New experimental data linking secondhand smoke exposure to lung cancer in nonsmokers

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ABSTRACT **Secondhand smoke (SHS) exposure is a known risk factor for lung cancer development in lifelong nonsmokers; however, the mechanistic involvement of SHS in the genesis of this malignancy remains elusive. The present study is the first comprehensive investigation of SHS mutagenicity *in vivo*, in which we have established the mutagenic effects of SHS in transgenic Big Blue mice, and subsequently found correlations between our experimental findings and those obtained from our analysis of the largest database of mutations in human *TP53*, which is the most frequently mutated gene in human lung cancer. We demonstrate that whole-body SHS exposure of mice for 5 h/d, 5 d/wk for a duration of 2 or 4 mo elicits a significant mutagenic response in the lung, trachea, and bladder of exposed animals, as reflected by the elevation of background *cII* mutant frequency in the respective organs. The organ-specific mutagenicity of SHS is most pronounced in the lung and remains persistent both in the lung and bladder of SHS-exposed animals after a 1-mo recovery in clean air. The induced *cII* mutagenesis in the lung of SHS-exposed mice perfectly recapitulates our analysis of the *TP53* mutations in human lung cancer in nonsmokers. Remarkably, the relative frequencies of all types of mutations in the *TP53* gene of nonsmokers' lung tumors and in the *cII* transgene of lung cellular DNA from SHS-exposed mice are indistinguishable from one another. We provide the first verification of a mechanistic mode of action for SHS of relevance for carcinogenesis and the first experimental evidence linking SHS exposure to lung cancer in nonsmokers.—Kim, S.-I., Yoon, J.-I., Tommasi, S., Besaratinia, A. New experimental data linking secondhand smoke exposure to lung cancer in nonsmokers. *FASEB J.* 26, 1845–1854 (2012). www.fasebj.org**

Key Words: carcinogenesis · mutagenicity · tobacco · TP53 *mutations*

SECONDHAND SMOKE (SHS), also referred to as environmental tobacco smoke, passive smoke, or involuntary smoke, is classified as a known human carcinogen by authoritative scientific bodies and regulatory agencies (1, 2). Worldwide, there are 1.2 billion smokers who impose an indiscriminate burden of SHS on billions of nonsmokers (3). In the past 3 decades, ample evidence has accumulated from human biomonitoring studies to help establish SHS as a risk factor for lung cancer development in lifelong nonsmokers (4). What remains to be elucidated, however, is the underlying mechanism of action of SHS in lung carcinogenesis (5). Determining the mechanistic role of SHS in the genesis of nonsmokers' lung cancer can help decipher the pathogenesis of this disease, which continues to take its toll as the leading cause of cancer-related deaths in the United States and throughout the world (6). Understanding the pathogenesis of lung cancer in nonsmokers can help devise future strategies for prevention, early detection, and treatment of this malignancy (7, 8).

SHS is a complex and dynamic aerosol, which comprises the exhaled mainstream smoke of active smokers, the sidestream smoke emanated from the smoldering cone of tobacco products, and the smoke diffused through the wrapping materials, *e.g.*, cigarette paper (9, 10). Being produced at a lower temperature relative to mainstream smoke (~600 *vs.* 900°C) and having undergone aging and dilution in ambient air, SHS differs from mainstream smoke in terms of quantities of its constituents and physicochemical properties (9, 11). Qualitatively, however, SHS contains essentially the same toxicants and carcinogens as those found in mainstream smoke (9–11). Many of the carcinogens present in mainstream smoke are known to exert their effects through a genotoxic mode of action, which is based on their ability to induce DNA damage and mutations (12), although an epigenetic mode of action, *e.g.*, through aberrant DNA methylation and histone modifications, is also beginning to emerge for a few of these carcinogenic compounds (7, 13). The genotoxicity of mainstream smoke carcinogens manifests as mutations occurring in key cancer-related genes, *i.e.,* protooncogenes or tumor suppressor genes that control crucial cellular functions, *e.g.*, growth and survival, in

Abbreviations: B[*a*]PDE, benzo[*a*]pyrene diol epoxide; CI, confidence interval; IARC, International Agency for Research on Cancer; SHS, secondhand smoke.

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doi: 10.1096/fj.11-199984

lung tumors of active smokers (14, 15). Of these, *TP53* mutagenesis causing abrogation of gene transcription, DNA synthesis and repair, cell cycle arrest, and senescence and apoptosis that can lead to genetic instability and progression to lung cancer, represents a unique avenue for investigating lung cancer etiology (16–18). More specifically, the frequency, pattern, and codon distribution of mutations in this tumor suppressor gene in smokers' lung cancer bear a striking resemblance to those found in experimental model systems that recapitulate exposure to tobacco smoke or its constituents (16, 19, 20). *TP53* mutagenesis is the most frequent genetic alteration in smoking-related lung cancer, with a unique mutation spectrum characterized by a predominance of G→T transversions, which cluster almost exclusively in several methylated CpG-containing codons and occur preferentially on the nontranscribed DNA strand (16, 19, 20). The same codon positions are the hotspots of DNA damage formation in cells treated *in vitro* with a number of tobacco smoke-derived carcinogens (21–23). The correspondence between preferential sites of DNA damage formation and mutational hotspots in the *TP53* gene in smoking-attributable lung cancer has provided significant clues to the etiology of this disease (16, 19, 20).

The comparatively uniform chemical compositions of mainstream smoke and SHS (6, 7) bear out the hypothesis that SHS possesses a genotoxic mode of action based on mutagenicity of some of its components. To date, however, no experimental study has comprehensively investigated the mutagenic potential of SHS in a relevant model system and under well-defined and controlled exposure conditions. In the present study, we have investigated the mutagenicity of SHS *in vivo* in transgenic Big Blue mice, an extensively validated model system for the analysis of experimentally induced mutations (24). Here, we have investigated the mutagenic consequences of exposure to SHS in transgenic Big Blue mice exposed whole body to SHS generated by a microprocessor-controlled smoking machine. We have determined the frequency of *cII* mutants in various target and surrogate organs of SHS-treated mice and characterized the spectrum of *cII* mutations in the lung of SHS-exposed animals. Subsequently, we have sought correlations between our experimental findings in SHS-treated mice and those obtained from the compilation of research on *TP53* mutagenesis in human lung cancer in known smokers and nonsmokers [International Agency for Research on Cancer (IARC) TP53 database (25)].

