Cigarette Smoke Induces DNA Deletions in the Mouse Embryo


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ABSTRACT

Cigarette smoking causes cancer and DNA mutations. However, long-term chronic exposure to smoke is believed to be necessary for carcinogenesis. Here, we investigate the relationship between short-term exposure to smoke and the frequency of deletions in the mouse embryo. Deletions and other genome rearrangements are associated with carcinogenesis and inheritable diseases. The pink-eyed unstable (pnull) mutation in the C57BL/6J mouse is the result of internal duplication of 70 kb of DNA within the p gene. Spontaneous reversion events in homozygous pnull/pnull mice occur by deletion of one copy of the duplicated sequence. Reversion events occurring in the embryonic premelanocytes of the developing fetus give rise to black spots on the gray fur of the offspring after birth. We investigated the effects of exposure of pregnant pnull mice to cigarette smoke and cigarette smoke condensate (CSC) on the frequency of black spots occurring in the offspring. Pregnant dams were exposed (whole body) to smoke generated by either filtered or unfiltered cigarettes for 4 h, or alternatively, mice were given a 15 mg/kg dose of CSC during their 10th day of gestation. TPM, CO concentration, and plasma nicotine and cotinine levels were determined to characterize the smoke exposure. There was a significant increase in the number of DNA deletions in the embryo as evidenced by spotted offspring in both smoke-exposed groups and in the CSC group. These results suggest that embryos are highly sensitive to the genotoxic activity of cigarette smoke following a single exposure of only 4 h.

INTRODUCTION

Cigarette smoking accounts for 30% of all cancer deaths in the United States as estimated by the American Cancer Society. The WHO estimates that globally, 3 million people die each year as a result of smoking. In the United States alone, it is calculated that cigarette smoking is related to 419,000 deaths per year (1).

Studies using cell culture, animal models, and human beings have demonstrated that cigarette smoking can lead to genotoxic effects. DNA adducts have been found in heart, aorta, and kidney of human lung cancer patients with a history of smoking (2), in the placenta of pregnant smokers (3), in human monocytes (4), and in the mitochondrial and nuclear DNA of lung tissue from rats exposed to cigarette smoke (5). Single-strand breaks in DNA occur in lymphocytes of pregnant smokers (6), in human monocytes (4), and in the mitochondria of mice (7). Increases in the frequency of dominant lethal mutations and micronuclei in mice (8) and increases in sister chromatid exchange in humans (9, 10) and mice (11) have also been observed (11). Smoking also induces oxidative stress as evidenced by a 50% increase in 8-oxodeoxyguanosine lesions in sperm DNA samples from human smokers (12). However, most of these studies used medium-term to long-term exposures of 40 days (8), 100 days (5), and 65–70 weeks (13) or chronic exposure (3, 9, 10, 12). Only a few studies used short-term exposures of 2 days (6, 11).

Smoking has also been documented to lead to a variety of abnormalities in testicular sperm. Low birth weight, with newborns of smoking mothers averaging 200 g smaller than newborns of nonsmoking mothers, is well documented (14). Sudden infant death syndrome and spontaneous abortions have also been linked to maternal smoking during pregnancy (14). DNA adducts have also been found in placenta and umbilical cord vasculature (3, 15), and newborns of smoking mothers have been observed to have trends toward increased DNA damage, as measured by single-cell electrophoresis (16). The potential health consequences that could arise from these genetic alterations in offspring of smoking mothers are of major concern.

The fact that smoking leads to various genotoxic effects has implications for carcinogenesis. Many studies show an association between genome rearrangements and tumor cells (17–20). Cell cultures from several human hereditary diseases, such as ataxia telangiectasia, Li–Fraumeni syndrome, Bloom’s syndrome, and Werner syndrome, demonstrate a high frequency of recombination events (21–24). These recombination events can cause DNA rearrangements, such as deletions. Spontaneous causes of deletions can be intrachromosomal recombination events that take place between repetitive sequences of DNA in the genome. About 25% of our genome is composed of repeated DNA elements that may be organized either interspersed or tandemly repeated (25). These repetitive sequences that are scattered throughout the human genome serve as substrates for intrachromosomal recombination events (see, for example, Ref. 26). Such events may lead to cancer or various genetic disorders if an essential locus is deleted or disrupted during the process. Indeed, those afflicted with the above hereditary diseases, which are associated with a high frequency of recombination events, also have a marked predisposition to cancer (21–24).

