On the Utility of Heteroplasmy in Genotoxicity Studies: An Example from Chornobyl

ROBERT J. BAKER, J. ANDREW DEWOODY, AMANDA J. WRIGHT, AND RONALD K. CHESSER

1Department of Biological Sciences, Texas Tech University, Lubbock, TX 79409
Email: hjrb@ttacs.ttu.edu
2Savannah River Ecology Laboratory and Department of Genetics, University of Georgia, Athens, GA

Received 24 February 1999; Accepted 29 July 1999

Abstract. We examine the utility of mtDNA heteroplasm in assessing genetic damage due to environmental insult. Site heteroplasmy was quantified in a 400 bp portion of the cytochrome b gene in voles from a contaminated area near Chornobyl, Ukraine and from a relative control site by examining pregnant females and their embryos. A four hundred base pair segment was sequenced from approximately ten clones from each mother and embryo. Taq and/or cloning mutations were evaluated to estimate technical error. Although the rates of substitutions in clonal variants for experimentals, biological controls and technical controls were 1 in 1,840 bp, 1 in 2,280 bp, and 1 in 3,333 bp, respectively, we could not reject the null hypothesis that the variants were the result of a single, combined, mutation. However, multiple substitutions and transversions were restricted to clones from the Chornobyl samples. Bootstrap analyses indicate that these aspects of variation were significantly different from the controls. Examination of the mitochondrial genome by cloning individual molecules for site heteroplasmy estimate effects of pollution on mutation rate in free-living organisms appears to warrant additional study.

Keywords: heteroplasmy; Chornobyl; ionizing radiation; mitochondrial DNA; Clethrionomys glareolus

Introduction

We explore the utility of heteroplasmy (intra-individual DNA sequence variation) in the mitochondrial genome as an indicator of the effects of pollution on mutation rates. Detection of mutants, somatic or germ line based, (Cotton 1997 and Chesser 1998) in individual molecules prior any action of genetic drift or purifying selection is critical to the success of studies documenting elevated mutation rates. This study is designed to examine such putative changes in individual molecules by estimating the amount of heteroplasmy in a segment of the mitochondrial cytochrome b gene. RFLP analysis of mtDNA has previously been used to assess the genetic effects of pollution (Murdock and Hebert 1994); however, because mutation rates of mtDNA are typically on the order of $10^{-5}$ substitutions/site/generation or lower, conventional mtDNA studies are unlikely to reveal elevated mutational spectra unless several hundred thousand nucleotides are surveyed. Site heteroplasmy (as opposed to the more apparent "length" heteroplasmy, Casane et al. 1994) is the occurrence of multiple mtDNA haplotypes within a single individual, each distinguishable by one or multiple nucleotide substitu-
tions. Site heteroplasy has been described in a variety of mammals including cattle, pigs, bats, rabbits, and humans (Laipis et al. 1988, Ghivizzani et al. 1993, Ivanov et al. 1996, Casane et al. 1994, Petri et al. 1996). Recently, it has been speculated that heteroplasy may be a valuable indicator of mutation rate (Chesser 1998, Gibbons 1998).

There are several reasons why the mitochondrial genome is well suited for studies of mutation and resulting heteroplasy. These include 1) the mitochondrial genome has no proofreading repair mechanism in the DNA replication process; therefore, the signature of an elevated mutation rate should be more evident than in nuclear DNA. 2) Mitochondrial DNA is maternally inherited in mammals (Lightowers et al. 1997) and thus the paternal gametic contribution (Meirelles and Smith 1998, Shitara et al. 1998) is minimized in analyses of wild-caught animals. Therefore, characterization of mtDNA in the mother and in the embryos provides an estimate of the mutation rate over a single generation (Baker et al. 1996b, 1997). 3) Because there are 10^3--10^4 copies of the mtDNA genome in every cell (Lightowers et al. 1997), mutant forms of a mitochondrial gene can supposedly be tolerated without loss of that cell (Piko and Matsumoto 1976). 4) The mitochondrial genome contains both rapidly evolving regions under weak selective constraints and more slowly evolving regions, permitting latitude in experimental design.

