Mutational Spectra of a 100-Base Pair Mitochondrial DNA Target Sequence in Bronchial Epithelial Cells: A Comparison of Smoking and Nonsmoking Twins

Hilary A. Coller, Konstantin Krhakpo, Alfonso Torres, Mark W. Frampton, Mark J. Utell, and William G. Thilly

Division of Toxicology and Center for Environmental Health Sciences, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139 [H. A. C., K. K., W. G. T.]; Division of Aging, Harvard Medical School, Boston, Massachusetts 02115 [K. K.]; and University of Rochester Medical Center, Rochester, New York 14642 [A. T., M. W. F., M. J. U.]

ABSTRACT

Seventeen separate mitochondrial hot spot mutations in a 100-bp target sequence (mitochondrial bp 10,030-10,130) were detected and measured in bronchial epithelial cell samples isolated from smokers and nonsmokers. Among the individuals sampled were three pairs of monozygotic twins in which one twin had never smoked and had a nonsmoking spouse, and the other had a smoking history of >10 pack-years. Individual point mutations present at frequencies as low as 10^-6 were detected. Partially denaturing electrophoresis was used to separate mutant from nonmutant sequences on the basis of their melting temperatures, and the target sequence was subsequently amplified via high-fidelity PCR with Pfu DNA polymerase. Tests were performed to determine whether mismatch intermediates or DNA adducts present in the cellular DNA were converted to mutants during PCR.

Hot spot mutations were clearly observed in bronchial epithelial cells, and the same hot spots were observed consistently in different samples. Significant numerical variability in the mutant fractions for individual twins was observed among samples and are ascribed to unequal mitochondrial segregation in stem and transition cells.

The mutational spectra in smokers' samples did not differ significantly from the mutational spectra in nonsmokers' samples for this 100 bp of mitochondrial DNA. No smoking-specific hot spots were detected. The overall mutant fractions in smokers' samples were not elevated compared to those of nonsmokers. As much variability was observed between two samples from the same individual's lung as between a sample from a smoker and a sample from a nonsmoker.

These findings demonstrate that inhaled tobacco smoke does not induce prominent point mutations in this 100-bp target mitochondrial sequence in smokers' bronchial epithelial cells. Endogenous factors (e.g., DNA replication errors or DNA damage by endogenous reactive chemicals) are suggested to be more likely to represent the most important contributors to mitochondrial mutagenesis.

INTRODUCTION

Although the hypothesis that chemicals in the environment induce mutations that transform normal human cells to a tumorous phenotype is plausible, except for a few indisputable cases, a causal link has not generally been demonstrated. Our interest in testing this hypothesis has guided our experimental goal of measuring the point mutational spectra in human tissues. Studies in phage, bacteria, and human cell culture have demonstrated that treating cells with different chemicals results in characteristic mutational spectra (1–4). We hypothesized that the most important mutagens in human tissues could be identified based on the mutational spectra observed.

In this report, we test whether smoking induces a significant change in the number or number of mutations in a 100-bp region of mitochondrial DNA isolated from smokers' bronchial epithelial cells. We selected cigarette smoke as a model for testing whether a particular environmental agent is inducing a characteristic mutational spectrum for several reasons. Smoking represents a clear case of high and continuous exposure to a mixture of chemicals containing known, powerful chemical mutagens. Also, the epidemiological association between smoking and lung cancer is particularly strong. A clear dose-response relationship between the number of cigarettes smoked per day, the duration of smoking, and lung cancer risk has been demonstrated by several large epidemiological studies (17).

Furthermore, the public health significance of lung cancer is clear: lung cancer is currently the leading cause of cancer deaths in the United States and is also the most common cause of cancer death worldwide. In 1994, there were an estimated 400,000 deaths from lung cancer in the United States alone, which represented 30% of all cancer deaths (18).

Another important factor is that dose information is easily obtainable for smoking. As opposed to environmental agents for which exposure is often difficult to assess, the number of packs smoked per day and the duration of the exposure can be determined by questionnaire.

Finally, the target cell for smoking-induced lung cancer, the bronchial epithelial cell, can be harvested for analysis. In the experiments described herein, bronchial epithelial cells were obtained from brush biopsy samples (approximately 10^5-10^6). Almost 10^3 mitochondria per cell permits greater statistical power for a given sample size (5). Single-bp point mutants present at fractions as low as 10^-6 are detectable with this technique. The methodology uses an initial separation of mutant sequences from wild-type DNA with CDGE (6) followed by high-fidelity PCR with Pfu DNA polymerase. For further enrichment of mutants and visualization of mutational spectra, CDCE, a variant of capillary electrophoresis that includes a high-temperature zone for separation of mutants (7), is used due to its speed, reproducibility, and excellent resolution.

