DEVELOPING A BIOLOGICAL DOSIMETER BASED ON MITOCHONDRIAL DNA

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ABSTRACT

Direct measurement of deoxyribonucleic acid (DNA) damage from ionizing radiation may be advantageous in determining radiation exposures and assessing their effects on atomic radiation workers. The mitochondrial DNA molecule is one potential cellular DNA target which is: fully defined and sequenced; present in many copies per cell; not vital to cellular survival; and less subject to DNA repair than nuclear DNA. A method is described to isolate and analyse normal mitochondrial DNA. We describe the developments needed to determine DNA damage in mitochondrial DNA. The target is to make a biological dosimeter.

INTRODUCTION

DNA is the blueprint of life and when DNA is damaged by ionizing radiation or by other environmental agents, it can lead to health effects such as cancer. Genetic factors, encoded in the DNA, determine an individual's ability to repair DNA damage and, therefore, are important in determining an individual's response to ionizing radiation.

Because each person is genetically unique, and because this uniqueness is coded in their DNA, the response of each person to an environmental variable may differ. This idea is summarized by inspection of the biogenetic equation (1):

$$P = G + E \quad (1)$$

This equation says that the characteristics, diseases, and structure we observe in others, which is their phenotype (P), is the result of interactions between their genes or genotype (G) and the environment (E). Thus, the effects of a particular environmental agent will differ from one person to another depending upon their exact "G" and their individual interactions with their individual "E". We are, of course, interested mainly in the effects of the environmental agent ionizing radiation. By examining DNA molecules directly, the effects of ionizing radiation can be measured over the pre-existing background of DNA variations.

Radiation monitoring and protection are currently based on instruments or devices that measure exposures to ionizing radiation. These dosimeters do not take into account the genetic and biological variation within a population, which may make individuals more or less susceptible to the effects of ionizing radiation. Advances in molecular biology may enable us to move from this current dose-based radiation protection approach to risk-based radiation protection, taking account of individual genetic makeup.

The cell nucleus contains DNA with genetic instructions encoded in the 6×10^9 DNA base pairs in each nucleus. A cell also contains mitochondria, structures that provide energy to the cell. Mitochondria have their own small DNA molecules, which code for specific parts of the energy system. Each mitochondrial DNA molecule is 16,596 base pairs in length (2) and each cell may have up to 5000 copies of this molecule. The total mitochondrial DNA in a cell accounts for up to 1% of the total cellular DNA, representing a damage target of up to 60 million

base pairs. At an expected double strand break yield of one per $1.5x10^{10}$ base pairs per cGy (3), the mitochondrial DNA from as few as 200 cells might contain one double strand break after 1 cGy.

The mitochondrial DNA target may provide a way to quantify an individual's cumulative exposure to ionizing radiation. Because mitochondria are deficient in DNA repair (4), they accumulate lifetime DNA damage. This raises the possibility that mitochondrial DNA can serve as an integrating DNA damage dosimeter, as well as an emergency dosimeter. Essentially, mitochondrial DNA may more faithfully reflect the "E" component from ionizing radiation damage than the nucleus, because the damage may remain unrepaired. If mitochondrial DNA damage persists, and is a good indicator of initial DNA damage, the effective biological dose may be defined by comparison with other measures of nuclear DNA damage.

Fragment Indexing by Ligating Ends (FILE) technology is a novel, direct method for the isolation and analysis of DNA molecules, invented and developed at the Chalk River Laboratories of AECL (5). The core idea of FILE technology is that DNA fragments can be prepared which have distinct end sequences. The information in these end sequences can be used to define DNA fragments for isolation and analysis. A complex mixture of DNA fragments can be broken down into subsets using FILE technology. In Figure 1 we give a brief description of this new molecular biological methodology. By using synthetic DNA indexing "tags", a particular, defined DNA fragment can be isolated and amplified. Thus, rather than the random approaches used in gene cloning, FILE provides a directed approach to DNA isolation and analysis. One advantage is that an entire molecule such as the mitochondrial genome can be isolated and fully analysed.

A further advantage, which we intend to use in developing a mitochondrial dosimeter, is that the DNA isolation strategy can be designed so as to eliminate all normal DNA molecules from a pool. If we can selectively eliminate normal molecules, then only mutant molecules will be left for analysis. Two factors, therefore, will govern the efficiency of a mitochondrial DNA based dosimeter: the limits to isolation and amplification of abnormal DNA molecules; and the frequency with which abnormal molecules arise in an individual's cells, both spontaneously and as a function of radiation dose.