MATERIALS AND METHODS

Animals

Transgenic Big Blue mice on a C57BL/6 genetic background were obtained from Stratagene (Santa Clara, CA, USA) and bred and maintained at the City of Hope Animal Resources Center under conditions approved by the institutional animal care and use committee in accordance with the recommendations of the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals. Eighty male adult Big Blue mice (6–8 wk old) hemizygous for λ*LIZ* were randomly divided into 2 groups, experimental (SHS exposure; $n=40$) and control (clean air sham-exposure; $n=40$), each subdivided into 4 categories ($n=10$), including 2 mo exposure, 2 mo exposure + 1 mo recovery, 4 mo exposure, and 4 mo exposure + 1 mo recovery. The mice assigned to each experimental or control group ($n=10$) were kept in polypropylene cages in groups of 3–4 animals/cage and housed in an air-conditioned animal room with ambient temperature of 21 ± 1°C and relative humidity of 55% with a 12-h light-dark cycle. Throughout all experiments, including the exposure and recovery periods, the mice had access to food (PicoLab Rodent Diet 20; PMI Nutrition International, LLC, Brentwood, MO, USA) and water *ad libitum*.

Smoking machine and SHS exposure

Detailed information on smoking machine and experimental exposure of the mice to SHS are available in ref. 26. In brief, we used a custom-made smoking machine (model TE-10; Teague Enterprises, Davis, CA, USA) to generate SHS for experimental exposure of Big Blue mice to SHS. All mice assigned to various experimental groups underwent an acclimatization period during which they were gradually exposed to incremental doses of SHS (see Fig. 1 in ref. 26). After the acclimatization period, the mice were maintained on a SHS exposure regimen, which included 5 h/d, 5 d/wk, and 2 or 4 mo whole-body exposure to SHS produced through continuous smoking of 7–9 cigarettes. Control mice were handled similarly to SHS-treated animals and were maintained in clean air after sham exposure to filtered high-efficiency particulate air. At the end of all experiments, the SHS-treated and control mice were euthanized by CO_2 asphyxiation; various target and surrogate organs, including the lung, trachea, bladder, heart, liver, spleen, and testis were harvested; and genomic DNA was isolated (27) and preserved at −80°C until further analysis. We note that the whole-body smoke exposure of mice used in the present study may result in transdermal and gastrointestinal absorption of smoke materials (from grooming) by the animals (28, 29). However, a more controlled exposure procedure, such as "nose-only, " can cause stress and discomfort for the animals, especially in long-term studies such as the present one (30). Thus, our choice of whole-body exposure of mice to SHS was based on tolerability and practicality of this approach for chronic treatment of the relatively large number of animals used in this study and the similarity of this approach to SHS exposure of humans, which occurs in real life through whole-body exposure (2, 3, 6, 7). We note that the amount of SHS taken up by inhalation as opposed to other uptake routes in our experimental mice was not determined because of the lack of "specific" internal dose markers for SHS; however, this important measurement can be performed in future studies, in which the quantities of SHS taken up through different routes of exposure will be calculated using sensitive, specific, and validated internal dose biomarkers for SHS (6, 7). At present, work is underway in our laboratory to develop unique biological markers that can be used for sensitive and specific detection of SHS exposure *in vivo*.

cII mutant frequency and mutation spectrum analyses

Genomic DNA of transgenic Big Blue mice contains multiple copies of the coliphage λ*LIZ* shuttle vector, which is integrated into murine chromosome 4 in a head-to-tail configu-

ration, while harboring 2 mutational target genes, the *cII* and *lacI* (31). For mutant frequency determination, the λ*LIZ* shuttle vectors containing the *cII* transgene were recovered from the genomic DNA of SHS-treated and control mice and packaged into viable phage particles using a Transpack Packaging Extract kit (Stratagene). After preadsorption of the phages to G1250 *Escherichia coli*, the bacterial culture was grown on special TB1 agar plates. To select for *cII* mutants, the plates were incubated at 24°C for 48 h. Alternatively, the plates were incubated under nonselective conditions, *i.e.,* 37°C overnight, to express both the wild-type and mutant *cII*. Verification of all putative *cII* mutants was achieved by replating under the selective condition. To determine a statistically valid mutant frequency, minimums of 3×10^5 rescued phages were screened in each experimental or control sample (24). For mutation spectrometry, all verified mutant plaques were amplified by PCR using λ select-*cII* sequencing primers according to the manufacturer's recommended protocol (Stratagene). The purified PCR products were then subjected to direct DNA sequencing using a Big Dye terminator cycle sequencing kit and ABI-3730 DNA Sequencer (ABI Prism; PE Applied Biosystems, Foster City, CA, USA).

Statistical analysis

Results are expressed as medians ± 95% confidence intervals (CIs). Comparison of all variables between 2 separate groups was done using the Wilcoxon rank-sum test. The frequencies of specific type of mutations (*e.g.*, transitions, transversions, and others) between 2 different groups were compared by the χ^2 test. All statistical tests were 2-sided. Values of $P \leq 0.05$ were considered statistically significant. S-Plus 7.0 for Windows software (Insightful Corp., Seattle, WA, USA) was used for all statistical analyses.

RESULTS

cII mutant frequency in various target and surrogate organs of mice

We determined the mutant frequencies of the *cII* transgene in the lung, trachea, bladder, heart, liver, spleen, and testis of mice exposed to SHS/clean air (control) for 2 or 4 mo and an ensuing 1-mo recovery period. Because the background frequency of *cII* mutants in each of the above organs in various control groups, including 2 mo sham exposure, 2 mo sham exposure plus 1 mo recovery, 4 mo sham exposure, and 4 mo sham exposure plus 1 mo recovery, did not differ significantly from each other, for brevity, we used only the data from control group 4 for all comparison purposes. As shown in **Fig. 1*A***, the background *cII* mutant frequency in the lung of control mice ($1.91 \pm 0.29 \times 10^{-5}$) was significantly increased to $3.98 \pm 0.91 \times 10^{-5}$ ($P=0.0001$) and $3.96 \pm 0.79 \times 10^{-5}$ ($P=0.0011$) in the lung of 2- and 4-mo SHS-treated mice, respectively. After 1 mo of recovery, the frequencies of *cII* mutant in the lung of both 2- and 4-mo SHS-exposed mice still remained significantly elevated relative to those of controls (2.91 ± 0.30 and $3.84 \pm 0.39 \times 10^{-5}$, respectively, $P=0.0007$). However, after 1 mo of recovery, the persistence of increase in *cII* mutant frequency in the lung of 4-mo SHS-exposed mice was more pronounced than that in the 2-mo SHS-exposed animals ($P=0.0007$).