The presence of several hundred to thousands of copies of mitochondrial DNA per cell permits greater statistical power for a given sample size (5, 8). Use of a mitochondrial DNA target has permitted reproducible detection of rare mutants from the small number of cells obtainable from brush biopsy samples (approximately 10^5–10^6). Although the accumulation of point mutations in mitochondrial DNA has been suggested as an important contributor to human diseases, including cancer (9–11) and aging (12–16), we recognize that the most important contributors to mutagenesis in mitochondrial DNA are not necessarily the most important contributors to mutagenesis in nuclear DNA.

In this report, we test whether smoking induces a significant change in the number or number of mutations in a 100-bp region of mitochondrial DNA isolated from smokers' bronchial epithelial cells. We selected cigarette smoke as a model for testing whether a particular environmental agent is inducing a characteristic mutational spectrum for several reasons. Smoking represents a clear case of high and continuous exposure to a mixture of chemicals containing known, powerful chemical mutagens. Also, the epidemiological association between smoking and lung cancer is particularly strong. A clear dose-response relationship between the number of cigarettes smoked per day, the duration of smoking, and lung cancer risk has been demonstrated by several large epidemiological studies (17).

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biopsy from several individuals, including three pairs of monozygotic twins discordant for smoking. We also obtained bronchial epithelial cells from whole, dissected lungs. We then applied a recently developed methodology that permits detection of low-frequency point mutations in a 100-bp target sequence of mitochondrial DNA (5) to the bronchial epithelial cell samples to determine whether the point mutational spectra in this sequence differed between smokers and nonsmokers.

MATERIALS AND METHODS

Human Tissue Samples

Brush Biopsies Obtained via Bronchoscopy. Epithelial cells were collected by bronchoscopy with brushings from eight healthy volunteers who gave informed consent. The protocol was approved by the Research Subjects Review Board at the University of Rochester and the Committee on Use of Humans as Experimental Subjects at MIT. Subjects were free of significant respiratory or cardiovascular disease determined by medical history and physical examination.

Among the volunteers were three pairs of monozygotic twins recruited from the Minnesota Twins Registry (19) who were discordant for smoking. For each pair of twins, one had a smoking history including >10 pack-years and the other was a lifelong nonsmoker. The nonsmokers had nonsmoking spouses and were engaged in occupations that would not be expected to cause significant smoke exposure. Twins were all females, 46–54 years old. Also analyzed were brush biopsy samples from a 28-year-old male volunteer and a 24-year-old female volunteer, both of whom are smokers.

Subjects were premedicated with 0.75 mg of atropine i.v. 5–10 min prior to the procedure. The oropharynx was topically anesthetized with atomized lidocaine. A fiber optic bronchoscope (PVA-1000 Pentax, Tokyo, Japan) with video capability was inserted orally. The vocal cords were topically anesthetized with lidocaine. The bronchoscope was then advanced to the airways. After gently wedging the bronchoscope in a subsegment of the lingula, four 50-ml aliquots of sterile normal saline were instilled and withdrawn under gentle suction. Brush biopsies were then obtained from several positions within the first three bifurcations of airway, with two brushes from each position. After each biopsy, the brush was gently shaken in 3 ml of sterile PBS on ice. Cells were pelleted and resuspended in 10 µl of PBS. Each brush yielded approximately 3 × 10⁶ to 2 × 10⁷ cells, of which about 95% were epithelial cells.

Dissected Lungs. Whole lungs were harvested from organ donors at the University of Rochester Medical Center or Brigham and Women’s Hospital. Procedures were approved by the Research Subjects Review Board at the University of Rochester, the Committee on Use of Humans as Experimental Subjects at MIT, and the Institutional Review Board of Brigham and Women’s Hospital. Informed consent was provided by the next of kin. Lungs were obtained immediately postmortem and were free of diseases affecting the upper respiratory tree. Two smokers’ lungs (one 47-year-old female and one 58-year-old male), each of whom smoked two packs per day at the time of death, and one nonsmoker’s lung (an 84-year-old male) were available.

Target Sequence and Isolated Mutants

Spoiled DNA was resuspended in water and treated with restriction enzymes RsaI and Ddel (New England Biolabs, Beverly, MA) to liberase the desired mitochondrial target sequence (mitochondrial bps 10,009–10,231; see Fig. 1).

