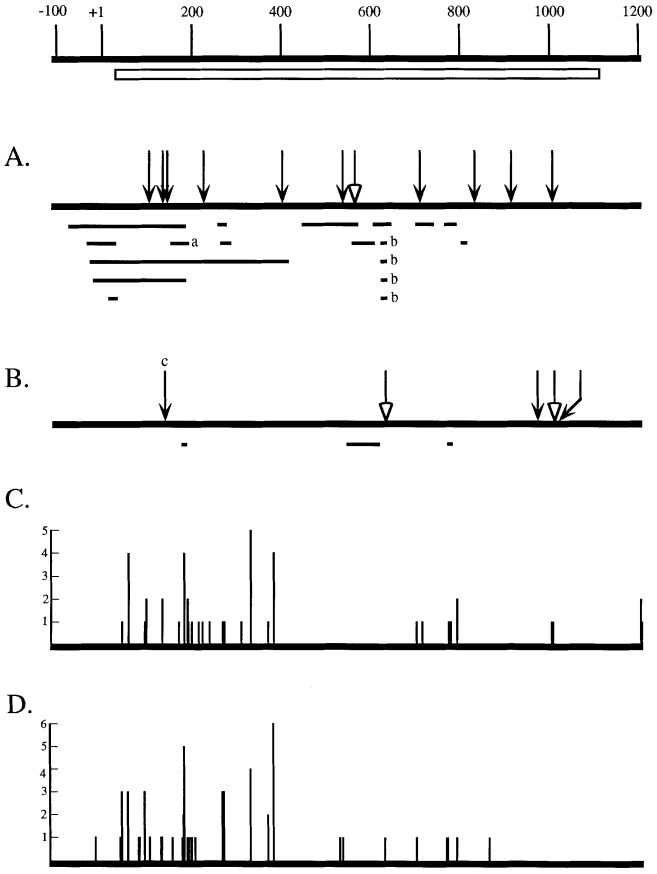

Fig 1. Distribution and frequency of *lacI* mutations. +1 indicates the position of the *lacI* transcription start site (35). The open bar indicates the extent of the *lacI* open reading frame. (**A**) Deletion and insertion mutations in BEM. (**B**) Deletion and insertion mutations in UCM. Solid arrows indicate the positions of single bp deletions. Open arrows indicate positions of insertions. Solid bars below the lines illustrate the extent of deletions. The letter 'a' indicates a deletion that is part of a complex mutation. The letter 'b' indicates possible hotspot deletion mutations. The letter 'c' indicates single bp deletions independently isolated from two different mice. (**C**) Point mutations of BEM. (**D**) Point mutations of UCM. Vertical bars indicate the positions of mutations. The height of the bar indicates the number of independently isolated mutations at a particular bp.

removed the sequence that encodes the ribosome binding site. The relative positions and frequencies of deletion mutations recovered from lung tissues of BEM and UCM are compared in Figure 1A and B. Deletion mutations of BEM were found at a variety of positions in *lacI*, and each deletion occurred at a different position except for an apparent hotspot mutation that was mapped within the tetranucleotide sequence 5′-CTGG-3′, which is repeated three times between base pairs 619 and 631. This 4 bp deletion was recovered from the lung tissues of four different BEM (Figure 1A and Table V).

The deletion mutations of UCM all caused reading-frame shifts that are predicted to result in synthesis of truncated *lacI* proteins. The seven deletion mutations recovered from UCM were each located at different positions in *lacI*, except for the deletion of an A:T bp in a run of five A's at base pairs 135–139, which was recovered from two different UCM and once from a BEM (Figure 1A and B, and Table V).

A total of 57% (4/7) of the deletion mutations in UCM were a result of a loss of a single base in a run of two or more identical bases, and 29% (2/7) resulted in loss of a dinucleotide at the site of a dinucleotide repeat (Table V).

