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Comparative Mutagenesis Of Human Cells
In Vitro And In Vivo

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Mitochondrial Mutagenesis

By combining the separation technology of CDCE (constant denaturing capillary electrophoresis) with high fidelity DNA amplification we devised a reliable means to measure mutant fractions of any and all point mutations in human cell or tissue mitochondrial DNA arising at mutant fractions at or above 10^{-6} .

Human cells in culture

We found that in the human mitochondrial DNA sequence bp 10,031 to 10,136 that point mutations are distributed as a reproducible spectrum of 19 non-random "hotspots". Of these 19, 17 are transition mutations, the other two, transversions. In human lymphoblastoid cells cultured *in vivo* the mutant fractions of these hotspots increase linearly with cell division. This fact and a series of specific tests for bias have persuaded us that these are the spontaneous mutations arising in this mitochondrial sequence and have not arisen as a result of experimental bias or as nonmutational lesions in the cells such as replication mismatch intermediates.

The rates of mutations for these hotspots range from 10^{-6} per cell division to about 5×10^{-8} per cell division in both TK6 and MT1 cells which differ in that the former has an active DNA mismatch repair system and the latter does not. The average mitochondrial point mutation rate can be calculated for this sequence to be 3×10^{-3} . In TK6 cells the average spontaneous point mutation rate per base pair in the nuclear hprt gene is about 3×10^{-11} . Thus the average spontaneous point mutation rate in human cell mitochondrial DNA appears to be 1000 times higher than for nuclear DNA.

With this new knowledge we considered the fact that no workers have been able to observe mutagen induced mitochondrial mutations in any mammalian cell system. We reasoned that with so high a spontaneous mutation rate that a single mutagen treatment sufficient to induce a mutant fraction higher than the accumulated spontaneous background fraction would probably be to cytotoxic to permit observation of surviving mutants. We overcame this problem by using our mismatch repair deficient MT1 cells which are resistant to killing but not nuclear mutation by alkylating agents such as MNNG or MNU. Using a single MNNG treatment that induced an hprt mutant fraction of 5×10^{-3} we have now observed a set of four induced G→A hotspot mutations within the target mitochondrial sequence. When we calculate the ratio of mitochondrial MNNG induced base pair mutations to the MNNG induced nuclear mutations we discover that they are essentially identical.

Human tissues

We completed studies of mitochondrial point mutational spectra in human colon, muscle and lung parenchymal cells. The same point mutational hotspots observed in human cells are observed in these tissues. The tissue samples show an approximately equal rate of G→A as A→G transitions which differs from the cultured cells in which A→G transitions arose at no more than 10% of the fractions of G→A transitions.

From these data we are persuaded that the same basic mechanisms create point mutations *in vivo* and *in vitro* and that these may be characterized as "spontaneous" mechanisms as opposed to induction by environmental mutagens. The mechanism of simple DNA replication misincorporation without mismatch repair is an attractive hypothesis. However, our data do not exclude a mechanism of reactions with endogenous cellular metabolites creating premutagenic lesions.

Because this conclusion contradicts the idea that the mitochondrial DNA reacts more readily with exogenous agents, especially alkylating agents and therefore would be particularly susceptible to exogenously induced mutations we sought a more rigorous *in vivo* test of our observations. We recruited four middle aged twins who were discordant for cigarette smoking throughout their lives. We imagined that cigarette smoking would mutate mitochondrial DNA if literally anything did. It did not. The kinds and frequencies of mitochondrial point mutational hotspots are not different in matched twins' bronchial epithelial cells.

We further analyzed this situation by taking samples from different anatomical regions of the lungs of the same donor by brush bronchoscopy. The data show that replicate analyses of the same sample give extremely high reproducibility but that each hotspot shows numerically significant differences among independent samples from the same lung. The variation among samples within the same lung is about the same as among the samples from lungs of different donors whether related or not. This is true of the mitochondrial mutational spectra in colon and muscle samples. It would appear that the same basic mechanism is driving mitochondrial point mutation in these organs and that the rates (mutations/cell year) are similar for these organs.