The copy number of mitochondrial target sequence in the restriction digestion was measured based on competitive PCR amplification with a known amount of a mutant PCR product of the same sequence. Purified mutant sequences were added to the samples at precise fractions of the total copy number to serve as internal standards (5).

Digested DNA with added internal standards was separated via CDGE (6). CDGE can be used to separate DNA sequences containing a high melting domain adjacent to a low melting domain based on the sequence of the low melting domain. Due to the cooperative melting of DNA as melting domains, the number of a mutant PCR product of the same sequence. Purified mutant sequences were added to the samples at precise fractions of the total copy number to serve as internal standards (5).

Mutational Spectrometry

DNA Isolation, Internal Standards, and CDGE. Mutational spectra were obtained as described previously (5). In brief, samples were digested with RNase A and proteinase K, and DNA was isolated by ethanol precipitation.
DNA sequences containing mutations that convert bp present in the low melting domain from AT to GC will have a higher $T_m$ than the wild type and will consequently migrate more rapidly through a gel under partially denaturing conditions, whereas sequences with mutations that convert GC to AT bp will have a lower $T_m$ than the wild type and will migrate more slowly under partially denaturing conditions. Homoduplexes with higher and lower $T_m$s than the wild type were excised from the gel positions below and above the wild type, respectively. High-$T_m$ mutants were enriched by this procedure approximately 100-fold, and low-$T_m$ mutants were enriched about 5–10-fold. The DNA was eluted from gel pieces as described previously (5) and resuspended in water.

PCR and CDCE. One-third of the eluted DNA sample was subjected to high-fidelity PCR with native Pfu DNA polymerase (Stratagene, La Jolla, CA) using 10-μl glass capillaries in an air thermocycler (Idaho Technology, Idaho Falls, ID). PCR conditions were as described previously (5). Primer sequences were CW7 (5’ ACC GTT AAC TTC CAÁ TTA AC 3’; bp 10,011–10,031 of the human mitochondrial genome) and J3 (5’ GCC GGC GCA GGA AAA GAG GT 3’; complementary to mitochondrial bp 10,196–10,215; see Fig. 1). In these experiments, the CW7 primer was labeled with biotin. The samples were subjected to PCR until the fluorescein-labeled primer was completely converted into product, thus forcing all mutant sequences into heteroduplexes with the remaining wild type, which was still present in substantial excess.

PCR product was separated based on sequence by CDCE (7), an adaptation of capillary electrophoresis that permits separation of DNA sequences with biphasic melting profiles. PCR products containing mutations in the low melting domain are separated based on differences in their velocity as they migrate through a heated section of the capillary based on the same principles as for CDGE. The CDCE instrument has been described previously (7, 23). Briefly, a 5-cm water jacket was positioned at the beginning of a 30-cm-long, 75-μm inner diameter fused silica capillary. The water temperature was approximately 65°C, and the precise temperature was determined for each set of heteroduplex collections. The inner surface of the capillaries was coated with linear acrylamide based on a procedure adapted from Hjertén (24) and described fully elsewhere (25). Capillaries were filled with a 5%, 5% linear polyacrylamide matrix polymerized in TBE as described elsewhere (25). About 10⁵ copies of PCR product were electroinjected into the capillary and run at approximately 200 V/cm. Samples were collected into tubes with 5 μl of 0.1× TBE, 0.1 mg/ml BSA. DNA was detected by laser-induced fluorescence of fluorescein moieties covalently linked to DNA primers used for PCR.

At a specific temperature, all heteroduplexes migrate through the heated zone within a definable range of mobilities, which is differentiable from that of the wild type. Heteroduplexes in the amplified PCR product could therefore be collected separate from the wild-type homoduplex, thus allowing enrichment of mutants. For samples with low mutant fractions, the collected heteroduplexes were PCR amplified, and the heteroduplex collection was repeated to further enrich for mutants. Heteroduplex fractions that were sufficiently enriched for mutants were amplified to completely deplete the primer. Aliquots of these PCR reactions were then subjected to a subsequent PCR amplification that was terminated prior to depleting the primer to create homoduplexes. Homoduplexes were visualized under high-resolution conditions, which include the use of an acrylamide matrix containing 30 mM Na⁺ in addition to TBE and a 15-cm water jacket as described previously (26). Signals were observed in the computerized output as peaks, each of which represents a different mutant. The area under each peak is proportional to the number of copies of the particular DNA sequence. The mutant fraction that each mutant represented in the original sample can be estimated by comparing the area under each mutant peak in the sample to the area under the internal standard.