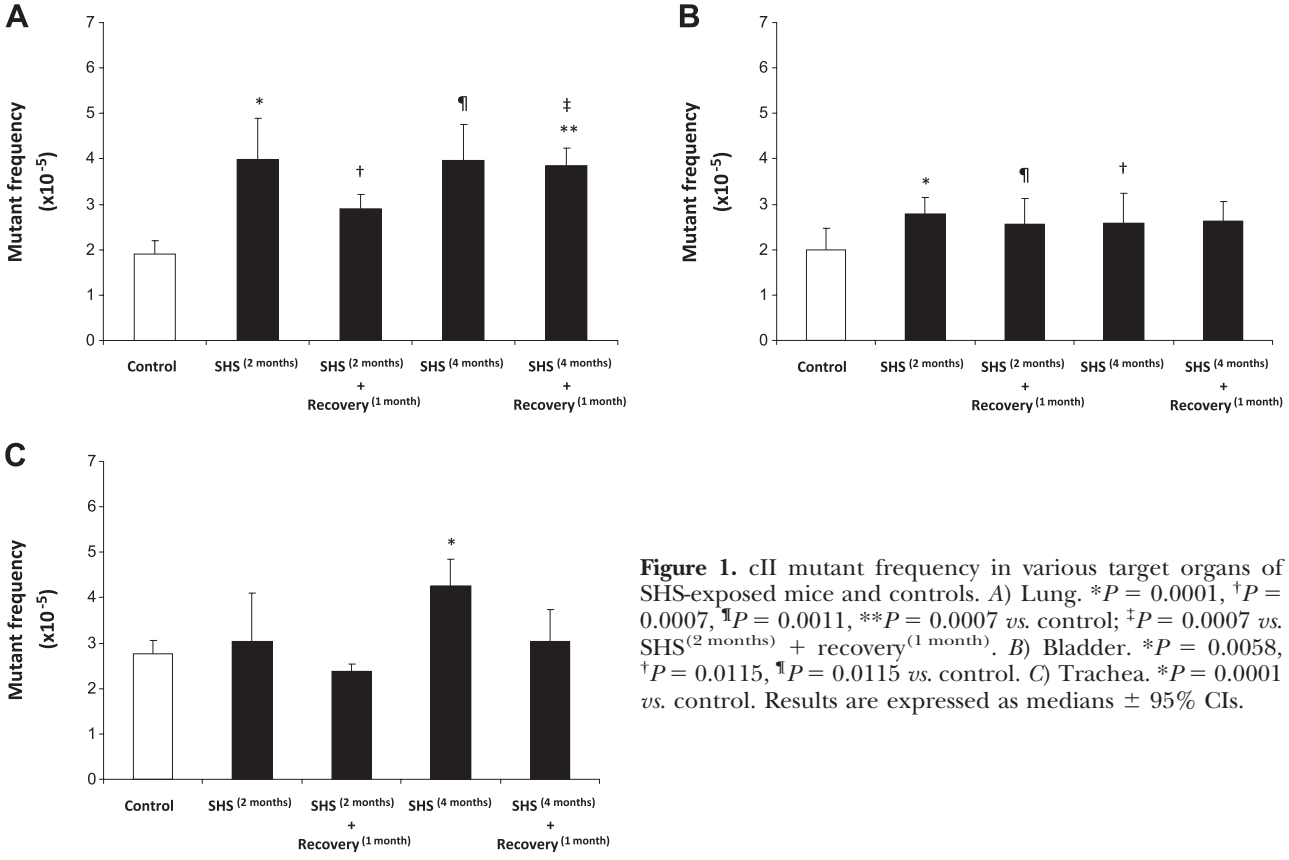

Figure 1. cII mutant frequency in various target organs of SHS-exposed mice and controls. *A*) Lung. $^*P = 0.0001$, $^\dagger P = 0.0007$, $^\P P = 0.0011$, $^{**}P = 0.0007$ *vs.* control; $^\ddagger P = 0.0007$ *vs.* SHS$^{(2\ \text{months})}$ + recovery$^{(1\ \text{month})}$. *B*) Bladder. $^*P = 0.0058$, $^\dagger P = 0.0115$, $^\P P = 0.0115$ *vs.* control. *C*) Trachea. $^*P = 0.0001$ *vs.* control. Results are expressed as medians ± 95% CIs.

Likewise, the background *cII* mutant frequency in bladder of control mice ($2.00\pm0.48\times10^{-5}$) was significantly raised to $2.78 \pm 0.36 \times 10^{-5}$ (P=0.0058) and $2.59 \pm 0.65 \times 10^{-5}$ (P=0.0115) in bladder of 2- and 4-mo SHS-treated mice, respectively (Fig. 1*B*). The frequencies of *cII* mutant in bladder of both 2- and 4-mo SHS exposed mice after 1 mo of recovery still remained higher than that in controls ($2.58\pm2.38\times10^{-5}$, P=0.0115 and $2.63\pm0.43\times10^{-5}$, P= 0.0553, respectively).

In addition to an increase in the *cII* mutant frequency in trachea of mice exposed to SHS for 2 mo relative to that in controls (3.04 ± 1.06 *vs.* $2.76\pm0.30\times10^{-5}$, P=0.0587), the relative frequency of *cII* mutants in trachea of 4-mo SHS-treated mice was significantly elevated ($4.26\pm0.58\times10^{-5}$, P=0.0001), which subsequently returned to almost the baseline level after a 1-mo recovery in clean air ($3.04\pm0.70\times10^{-5}$, $P<0.6$; Fig. 1*C*). As shown in **Fig. 2**, despite the slight increases in relative *cII* mutant frequencies in heart, liver, spleen, and testis of the mice exposed to SHS for 2 or 4 mo or after 1 mo of recovery, the *cII* mutant frequency was only significantly elevated in liver of 4-mo SHS-exposed animals after 1 mo of recovery (2.43 ± 0.30 *vs.* $4.79\pm1.10\times10^{-5}$, P=0.0007; Fig. 2*B*). We note that the administered doses of SHS in the present study were well tolerated by the mice as reflected by the 100% survival rate of the animals in various experimental groups, which was similar to that in counterpart sham-exposed mice (data not shown).

cII mutation spectrum in the lung of mice

We established the spectra of mutations in the *cII* transgene in the lung of SHS-treated mice and controls by DNA sequencing of the *cII* mutants obtained from the analysis of lung genomic DNA from 4-mo SHS-exposed mice and controls. We randomly selected 30 mutant plaques derived from the lung DNA of each of the 10 SHS-exposed mice and controls and performed DNA sequencing analysis, which resulted in the establishment of the first comprehensive database of SHS-induced mutations *in vivo*. Detailed information on the type and frequency of mutations in the *cII* transgene in the lung genomic DNA from SHS-treated mice and controls is shown in **Tables 1** and **2**. Distribution of these mutations along the nucleotide positions of the *cII* transgene is also outlined in **Figs. 3** and **4**. In all cases, calculations were made both with and without the sibling mutations, which are defined as the identical mutations that occur repeatedly at the same nucleotide positions in the same sample from an individual animal and may or may not be independent events from one another. The overall contributions of the sibling mutations to the SHS-induced and control mutation spectra were 34.1 and 38.1%, respectively, which are not significantly different from one another. We note that neither the spectrum of mutations produced by SHS nor that of the