The association of genomic rearrangements with cancer prompted Schiestl et al. (27) to construct a system in the yeast Saccharomyces cerevisiae that selects for deletions by intrachromosomal recombination, termed the DEL assay. DEL recombination is inducible by a wide variety of carcinogens, including those that are negative in most other short-term assays (28–31). These carcinogens include many of those that are present in cigarette smoke. In addition, deletion events between duplicated regions within the hprt gene are inducible by several carcinogens in Chinese hamster cells (32) and in human cells (33). Finally, reversion of the 70-kb internal duplication at the pink-eyed dilution (p) gene in the C57BL/6J pnull/pnull mouse is inducible by X-rays (34) and by chemical carcinogens (35).

We designed this study to determine effects of a very short-term cigarette smoke exposure on the frequency of DNA deletions in the mouse embryo. The C57BL/6J pnull/pnull mouse cited above was used as the experimental model. The p gene normally encodes a melanosomal integral membrane protein responsible for the assembly of a high molecular weight melanin complex that produces the black coat color of wild-type mice (36). Phenotypically, the pnull mutation results in a dilute, light gray coat color and pink eye color. Deletion events that remove one copy of the gene duplication (Fig. 1) lead to a reversion of the pnull mutation to the wild-type p gene. One deletion event occurring in a premelanocyte in the embryo will cause a visible black spot on the gray fur of the offspring after amplification of the premelanocytes (Fig. 2). The loss of the duplicated region of the p gene and subsequent reversion to wild type may be caused by an intrachromosomal recombination event (37). Spontaneous reversion of a pnull
allele leads to spots on the fur of about 2–11% of the offspring (34, 35, 38, 39).

The frequency of black spots on the fur of p<sup>mm</sup>/p<sup>mm</sup> offspring is elevated following exposure of the dams to X-rays and the animal carcinogens benzo(a)pyrene, benzene, ethylmethane sulfonate, methylnitrosourea, ethyl nitrosourea, trichloroethylene, aroclor 1221, aroclor 1260, and 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD) (34, 35). These carcinogen-induced spots are due to intrachromosomal recombination resulting in deletion of one copy of the gene duplication (35). Because several of these carcinogens, such as benzo(a)pyrene and benzene, are present in cigarette smoke, the p<sup>mm</sup>/p<sup>mm</sup> mouse appeared to be an appropriate system to determine effects of cigarette smoke exposure.

Mice homozygous for the p<sup>mm</sup> mutation were exposed to cigarette smoke or CSC, and the frequency of spots on the fur of the offspring was determined. To characterize exposure levels we determined TPM and carbon monoxide (CO) levels in the smoking chamber and plasma nicotine and cotinine levels in the exposed mice.

**MATERIALS AND METHODS**

**Mouse Model, Determination of p<sup>mm</sup> Reversion, and Smoke Exposure Protocol.** Mice homozygous for the pink-eyed dilution-unstable C57BL/6J p<sup>mm</sup>/p<sup>mm</sup> mutation were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred in our facility. Male and female mice were allowed to mate, and pregnancy was timed from the discovery of a vaginal plug. Because fertilization usually occurs in the early morning hours of the day that a vaginal plug was discovered, noon of that day was defined as 0.5 days postconception (40, 41). The mice were given a whole-body exposure to various concentrations of the gas inlet and outlet of the chamber. The chamber was fitted with a small fan at the center of the top to keep the mice ventilated and to keep the aerosol well mixed in the chamber.

The cigarette smoke was generated using a smoking machine (ADL II Smoking System, Arthur D. Little Electronics, Cambridge, MA). Briefly, the smoke machine generated a 35-ml puff of smoke at regular intervals of 58 s, which was ejected into a 1-liter cylindrical bottle. The smoke in the bottle was mixed with fresh air, drawn from a compressed air tank, and the mixture was introduced into the exposure chamber. The mice were exposed to the air containing cigarette smoke for a predetermined period of 4 h during the 10.5th day of gestation. The smoke particle concentrations and carbon monoxide levels in the exposure chamber were adjusted to the desired values by adding fresh air to the chamber.