Studies investigating mutation rates and other forms of genotoxicity depend on the development and application of experimental designs incorporating biomarkers yielding an interpretable effect (Shugart et al. 1992, Shugart and Theodorakis 1998). Thus, mtDNA is more suitable than the nuclear genome for studies of the potential genotoxic impact of mutagenic pollutants (Bickham and Smolen 1994). Specifically, the cytochrome b gene may be informative for at least three reasons. Firstly, the molecular evolution of the cytochrome b gene is well understood in mammals (Irwin et al. 1991). Secondly, cytochrome b is a protein coding gene which evolves at an intermediate rate as compared to other regions of the mtDNA genome (e.g. D-loop and 12s). If there are detectable differences in the mutational profile of this gene between individuals from polluted and nonpolluted habitats, then several regions of the mitochondrial genome should be a valuable research tool for genotoxicological studies. Thirdly, DNA sequence variation was observed within individuals (i.e., clonal variation was detected) in voles (genus Microtus) living near Reactor 4 of Chornobyl (Baker et al. 1996b, 1997). Thus, these voles are an appropriate model system to explore the utility of heteroplasy in genotoxicity studies. Herein, we quantify the variability among clones from each individual from a mother and her embryos. Additionally, we estimate artificial variation resulting from the experimental procedure (such as Taq error).

Materials and Methods

Biological series; heteroplasy

In this study, we examine mother/embryo sets of wild caught voles (Microtus arvalis) for variation in clones of a four hundred base pair segment of the mitochondrial cytochrome b gene (Fig. 1). Two sets (each consisting of a female and five embryos) were studied. The experimental mother was collected from a highly polluted area in the Red Forest region, 1 km west of Reactor 4 at the Chornobyl Nuclear Power Station (Baker et al. 1996a). At the time of capture, this mother emitted 30,000 disintegrations per minute as measured by a hand held Geiger counter. This mother/embryo set was previously reported in Baker et al. (1996b, 1997). The control female was collected from a region 32 km southeast of Reactor 4 (near Stracholessye) that has little pollution resulting from the Chornobyl accident. Both sets of embryos were at comparable stages of development near parturition at the time of sacrifice.

Whole cell DNA was isolated from liver tissue for mothers and from whole embryos. The entire cytochrome b gene was amplified from genomic DNA of the 12 individuals (2 mothers and 10 embryos) using the method of Kocher et al. (1989) L14724 and H15915 primers of Bickham et al. (1995) LGL765 and LGL287 primers (Table 1). PCR conditions were as follows: approximately 100 ng of genomic DNA, Perkin-Elmer 10X II buffer, 2.0 mM MgCl_2, 200 µM each dNTP, 0.5 µM each primer, and 1 unit Taq DNA poly-
erase (AmphiTaq; Perkin-Elmer) for an initial 2 minute denaturation at 94°C, followed by 35 cycles of 1 min at 94°C, 30 sec at 55°C, 1.5 min at 72°C, and finally, a single 7 min incubation at 72°C. PCR products were visualized in agarose and then cloned in a T/A cloning vector. Ones were picked from a plate with sterile nitpicks, swished in 100 μl sterile water, and unestered to a microtiter plate with growth medium. Plates were incubated at 37°C overnight, celol was added to a final concentration of 1%, and the plates were frozen in a -80°C freezer. The cell/water suspension (5 μl) was used as a template for PCR amplification in place genomic DNA in the protocol above. PCR products were verified as full-length amplions, pleted via Qiagen PCR purification columns and eluted in 30 μl of water, and sequenced with th amplification and internal primers (Kocher et al. and Table 1) using ABI's dye terminator chemistry and analyzed on an ABI 310 automated sequencer. Efforts were made to archive and sequence ten clones from each individual.

If a clone in the initial sequencing did not contain any changes relative to the consensus sequence for that individual, it was not further analyzed. Clones with apparent apomorphic sites (unique nucleotides compared to other clones and results from direct sequencing for that individual) when sequenced with the L14724 primer, were sequenced in the opposite direction with the internal H15149 primer to verify the change. Thus, all “mutations” reported herein were confirmed by sequencing in both directions. In some cases, sequencing a clone in the opposite direction from the same amplification failed to confirm the presence of the supposed point mutation. Such clones were reamplified from the archived stock and sequenced again in both directions. In all cases where this occurred, the initial nucleotide substitution was not found on either strand of the reamplification.