We report here preliminary studies on the isolation of mitochondrial DNA fragments as part of the process leading to the development of a mitochondrial DNA dosimeter.

RESULTS

Mitochondria from cultured human cells are being used as a model system to develop this dosimeter. Mitochondrial DNA was cut into discrete fragments and each fragment was isolated using FILE technology. Figure 2 shows a map of the mitochondrial genome, and a gel on which all 23 mitochondrial DNA fragments were separated. We have shown that these mitochondrial DNA fragments can be sequenced; 6 out of 25 fragments have been sequenced so far. An example of sequencing data is shown in Figure 3. This sequence information represents the fundamental genetic information in the cell or mitochondrion.

DISCUSSION

When DNA is damaged due to ionizing radiation, it can be repaired either correctly or incorrectly. It is only the incorrectly repaired DNA molecules that are of concern. We are developing methods to look for aberrant mitochondrial DNA fragments, either pre-existing or caused by ionizing radiation. In particular, we are developing strategies to detect deletions and base mutations. Direct assays for double strand breaks and for single strand breaks depend on advances in analytical methods for single molecules.

To detect deletions, indexers can be used which define molecules in which intervening sites have been lost. For example, from Figure 2A, using indexers to define the A-C fragment will isolate molecules which have lost the

"B" site. These molecules will be smaller than A-C and can be further analysed by direct sequencing. Suitable internal controls are being developed to test sensitivity and fidelity.

DNA base damage may be assessed either by direct sequencing, or by searching for molecules in which the end sequences have mutated. A different indexer is then required to isolate the mutated fragment.

Finally, the development process needs to be validated using mitochondrial DNA from irradiated cells in tissue culture and extended to whole animal studies.

CONCLUSION

We have demonstrated that the mitochondrial fragments can be isolated from very small amounts of blood. In theory, the mitochondrial DNA isolated from a drop of blood, or even the root of a hair, may be sufficient to detect very small radiation doses, suggesting that this approach has potential for emergency dosimetry where no external dosimetry is available.

Whereas an external dosimeter can measure an individual's exposure to ionizing radiation, a biological dosimeter can be used to measure the initial amount of DNA damage, giving a more direct estimate of potential health effects. The next phase in development will be to calibrate the assay using DNA with known deletions, and then to investigate the effects of ionizing radiation on deletions in mitochondrial DNA from cells in tissue culture. This will be a system in which both "G" and "E" can be measured in assessing risk; currently, we can only measure the "E" component.

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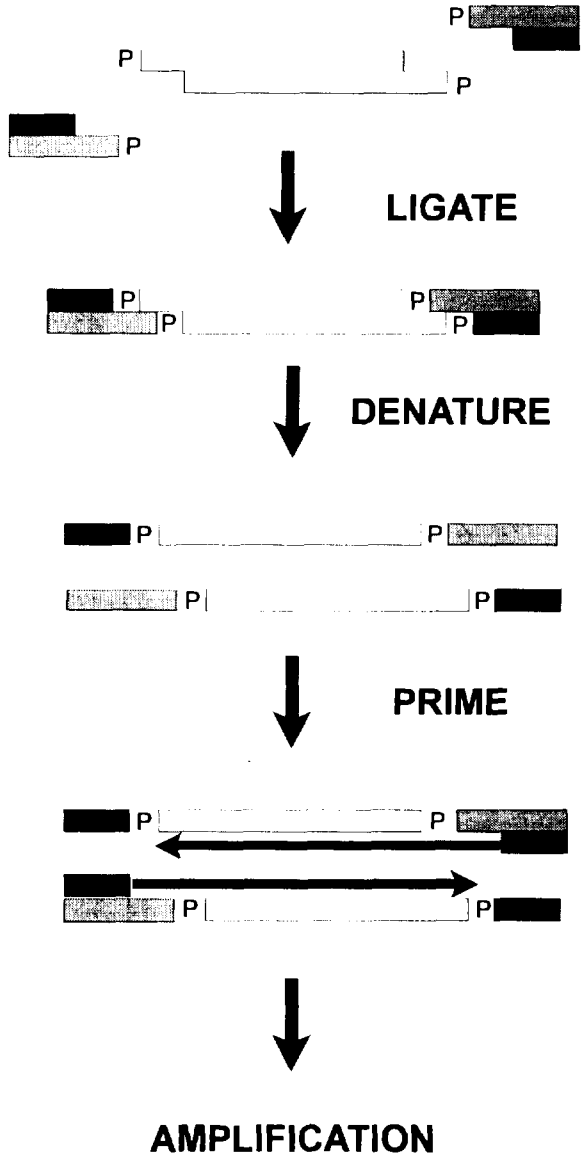
APPENDIX 1