We do not know the reason for these significant numerical differences. We extended our studies to tumors derived from muscle and colon. Again the same set of hotspot mutations were found. When we dissected a muscle tumor we found significant differences in individual hotspot mutant fractions. Samples anatomically juxtaposed showed smaller differences than samples more widely separated in the tumor.

Unfunded proposed research

Had we been funded, we would've liked to distinguish between DNA replication error and endogenously induced mutation. Since the process occurs in our cell cultures we have a well controlled experimental system for the studies. We have formed a collaboration to use cloned human mitochondrial DNA polymerase in a "PCR fidelity" assay to discover if these same point mutations are created by the polymerase itself.

The reasons for the distinct numerical variations in hotspot fractions within an organ or tumor not clear. *In vivo* we would expect that only mutations that remain in a stem cell would be "remembered". Most mutations would by chance go to transition cells in the tissue "turnover units" and be lost to cell death. This process would give rise to a distribution of mutations in all copies of the mitochondrial DNA in a stem cell and descendant transition cells, given that the mutation were not lethal. Such turnover units appear to us to be about 128 cells in colon and lung epithelium so with 500 copies per cell this would lead to infrequent "colonies" of some 500×128 mutants distributed in a tissue. Such a process could account for our observations but it is only one reasonable possibility.

Nuclear Mutagenesis

Measurements in nuclear genes are more difficult than in mitochondrial genes. First, the average mutant fractions per base pair in middle-aged human T cells' hprt gene are about 10^{-8} which is much lower than the approximately 3×10^{-6} we have found for mitochondrial point mutations in several human tissues. To see point mutational nuclear hotspots we will need an analytical procedure which is reliable at mutant fractions of 10^{-7} and higher. Fortunately we are close to that goal. At this writing reconstruction experiments with human cells indicate we have achieved a sensitivity at least as low as 5×10^{-7} so we are optimistic that we can reach the required criterion.

A second difference between nuclear and mitochondrial sequences is that the former we find at between 300 and 1000 per cell while the latter are at 2 per cell for autosomal alleles. This means we have to work with much larger cell samples and "fish out" the desired sequences from a much larger excess of "other" DNA. This problem has been wholly overcome in the past year.

Human APC (adenomatous polyposis coli) gene: first example

The sequence we have chosen comprises bp 8429 to 8683 in the human APC gene which is suitable for or separations of mutants from wild type sequences using CDCE. We constructed mutant sequences and determined suitable CDCE separation conditions. Next we applied high fidelity PCR to the sequence and noted the specific Pfu DNA polymerase created errors.

The problem of reducing the total DNA amount was achieved using 10^5 excess biotin labeled probes for both strands of the desired sequence, annealing these with the whole restriction digest of 10 milligrams of human cellular DNA and removing the probe bound DNA from the solution with porous glass beads carrying a streptavidin moiety. When the strands were removed by gentle elution and allowed to reanneal we found we had a yield of approximately 70% of our starting sequences and about 5 micrograms of total DNA.

Because the 75 micron capillaries used in CDCE could not be used with DNA samples greater than 100 nanograms, even 5 microgram samples were too big. However, we devised a means of overcoming the intrinsic problems of heat generation and dissipation to permit use of 540 micron capillaries with a loading capacity of up to 10 micrograms of DNA in the form of a restriction digest. With this "fat" capillary system we were able to achieve a 50 fold enrichment of mutant/wild type sequences in a single pass. This resulted in less than 100 nanograms of DNA containing the desired mutant heteroduplexes which were loaded on a "skinny" high resolution CDCE system for final enrichment before high fidelity PCR.

With this approach we now see doped mutants at 10^{-6} as peaks much greater than the system noise when we begin with milligram quantities of DNA in human cells.