Technical series; procedural error

In order to test for “technical error” as opposed to “biological error,” six clones that had at least one unique nucleotide position (autapomorphies) relative to all other clones from the same individual were reamplified from archival stock, cloned in a T/A vector, and sequenced as described above (Fig. 1). For each of these six original clones we sequenced ten subclones. We chose distinguishable clones in order to identify possible
contamination with wild-type sequences. Any other deviation could be attributed to technical error (e.g. Taq infidelity or cloning artifact), not biological variation such as heteroplasmacy.

Analytical and statistical methods

The number of unique variants (potential mutations) observed for technical controls, biological controls, and experimental animals was compared using a likelihood ratio test for Poisson distributed data. However, because observed "mutations" were such rare events, the distribution properties and power of this statistic were questionable. We therefore chose to perform a series of bootstrap tests. The basic question addressed by the bootstrap analysis was: Could the observed differences in mutation events for the three classes be explained by sampling error alone? This question was addressed using the observed mutation rate for a particular class (technical controls, biological controls, and experiments). The null hypothesis was that greater than 5% of the generated values would overlap the observed values for all classes, and for all mutation rates. The bootstrap tests used the observed probability of visualizing an apomorphy for either the technical controls, biological controls, or the experimental data. For each of these classes the mutation probability was the number of variants observed divided by the total number of base pairs examined. A total of 5,000 replicates was generated using a FORTRAN-77 program running on a SUN-Sparc-20 Microsystems computer. Each replicate contained the total number of base pairs examined for technical controls (50 x 400 = 20,000), biological controls (23,000) and experimental (27,600), yielding a total of 70,600 base pairs for each replicate. 5,000 replicates were performed. A random number (additive pseudo random number method) was generated to correspond with each base pair, for all individuals in each class. If the random number was less than the observed mutation rate used in that particular trial, then it was considered that a mutation had occurred for that base pair. The number of mutations for each replicate was tracked for each class. Deviation in the number of mutations between classes was considered significant if the number of generated mutations per replicate exceeded or was less than the observed number of mutations for a class in 250 or fewer of the replicates ($P < 0.05$).

A similar bootstrap analysis was performed to assess the likelihood that chance was responsible for the observed numbers of multiple mutations occurring in individuals of experimental and biological and technical control classes. Bootstrap analyses were performed using observed mutation rates for each class as the baseline for all classes, using the mutation rate of each class to determine frequencies for each class separately, and using the mutation rate for the experiments for individuals within that class while assigning the mutation rate for the technical controls to the other two classes. Finally, a simple bootstrap program was run to assess the probability that random chance was responsible for the observed distribution of transitions and transversions in the three classes.

Probabilities of double and triple base pair substitutions in a single clone were calculated using the binomial below, where $r$ is the number of hits in a 400 bp segment and $p$ is the mutation rate ($q = 1 - p$).

$$P = \frac{400!}{r!(400 - r)!} p^r q^{(400 - r)}$$

Results

Biological series

Two families of mice were studied, one from a radioactive area (experimental sample) and one from the reference area (biological control). In the experimental samples, twenty clones were sequenced from the mother, and fifty from the five embryos (11, 10, 10, 10, 9 respectively). In the reference family, ten clones were sequenced from the mother, and forty-seven from the five embryos (10, 10, 9, 9, 9 respectively). Unique clones, the individual from which they were derived, and that individual's collecting locality are listed in Table 2. Table 3 lists the wild-type sequences, the apomorphic sequences, nucleotide position and the type of change (transition or transversion).
Table 2. Individual animals from (A) radioactive regions and (B) reference regions and their corresponding clones which contain apomorphic sites. JAD43 appears to be a nuclear copy of a pseudogene and is not included in any analyses. Embryo TK44920 is represented by nine identical clones.