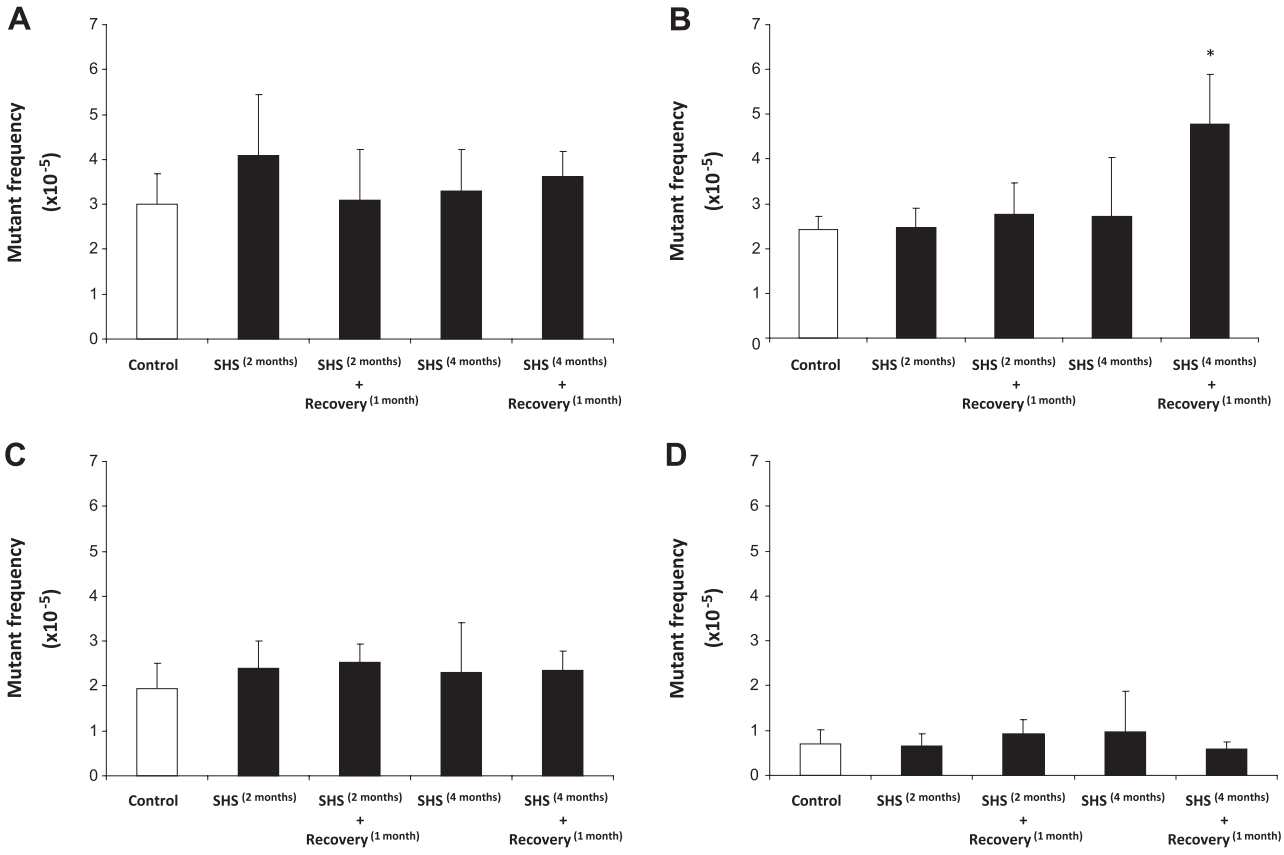

Figure 2. cII mutant frequency in various surrogate organs of SHS-exposed mice and controls. *A*) Heart. *B*) Liver. *P = 0.0007 *vs.* control. *C*) Spleen. *D*) Testis. Results are expressed as medians ± 95% CIs.

TABLE 1. *Comparative mutation spectra of the cII transgene in the lung of Big Blue mice chronically treated with SHS or control clean air*

Mutation type	Mutations (*n*)				Mutations (%)				Absolute mutant frequency ($\times 10^{-5}$)			
	Nonadjusted		Adjusted		Nonadjusted		Adjusted		Nonadjusted		Adjusted	
	SHS	Control	SHS	Control	SHS	Control	SHS	Control	SHS	Control	SHS	Control
G:C→C:G	20	6	15 (4)	6 (2)	6.9	2.0	7.7 (2.1)	3.2 (1.1)	0.28	0.04	0.20 (0.06)	0.04 (0.01)
G:C→T:A	34	22	30 (4)	21 (3)	11.6	7.3	15.5 (2.1)	11.2 (1.6)	0.46	0.14	0.41 (0.06)	0.13 (0.02)
G:C→A:T	156	188	84 (72)	97 (86)	53.4	62.1	43.3 (37.1)	51.6 (45.8)	2.13	1.19	1.15 (0.98)	0.61 (0.54)
A:T→T:A	16	7	15	7	5.5	2.3	7.7	3.7	0.22	0.04	0.20	0.04
A:T→G:C	17	13	17	12	5.8	4.3	8.8	6.4	0.23	0.08	0.23	0.08
A:T→C:G	11	18	9	17	3.8	5.9	4.6	9.0	0.15	0.11	0.12	0.11
Deletion	23	32	16 (6)	19 (1)	7.9	10.6	8.3 (3.1)	10.1 (0.5)	0.32	0.20	0.22 (0.08)	0.12 (0.01)
Insertion	15	17	8	9	5.1	5.6	4.1	4.8	0.20	0.11	0.11	0.06

Male adult Big Blue transgenic mice were exposed whole body to SHS or control clean air (filtered high-efficiency particulate air) for 5 h/d and 5 d/wk for a duration of 4 consecutive mo and sacrificed immediately afterward. Adjusted results indicate adjustment after exclusion of sibling mutations that occurred more than once at the same nucleotide position in the same animal. Values in parentheses indicate mutations occurring specifically at CpG dinucelotides.

control changed significantly after the exclusion of sibling mutations (Table 1).

As shown in Table 2, single base substitutions comprised the vast majority of *cII* mutations found in the lung genomic DNA from both SHS-treated mice and controls (85.2 *vs.* 83.8%). Of these, mutations occurring at G:C base pairs predominated the SHS-induced and control mutation spectra alike (69.6 *vs.* 66.5%), with G:C→A:T transition mutations being the most frequent type of mutations found in both spectra (43.3 *vs.* 51.6%, $P<0.1$; Table 1; for brevity, all comparisons henceforth are made with the exclusion of sibling mutations). The frequency of mutations occurring at G:C base pairs or that of G:C→A:T transition mutations, which were targeted to 5′-CpG dinucleotides, did not differ significantly between SHS-exposed and control mice (44.4 *vs.* 49.0%, $P<0.5$ and 37.1 *vs.* 45.8%; $P<0.1$, respectively). Thus, the induced *cII* mutations in the lung of SHS-exposed mice are not biased toward 5′-CpG-containing sequences (discussed below).

