COMPARATIVE MUTAGENESIS OF HUMAN CELLS
IN VIVO AND IN VITRO.

Our goal is to develop the tools of mutational spectrometry in order to discover the cause(s) of genetic change in somatic and germinal cells in humans. This past year has been remarkable progress in both tool development and primary observations of mutational spectra in endogenous human genes both in vitro and in vivo.

1. Human Mitochondrial Mutation (John Hanekamp, Gengxi Hu and Ahmad Chaudhry)

Potentially the most important results come from our study of the spectrum of point mutations in human mitochondrial DNA sequences. This effort led by John Hanekamp, a graduate student, has discovered that there are multiple point mutation hotspots in each of four separate sequences in the mitochondrial genome. These spectra were revealed by a combination of high fidelity PCR (modified T7 polymerase) and denaturing gradient gel electrophoresis which has a limit of detection of about 10^-3. There appear to be identical hotspot mutations in both cultured B cell and fresh human blood T cell samples.

As may be seen in Figure 1 the TK-6 human B cell line shows a series of mutant/wild type heteroduplex bands in each of the four sequences examined for the sequence designated CW-1. Dr. Gengxi Hu a post doctoral scientist has already determined the most prominent mutations to be:

<table>
<thead>
<tr>
<th>bp</th>
<th>freq. (approx.)</th>
<th>sequence change</th>
</tr>
</thead>
<tbody>
<tr>
<td>9959</td>
<td>1 x 10^-3</td>
<td>T --&gt; A</td>
</tr>
<tr>
<td>9966</td>
<td>5 x 10^-3</td>
<td>G --&gt; A</td>
</tr>
<tr>
<td>9984</td>
<td>5 x 10^-3</td>
<td>G --&gt; A</td>
</tr>
<tr>
<td>10,047</td>
<td>1 x 10^-3</td>
<td>C --&gt; G</td>
</tr>
<tr>
<td>10,088 and 10,184</td>
<td>1 x 10^-3</td>
<td>C --&gt; T and C --&gt; G</td>
</tr>
<tr>
<td>10,194</td>
<td>1 x 10^-3</td>
<td>C --&gt; A</td>
</tr>
</tbody>
</table>

both by direct sequencing of PCR products and sequencing of multiple M13 clones. We were more than mildly surprised to observe such high mutant fractions in mitochondria. It was already known that in yeasts the mitochondrial mutation rate was about 20 fold higher than in nuclear genes and that the human polymorphism frequency was greater in mitochondria than in nuclear genes. Our observations however appeared to imply a plasticity of unforeseen degree. The TK-6 cells used in this experiment had grown exponentially as stocks for about 1000 generations (~ 2 years) without subcloning. The sum of observed hotspot mutants in this 250 bp CW-1 sequence was about 1.5 x 10^-2. Calculating an average mutation rate per bp per generation (1.5 x 10^-2 + 10^3 + 250) yields 6 x 10^-7 mutations / bp generation. In these same cells the spontaneous hprt (TGR) mutation rate is 6 x 10^-8 point mutations per generation which if averaged over a hypothetical 1000 bp target gene is 6 x 10^-11 point mutations / bp generation. Allowing a factor of 10 for the ratio of total / selected mutants and a factor of 20 in analogy to the yeast mitochondrial / nuclear point mutation rate would lead to the expectation that the average mitochondrial mutation rate would be about 6 x 10^-11 x 20 x 10 = 1.2 x 10^-8. This is 50 fold lower than what we observe. Furthermore it appears that the other mitochondrial sequences contain hotspots with...
DGGE
lanes

J. Hanekamp

Spontaneous B-Cell Mutational Spectra

lane 1 – cytochrome c oxidase subunit III / tRNA glycine / NADH dehydrogenase subunit III (base pairs 9911 – 10146)
lane 2 – single strand (lane 1)
lane 3 – origin / membrane attachment site (base pairs 16340 – 16460)
lane 4 – single strand (lane 3)
lane 5 – cytochrome c oxidase subunit I (base pairs 6615 – 6750)
lane 6 – single strand (lane 5)
lane 7 – NADH dehydrogenase subunit I (base pairs 3995 – 4125)
lane 8 – single strand (lane 7)
frequencies of several percent of the total implying a still greater discordance between expectation and observation.