<table>
<thead>
<tr>
<th>A. Radioactive regions</th>
</tr>
</thead>
<tbody>
<tr>
<td>C44605 other</td>
</tr>
<tr>
<td>D24</td>
</tr>
<tr>
<td>D81</td>
</tr>
<tr>
<td>D82</td>
</tr>
<tr>
<td>D86</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B. Reference regions</th>
</tr>
</thead>
<tbody>
<tr>
<td>C44916 other</td>
</tr>
<tr>
<td>D91</td>
</tr>
<tr>
<td>D92</td>
</tr>
<tr>
<td>D93</td>
</tr>
</tbody>
</table>

Table 3. Clones which contain an apomorphic position relative to the consensus sequence, the consensus (wild-type) sequence, the derived sequence, and the nature of the change.

<table>
<thead>
<tr>
<th>one #</th>
<th>Wild-type sequence</th>
<th>Positiona</th>
<th>Apomorphic sequence</th>
<th>TS/TVb</th>
</tr>
</thead>
<tbody>
<tr>
<td>D5</td>
<td>CGAAAAAACA</td>
<td>14765</td>
<td>CGAAATAAACA</td>
<td>TV</td>
</tr>
<tr>
<td>D5</td>
<td>CAGCΑTCTATΑ</td>
<td>15130</td>
<td>CAGCΑTCTATΑ</td>
<td>TS</td>
</tr>
<tr>
<td>D8</td>
<td>AAAΑΤΤαTCACA</td>
<td>14883</td>
<td>AAAΑΤΤαTCACA</td>
<td>TV</td>
</tr>
<tr>
<td>D35</td>
<td>CACAGΓΑTΓAT</td>
<td>14890</td>
<td>CACAGΓΑTΓAT</td>
<td>TS</td>
</tr>
<tr>
<td>D35</td>
<td>AΑАGGΑΓΑΓΓΤΓCTC</td>
<td>15005</td>
<td>AΑΑGGΑΓΑΓΓΤΓCTC</td>
<td>TV</td>
</tr>
</tbody>
</table>
| D44   | ΓΑΤΑΤΑΓΓΓΓΓΓCTA    | 14895     | ΓΑΤΑΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓGamma = transition and TV = transversion.

| D5    | ACAGCTACTCAT       | 15129     | ACAGCTACTCAT        | TS     |
| D5    | ATTAAAACCC          | 14842     | ATTAAAACCC          | TS     |
| D91   | CACTAACA            | 15069     | CACTAACA            | TS     |
| D91   | GCCCCΓΑΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓGamma = transition and TV = transversion.
| D98   | CΑΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓGamma = transition and TV = transversion.
| D106  | CΑΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓGamma = transition and TV = transversion.
| D112  | CΑΓΓΓΓΓΓΓΓΓΓΓΓΓΓΓGamma = transition and TV = transversion.
| D114  | CΑΓΓΓΓΓΓΓΓΓΓΓGamma = transition and TV = transversion.
| D115  | CΑΓΓΓΓΓΓΓGamma = transition and TV = transversion.
| D161  | CΑΓΓΓΓΓΓΓΓΓGamma = transition and TV = transversion.
| D123  | AGGGGGTΓΓΓΓGamma = transition and TV = transversion.
| D128  | GAAAACCTAA          | 14743     | GAAAACCTAA          | TS     |
| D131  | CTACΓΓΓΓΓΓΓΓGamma = transition and TV = transversion.

Position refers to the nucleotide position of the substitution in reference to the human mitochondrial genome (Anderson et al. 1981).
An average of ~400 bp of reliable sequence was obtained from each clone. In the experimental samples, 70 clones were sequenced. One clone differed substantially in sequence from all other clones (only 83% sequence identity) and is probably a nuclear pseudogene. If this clone is a nuclear fragment, its inclusion in our analyses would artificially inflate the variation in the experimental sample. Therefore, this clone was not included in further analysis and is described elsewhere (DeWoody et al. 1999). The remaining 69 clones from the experimental family, at 400 bp each, resulted in 27,600 bp of sequence data. Fifteen apomorphous sites were discovered among these clones, resulting in an average of one change per 1,840 bp. Each base pair substitution detected was unique. Fifty-seven clones were sequenced from the control family (22,800 bp). Ten unique apomorphies were discovered, resulting in an average of one per 2,280 bp.