Table V. Nucleotide sequence features flanking insertion and deletion mutations

Mutant	Treatment	Mutation	Wild-type and mutant sequences
			130 145
1-11	UCM	1 bp deletion	ACGCGGG-AAAAA-GTGGAAGCGGC
			ACGCGGG-AAAA-GTGGAAGCGGC
			1005 1020
2-8	UCM	1 bp insert	GAAAAG-AAAAA-CCACCCTGGCG
			GAAAAG-AAAAAA-CCACCCTGGCG
			770 785
3-3	UCM	2 bp deletion	ACGATCAGATG-GCGC-TGGGCGCAA
			ACGATCAGATG-GC-TGGGCGCAA
			965 980
5-7	UCM	1 bp deletion	GGCGGTGAA-GGG-CAATCAGCTGT
			GGCGGTGAA-GG-CAATCAGCTGT
			130 145
6-29	UCM	1 bp deletion	ACGCGGG-AAAAA-GTGGAAGCGGC
			ACGCGGG-AAAA-GTGGAAGCGGC
			175 190
8-20	UCM	2 bp deletion	CATTCCCAAC-CGCG-TGGCACAACA
			CATTCCCAAC-CG-TGGCACAACA
			545 620
8-7	UCM	67 bp deletion	TGGCA-TCTGGTC....CGTCTG-CGTCT
			TGGCA-CGTCT
			619 635
8-16	UCM	4 bp insert	CGT-CTGGCTGGCTGG-CATAA
			CGT-CTGGCTGGCTGGCTGG-CATAA
			1005 1020
8-18	UCM	1 bp deletion	AAAAGAAAAA-CC-ACCCTGGCGCC
			AAAAGAAAAA-C-ACCCTGGCGCC
			260 285
20-12	BEM	13 bp deletion	CGCAAA-TTGTCGCGGCGATT-AAATCTCG
			CGCAAA-T-AAATCTCG
			610 655
20-23	BEM	34 bp deletion	GCGTCT-GCGTCTG.....CACTCGC-AATCAAA
			GCGTCT-AATCAAA
			615 635
20-25	BEM	4 bp deletion	CTGCGT-CTGGCTGGCTGG-CATAAATA
			CTGCGT-CTGGCTGG-CATAAATA
			130 145
21-3	BEM	1 bp deletion	ACGCGGG-AAAAA-GTGGAAGCGGC
			ACGCGGG-AAAA-GTGGAAGCGGC
			155 195
21-6	BEM	Complex	ATGGCGG-AGCTG.....AACAA-CTGGCG
			ATGGCG-T-ACTGGCG
			450 575
21-8	BEM	119 bp deletion	CACT-AATGTTC.....AGCAAAT-CGCG
			CACT-AAT-CGCG
			535 550
21-4	BEM	1 bp deletion	GACTGGGCGT-GG-AGCATCTGGTC
			GACTGGGCGT-G-AGCATCTGGTC
			710 725
21-10	BEM	1 bp deletion	TTTCAACAAA-CC-ATGCAAATGCT
			TTTCAACAAA-C-ATGCAAATGCT
			765 795
21-12	BEM	18 bp deletion	CCAACG-ATCAGA.....CGCAAT-GCGCGCC
			CCAACG-AT-GCGCGCC
			830 845
21-7	BEM	1 bp deletion	GGATATCTCGC-T-AGTGGGATACG
			GGATATCTCGC-AGTGGGATACG
			615 635
22-9	BEM	4 bp deletion	CTGCGT-CTGGCTGGCTGG-CATAAATA
			CTGCGT-CTGGCTGG-CATAAATA
			705 745
22-15	BEM	34 bp deletion	GTTTTCA-ACAAA.....TCGTT-CCCACTG
			GTTTTCA-CCCACTG
			910 925
22-7	BEM	1 bp deletion	CGCCTGCT-GGGG-CAAACCAGCGT
			CGCCTGCT-GGG-CAAACCAGCGT
			–20 190
23-5	BEM	198 bp deletion	CGGTA-TGGCATGA.....CGTGGCA-CAAC
			CGGTA-TGGCA-CAAC