The B cell study was soon complemented by observations in the uncultured T cell samples drawn from four healthy young adults. The results with sequence, CW-7, a subsection of CW-1, is shown in Figure 2. It is clear that the kind and approximate frequency of mutations found in the TK-6 human B cells are also appearing in each and all of the four human donors. Idiosyncratic bands do appear at low frequency in the individual donors but the spectra are striking by their similarity. Of note with regard to the obvious question of cross contamination is the fact that the wild-type sequence of one of the donors showed a point mutant polymorphism relative to the TK-6 and consensus human sequence but still shows the same set of mutations!

A human is conceived with $2 \times 10^5$ mitochondrial copies in the ovum and in growing to adulthood of $10^{14}$ cells with $10^4$ copies per cell must create at least $10^{18}$ mitochondrial DNA copies. Does the embryo start as a single nonplacental cell ($10^5$ copies) and in a $10^{13}$ fold expansion accumulate hotspot mutants as high as 1 or 2% ?? $10^{13}$ is equivalent to about $2^{43}$. If we make the simplifying (stupid?) assumption that humans are created by exponential cell growth this implies an average *in vitro* mutation rate of $1.5 \times 10^{-2}$ observed mutant fraction in 250 bp over 43 generations or about $1.5 \times 10^{-6}$ point mutations per bp per generation which is darned high no matter how you look at it.

Another puzzling thing is the similarity of the TK-6 mutational spectrum to the human T cell samples and the similarity among T cell samples. Could this possibly mean that the primary mode of mitochondrial mutation is identical between human cells grown *in vivo* and *in vitro*? among cells grown in different humans? We don’t really think so.

Nuclear gene mutation rates *in vivo* are quite low ($6 \times 10^{-11}$ bp$^{-1}$ generation$^{-1}$) and the *in vivo* nuclear hpmt exon 3 “spontaneous” *in vitro* spectrum (see Figure 3) is distinct from that arising from treatment with any of a growing list of mutagenic chemicals UV light and ionizing radiation or that observed as somatic or germinal human *in vivo* mutations.

**First, have these mitochondrial mutants really arisen as a result of inherited or accumulated mutations?**

*In vivo* they could have been inherited from the maternal mitochondrial complement. *In vitro* they could have been inherited from the original donor of the W1-L2 parent line of TK-6. However, what little we know about the segregation of sister DNA copies of new mutations in mitochondria would seem to argue against this explanation. One would expect a rare new mutation to be segregated into a small subfraction of the descendant cell population. What changes this thinking is that we must now consider a particular mitochondrial mutation arising with in cells as frequently as $10^4 \times 1.5 \times 10^{-6}$ or 1.5% per cell generation. Now “mixing” can occur as a single cell can contain identical descendent mutants of independent mutations.

We have worked out limit models of zero segregation and complete segregation for sister mitochondrial DNA copies and note that the more complicated models involving partial mixing are, however, calculable. To test these required but simple models we have set up a series of single cell clones of the TK-6 population and are examining the distribution of mutant sequences among individual cells. If either the “complete mixing” or “fully inherited” models are correct then cell clones will yield the same spectra. The experiment will then be extended to T cell clones of the
Spontaneous Mutational Spectra

tRNA glycine and NADH dehydrogenase subunit III (base pairs 10,009 - 10,119)

lane 1 – TK6 B-cell line
lane 2 – T-lymphocyte donor (34521)
lane 3 – T-lymphocyte donor (25361)
lane 4 – T-lymphocyte donor (26421)
lane 5 – T-lymphocyte donor (69691)

Figure 2
human donors. If they too show identical mutant patterns within single cells, it would appear that the mutations we are seeing have accumulated not in a single human life time but over the approximately $10^5$ human generations since forming a new species. This is not an unlikely outcome. The accumulation of null mutants in pharmacogenetic studies in nuclear genes indicates that they exist at levels of around 1% in the general human population and thus for a 1000 bp gene target one can estimate a germinal nuclear mutation rate of roughly $10^{-2} \times 10^3 \times 10^5 = 10^{-10}$ expressed nuclear mutations / bp human generations. A similar calculation based on a 2.5% sum of hotspots distributed over 250 bp in mitochondria in $10^5$ generations leads to an estimate of $2.5 \times 10^{-2} + 2.5 \times 10^2 + 10^5 = 1 \times 10^{-9}$ total point mutations in mitochondria / bp generation which is not unreasonable and is in fact about 10 fold higher than calculated for human nuclear mutations.