Materials and Methods

Mitochondrial DNA was extracted from a normal lymphoblastoid cell line ATCC 2184 by the methods described by Anderson *et al.* (2). The cells were cultured in RPMI medium supplemented with fetal calf serum (20%). 10^8 cells were spun down into a pellet at 1800 rpm. The mitochondrial DNA was extracted and resuspended in 20 µl Tris-EDTA. The mitochondrial DNA was then digested with 1 unit of *Sfa*N1 (NEB) and 10 µg RNase A (Sigma) at 37°C for 30 min in a total of 60 µl, followed immediately by enzyme inactivation at 65°C for 20 min. The digested DNA was ligated to the specific indexers using 400 units of T4 DNA ligase (NEB) at 37°C for 60 min in a volume of 10 µl. The ligase was then inactivated at 65°C for 15 min followed by PCR amplification of the product. The amplification buffer was 2 mM $MgCl_2$, 15 mM $(NH_4)_2SO_4$, 60 mM Tris-HCl (pH 9). Amplification conditions were: 95°C - 5 min, 80°C hot start with Ampli*Taq* (ABI/Perkin Elmer Canada Ltd.), 94°C - 2 min, 94°C - 1 min, 55°C - 2 min, 72°C - 3 min; the last three steps were repeated 29 times, followed by 7 min at 72°C. The amplifed products were loaded onto a 1.5% agarose gel and electrophoresed. Ethidium bromide was used to stain the DNA, which was then visualized with ultraviolet illumination. The mitochondrial DNA fragments to be sequenced were purified by extraction from 0.8% polyacrylamide gels using the crush and soak method described in Maniatis (6). The DNA fragments were sequenced by the Core Facility for Protein and DNA Chemistry at Queen's University, Kingston, Ontario.

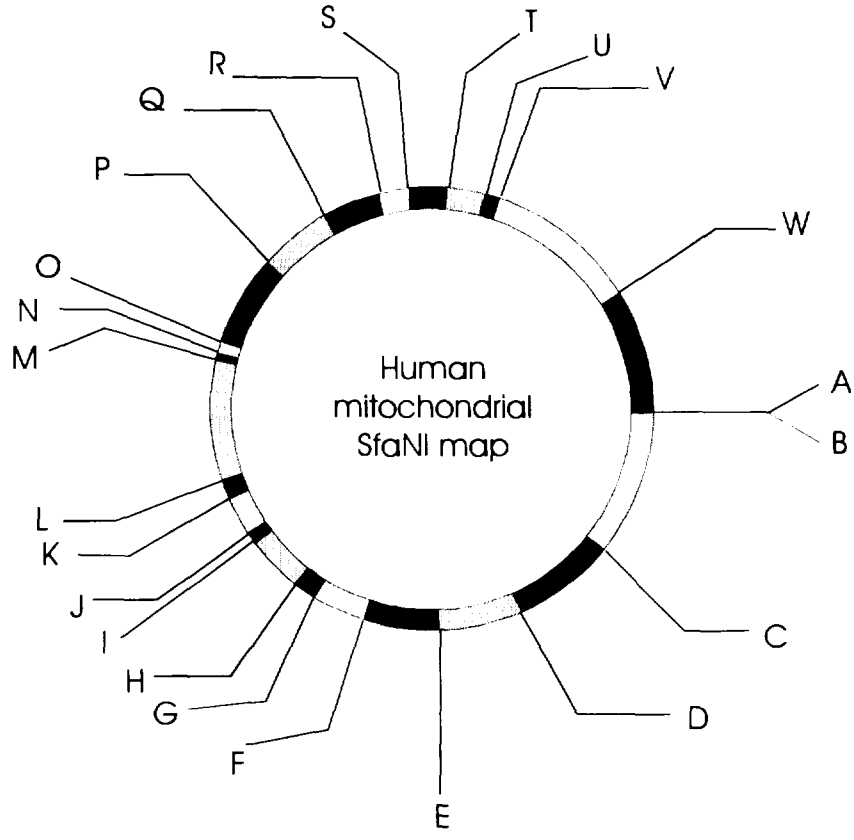


COMMON COMPLEMENT/ PRIMER

4-NT, 5'-P INDEXER STRAND

FIGURE 1. INDEXED AMPLIFICATION

a)



b)