Table V. *continued*

Mutant	Treatment	Mutation	Wild-type and mutant sequences
			140 155
23-8	BEM	1 bp deletion	AAGTGGAAGC-GG-CGATGGCGGAG
			AAGTGGAAGC-G-CGATGGCGGAG
			410 425
23-4	BEM	1 bp deletion	CGCTGGATGA-CC-AGGATGCCATT
			CGCTGGATGA-C-AGGATGCCATT
			560 575
23-2	BEM	5 bp insert	GGGTCA-CCAGC-AAATCGCGCTG
			GGGTCA-CCAGCCCAGC-AAATCGCGCTG
			1005 1020
23-6	BEM	1 bp deletion	GAAAAG-AAAAA-CCACCCTGGCG
			GAAAAG-AAAA-CCACCCTGGCG
			–75 190
24-5	BEM	254 bp deletion	CCTAGTC-TCGAG.....GTGGC-ACAACAA
			CCTAGTC-ACAACAA
			220 235
24-19	BEM	1 bp deletion	ATTGGCGTTG-CC-ACCTCCAGTCT
			ATTGGCGTTG-C-ACCTCCAGTCT
			615 635
24-8	BEM	4 bp deletion	CTGCGT-CTGGCTGGCTGG-CATAAATA
			CTGCGT-CTGGCTGG-CATAAATA
			–25 415
25-1	BEM	433 bp deletion	CCTTTCG-CGGTA.....GCTGG-ATGACCA
			CCTTTCG-ATGACCA
			265 290
25-6	BEM	16 bp deletion	AAATTG-TCGCGGCG.....TCTCGCG-CCGA
			AAATTG-TCGCG-CCGA
			615 635
25-7	BEM	4 bp deletion	CTGCGT-CTGGCTGGCTGG-CATAAATA
			CTGCGT-CTGGCTGG-CATAAATA
			800 820
25-5	BEM	7 bp deletion	ATTACCGAGTC-CGGGCTGC-GCGTTGG
			ATTACCGAGTC-C-GCGTTGG
			100 115
26-13	BEM	1 bp deletion	GTGAACCAGG-CC-AGCCACGTTTC
			GTGAACCAGG-C-AGCCACGTTTC
			–20 35
27-4	BEM	43 bp deletion	CGCGG-ATGCGATG.....GAATG-TGAAAC
			CGCGG-ATG-TGAAAC
			10 35
27-22	BEM	13 bp deletion	TCAATTCA-GGGT.....TGTG-AAACCAG
			TCAATTCA-G-AAACCAG
			565 615
27-6	BEM	44 bp deletion	TCACCAG-CAAAT.....CGCG-TCTGCGT
			TCACCAG-TCTGCGT

Mutant sequences are shown below the wild-type sequences. Dotted lines below the wild-type sequence illustrate mononucleotide runs at the deletion site. Arrows mark direct repeat sequences. Dotted lines within sequences indicate intervening sequences that are not shown. A thick underline below a base indicates a base substitution at one of the deletion endpoints.

Mutation 8-7 is a 67 bp deletion and it is the only deletion mutation of the UCM that is longer than 2 bp (Table V).

In BEM, 33% (9/27) of the deletion mutations resulted from the loss of a single base in a run of two or more identical bases (Table V). Four deletion mutations recovered from the BEM occurred at the site of three adjacent tandem direct repeats of the nucleotide sequence 5′-CTGG-3′ and resulted in the loss of one of the tetranucleotide repeat sequences (Table V). In *E.coli*, ~70% of all spontaneous mutations in *lacI* are insertions and deletions in this 5′-CTGG-3′ repeat sequence (32,36,37). Six deletion mutations of the BEM occurred at the site of a pair of short hyphenated direct repeat sequences and resulted in the loss of one of the repeats and the bases between the repeats (Table V). The remaining eight *lacI* deletion mutations recovered from BEM occurred in several different nucleotide sequence contexts (Table V).

The only insertion mutation found in BEM resulted in a tandem direct repeat of a 5 bp sequence 5′-CCAGC-3′ (Table V). In UCM, two insertions were isolated, one of which resulted in insertion of an A in a run of five A's. The other insertion mutation resulted in insertion of 4 bp within the apparent deletion hotspot sequence identified in the BEM (Table V).

Spectrum of lacI point mutations in lung tissues of BEM and UCM

G:C→A:T transitions accounted for ~73% (40/55) and 85% (58/68) of the point mutations recovered from lung tissues

Table VI. Positions of apparent point mutation hotspots

Unexposed control mice			Benzene-exposed mice		
Base	Mutation	No. of mutants	Base	Mutation	No. of mutants
42	G:C→A:T	5	56	G:C→A:T	5
56	G:C→A:T	5	180	G:C→T:A	1
93	G:C→A:T	3		G:C→A:T	3
180	G:C→A:T	5	329	G:C→A:T	6
269	G:C→A:T	3	381	G:C→A:T	6
270	G:C→A:T	4		G:C→C:G	2
329	G:C→A:T	8			
381	G:C→A:T	8			

of BEM and UCM, respectively (Table III). Ninety percent (36/40) of the G:C→A:T mutations in BEM and 84% (49/58) of those in UCM occurred at 5′-CpG-3′ dinucleotides.