TABLE 2. *Types of mutations in the cII transgene in the lung of Big Blue mice chronically treated with SHS or control clean air*

Mutation type	Nonadjusted		Adjusted	
	SHS	Control	SHS	Control
Single mutation	284 (99.0)	295 (98.7)	186 (98.4)	182 (98.4)
Multiple mutations	3 (1.0)	4 (1.3)	3 (1.6)	3 (1.6)
Base substitution				
Single	245 (85.4)	248 (82.9)	161 (85.2)	155 (83.8)
Tandem				
CC→AA	1 (0.3)	0 (0)	1 (0.5)	0 (0)
GA→CT	1 (0.3)	0 (0)	1 (0.5)	0 (0)
Multiple				
G→A and C→A	1 (0.3)	0 (0)	1 (0.5)	0 (0)
T→A and T→A	1 (0.3)	0 (0)	1 (0.5)	0 (0)
C→G and (A) Ins.	1 (0.3)	0 (0)	1 (0.5)	0 (0)
G→A and G→T	0 (0)	1 (0.3)	0 (0)	1 (0.5)
A→C and G→T	0 (0)	1 (0.3)	0 (0)	1 (0.5)
G→A and (ACA) Del.	0 (0)	2 (0.7)	0 (0)	1 (0.5)
Deletion				
Single	21 (7.3)	28 (9.4)	14 (7.4)	16 (8.7)
Multiple	2 (0.7)	2 (0.7)	2 (1.1)	2 (1.1)
Insertion				
Single	14 (4.9)	17 (5.7)	7 (3.7)	9 (4.9)
Multiple	0 (0)	0 (0)	0 (0)	0 (0)

Male adult Big Blue transgenic mice were exposed whole body to SHS or control clean air (filtered high-efficiency particulate air) for 5 h/d and 5 d/wk for a duration of 4 consecutive months (5 h/d and 5 d/wk) and sacrificed immediately afterward. Adjusted results indicate adjustment after exclusion of sibling mutations that occurred more than once at the same nucleotide position in the same animal. Values in parentheses indicate mutations occurring specifically at CpG dinucelotides.

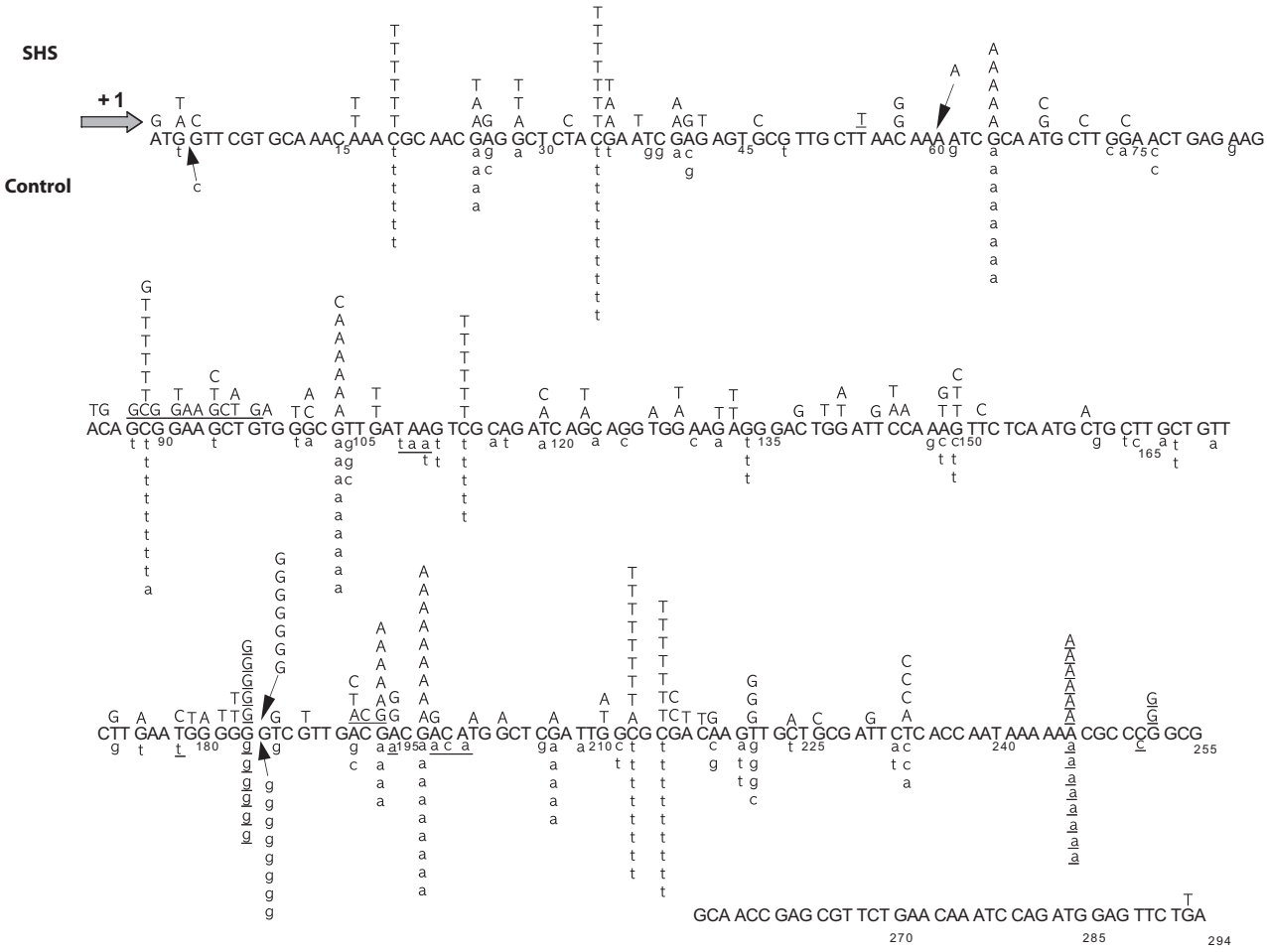

Figure 3. Detailed mutation spectra of *cII* transgene in the lung of SHS-exposed mice and controls. SHS-induced mutations are set in capital letters above the reference *cII* sequence; control mutations are set in lowercase letters below the reference *cII* sequence. Deleted bases are underlined. Inserted bases are shown with an arrow. Numbers below the bases are the nucleotide positions.

To find what specific types of mutation have caused the significant increase in *cII* mutant frequency in the lung of SHS-exposed mice relative to controls, we computed the absolute mutant frequency of each type of mutation (*i.e.,* transitions, transversions, deletions, and insertions) in the *cII* transgene in the lung DNA of both the SHS-exposed mice and controls. As shown in **Fig. 5*A,*** *B* and Table 1, the absolute mutant frequencies of G:C→C:G transversions, G:C→T:A transversions, G:C→A:T transitions, A:T→T:A transversions, A:T→G:C transitions, A:T→C:G transversions, deletions, and insertions were all increased, although to different extents, in the *cII* transgene of lung genomic DNA from SHS-exposed mice. The percentage contributions of the respective types of mutation to the overall increase in relative *cII* mutant frequency in the lung of SHS-exposed mice were 11.5, 15.4, 45.2, 8.7, 7.2, 1.9, 5.8, and 4.3. Therefore, G:C→A:T transition mutations account for the majority of induced *cII* mutations in the lung of SHS-exposed mice (Fig. 5*C, D*).