In the initial experiments performed with this technique, the mutants observed in human tissue samples were isolated and sequenced. As additional samples were examined, we observed that the same mutants were present repeatedly in multiple samples. To determine whether a peak in a given sample was the same as a previously sequenced peak or whether it represented a new mutant, we adopted a dual dye detection system (5). Fluorescein-labeled samples were coinjected into the capillary with authentic standard mutant peaks amplified with PCR primers labeled with TMR. Sample peaks that comigrated with known standards were preliminarily identified and measured.

Capillary Hybridization

To identify individual peaks more rigorously, capillary hybridization was developed as a test for whether a specific peak actually represents a known mutant with which it comigrates. This procedure will be described in detail elsewhere. Briefly, the fluorescein-labeled sample is cojected with an excess of TMR-labeled standard mutants. The sample is then heated to denature the DNA and the temperature is then lowered to allow reannealing. If a sample peak is identical to a mutant in the standard set, hybridization will result in a homoduplex. Nonidentity would be recognized by a change in mobility associated with the resultant heteroduplexes.

The CDCE instrument used for identification by hybridization included consecutive heating jackets, each bathed by a separate water bath. The fluorescein-labeled sample and a panel of TMR-labeled standards were coseparated in the first jacket at the appropriate temperature (~71.5°C) under high-resolution conditions (26). Approximately 3 × 10⁵ copies of each standard mutant and about 10⁶ copies of fluorescein-labeled sample, in which each mutant represented approximately 10% of the copies, were injected. The separated set of peaks was stopped; the temperature in the second jacket was increased to denature the DNA (~80°C) and then decreased and maintained for 10 min to permit reannealing of single strands (~55°C). The sample was then electrophoresed to the detector at a temperature that retards heteroduplexes as compared to homoduplexes (~70°C). Peak identity was verified when individual fluorescein-labeled peaks were observed as homoduplexes, reflecting the fact that they had reannealed with known TMR-labeled mutants. Sample peaks that did not correspond to any TMR-labeled standards formed single strands or heteroduplexes; such species were retarded as compared to the homoduplex standards and consequently “disappeared.”

Test for Asymmetrical Species

A test was designed to distinguish between signals that resulted from true, double-stranded mutants present in cells and those that originated from cellular premutagenic species, such as adducts and heteroduplexes, that were converted to mutants during PCR. Linear PCR was performed with a single primer to enrich for sequences synthesized from one or the other strand of the double helix prior to exponential PCR. For true mutants, the mutant fraction of a particular peak is the same regardless of which primer was used for preamplification. For DNA adducts or mismatches, one strand is wild type and the other is “premutagenic.” Therefore, preamplification from one primer to enrich for sequences synthesized from a specific strand would result in a different mutant fraction than preamplification from the other primer.

For these experiments, DNA eluted from a CDGE was linearly amplified with either the upstream or downstream primer for 20 cycles. Instead of J3, primer J16, a 16-bp variant of J3 truncated to exclude 4 bp on the 3’ end, was used for this linear amplification. Eluted DNA was also amplified with both primers for 5 cycles. Exonuclease deficient Pfu DNA polymerase was used for this first amplification for all three samples, and conditions were otherwise the same as for other PCR reactions. In some cases, a heteroduplex was added to the sample prior to linear amplification to serve as an asymmetrical control. Samples were then subjected to exponential amplification with exonuclease-proficient Pfu DNA polymerase. Heteroduplexes were collected by CDCE and reamplified to create homoduplexes. Homoduplexes were visualized by high-resolution CDCE as described above. Comparison of the mutant fractions as compared to an internal standard was performed for individual peaks to determine whether each signal was significantly different depending upon the primer used for preamplification.

RESULTS

Sample Mutational Spectra. Shown in Fig. 2 are mutational spectra for mutants with a lower $T_m$ than the wild type in two sections

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of the lungs for the smoking and nonsmoking members of one pair of twins. Peaks represent mitochondrial hot spot mutations that are clearly detectable above background levels. Fig. 2A shows the results for cells sampled from the right side of the main carina of the nonsmoking twin. The DNA was split after restriction digestion, and internal standards were almost always within 2-3-fold. In essence, the variability observed within a single lung was as large as any differences observed between smokers and nonsmokers.