The data in Table III show that the relative frequencies of three classes of transversions are >3-fold higher in BEM than in UCM. A:T→T:A and G:C→C:G transversions each account for 7% (4/55) of the point mutations in BEM compared with ~1% (1/68) for each in UCM. G:C→T:A transversions accounted for ~9% (5/55) of the point mutations in BEM and 3% (2/68) of the mutations in UCM. In the case of A:T→C:G mutations, the frequency was ~3-fold higher in the UCM than in the BEM. Although the number of mutants examined by nucleotide sequence analysis is relatively low, the data suggest that benzene exposure may alter the relative frequency of transversion mutations.

Distribution of point mutations in lacI

The point mutations recovered from the BEM and UCM were distributed throughout the *lacI* structural gene; however, most of the mutations were found in the first 400 bp (Figure 1C and D, and Tables I and II). Within the first 400 bp of *lacI* are eight positions where the same mutation was independently isolated three or more times from different UCM (Table VI). Four of these eight apparent mutational hotspots were also found multiple times in different BEM (Table VI).

Four complex lacI mutations were isolated from lung tissues

Mutations were classified as complex if they consisted of more than one point mutation, or a combination of a point mutation and a deletion. Two of the complex mutations were comprised of point mutations at two adjacent bases: one such mutant was from a BEM and the other was from an UCM (Tables I and II). The other two complex mutations were isolated from BEM: one consisted of a deletion of 34 bp and a point mutation at one of the deletion endpoints (Table V), and the other consisted of point mutations at base pairs 242 and 467 (Table I).

Discussion

Studies from several laboratories have provided convincing evidence that exposure to benzene induces chromosome breakage and aneuploidy in the lymphocytes of humans (20–24), rodents (26–28) and in cultured cells (29,30). Benzene was generally regarded as non-mutagenic or weakly mutagenic in most short term *in vitro* gene mutation tests (38,39); however, using the Big Blue™ mouse genotoxicity assay, we found that inhalation of benzene at a level of 300 p.p.m. for 6 h/day×5 days/week for 12 weeks was accompanied by a statistically significant 1.7-fold elevation of the mutant frequency of *lacI* transgenes in lung tissues (31).

The data presented in this paper provide direct evidence that benzene is a gene mutagen in lung tissues, because exposure resulted in a 1.8-fold increase in the mutation frequency, and the pattern of *lacI* mutations in BEM was significantly different from that of UCM. In particular, we found that benzene exposure resulted in an increase in the frequency and length of deletion mutations.

The frequency of three classes of transversions is higher in BEM than in UCM (Table III); however, our data set is relatively small and more point mutational data will be required before we can evaluate the statistical significance of the increased transversion frequency in BEM.

The genotoxicity assay used in this work allowed detection of localized mutations, such as point mutations and small insertions or deletions; however, it did not allow us to measure loss or gain of chromosomes, large scale chromosome rearrangements, silent mutations in *lacI* or mutations that prevent packaging of phage λ. Thus, the 1.8-fold increase in the *lacI* mutation frequency is probably an underestimate of the genetic damage caused by benzene in lung tissues.

How might benzene exposure increase the frequency of deletion mutations? Alterations in the structure of DNA and/or altered activities of DNA metabolic enzymes are generally assumed to play a role in the formation of deletion mutations, and most models that seek to explain the formation of deletions are based on sequence features found at or near the site of mutations (for a review see 40). For example, the strand slippage model (SSM) proposed by Streisinger *et al.* (41) offers an explanation for deletions and insertions that occur at the site of nucleotide sequence repeats. The SSM predicts that the template and primer strands slip out of their normal register and one or more bases of one DNA strand become unpaired because of alternative sequence annealing. Extension of the misaligned primer by DNA polymerase can result in deletion (Figure 2A and B) or insertion mutations (Figure 2C).

The SSM can account for the formation of each of the spontaneous deletion and insertion mutations of the UCM, except for mutation 8-7. Although the mechanism by which mutation 8-7 formed is unknown, one type of mechanism that could account for this 67 bp deletion is the dislocation mutagenesis model (DMM) proposed by Kunkel and Soni (42). In the DMM, a base at one position can code a mutation at another position, and this mechanism is proposed to operate over large distances. Figure 2D illustrates one possible mechanism by which the DMM can explain how mutation 7-8 may have formed. In the first step, the sequence 5′-ATC-3′ becomes unpaired and anneals to a distal complementary sequence in *lacI* where it functions as a primer for DNA synthesis. Next, DNA polymerase adds the sequence 5′-GCG-3′ in a template-directed reaction, and a second slippage reaction displaces the 3′ end of the primer strand to another distal complementary sequence. Finally, DNA synthesis and local processing fixes the deletion mutation in place.