**Determination of TPM Concentrations and Measurement of Mass Particle Size Distribution.** TPM concentrations were measured as a function of time using a RAM (model RAM-1; GCA Corp., Bedford, MA). This instrument is a portable, self-contained aerosol monitor that measures the concentration of particles (having a size of 0.1–10 μm) in the chamber air almost instantaneously by using a light-scattering technique. A sampling pump draws the air at the rate of 2 liters per min into a sensing chamber of the RAM, where it passes through the path of the light beam. The particles in the air scatter some of the light to a detector, which produces an electrical signal proportional to the intensity of the light. The instrument was calibrated to the smoke particles by

![Diagram](image-url)

**Fig. 1.** Structure of the tandem head to tail duplication of the p<sup>mm</sup> mutation and spontaneous reversion event as described and shown in Ref. 37. The order of linear information in the wild type (or revertant) is disrupted in the p<sup>mm</sup> duplication (A, numbers below the boxed regions). The reversion event deletes one copy of the duplication and restores the linear information at the p locus (B and C). C, intrachromosomal recombination via single-strand annealing as a hypothesized mechanism for deletion of duplicated portion of p<sup>mm</sup> mutation and reversion to wild type. In this scenario, a DNA damaging agent causes a single- or double-strand break in DNA that is followed by exonuclease activity of the ends and single-strand annealing in the regions of homology. Another possible but less likely mechanism for p<sup>mm</sup> reversion is intrachromatid exchange (8).

![Diagram](image-url)

**Fig. 2.** The pink-eyed dilution-unstable C57BL/6J p<sup>mm</sup>/p<sup>mm</sup> mouse. This mutation consists of a 70-kb duplication of a region in the pink-eyed dilution (p) gene and phenotypically results in a light gray, or dilute, coat color. A reversion event occurs when there is a deletion of the duplicated portion within the gene during embryonic development resulting in reversion to the wild-type p gene and expression of the wild-type coat color. The mouse on the right shows two spots of dark fur derived from an embryonic DNA deletion event caused by carcinogens exposure. The mouse on the left has not been exposed to any carcinogen and shows uniformly gray fur.
gravimetric analysis of the samples collected onto 0.4-μm membrane filters (Costar-Corning Corp., Cambridge, MA) during each exposure experiment.

Particle size distribution was determined using a SMPS (model 3934, TSI Inc., St. Paul, MN). This is a current version of a widely used instrument in aerosol research, known as an electrostatic classifier. The basic principle of the classifier is that the velocity of a charged particle in an electric field is directly related to the diameter of the particle. A charged aerosol enters near the outer circumference of the classifier and particles with a narrow range in electrical mobility exit through a slit in the center electrode. The mobility distribution is determined by measuring the number concentration exiting the slit as a function of the electrode voltage. The number concentration of the aerosol in each range is measured with a condensation particle counter (model 3022A, TSI Inc.). The condensation particle counter detects particles in the size range of 5–1000 nm and particle concentrations of 107/cm3 per electrical mobility range. The instrument is calibrated using Polysciences Latex Beads.

Because the cigarette smoke particle concentrations during our exposure experiments were expected to be higher than the allowable range of the instrument, the aerosol were diluted severalfold (dilution ratios of 750–40,000) before being sampled by SMPS. The dilution system was well calibrated using known aerosol concentrations and also cross checked with concentration measurements by RAM and gravimetric analysis during the measurements. Smoke generated from unfiltered cigarettes had a count median diameter of 52.6 nm, with a ΩG of 2.34 (measured mass median diameter was 341.8 nm, with a ΩG of 1.72). The measurements were carried out at particle concentrations of 47.26 mg/m3 as determined by gravimetric analysis and RAM (SMPS measurements determined the particle concentrations to be 43.05 mg/m3). The smoke generated from filtered cigarettes had a count median diameter of 120.4 nm, with a ΩG of 3.87 (mass median diameter was 562.3 nm with a ΩG of 1.335). The filtered cigarette smoke particle size distribution was determined at concentrations of 49.73 mg/m3, as determined by RAM and gravimetric analysis (SMPS determined the particle concentration as 39.54 mg/m3).

Measurement of Carbon Monoxide Concentrations. Carbon monoxide concentrations in the exposure chamber were measured using an EcoLyzer brand portable monitor (EcoLyzer Series 2000, National Draeger Corp., Pittsburgh, PA). The instrument functions are based upon the electrochemical potential changes of a sealed wet cell when exposed to CO. This instrument is extremely versatile, comes with dual ranges, and can measure CO concentrations in the range of 0–1000 ppm. The air sampling rate to the instrument was 0.71 liters per min. The instrument was calibrated before each exposure experiment using CO concentrations of 56.7 ppm from a certified span air tank.