Fig. 3 provides the mutational spectra for this target sequence, including both low- and high-$T_m$ mutants, for a representative sample from each of the six twins. Fig. 3 includes the TMR-labeled "standard set" of previously purified mutants that was coseparated with each sample and recorded in a separate channel. Observations similar to those reported for Fig. 2 are here extended to the high-$T_m$ mutants and other twin pairs. The same mutants were repeatedly observed in all of the samples, although a particular mutant may not be detectable in a given sample. An extra mutant not included among the TMR-labeled standards was occasionally observed at a significant frequency in a particular sample (for instance, Fig. 3A, peak x). No specific peaks were observed to be present in smokers and absent in nonsmokers. Furthermore, the overall mutant fraction, based on comparison with internal standards, was not significantly increased in smokers for this sequence.

The mutational spectra observed in bronchial epithelial cells from dissected lungs provided an opportunity to test whether smoking induces mutations below the second bifurcation of airway, the most distal position achieved with brush biopsies. Mutational spectra derived from dissected lungs, including samples from both smokers and nonsmokers through the eighth generation of airways, were similar to spectra from brush biopsy samples. The same mutants observed in brush biopsy samples were also represented in the dissected lungs, and the mutant frequencies were similar as well. No significant differences were observed in smokers compared to nonsmokers. Fig. 4 provides a comparison of sample mutational spectra from dissected lungs of a smoker and a nonsmoker.

**Capillary Hybridization.** The dual dye detector method for peak identification is based on comigration of fluorescein-labeled peaks with TMR-labeled standards run simultaneously in the same capillary but recorded in separate channels. Development of a dual-dye detection method significantly improved our ability to identify mutants and supported our preliminary conclusion that the same specific mutants appeared repeatedly in all samples tested. However, comigration is not proof of identity. Two distinct mutants could theoretically comigrate under a specific set of conditions. To test more rigorously the identity of specific peaks, we developed an identification procedure based on capillary hybridization of sample peaks with the panel of previously isolated mutants.

If a sample peak were identical to the standard with which it comigrated, then its reallocation would be forced by the high concentration of the standard mutants labeled with TMR, and the result would be homoduplexes. In this case, the position of the fluorescein-labeled peak with respect to the TMR-labeled homoduplexes, which reanneal efficiently due to their high concentration, would be retained. In contrast, if the peak were different from any of the standard
Fig. 3. Mutational spectra of bronchial epithelial cell samples from three pairs of twins discordant for smoking. Depicted is a representative sample from each of the six individuals. Both high-"T_m homoduplexes (left) and low-"T_m homoduplexes (right) are shown. A, results for the first pair of twins; in each case, a sample biopsied from the right upper lobe is shown. B, samples taken from the second pair of twins. For the smoking twin, the sample was taken from the right upper lobe, and for the nonsmoking twin the sample was taken from the left lower lobe. C, samples were taken from the third pair of twins, in both cases from the right lower lobe. Cells were resuspended in 100 μl of PBS, and the mutational spectra were determined for each sample as described in “Materials and Methods.” The artificial mutant "p13 were added as internal standards (is) at 10^{-4} except in the case of twin pair 3, nonsmoking sample, in which "p13 was added at 10^{-5}. Shown are fluorescein-labeled homoduplexes and the TMR labeled standard set with which they were coseparated. Peak identity was confirmed by capillary hybridization. The wild-type peak is not shown. Numbers indicate the specific mutation represented by each peak. bp changes for each mutant are given in Fig. 1.

_mutants, the hybridization procedure would convert the sample mutant into the single stranded form or into two heteroduplexes with a standard mutant present in excess. Such heteroduplexes are always less stable than the homoduplexes from which they were derived, and they move to a different position during the posthybridization portion of CDCE separation. This results in the “disappearance” of the sample peak from its original position in the spectrum.

Capillary hybridization was performed for high-"T_m mutants from 16 brush biopsy samples from six twins and three dissected lung samples. The specific mutants tested by capillary hybridization were 1, 3, 5, 5.5, 6, 7, 11, 11.3, 11.5, 12, 13, 13.5, 15.5, 16, 14, 18, 18.5, and 19. Approximately 90% of peaks identified by comigration were confirmed by capillary hybridization.

An example of the mutational spectra observed before and after capillary hybridization is shown in Fig. 5. In Fig. 5A, the high-"T_m mutational spectra of a sample biopsied from a nonsmoking twin’s left lower lobe is shown. The lower curve of the top panel represents several TMR-labeled mutants that were electrophoresed simultaneously with the sample. In Fig. 5B, the same sample is shown after capillary hybridization. Peaks that remain are identical to the standard with which they comigrate and are numbered. All sample peaks that were not identical to one of the TMR-labeled mutants injected in this run were converted to single-stranded DNA or heteroduplexes. Such species were retarded during migration through the second water jacket and hence passed the detector later, after the hybridized homoduplexes. These species “disappeared” from the spectrum. As an example, the peak between mutant 11 and mutant 11.5 (Fig. 5A, x) was not identical to any of the TMR-labeled standards included in this run and was consequently not observed in the spectrum after capillary hybridization. This particular peak represents mutant 11.3.