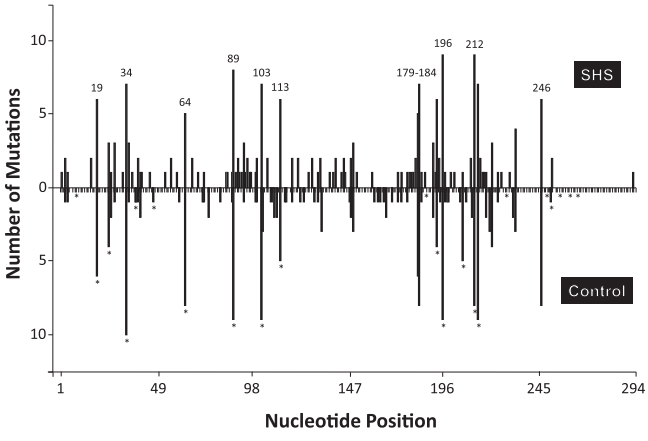

Figure 4. Distribution of mutations in the *cII* transgene in the lung of SHS-exposed mice and controls. Asterisks in the control indicate 5′-CpG-containing sequences.

TP53 mutation spectrum in human lung cancer in smokers and nonsmokers

We analyzed the largest and most updated database of *TP53* mutagenesis in human lung cancer in both smokers and nonsmokers (IARC TP53 database; ref. 25) and sought correlations between these compiled data and those obtained from our mutagenicity exper-

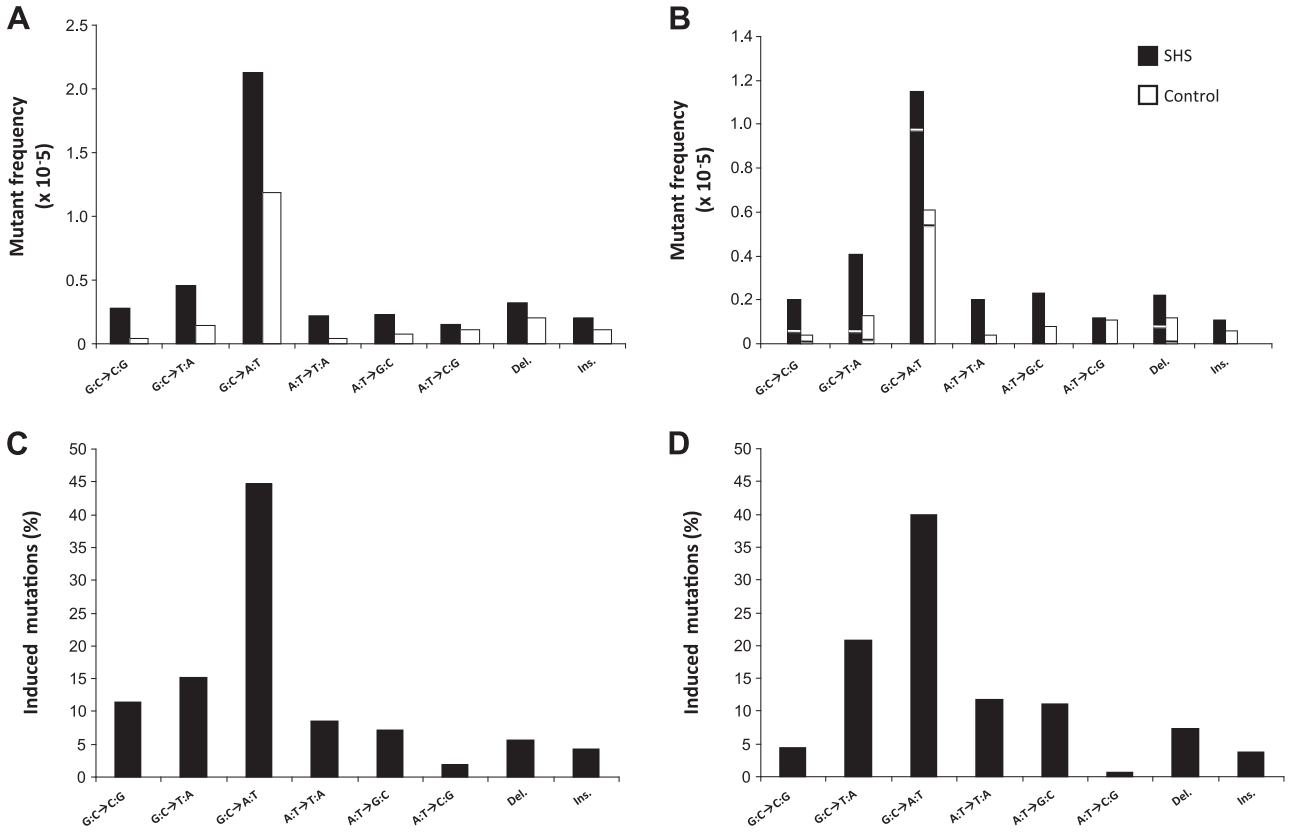

Figure 5. Spectrum of induced *cII* mutations in the lung of SHS-exposed mice. *A*, *B*) Absolute mutant frequency of each specific type of mutation in the *cII* transgene of lung cellular DNA from SHS-exposed mice. *A*) Sibling mutations are included (see text for a definition of these mutations). *B*) Sibling mutations are excluded. Horizontal lines within bars represent the contribution of mutations occurring specifically at 5′-CpG dinucleotides. *C*, *D*) Percentage increase in the frequency of each specific type of induced mutation in the *cII* transgene of lung cellular DNA from SHS-exposed mice relative to control. *C*) Sibling mutations are included. *D*) Sibling mutations are excluded. Horizontal lines within bars represent the contribution of mutations occurring specifically at 5′-CpG dinucleotides.

iments in mice exposed to SHS *in vivo*. Because exposures to asbestos, coal, mustard gas, or radon are established risk factors for lung cancer development (2, 6, 16), we excluded all the entries with known exposure to these agents from our analyses. The R15 version of the IARC TP53 database contains 27,580 somatic mutations, of which 2860 mutations are of lung topography. Of these, 1083 and 220 mutations are recovered from the lung tumors of "known" smokers and nonsmokers, respectively (after exclusion of the entries with documented exposure to asbestos, coal, mustard gas, or radon; ref. 25).