1 AB BC CD DE EF FG GH HI IJ JK KL LM 2 MN NO OP PQ QR RS ST TU UV VW WA 3

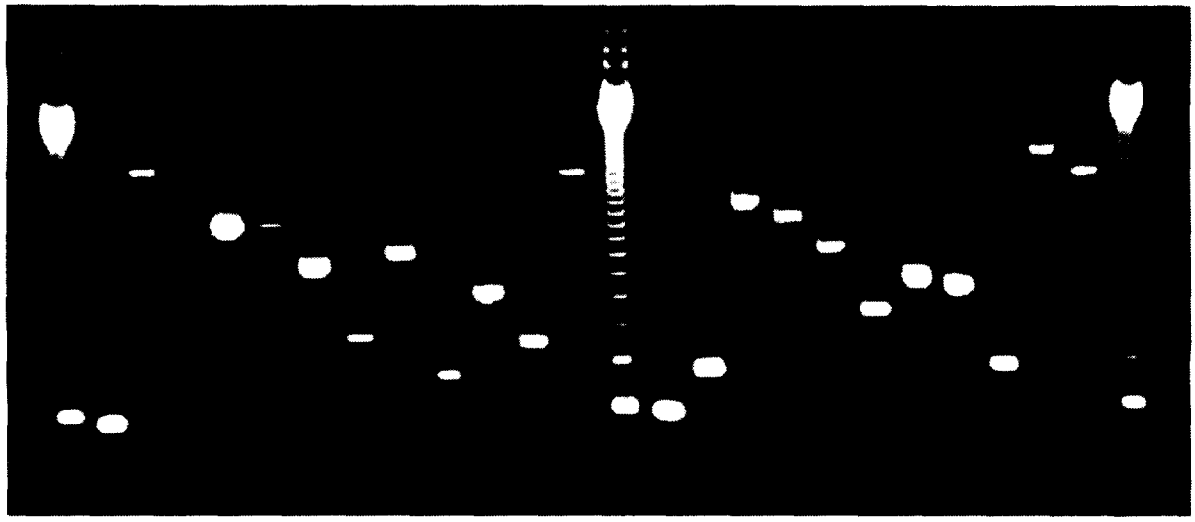


FIGURE 2. MITOCHONDRIAL *SFAN1* FRAGMENTS. (A) *SFAN1* CUTS HUMAN MITOCHONDRIAL DNA AT 23 SITES (A-W). (B) EACH OF THE *SFAN1* FRAGMENTS (AB-WA) HAVE BEEN INDEXED AND AMPLIFIED. LANES 1, 2, 3: 123-BP LADDER MOLECULAR WEIGHT STANDARD.

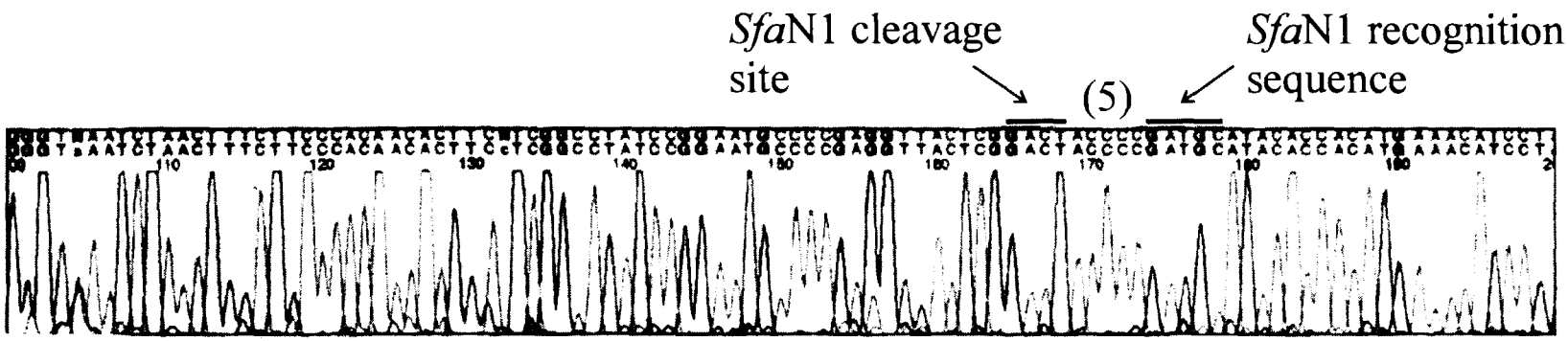


FIGURE 3. DNA SEQUENCE OF A HUMAN MITOCHONDRIAL DNA FRAGMENT, SHOWING THE "I" *SFA*N1 RECOGNITION AND CLEAVAGE SITES.