**Tests for Experimental Errors and Asymmetric Species.** In addition to confirming the identity of specific mutants, we also performed tests to determine the origin of the signals observed. We have previously described the experiments that demonstrated that almost all of the mutational hot spots detected by this procedure represent mutants present in human cells and not artifacts of the procedures performed (5). Important among these were controls in which internal
Fig. 4. Mutational spectra from dissected lungs. Bronchial epithelial cells were obtained from dissected lungs of a lifetime nonsmoker and a smoker. Samples were taken from the right lower lobe (second bifurcation of the bronchial tree) from the smoker and the right upper lobe (fifth bifurcation of the bronchial tree) from the nonsmoker. DNA was isolated from resuspended cells and subjected to mutational spectra as described in "Materials and Methods." Internal standards (ii) were p13 or artificial mutant and were added at 10^{-4} and are designated with an arrow as needed. Shown are the resulting homoduplexes along with the coseparated TMR-labeled standards. The identity of peaks was confirmed by capillary hybridization.

standards were introduced into purified wild-type DNA and carried through the procedure. The lack of prominent mutants in such samples, which were routinely performed along with the twins' samples, demonstrated that the signals observed in human samples were likely to reflect mutants actually present in tissues and not a byproduct of the procedures. Additional experiments were also performed to test even further whether misinsertion errors introduced during PCR with Pfu DNA polymerase could have contributed to the mutants observed. The mutational spectrum of Pfu DNA polymerase in this target sequence was determined (27). The overall error rate was discovered to be extremely low (approximately 6 × 10^{-7}). Furthermore, the individual mutants created by Pfu were isolated and sequenced. One of the mutants observed consistently in human tissue samples, p6, was discovered among Pfu-induced mutants, but none of the other 16 mutants that were repeatedly observed in human tissue samples was present among the Pfu-induced mutational hot spots.

Another possible source of error was also specifically tested. Individual peaks present in homoduplex spectra could have originated from true, double-stranded mutants or from PCR past cellular premutagenic species. Examples of premutagenic lesions include DNA adducts and bp mismatches created by replication errors. To distinguish between true mutants and premutagenic lesions, a test for symmetry between the Watson and Crick strands of the DNA helix was used. For true mutants, the mutation is present on both strands of the molecule, and therefore, the mutant fraction represented by a particular mutant should be constant regardless of whether the sequence is linearly amplified from the Watson or the Crick template prior to exponential PCR. For DNA adducts or mismatches, one strand of the sequence is wild type, whereas the other is premutagenic. Therefore, linear amplification with one strand as a template would result in a different mutant fraction for a given premutagenic lesion than linear preamplification with the other strand as a template.

We discovered that for high-T_m mutants in the sample tested, the pattern was essentially constant whether the sample was preamplified with one primer, the other primer, or both primers. Fig. 6A shows the 

Fig. 5. Capillary hybridization to confirm peak identification. The high-T_m homoduplex mutational spectrum was determined for the left lower lobe brush biopsy sample of a nonsmoking twin. A. curves reveal the set of homoduplexes observed and several TMR-labeled mutants from the standard set, specifically 11, 11.5, 13, 16, 18, and 19, prior to capillary hybridization. B. curves represent the same sample after capillary hybridization. Individual peaks that are still present in the sample are considered confirmed to represent specific mutants and are numbered. x, peak 11.3. This mutant disappeared after hybridization because there was no excess 11.3 TMR-labeled mutant with which it could reanneal.
We have used a combination of constant-denaturant electrophoresis and high-fidelity PCR to detect low-frequency point mutations in a 100-bp mitochondrial target sequence in human tissue samples without phenotypic selection. The mutational spectra of this mitochondrial target sequence were determined in bronchial epithelial cells sampled from several positions in multiple individuals, including both smokers and nonsmokers. Clear hot spot mutations were observed above background and could be measured. Tests were performed to confirm the identity of hot spot mutations and to verify that signals originated from true mutants as opposed to adducts converted to mutations during in vitro PCR.