As shown in **Fig. 6*A***, the mutation spectrum of *TP53* in lung cancer of smokers differs from that of nonsmokers in that the smokers have a significantly increased frequency of G:C→T:A transversion mutations relative to nonsmokers (31.7 *vs.* 17.7%, $P<0.00005$), which occur preferentially on the nontranscribed DNA strand (89.5%) and are strongly biased toward 5′-CpG-containing sequences. The targeting of mutations at 5′-CpG dinucleotides in the *TP53* gene in smokers' lung cancer is best represented by the clustering of mutational hotspots almost exclusively in 5′-CpG-containing codons in this tumor suppressor gene in smokers' lung tumors, *i.e.,* 5 of 6 mutational hotspots in lung cancer of smokers are found in codons with 5′-CpG in their sequence context (Fig. 6*B*). Conversely, the mutation spectrum of *TP53* in nonsmokers' lung cancer is characterized by a significantly increased frequency of G:C→A:T transition mutations (40.5 and 25.3% in nonsmokers and smokers, respectively, $P=0.000007$), which occur indiscriminately on both DNA strands (transcribed 48.3% and nontranscribed 51.7%, $P<0.6$) and are not biased toward 5′-CpG-containing sequences (Fig. 6*C*). In addition, the ratio of G:C→A:T transitions to G:C→T:A transversions in the *TP53* gene in nonsmokers' lung cancer is significantly elevated relative to that in smokers' lung cancer (2.3 *vs.* 0.79, $P=0.000001$).

The above-specified mutation spectrum of the *TP53* gene in lung cancer of nonsmokers is virtually identical to the *cII* mutation spectrum in the lung of SHS-exposed mice *in vivo* (Fig. 6*A*). Whereas the characteristic mutation spectrum of the *TP53* gene in nonsmokers' lung cancer is perfectly duplicated in the spectrum of mutations found in the *cII* transgene in lung DNA from SHS-exposed mice, the frequencies of all other types of mutation in the two mutation spectra are nearly identical. More specifically, the frequencies of G:C→A:T transition mutations in the *TP53* gene of

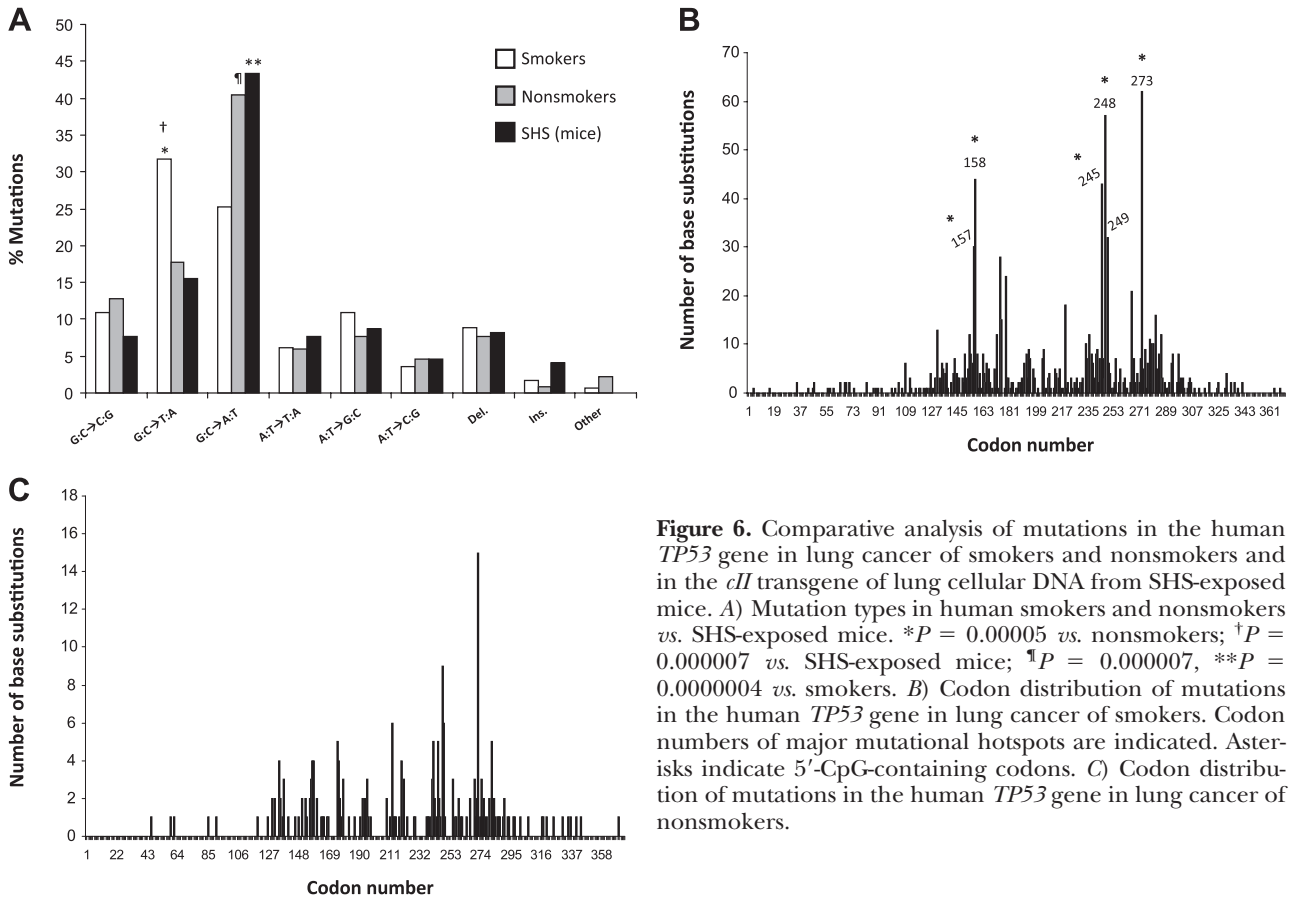

Figure 6. Comparative analysis of mutations in the human *TP53* gene in lung cancer of smokers and nonsmokers and in the *cII* transgene of lung cellular DNA from SHS-exposed mice. *A*) Mutation types in human smokers and nonsmokers *vs.* SHS-exposed mice. $*P = 0.00005$ *vs.* nonsmokers; $^{\dagger}P = 0.000007$ *vs.* SHS-exposed mice; $^{\P}P = 0.000007$, $**P = 0.0000004$ *vs.* smokers. *B*) Codon distribution of mutations in the human *TP53* gene in lung cancer of smokers. Codon numbers of major mutational hotspots are indicated. Asterisks indicate 5′-CpG-containing codons. *C*) Codon distribution of mutations in the human *TP53* gene in lung cancer of nonsmokers.

nonsmokers' lung tumors and in the *cII* transgene of the lung DNA from SHS-exposed mice are 40.5 and 43.3%, respectively ($P<0.6$). Likewise, the ratios of G:C→A:T transitions to G:C→T:A transversions in the respective groups closely resemble one another (2.3 *vs.* 2.8, $P<0.6$), while both being significantly higher than that in smokers' ($P<0.000007$; Fig. 6*A*). Furthermore, neither the spectrum of mutations in the *TP53* gene in nonsmokers' lung cancer nor that in the *cII* transgene of lung cellular DNA from SHS-treated mice shows a bias of mutagenesis at 5′-CpG dinucleotides. The latter is in sharp contrast to that of smokers' *TP53* mutation spectrum in lung cancer, which is highly biased toward 5′-CpG-containing sequences (Figs. 3 and 6*B*, *C*).