Technical series

We sequenced fifty subclones (see below) at ~ 400 bp each from 6 of the original clones and found six substitutions, all of which were transitions (Table 4). This averages to one change per 3,333 bp. To obtain these results, we sequenced a total of fifty-four subclones. Four of these subclones were originally from the same clone and appeared to contain the same single base change. This "change" occurred in the reverse direction at the site that was apomorphic in the original clone, so that each of these four clones appeared to have reverted to the wild-type sequence. Resequencing the same aliquot of DNA confirmed this result, and there are at least two explanations. First, repeated reversals to the wild-type sequence could have occurred. Although we cannot eliminate this possibility, multiple reversals occurring in over 50% of the subclones from a single piece of DNA seems improbable unless nearby base composition affects nucleotide selection during replication. A second explanation is cross-contamination from adjacent microtiter wells during the archivial process. This is the most parsimonious explanation, and we have not included these four clones in our analyses.

Statistical analyses

Likelihood ratio tests were not significant when mutation events were compared for all three classes simultaneously ($X^2 = 1.62$) or when each pair of classes were compared (maximum $X^2 = 0.53$). Bootstrap analyses confirmed the results of the likelihood ratio tests. When the mutation rate for all data combined was used, 686 of the trials produced results equal to or more deviant than those observed ($P = 0.14$). Therefore, we could not reject the hypothesis that the experiments, biological controls, and technical controls had the same mutation rate.

Transitions, transversions, and multiple base changes

Within this data set, there are 4 transversions and 21 transitions. Also, there are 3 clones with multiple base pair substitutions (two clones with 2 substitutions and 1 clone with 3 substitutions). Both the transversions and multiple hits were restricted to clones from the experimental mother/embryo set. The observation that all clones containing multiple mutations were in the experimental group was found to be significant. In the bootstrap analyses using all combinations of observed mutation rates, a maximum of 18 of 1000 replicates resulted in multiple mutations being confined to only one class ($P_{max} = 0.018$). This maximum probability was observed when the mutation rate of the technical controls was used for all classes. Similarly, only four of 10,000 replicates
resulted in all transversions being restricted to the experimental group ($P = 0.0004$).

**Discussion**

**Biological significance of heteroplasmy**

Here are several aspects of our results that have implications for heteroplasmy and its application in genotoxicology. First, the number of base substitutions resulting from the technical procedure suggests that this source of error is a potential problem. This could be partially addressed by using a proofreading enzyme (such as *Pfu*) with greater fidelity than the polymerase (*Taq*) used in this study (Cline et al. 1996). However, this study demonstrates the need for baseline data (our technical controls) in genotoxicity studies designed to detect variation in mutation rate. Most mtDNA cloning studies, all within-individual substitutions have been attributed to PCR or cloning artifacts (Arentander 1995, Janek et al. 1996, Kriegers et al. 1997). Our data illustrate that single base pair substitutions in both our experimental and control groups result from two sources of variation, 1) PCR or cloning artifacts and 2) heteroplasmy.

Another interesting aspect of our study concerns the experimental design used to document the presence or absence of heteroplasmy. In studies of humans, PCR-amplified products are usually sequenced without cloning, frequently by means of an automated DNA sequencer. Individual peaks within a chromatogram can then be sequenced for a mixture of bases at a particular position. However, the effectiveness of this procedure can be enhanced in studies of humans because mitochondrial DNA hotspots have been identified (Srinivasan et al. 1996, Khrapko et al. 1997) and can be preferentially sequenced for heteroplasmy (reviewed in Wallace 1993). As part of another study, directly sequenced PCR products from all of the individuals (mothers and embryos) and no variation among mother/embryo combinations was detected (GenBank Accession #). It is significant that none of the heteroplasmic variation documented in these voles was of such frequency that it could be detected using direct sequencing. Furthermore, this study failed to reveal mtDNA hotspots in *Microtus*, as all nucleotide substitutions among clones were unique.

Mutations in individual molecules are the initial signature of mutagens (Casane et al. 1994, Cotten 1997) on the genome and need to be observed before the action of genetic drift or purifying selection (Lighthiders et al. 1997, Chesser 1998, Meirelles and Smith 1998, Shitara et al. 1998) can eliminate new heteroplasmy. Our methods have detected variation among cloned PCR products that appears to result, at least in part, from low frequency point mutations. The technology of direct sequencing is currently incapable of detecting the type of variation in individual molecules that was observed using our experimental design. Thus, cloning appears to be a reliable method of estimating such mutations.