The 100-bp target sequence used in these experiments was selected because its behavior under partially denaturing electrophoresis conditions permits high-resolution separation of mutants. For the target sequence studied, there are several reports in the literature of individuals with point mutations in this sequence representing their major mitochondrial sequence (28). A TA→CG transition at bp 10,034 has been reported as one of many normal sequence variants in a myoclonic epilepsy and ragged red fibers syndrome (MERRF) patient (29, 30). A CG→TA mutation at bp 10,100 was observed, among several other variations, in a patient with dilated cardiomyopathy (31, 32).

Neither of these mutations is among those that we have observed repeatedly in human tissues.

We discovered that the same specific mutants were present as hot spots in all of the samples analyzed, although with considerable variability. Although in the vast majority of cases, the mutant fraction of a given mutant in a particular sample was less than four times the average mutant fraction for that particular mutant in all samples, in approximately 2% of cases, the mutant fraction for a particular mutant in a given sample was >5-fold larger than the average mutant fraction for that mutant. As will be discussed elsewhere, we consider it likely that these unusual cases represent instances in which a particular mutant came to represent many of the copies in a bronchial epithelial stem cell. The descendants of such a mutant-rich stem cell would then be expected to also contain many mutant mtDNA copies, leading to a cluster of mutant-rich cells. It is possible that the accumulation of mutants represented selective amplification of an initial mutated mitochondrial DNA copy as has been suggested by others (33–35). The data generated by these experiments do not allow us to conclude, however, whether selective amplification occurred or whether individual cells were homoplasmic or heteroplasmic for the mitochondrial mutant.

We discovered that the same specific mutants were consistently present as hot spots in this mitochondrial target sequence in both smokers' and nonsmokers' bronchial epithelial cell samples. Although the mutant fractions varied considerably, there was no significant increase in the smokers' samples as compared with the nonsmokers' samples. A close association between cigarette smoking and lung cancer has been thoroughly documented (17, 36–39). In addition to its association with lung cancer, smoking has also been shown to induce this mutant (44, 45). In addition, the in vitro adduct hot spots generated by each primer were compared to the standard set, the ratio of the mutant fractions in a sample preamplified with one primer versus the other was less than 2. Under similar conditions, the ratio of mutant fractions for a heteroduplex introduced prior to preamplification as an asymmetrical control was 6 when samples preamplified with one or the other primer were compared. These results support the conclusion that high-T_m signals represent true, double-stranded mutants. Similar results were also observed for samples from other organs besides lung.

Among low-T_m mutants, the signal designated p3 was significantly different in mutant fraction as compared to the internal standard when samples amplified with CW7 and J16 were compared (see Fig. 6 of Ref. 5). We conclude that this mutant is likely to represent an adduct or mismatch. The behavior of p1, on the other hand, is consistent with it representing a true mutant because its fraction was similar with respect to the internal standard when preamplified with one or the other primer. We also observed that peaks generated by PCR noise vary in mutant fraction between the two preamplification samples. This finding is as expected, given that PCR noise reflects a mutagenic interaction with the polymerase and one, but not the other, strand of the double helix.

**DISCUSSION**

5H. A. Coller, K. Khrapko, P. Herrero, and W. G. Thilly. Distribution of mitochondrial mutants in human cell and tissue samples, manuscript in preparation.
treat ing the p53 gene with benzo[a]pyrene diol epoxide (BPDE) were found to correlate with the hot spot positions observed in human lung tumors (46).

Smoking has also been suggested to increase the level of mitochondrial deletions. Ballinger et al. (43) examined the levels of a 4.9-kb common deletion of mitochondrial DNA that results from recombination between two 13-bp direct repeats (14, 47, 48). In alveolar macrophages, smokers contained almost 7 times the level of the common deletion as compared with nonsmokers (0.016 versus 0.0023%), although the differences were not statistically significant because interindividual values were large. A smoking-induced increase in the frequency of the same deletion was also reported in hair follicle mitochondrial DNA in another study (49).

In contrast to these findings, we discovered that there was no significant increase in the mutant fraction or individual mutants in bronchial epithelial cells sampled from smokers compared with nonsmokers for the mitochondrial sequence investigated. Any mutations induced in this mitochondrial target sequence by smoking must be small (<10^-5) compared to those that we observe easily at frequencies as high as several times 10^-4. The prominent mutants observed apparently derive from a different and presumably endogenous source. We suggest several possible explanations for our findings.