DISCUSSION

We determined the mutagenic potency of SHS in our experimental mice by quantifying the mutant frequency of the *cII* transgene in various organs of chronically treated mice relative to controls. Overall, SHS exposure elicited a significant mutagenic response in the lung, trachea, and bladder of exposed animals as reflected by the elevation of background *cII* mutant frequency in the respective organs (Fig. 1). This organ-specific mutagenicity of SHS was most pronounced in the lung and remained persistent both in the lung and bladder in SHS-exposed animals after 1 mo recovery in clean air. Organs of the respiratory tract and urinary tract are known targets of tumorigenesis in smoking-related malignancies (6). Although the lung is an established target organ for SHS-associated carcinogenesis (5), the mutagenic response found in bladder of the SHS-exposed mice deserves special attention. Of relevance, SHS is highly rich in aromatic amines, such as 4-aminobiphenyl, *o*-toluidine, and 2-naphthylamine that are known bladder carcinogens (6, 7, 20).

Notwithstanding the lack of mutagenicity of SHS in different surrogate organs, including heart, spleen, and testis of SHS-exposed mice, there was a delayed mutagenic response in liver of 4-mo SHS-exposed animals after 1 mo recovery in clean air (Fig. 2). Although the exact mechanism of this delayed effect is currently unknown, the proliferatively slow hepatic cells may require prolonged time for mutation fixation. In addition, the liver is a major site for metabolic activation/detoxification of xenobiotics, many of which are known to induce/suppress the expression or activity of some of its metabolizing enzymes (32). Thus, it is conceivable that on removal of exposure, the biological response to SHS in hepatic cells may manifest after a latency period.

DNA sequencing analysis of lung cellular DNA from SHS-exposed mice and mutation spectrum analysis of *TP53* in human lung cancer of known nonsmokers revealed that the two mutation spectra bear a striking resemblance to one another while both being signifi-

cantly different from the mutation spectrum of smokers' *TP53*. Specifically, the frequencies of G:C→A:T transition mutations in the *TP53* gene of nonsmokers' lung tumors and in the *cII* transgene of lung cellular DNA from SHS-exposed mice are virtually identical, and the frequencies of all other types of mutation in the two mutation spectra are indistinguishable from one another (Fig. 6*A*). In addition, the ratio of G:C→A:T transitions to G:C→T:A transversions in the respective groups follows the same pattern of being significantly elevated relative to that in smokers. In stark contrast to smokers' *TP53* mutation spectrum in lung cancer, which is highly biased for mutagenesis at 5′-CpG dinucleotides (19, 20), the spectra of mutations in the *TP53* gene in nonsmokers' lung cancer and in the *cII* transgene of lung cellular DNA from SHS-treated mice do not favor mutagenesis at 5′-CpG dinucleotides (Figs. 3 and 6*B*, *D*). The virtually identical mutation spectra of *cII* transgene in the lung of SHS-exposed mice *in vivo* and the *TP53* gene in lung cancer of nonsmokers is of importance because it provides the first mechanistic link between exposure to SHS and lung cancer in nonsmokers.

We acknowledge that in the present study, we have investigated SHS mutagenicity in a mouse model, which may lack complete comparability to humans (24). For example, the *cII* mutational target gene in the transgenic Big Blue mouse system is a transcriptionally inactive reporter gene (24, 31). In humans, however, DNA damage-derived mutagenesis in endogenous cancer-related genes can be modulated by transcription-coupled DNA repair, which preferentially removes DNA lesions from the transcribed strand of the actively expressed genes (33). In addition, other determinants of DNA damage formation and mutagenesis, such as global DNA repair, replicative DNA polymerases, and metabolic activation and/or detoxification, are known to have varying efficiencies in different species (32, 34, 35). Nonetheless, when used properly, the transgenic Big Blue mouse model system has been shown to accurately portray many aspects of human carcinogenesis and provide invaluable information on the underlying mechanisms of malignant cell transformation (31, 36–38). For example, Yoon *et al.* (37) have demonstrated that the tobacco-derived carcinogen, benzo[*a*]pyrene diol epoxide (B[*a*]PDE), forms DNA lesions at specific nucleotide positions in the *cII* and *lacI* transgenes of this same model system, which correspond to the sites of B[*a*]PDE-induced mutations in the respective transgenes (37). The patterns of B[*a*]PDE-induced DNA adduction and mutagenesis in both transgenes in this model system perfectly mirror those found in the *TP53* gene in smoking-related lung cancer (37). Likewise, we have shown that other carcinogens, such as solar ultraviolet radiation, can produce characteristic DNA damage-targeted mutagenicity in this model system, which recapitulates that found in the *TP53* gene of sunlight-associated skin cancer (36).

Although the association between SHS exposure and lung cancer development in nonsmokers has been established in epidemiological studies (7), no experimental study has mechanistically verified this association in a validated model system and under strictly controlled exposure conditions. Demonstrating a causal link between SHS exposure and lung carcinogenesis in experimental model systems can help elucidate the underlying mechanism of this malignancy, which, may, in turn, help improve preventive, diagnostic, and therapeutic strategies against this disease. Our study is unique in that it not only provides the first verification of a mechanistic mode of action for SHS of relevance for carcinogenesis, but it also offers the first piece of evidence to link SHS exposure to lung cancer development in nonsmokers. Our findings have important public health relevance because they provide evidence revealing the etiological role of SHS in human lung carcinogenesis. The findings also support our recently proposed model of lung carcinogenesis in nonsmokers (Fig. 2 in ref. 7), in which SHS exposure can cause DNA damage leading to genetic alterations, such as mutations in oncogenes and tumor suppressor genes, as well as epigenetic changes, such as aberrant DNA methylation, histone modifications, chromatin remodeling, and microRNA-derived modulation of gene expression (39–43). These SHS-induced genetic changes in conjunction with other epigenetic alterations can confer lung cancer susceptibility in SHS-exposed nonsmokers (7). FJ

The authors thank Dr. Yong Jiang for technical assistance in the conduct of our experiments, Dr. Fong-Fong Chu for help with mouse breeding and colony expansion, Dr. Walter Tsark for helpful discussion on institutional animal care and use committee protocol preparation, and their department chair for general support. Special thanks to the dedicated staff and management of the City of Hope Animal Resources Center, in particular, Armando Amaya, Marie Prez, Lauren Ratcliffe, Yvonne Harper, Donna Isbell, Kenneth Golding, and Dr. Richard Ermel. This study was supported by a grant from the University of California Tobacco Related Disease Research Program (18KT-0040) to A.B. The authors declare no conflicts of interest.

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Received for publication November 7, 2011.
Accepted for publication January 30, 2012.