Administration of CSC. pmm mice were treated with CSC after 10.5 days of gestation. A 7% solution of CSC suspended in DMSO was kindly provided by Dr. Gary Gria Rita of the University of Kentucky. This solution contained 50 μg/μl of CSC and was administered to the mice in a dose of 15 mg/kg of body weight. Because mice are obligate nose breathers, the method of insufflation was used to administer CSC. Control mice were treated in the same fashion using DMSO as the vehicle control. ppm mice were anesthetized with inhaled methoxyflurane and checked for weight gain as an indication of pregnancy, and CSC was given very slowly through one nostril using a P20 pipettor-equipped with a thin pipette tip. Mice were monitored closely and allowed to recover before being placed back in their pens. The final volume of CSC administered to each mouse was in the range of 6–7.5 μl. Offspring were scored for fur spots as detailed above.

Plasma Nicotine and Cotinine Determination. Plasma nicotine and cotinine were determined to obtain a biological parameter for cigarette smoke exposure and relative dose received. pmm mice were subjected to whole-body exposure of filtered cigarette smoke at a concentration of 14.5 ± 0.4 mg/m3 TPM and unfiltered cigarette smoke at concentrations at 30, 45, and 102 mg/m3 TPM. After 3 h of exposure, mice were removed from the chamber, one at a time, for blood sampling. Blood was immediately collected after mice were removed from the smoking chamber to avoid excessive breathing of fresh air outside the chamber. Mice were anesthetized with inhaled methoxyflurane, and blood was immediately drawn from the inferior vena cava and placed in a heparinized vacuum tube on ice. Plasma was separated from blood by centrifugation and stored at −20°C. Samples were shipped on ice to an independent toxicology laboratory (Medox, Minneapolis, MN), where nicotine and cotinine levels were assayed using a gas chromatograph equipped with a flame ionization detector.

Statistical Analysis. Statistical significance between spotting frequency of exposed versus control mice was calculated using a χ² test with the statistical software package Systat 5.2 (SYSTAT, Inc., Evanston, IL). Significance was accepted at 0.05 for all tests.

RESULTS

Whole-Body Exposure to Unfiltered and Filtered Cigarette Smoke. Pregnant dams were exposed to filtered and unfiltered cigarette smoke for 4 h during the 10.5th day of gestation. For unfiltered cigarettes, Kentucky reference cigarettes 2R1 were used, and for filtered cigarettes, 2R1F cigarettes were used. The filtered and unfiltered cigarettes were identical in composition, differing only by the presence of a filter. Whole-body exposures with unfiltered cigarette smoke were conducted at three different concentrations of TPM. A concentration of 31 ± 2 mg/m3 of TPM raised the spotting frequency from the control value of 12.6 ± 23.3%, a significantly increase (P < 0.05; Table 1). Exposures at a TPM concentration of 57 ± 7 mg/m3 resulted in a similar increase to 23.3%, but was not statistically significant, due to the smaller sample number of offspring (Table 1). Finally, exposures at 96 ± 15 mg/m3 TPM resulted in 28% of offspring showing spots, again showing a significant increase (P < 0.05; Table 1).

Statistical analysis was done after pooling all mice exposed to unfiltered cigarette smoke, regardless of TPM concentration, versus nonexposed control mice. This analysis revealed that the three exposure groups pooled together had a significantly higher spotting frequency of 25% in the offspring as compared to 13% in control mice (P < 0.05; Table 1).

Filtered cigarette smoke exposure at concentrations of 12 ± 0

Table 1: Spoting frequency in pmm mice exposed to cigarette smoke generated from filtered and unfiltered cigarettes.