The SSM can explain the formation of the 5 bp insertion mutation 23-2 (Figure 2C) and 20 of the 28 deletions in BEM. Deletion mutations 20-12, 20-23, 21-7, 22-15, 24-5, 25-1, 27-6 and 27-22 cannot be explained by the SSM. Although other explanations are possible, the DMM can explain the formation of each of these eight deletions.

Based on examination of the deletions in BEM and UCM we propose that one of the consequences of benzene exposure

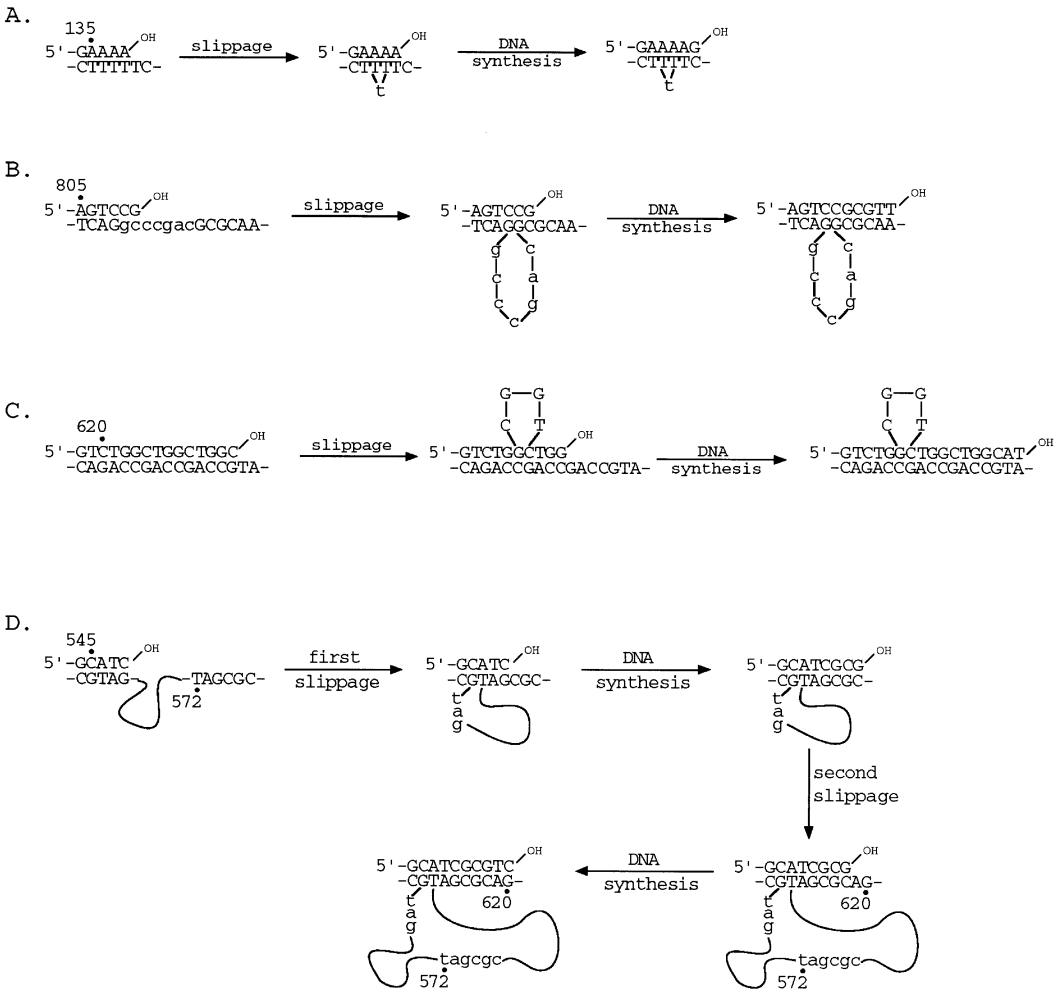

Fig 2. Models illustrating how selected deletion and insertion mutations may have formed. (**A**) Strand slippage in a mononucleotide run can result in a single bp deletion, as in mutation 6-29. (**B**) Strand slippage at a hyphenated direct repeat resulting in a 7 bp deletion as in mutation 25-5. (**C**) Slippage at a tandem direct repeat of a 4 bp sequence results in an insertion of 4 bp, as in mutation 8-16. (**D**) Dislocation mutagenesis mechanism that can account for the deletion of 67 bp, as in mutation 8-7.

is an increase in strand slippage reactions. An increased frequency of strand slippage may be part of the explanation for the increased frequency and length of deletions.