Finally, the mutations detected by the methods described in this study are most likely somatic in origin and would not be transmitted genetically to future generations however mutations arising in the maternal germ line are possible and cannot be eliminated from consideration. Although there are no data to indicate that an elevated mutation rate in somatic tissues will serve as an accurate predictor of the mutation rate in germ tissue such a possibility cannot be eliminated.

**Heteroplasmy at Chernobyl**

Levels of heteroplasmy are greater in the sample from the radioactive site than in the samples from the control site (an average of 1 heteroplasmic site per 1,840 bp as opposed to 1 per 2,280 bp). How robust is the evidence that this difference in frequency is the result of exposure to the pollution in the Chernobyl environment? We employed several tests for significance, and the results are mixed. In our most powerful test for significance (bootstrap analyses), the number of mutations per base pair sequenced in the experimental family was not significantly different from that of the control family. Studies of nuclear minisatellite and microsatellite repeats in humans and swallows respectively (Dubrova et al. 1996, Ellegren et al. 1997) have demonstrated significantly elevated mutation rates in individuals exposed to Chernobyl-related pollution. In
light of these data from the nuclear genome, it would be an important result to document that the mitochondrial genome of voles living in the radioactive regions does not have a mutation rate significantly different from that of the voles from control regions.

Two aspects of the data do show significant differences between the experimental and control samples. These are distributions of 1) transversions and 2) presence of multiple base pair substitutions in a single fragment. That the occurrence of transversions was restricted to the Chornobyl sample is significant in the bootstrap analyses (in 4 of 10,000 replicates, the transversions were restricted to the experimental group), especially when the substantial predominance of transitions in the cytochrome b mutational spectrum (Irwin et al. 1991, Khrapko et al. 1997) is considered.

The bootstrap values documenting the frequency at which chance alone could account for the multiple mutations being restricted to the experimental group provides a value of p = 0.018. Furthermore, using the highest mutation rate in our study (μ = 0.00543), the probability of two substitutions occurring is 0.020 and the probability of a triple substitution occurring is 0.001.

In combination, the data on frequency and distribution of transversions and multiple mutations in single clones indicates that the Chornobyl samples may be experiencing a unique mutational profile relative to that observed for the samples from the biological control and from the technical procedure (Otto and Oldiges 1980, Bickham et al. 1998). We conclude that heteroplasmy merits more study to determine its effectiveness as a measure of the effects of pollution on mutation rate. Although this study documents a significant change in only minor aspects of site heteroplasmy for animals living in the Chornobyl region, experimental refinements may enhance the likelihood of detecting elevated mutation rates. These include: 1) more labile regions of the mitochondrial genome, such as the D-loop, rather than a protein-coding region might reveal greater variability; 2) the study of clones from different tissues may increase the probability of observing heteroplasmy that has segregated during early stages of ontogeny (Chesser 1998); and 3) examination of tissues that are more likely to harbor heteroplasmonic conditions (e.g., brain, Jazin et al. 1996).

Acknowledgements

We thank R. Van Den Bussche, S. Gashak, E. Buntova, and G. Rudenskaya for their assistance in collecting and preparing specimens, N. Arkhipov for providing laboratory space in the Chornobyl Exclusion Zone, and J. Bickham, J. Bull, H. Wichman, L. Wiggins, and J. Wickliffe for preliminary reviews of prior versions of this manuscript. We appreciate the cooperation and facilitation of V. Kholosha, A. Grebanyuk, and M. D. Lomakin for help in gaining access and establishing study areas in the Chornobyl region. Financial assistance for this study was provided by contract DE-FC09-96SR18546 between the U.S. Department of Energy and the University of Georgia, by Texas Tech University, and by the Ukrainian Ministry of Emergencies and Protection of the Population from the Consequences of the Chornobyl Nuclear Disaster.

References


Heteroplasmy in Wild Rodents: A Chornobyl Study 309