<table>
<thead>
<tr>
<th>Smoke concentration*</th>
<th>CO (ppm)</th>
<th>TPM (mg/m³)</th>
<th>Exposed mice (n)</th>
<th>Mice with litters (n)</th>
<th>Live pups (n)</th>
<th>Average litter size (n)</th>
<th>Spotted pups (n)</th>
<th>Frequency of deletions (%)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>0</td>
<td>0</td>
<td>62</td>
<td>61</td>
<td>475</td>
<td>7.7</td>
<td>60</td>
<td>12.6</td>
<td></td>
</tr>
<tr>
<td>Low smoke concentration with unfiltered cigarettes</td>
<td>32.5 ± 2.5</td>
<td>30.9 ± 2.0</td>
<td>10</td>
<td>7</td>
<td>43</td>
<td>6.1</td>
<td>10</td>
<td>23.3 (P &lt; 0.05)</td>
<td></td>
</tr>
<tr>
<td>Moderate smoke concentration with unfiltered cigarettes</td>
<td>51.5 ± 8.5</td>
<td>57.0 ± 7.0</td>
<td>8</td>
<td>5</td>
<td>30</td>
<td>6.0</td>
<td>7</td>
<td>23.3 NS*</td>
<td></td>
</tr>
<tr>
<td>High smoke concentration with unfiltered cigarettes</td>
<td>99 ± 4</td>
<td>96 ± 15.1</td>
<td>6</td>
<td>6</td>
<td>36</td>
<td>6.0</td>
<td>10</td>
<td>27.8 (P &lt; 0.05)</td>
<td></td>
</tr>
<tr>
<td>Totals for all unfiltered smoke exposures</td>
<td>40 ± 14.0</td>
<td>12 ± 0.0</td>
<td>24</td>
<td>18</td>
<td>109</td>
<td>6.0</td>
<td>27</td>
<td>24.8 (P &lt; 0.05)</td>
<td></td>
</tr>
<tr>
<td>Filtered cigarette exposure</td>
<td>40 ± 14.0</td>
<td>12 ± 0.0</td>
<td>10</td>
<td>4</td>
<td>35</td>
<td>8.8</td>
<td>11</td>
<td>31.4 (P &lt; 0.05)</td>
<td></td>
</tr>
</tbody>
</table>

* Values are averages of two independent trials ± range.

NS, not significant.
mg/m³ TPM resulted in 31.4% of offspring with spots on their fur, again showing a significant induction (P ≤ 0.05; Table 1).

**Plasma Nicotine and Cotinine Levels.** p<sup>+</sup>/p<sup>−</sup> mice were subjected to whole-body exposure of filtered cigarettes consisting of concentrations of 14.5 ± 0.4 mg/m³ TPM and unfiltered cigarette smoke at 29, 45, and 102 mg/m³ TPM. Plasma nicotine and cotinine levels were used as biological parameter of total smoke exposure, which allowed us to estimate the total smoke dose received. These concentrations were chosen to correspond as closely as possible to the TPM used in the exposures of pregnant p<sup>+</sup>/p<sup>−</sup> mice. The levels of plasma nicotine and cotinine were significantly higher in all smoke-exposed mice when compared to unexposed controls (P ≤ 0.05; Table 2). There appeared to be a slight linear relationship between increases in TPM concentrations and nicotine/cotinine levels. However, levels of nicotine/cotinine appeared to reach a maximum at 45 mg/m³ of TPM (Table 2). This may be due to metabolic differences between mice, or it is possible that mice at the higher TPM concentrations may have adjusted their breathing pattern to minimize exposure. Whichsoever the case may be, based on the nicotine/cotinine data, it appears that mice exposed to 45 and 102 mg/m³ TPM may have experienced physiologically similar doses.

**Administration of CSC.** Administration of 15 mg/kg dose of CSC via insufflation was performed on day 10.5 of gestation. This one-time treatment of pregnant dams resulted in a statistically significant increase of spotting frequency in offspring to 28.8% (Table 3). The solvent DMSO alone did not cause any significant difference in the frequency of offspring with spots as compared to unexposed controls. The reversion frequency induced by CSC corresponded well with the level of induction observed with cigarette smoke.

**DISCUSSION**

In the present study, pregnant C57BL/6J p<sup>+</sup>/p<sup>−</sup> mice were exposed to various concentrations of cigarette smoke for 4 h during the 10.5th day of gestation. This exposure was carried out using filtered and unfiltered cigarettes as well as CSC. Cigarette smoke was characterized by measuring TPM and CO in the smoke chamber. TPM contains the tar fraction of cigarette smoke, and CO is found in the gaseous fraction. The number of spotted offspring was determined to measure the frequency of DNA deletion events during embryonic development. Exposures using unfiltered cigarettes at TPM concentrations of 31–96 mg/m³ led to an increase in the spotting frequency compared with control mice. Filtered cigarette smoke exposure at 12 mg/m³ TPM also caused an increase in the frequency of deletions, as did 15 mg/kg of CSC. Increases in the frequency of deletions resulted from single smoke exposures 4 h in duration.