If it turns out that we have discovered an array of mitochondrial mutations inherited through heteroplasmic ova we will press on to measure lower mutant frequencies (10$^{-5}$ to 10$^{-7}$) expected for induced somatic mutational hotspots and use these high frequency inherited mutations as convenient internal standards. We should know which path to take by August 1991.

**Nota Bene:** We are well aware of the strong implications of such observations and maintain pro tempore the strong suspicion that something may be wrong with our analyses. Appropriate tests for bias, contamination and DNA handling artifacts have not yielded any indication of such error but the following discussion is put forward with this caveat in mind.

2. Human Nuclear Mutation

a. hprt Exon 3

Our general procedure of selecting 6TG$^R$ human B cells *en masse*, amplifying exon 3 with suitable primers and separating and enumerating individual mutant sequences has fulfilled all of the criteria for success we could have hoped for in 1984 when we first proposed the procedure. A summary of spectra obtained to date is shown in Figure 3.

In order to obtain a broader range of spectra new researchers are simultaneously learning the technology and obtaining spectra (in triplicate) from the following mutagens and cell lines

- bromodeoxyuridine (W1-L2)
- methyl nitrosourea (TK-6)
- ethylene oxide (TK-6)
- styrene oxide (TK-6)
- benzo(a)pyrene (MCL-5)
- fluoranthene diolepoxide (TK-6)
- aflatoxin B1 (MCL-5)
- smokeless tobacco extract (TK-6)
- radon (TK-6)

Low LET radiation (X-rays) are being studied in collaboration with Dr. Richard Okinaka at Los Alamos National Laboratory who after a short period of study with us has successfully set up this approach in his own laboratory.
Figure 3

- ICR - 191 +1G
- MNNG G → A
- UV G → A
- BPDE G → T
- Spontaneous
- O₂ 960 µM
- VACANT
- H₂O₂
- X-ray

% mutants

base-pair position

R. Okinaka (LANL)
b. Other hprt Exons (Alexandra Kat and Jacklene Goodluck-Griffith)

Using the TK-6 derivative MT-1 Alexandra Kat has devised suitable primers for examining 594 bp of the 654 bp reading frame and about 400 bp of the intron regions flanking the exons. Using MNNG as a mutagen she has obtained the induced spectrum of hotspots which constitute fully 36.5% of the total 6TG\textsuperscript{R} mutants in her experiments. Ms. Kat is writing up her doctoral thesis at this time. See Figure 4.

c. Multi-copy Nuclear Genes (Hilary Coller)

We have previously reported the ability to examine the multicopy (n = 400 approx) r RNA genes in human TK-6 cells and note that no mutants were found at the level of 10\textsuperscript{-3}. Ms. Hilary Coller will proceed to look at mutations in these sequences in mutagen treated cell populations in the coming year. Given an \textit{in vivo} hprt mutant fraction of 5 x 10\textsuperscript{-6} some 10\textsuperscript{9} copies of the gene is needed for a statistically reproducible mutational spectrum. Some 10\textsuperscript{9} / 400 = 2.5 x 10\textsuperscript{6} human T cells or about 2.5 ml of blood should be sufficient for her studies.

3. \textit{Technological Development}

a. Mismatch Amplification Mutation Assay (MAMA) (Rita Cha)

We are currently working on a DGGE-independent method of detecting specific predetermined point mutations occurring at a frequency of 10\textsuperscript{-5} or less. We have been using the transforming allele of rat c-H-ras gene found among NMU induced mammary tumors (G to A transition at the 12th codon) for the development of our technique. Using PCR primers that have mismatches at the 3' positions, we have succeeded in detecting 10 copies of transforming allele present amongst 10\textsuperscript{6} copies of non-transforming wild type alleles. (Figure 5). Although the MAMA technique is limited to detecting one mutation at a time (unlike DGGE which detects virtually all point mutations present within a given piece of DNA), the simple and rapid nature of the technique will be invaluable for the screening for putative mutational hotspots without phenotypic selection. For instance one can use the procedure to measure induced c-ras codon 12 mutations in animals immediately after treatment and follow clonal expansion to tumor formation.

b. Automated PCR using Heat Labile Enzymes.