We have applied the analysis to human MT1 cells treated with MNNG so as to produce a mutant fraction of 5×10^{-3} at the hprt locus. This treatment produces a set of 12 hprt point mutant hotspots ranging in mutant fractions from 3×10^{-4} to 1.5×10^{-5} when 8 exons and contiguous sequences totaling 912 bp are examined. Had there been an MNNG induced mutant hotspot in our APC sequence we should have detected it. There was no hotspot. Our present interpretation is that the MNNG induced spectrum for these 100 bp is "vacant" in that it contains no hotspot. The chances of this occurring was about $e^{-12/912}$ or about 27% so the result is not particularly unexpected. We are now examining cells treated with other mutagens in order to find an induced hotspot for use as an internal standard and for further reconstruction studies.

Planned but unfunded research

Had we been funded we would've wished to improve the yields of mutant sequences, the enrichment of mutant sequences and the fidelity of PCR in order to reach our desired sensitivity. We have data from some inventors with nonporous cellulose beads carrying avidin side chains that suggest a significant improvement over porous glass is possible in isolating our sequences in a minimum of nonspecific DNA. We find that much of our interfering wild type sequences are not "tails" in our CDCE separation but products of undesired terminal transferase and exonuclease reactions in the low melting domain or target sequence. We are exploring ways to either reduce the rates of these reactions or removing the undesired products prior to a subsequent PCR step. Our data on PCR infidelity suggest to us that it is created by a minority of polymerase molecules which lack an active editing exonuclease. This is the general problem of microheterogeneity in proteins. Among the things we we would've tried was differential inactivation of polymerases lacking exonuclease function.

One important application of this work is that it would permit determination of whether or not ionizing radiation contributes in any significant way to mutations in human tissues.

Developing a General Procedure for All DNA Sequences

Our strategy depended on the use of a 100 bp target sequence which acts as an isomelting domain in a cooperative equilibrium between melted and helical forms. In our work this target must be adjacent to an isomelting domain with a melting temperature at least 5C higher than the target. This high melting sequence is called a "clamp" because it is not melted in our separation procedure and thus "clamps" the low melting sequence together even when it is in a fully melted form. There is no shortage of suitable sequences of this sort in the genomes of all species, but certain sequences of public health importance, such as many stretches of the human tumor suppressor genes and oncogenes are not in this form.

We believe we can overcome this limitation. We found that we could alter our CDCE tactics and achieve separation of mutant/wild type heteroduplexes from wild type homoduplex in the complete absence of a clamp. We are not sure of the fraction of all mutants which could be detected in a given sequence by this new tactic but we will find out with suitable reconstruction experiments. If the approach is successful it would extend this approach to some 95-99 % of the human genome. It also appears to offer means of scanning up to 200 or more base pairs instead of the present 100bp target size limitation which assures that all point mutants are detected.

Application of the new technology in other fields and research centers

It is clear that the ability to see and measure any and all point mutations in 100 bp sections also provides a means to screen human populations for genetic polymorphisms in simultaneous batches. One could take 1 microliter of blood from each of 1000 people and screen any and all DNA sequences desired as a single sample using the technology we have developed. Our laboratory has received visiting scientists from all over the world who have built our CDCE apparatus from scratch, learned the basics from us and set it up at home. The following groups have already installed their apparatus and started independent research.

Anne-Lise Borressen
Radium Hospital, Oslo, Norway

Jonathan Howard
Institute of Genetics, Cologne, Germany

Jan-Ake Gustavson
Bo Lambert
Kari Hemminki
Karolinska Institute, Stockholm, Sweden

Alexander Zehnder
ETH, Zurich, Switzerland

Steven Akman
Wake Forest Medical School, North Carolina

Helmut Zarbl
Fred Hutchinson Cancer Center, Seattle,
Washington

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These publications have resulted from the research supported by this grant during the final year.

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