Effects of CSC on cellular processes have been investigated in the past. Zhang and Jenssen (32) did not observe any effect of CSC treatment on intrachromosomal recombination in Chinese hamster cells containing a partial duplication in the hprt gene. On the other hand, CSC from both mainstream and side stream smoke has been shown in mice to result in the formation of DNA adducts in the major organs and in the skin when applied topically (42) and to have clastogenic effects in bone marrow (43). Carmichael et al. (42) have observed adducts in human skin cells treated with CSC as well.

**Possible Candidate Compounds Responsible for Induction of Deletions.** The fact that both cigarette smoke and CSC increase the frequency of DNA deletions suggests that the tar fraction of cigarette smoke contains the responsible compounds. Possible candidate chemicals may lead to p<sup>+</sup> reversion and are that found in the particulate phase of smoke include benzo(a)pyrene, cadmium, acetamide, aniline, o-toluidine, acrylonitrile, and catechol (44). Benzo(a)pyrene is an animal carcinogen (45), a potent inducer of p<sup>+</sup> reversion (35), and an inducer of intrachromosomal recombination in Chinese hamster cells (32). Some DNA adducts caused by cigarette smoke exposure are derived from an aromatic carcinogen, possibly benzo(a)pyrene (3). There is preferential formation of benzo(a)pyrene adducts at the lung cancer mutational hot spots of p53 in HeLa cells and in normal human bronchial epithelial cells that are treated with (±)-anti-7ß,8a-dihydroxy-9a,10a-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene, a very potent carcinogen metabolite of benzo(a)pyrene (46). Thus, it is likely that benzo(a)pyrene is one of the primary compounds responsible for p<sup>+</sup> reversion observed in smoke-exposed animals.

Other compounds found in cigarette smoke (cadmium, acetamide, aniline, o-toluidine, acrylonitrile, and catechol) have all been shown to induce intrachromosomal recombination in yeast (28, 29, 47). Therefore, these compounds may also play a role in the reversions observed in CSC and smoke-exposed p<sup>+</sup> mice. Because cigarette smoke is an exceedingly complex mixture of over 4000 compounds, the above chemicals that may lead to p<sup>+</sup> reversion and are that found in the panic substances are derived from an aromatic carcinogen, possibly benzo(a)pyrene (3).

**Mechanisms of p<sup>+</sup> Reversion.** Increasing levels of cigarette smoke concentrations, as measured by TPM and CO, resulted in...
greater plasma nicotine/cotinine levels but not in a steadily increasing spotting frequency. The percentage of spotted offspring born to smoke-exposed dams remained somewhat in spite of increasing TPM concentrations. The mice were receiving a higher dosage of cigarette smoke as evidenced by plasma nicotine/cotinine levels, but a "ceiling effect" was observed for p" reversions. This ceiling effect has been observed previously for chemical carcinogen induced p" reversions. Treatment of p" mice with X-rays, ethylmethane sulfonate, methylmethane sulfonate, trichloroethylene, benzene, and sodium arsenate at the maximum tolerated dose all result in a spotting frequency of around 30% (35). Some of the above carcinogens contained in cigarette smoke are clastogens, and p" reversion events are most likely induced by DNA strand breaks (see below), which can also lead to cell death. Thus, the ceiling effect may be caused by cytotoxicity that at higher doses may be lethal to the fetus. This is evident from a decreased number of mice bearing litters after exposure. Of the control mice, 98% had litters, compared with 79% of the mice exposed to unfiltered cigarettes and 40% of the mice exposed to filtered cigarettes (Table 1). Furthermore, embryotoxicity was also evident from the average litter size, which was 7.7 in the controls and an average of 6.5 in the exposed group.