Unselected mutational spectrometry requires high fidelity PCR. Under commonly used conditions Taq polymerase has an error rate of 2 x 10\textsuperscript{-4} mutations / bp doubling. In the past year we have shown that a different heat stable enzyme, Vent, has an error rate of 4 x 10\textsuperscript{-5}, about the same as the heat labile T7 DNA polymerase. We have switched to VENT for all studies of selected spectra where a limit of detection of 10\textsuperscript{-3} is satisfactory.

This is not satisfactory for unselected mutational spectrometry in which resolution at 10\textsuperscript{-7} is desired. Dr. Phouthone Keohavong has undertaken a major study of anti-mutator T\textsubscript{4} DNA polymerase and its accessory proteins, especially the gene 32 product, in an effort to achieve a PCR error rate of 10\textsuperscript{-10} or lower. This involves a collaboration with Professors William Konigsberg (Yale University) and Linda Reha-Kranz (University of Alberta).
MNNG HOTSPOTS IN THE HPRT CODING REGION AND FLANKING INTRONS

<table>
<thead>
<tr>
<th>NUMBER OF ALTERATIONS</th>
<th>PERCENT OF POPULATION</th>
<th>NATURE</th>
<th>EXON SIZE/BP SCANNED</th>
</tr>
</thead>
<tbody>
<tr>
<td>EX 2 3</td>
<td>4.5 %</td>
<td>G.C → A.T</td>
<td>107/71</td>
</tr>
<tr>
<td>EX 3 3</td>
<td>20 %</td>
<td>G.C → A.T</td>
<td>184/184</td>
</tr>
<tr>
<td>EX 4 1</td>
<td>0.5 %</td>
<td>A.T → G.C</td>
<td>66/66</td>
</tr>
<tr>
<td>EX 5 2</td>
<td>5.5%</td>
<td>G.C → A.T</td>
<td>18/18</td>
</tr>
<tr>
<td>EX 6 1</td>
<td>2%</td>
<td>G.C → T.A</td>
<td>83/83</td>
</tr>
<tr>
<td>EX 7 1</td>
<td>3%</td>
<td>G.C → A.T</td>
<td>47/42</td>
</tr>
<tr>
<td>EX 8 0</td>
<td></td>
<td></td>
<td>77/77</td>
</tr>
<tr>
<td>EX 9 1</td>
<td>1%</td>
<td>G.C → A.T</td>
<td>48/48</td>
</tr>
</tbody>
</table>

28.5% of 6TG R alterations in 90.8% of the coding region
8% of 6TG R alterations in flanking intron sites

Distribution of Hotspots

Figure 4
Since preliminary experiments showed an efficiency of nearly 100% when T₄ DNA polymerase was used with its accessory proteins, we have designed and built an automated system for PCR with heat labile components so that advances may be brought rapidly on line in research. The original instrument was constructed to our specifications by the Zymark Corporation. Problems were encountered in design and are now being addressed by Mr. Sam Lippert an undergraduate in the Department of Mechanical Engineering.

The Device: Our automated PCR device (shown below) consists of two 96 well temperature controllers, a mechanical arm and a computer controller. One well holds the samples the PCR is being performed on, and cycles it through temperatures from 25°C to 100°C. The second well holds the enzyme used in the process at 25°C. The mechanical arm transfers the enzyme in response to the temperature cycles and mixes the components rigorously upon addition.

Progress: When work began on the PCR machine there were problems coordinating the enzyme transfer with the temperature cycles. This has been repaired by a small rewrite of the controlling software. Our current problem is controlling the evaporation of the samples during the PCR. Experiments have determined the exact amount of evaporation some 2.5 μl / cycle. The favored method of over-coming this problem is simply deluting the enzyme / accessory protein mixture to replace the 2.5 μl while adding the enzyme mixture. This requires attention to problems of loss of enzyme activity by simple dilution. We have however now found a recipe which allows DNA polymerase to be added in volumes to 4 μl per sample.

Figure 5: Automated PCR machine.
c. Simplification of DNA Sequencing.

Dr. Gengxi Hu has simplified our approach to using the Sanger sequencing procedure with PCR products. A common problem was unsatisfactory sequencing reactions. Dr. Hu reasoned that the conditions giving maximum efficiency for modified T7 polymerase in PCR should be optimal for the single primer extension of the Sanger dideoxy termination reactions. This proved to be the case. We have reliable and facile sequencing protocols tailored to each DNA sequence studied in our lab.

Ms. Jacklene Goodluck-Griffith, previously a cell culture technician, has trained in molecular biological techniques in the past year and is now a full-time team member in molecular analyses.

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