The SSM predicts that insertions may form at sequence repeats through looping out of a repeated sequence on the primer DNA strand (Figure 2C); however, we did not find an increase in the frequency of insertion mutations in BEM. This last finding suggests that if benzene exposure increases the frequency of strand slippage then the products of reactions in which the template strand looped out are obtained preferentially.

In light of the convincing mutagenicity of some benzene metabolites (43–45), perhaps it should not be surprising that benzene can cause gene mutations. The benzene mutagenicity that we detected in mouse lung tissues may depend on metabolism and transport processes that were not readily reproduced in the *in vitro* assays that failed to detect gene mutations induced by benzene.

Lung tissues express a variety of enzyme systems capable of metabolically activating and/or detoxifying environmental pollutants including benzene (46,47). Cytochrome P450 2E1 (CYP2E1) is an enzyme that can be induced in several tissues of mice, including lung (48), and is able to catalyze oxidization of benzene to reactive metabolites *in vitro* (14,49,50). Mice that fail to express CYP2E1 because of a *cyp2e1*$^{-/-}$ null mutation (51) have dramatically reduced levels of benzene metabolites in their urine and reduced levels of micronuclei after exposure to benzene (15). Indirect evidence for a role of CYP2E1 in benzene-induced genotoxicity was provided by the finding of Tuo *et al.*, that inhibition of CYP2E1 with propylene glycol resulted in a decrease in DNA damage as measured by an alkaline comet assay on nucleated bone marrow cells (52).

The benzene metabolite *p*-benzoquinone reacts *in vitro* with deoxyadenosine-3′-phosphate to form the 3′-hydroxy-1,N^6-benzetheno-2′-deoxyadenosine-3′-triphosphate adduct (53), with deoxycytosine to form 3′-hydroxy-3, N^4-benzetheno-2′-deoxycytidine (54), and with deoxyguanosine to form the 3′-hydroxy-1,N^2-benzetheno-2′-deoxyguanosine adduct (55). Previous reports have shown that metabolites of benzene bind covalently to proteins and DNA (56,57) and some metabolites of benzene form adducts with DNA in a variety of different cell lines (58,59).

Although there have been contradictory findings with respect to benzene metabolite DNA adduct formation *in vivo* (60), Pathak *et al.* (18) and Lévay *et al.* (61) detected adducts in bone marrow and white blood cells of B6C3F1 mice injected i.p. with benzene. The major adduct appears to result from a

reaction of DNA with benzoquinone, and a minor adduct results from reaction with 1,2,4-trihydroxybenzene (18). In addition, covalent *N*-7-phenyl-guanine adducts have been detected in rat urine after i.p. injection of benzene (62). It is not known if any of the DNA adducts detected *in vitro* or *in vivo* are mutagenic.

Another possible mechanism by which benzene metabolites could cause gene mutations in DNA is through generation of active species of oxygen, including hydroxyl radicals and singlet oxygen (for a review see 63), which can react with DNA and cause strand breakage (64,65) and hydroxylation of bases (66). Single strand breaks in DNA might serve to initiate sequence slippage reactions that could lead to deletions. Evidence for the ability of benzene metabolites to undergo redox cycling and generate active oxygen in a human cell culture line and *in vivo* in mouse bone marrow is suggested by increases in the level of 8-hydroxy-2′-deoxyguanosine in the presence of benzene metabolites (19).

Mutations are generally accepted as being critically important in the process that transforms a normal cell into a malignant cell (67,68). Chronic whole body exposure to benzene is known to increase the frequency of a variety of tumors in mice including lung tumors (10,12), and the findings in this work suggest the possibility that deletions and point mutations are part of the mechanism by which benzene increases the frequency of tumors in mice.

In summary, the results presented in this paper demonstrate that the spectrum of mutations in *lacI* transgenes recovered from lung tissues of BEM is different from the spontaneous spectrum of mutations observed in UCM. Our data show that inhalation of benzene at a level of 300 p.p.m. for 6 h/day×5 days/week for 12 weeks resulted in a significant increase in both length and frequency of deletion mutations. The data also suggest the possibility that benzene may alter the frequency of transversion mutations. The increase in *lacI* mutation frequency in lung tissues of mice exposed to benzene, and the shift in the *lacI* mutational spectrum offers strong support for our proposal that benzene is a gene mutagen.

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