The p" duplication revert to wild type following an intrachromosomal recombination event (Refs. 35 and 37; Fig. 1). This could be mediated through intrachromatid exchange (Fig. 1B) or by single-strand annealing (Fig. 1C). The most likely mechanism for induced DEL recombination in yeast is the single-strand annealing pathway, which uses a double-strand break to initiate recombination (Fig. 1C; Ref. 48). In fact, it has been shown that DEL recombination can be induced by a specific DNA double-strand break in the sequence between a gene duplication (49, 50). Additionally, a yeast DNA ligation temperature sensitive mutant, which has increased levels of DNA strand breaks, shows a 100-fold increased level of DEL recombination (27). Deletions by intrachromosomal recombination in yeast (28), mammalian cells (33), and p" mice (34) have resulted after exposures to either X-rays or γ-rays, both producing double-strand breaks.

Smoking has been documented to result in DNA strand breaks. Human lung carcinoma cells treated with smoke-exposed PBS have a high frequency of DNA single-strand breaks (7). DNA single-strand breaks are detected in lymphocytes of smokers after a smoking session, but not in nonsmokers passively exposed in the same room as the smokers (6). Smoking also causes an increase in the frequency of dominant lethal mutations and micronuclei, indicative of clastogenic activity (8). Thus it seems that DNA strand breaks are likely to occur during smoke exposures and may be inducing intrachromosomal recombination at the p" locus in the offspring of smoke-exposed mice. Active oxygen species generated from the cigarette smoke may be responsible for the DNA strand breaks (7). Addition of superoxide dismutase and catalase to cell cultures dramatically decreases the amount of cigarette smoke induced single-strand breaks (7). Furthermore, manganese superoxide dismutase protects against smoke induced toxicity (51). Interestingly, oxidative mutagens are potent inducers of DEL recombination events in yeast (30). Furthermore, cadmium (52), as well as aniline and o-toluidine (53), which are constituents of cigarette smoke, cause oxidative stress and DEL recombination in yeast. Thus, it is likely that oxidative stress caused by cigarette smoke may also have a role in inducing p" reversions, which we will test in future studies.

Biological Consequences of Smoke Exposure. An increase in the frequency of p" reversion events indicates an increase in the frequency of deletions of 70 kb of DNA between a gene duplication constituting a genome rearrangement. Many studies show an association of genome rearrangements with cancer (17–20) and cells from patients affected with several "cancer-prone diseases" show a high incidence of genome rearrangements (21–24). As deletions occur repeatedly in DNA over time, it becomes possible that a critical gene, such as a tumor suppressor or cell cycle gene, may be mutated. Such a mutation may lead to loss of cell cycle control and subsequently to cancer. Furthermore, pregnant mothers who smoke may be placing their fetuses at risk of genotoxic events, such as deletions, that could manifest as developmental abnormalities and/or spontaneous abortion.

Because the mice in our experiments were exposed to mainstream smoke generated by a smoke machine, these results might be comparable with active smoking in humans. There are obvious differences between the smoking habits of humans and the exposure protocol for the mice; however, comparisons may still be appropriate based on plasma nicotine levels as a physiological parameter of exposure. The nicotine levels are probably not causing any genotoxic effect, but they provide a convenient in vivo biomarker of total body exposure to other genotoxic constituents of cigarette smoke. A controlled study performed by Grassi et al. (54) using nine human subjects showed plasma nicotine levels of 44.2 ng/ml after smoking one cigarette and 48.9 ng/ml after smoking a second cigarette. Another study, done by Kurihara (55) on two separate groups (17 people under 40 years old and 17 people over 40 years old), showed plasma nicotine levels of 50.4 and 53.1 ng/ml, respectively. In this study it was also noted that plasma nicotine levels remained at 31.3 ng/ml 30 min after smoking. Upon examination of these plasma nicotine levels, it appears that our exposures, which produced plasma nicotine in the range of 21.2–92.7 ng/ml, fall well within the range of nicotine burden found in humans after smoking a cigarette. In light of these data, it is possible that genotoxic events, such as DNA deletions, may be taking place in humans with similar plasma nicotine levels. It is a sobering thought that the lowest TPM exposure, which resulted in plasma nicotine of 21.2 ng/ml in the pregnant dams, led to a significantly increased frequency of DNA deletions in the embryos.

On the basis of our results, we believe that the p" mouse is a suitable model to detect inhalation effects of smoke on embryos. We further conclude that embryos are highly sensitive to the genotoxic activity of cigarette smoke as demonstrated by increases in deletion frequency observed at the p gene following a single 4-h smoke exposure.

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CIGARETTE SMOKE INDUCES DNA DELETIONS

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