One possibility is that this particular DNA sequence is "vacant" for smoking-induced mutations. Our extensive experience with the hprt gene (3, 4, 50-52) has shown that in approximately 15% of cases, a particular compound does not induce a prominent hot spot in a given 100-bp region. Indeed, the relatively low fraction of GC bp in the low melting domain of the 100-bp target sequence (27%) makes it a relatively poor target for compounds that specifically adduct at guanines. It remains possible that smoking-induced hot spots exist in other regions of mitochondrial DNA and would be detected by performing similar experiments on a different mitochondrial sequence.

A second possibility is that only a fraction of individuals are susceptible to smoking-induced mutagenesis. Genetic factors are likely to play a role in determining which smokers actually develop tumors (53) and could exert an influence in any of several aspects of lung cancer susceptibility, including the rate of particle clearance (54-56) or the activity of drug-metabolizing enzymes that convert tobacco-derived promutagens to their DNA-binding form (57-59). It could be argued that smoking-induced mutations would have been observed if our study had included members of a smaller, at-risk population and that the small size of our sample resulted in exclusion of such individuals. To test this hypothesis, healthy lung tissue from smokers who have actually developed lung cancer would need to be tested for smoking-specific mutational patterns.

Finally, smoking may induce mutations, but in contrast to nuclear genes, those mutations may be masked by the generally higher level of endogenous mutations in mitochondrial DNA. Indeed, the mutant fractions detected in these mitochondrial sequences are significantly higher than for nuclear sequences (60). A typical mutant fraction for one of the samples was 3 x 10^-4 for all detectable mutants in 100-bp in a 50-year-old individual, or 3 x 10^-6 per bp. This value can be compared with that discovered for nuclear sequences. The 6-thioguanine-resistant mutant fraction for a 50-year-old is approximately 10^-5 for the hprt or HLA-A genes (61, 62). Assuming approximately 1000 bp at risk, the mutant fraction is 10^-6 per bp. Although the hprt and HLA-A mutant fractions include only selectable mutants, it nevertheless appears that the background mutant fractions in mitochondrial DNA are more than 2 orders of magnitude higher than in nuclear DNA.

Studies performed by L. Marcelino et al.6 also support the possibility that smoking does not induce a significant increase in the mutant fraction in mitochondrial DNA due to a high level of endogenously induced mutations. Marcelino et al. demonstrated that to induce a detectable mitochondrial mutant fraction in this sequence, treatment with an unusually high dose of the alkylating agent N-methyl-N’-nitro-N-nitroso guanidine was required. To achieve such a dose, a human lymphoblastoid cell line deficient in mismatch repair, M’-1 (63), was treated with a 4 μM dose of N-methyl-N’-nitro-N-nitroso guanidine. On the basis of plating in the presence and absence of 6-thioguanine (64), the induced mutant fraction in the hprt gene was determined to be 8.7 x 10^-3. Using the methodology for determining mutational spectra in mitochondrial DNA as described in "Materials and Methods," mitochondrial point mutants were observed. These mutants were present at mutant fractions of approximately several times 10^-4, levels not significantly different from those observed for prominent hot spots in lung samples. The high doses of exogenous agents required to induce these mutants further supports the possibility that smoking-induced mitochondrial mutations are insignificant compared to those that result from endogenous pathways (60). In a recent, independent report, Pascucci et al. (65) concluded that mitochondrial mutations were induced by 20 J/m^2 UV light.

The reason for the difference between the levels of endogenous mutant fractions in nuclear versus mitochondrial DNA is not known. One possibility is that mitochondrial DNA is heavily mutated by free radicals generated during oxidative phosphorylation in the inner mitochondrial membrane. Also possible is that the mutations are generated during replication by the y polymerase. The predominance of transitions observed is reminiscent of the spectra induced by Klenow fragment (66). Mismatch repair activity has not been reported for mammalian mitochondrial DNA, which has been documented to lack repair of bulky adducts, such as pyrimidine dimers (67). Repair of nonblocking lesions has been demonstrated in mitochondrial DNA, however (68-70). Furthermore, mammalian mitochondria possess apurinic/apyrimidinic endonuclease activity (71), uracil DNA glycosylase activity (72, 73) and homologous recombination activity (74).

The demonstration that mitochondrial point mutations in this target sequence are not elevated in smokers’ bronchial epithelial cells suggests that the most important mutagens for mitochondrial DNA are likely to be endogenous factors, e.g., DNA replication errors or adducts caused by reactive species. Such pathways should be further investigated to better understand the contributors to mitochondrial mutagenesis.

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Hilary A. Coller, Konstantin Khrapko, Alfonso Torres, et al.


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