

REPLICATION OF MURINE MITOCHONDRIAL DNA FOLLOWING IRRADIATION

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Abstract: The effect of radiation on the mitochondrial genome *in vivo* is largely unknown. Though mitochondrial DNA (mtDNA) is vital for cellular survival and proliferation, it has little DNA repair machinery compared with nuclear DNA (nDNA). A better understanding of how radiation affects mtDNA should lead to new approaches for radiation protection. We have developed a new system using real-time PCR that sensitively detects the change in copy number of mtDNA compared with nDNA. In each sample, the DNA sequence coding 18S rRNA served as the nDNA reference in a run simultaneously with a mtDNA sequence. Small bowel collected 24 hours after 2 Gy or 4 Gy total body irradiation (TBI) exhibited increased levels of mtDNA compared with control mice. A 4 Gy dose produced a greater effect than 2 Gy. Similarly, in bone marrow collected 24 hours after 4 Gy or 7 Gy TBI, 7 Gy produced a greater response than 4 Gy. As a function of time, a greater effect was seen at 48 hours compared with 24 hours. In conclusion, we found that radiation increased the ratio of mtDNA:nDNA and that this effect seems to be tissue independent and seems to increase with radiation dose and duration following radiation exposure.

1. INTRODUCTION

Breakage of cellular DNA following radiation is a dose dependent phenomenon and occurs in both the nuclear and extra-nuclear DNA. Thus, while the vast majority of DNA is located in the nucleus (nDNA), on a mass basis, mitochondrial DNA (mtDNA) is equally affected. After radiation damage, the nuclear DNA has many advantages over

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mtDNA. These have been reviewed previously¹ and include 1) rapid and efficient double strand break repair,^{1,2} and 2) rapid and near perfect single strand break repair.³ Following irradiation, mtDNA has few repair mechanisms and continued mitochondrial function is preserved primarily due to its high copy number.^{4,5}

The cellular response to radiation with regard to the protection of mtDNA has rarely been studied. It is predictable that unchecked mtDNA damage will prevent the replication of properly functioning mitochondria over time, leading to mitochondrial depletion, and then late cell dysfunction.⁶ The latter would be most pronounced for long-lived cell types such as myocardial, renal, or neuronal cells. Consistent with this hypothesis, these tissues begin to demonstrate serious dysfunction 2 to 8 years after radiation exposure.⁷ Thus, as a means of self-protection, cells will want to respond to radiation to reduce the frequency and delay the onset of lethal radiation damage to the mitochondria. One such mechanism is enhanced replication of mtDNA.

In this study, we examined the early effects of radiation on the mtDNA copy number of different cell types using doses similar to those that might be experienced by bioterror victims or patients undergoing a bone marrow transplant. Two sets of primers were designed: one set coding for the 12S rRNA gene of the murine mitochondrial genome, and the other set coding for the 18S rRNA gene, an abundant housekeeping gene in the nDNA. The ratio of these two gene frequencies was compared at different times following different radiation doses to look for evidence of enhanced replication of mtDNA following irradiation.

2. MATERIALS AND METHODS

2.1. Animals and Treatments

Male Balb/c mice, 6 to 8 weeks old, were divided into groups of 5 animals. Control mice were not irradiated, while other groups received total body irradiation (TBI) via a J. L. Shepherd Irradiator with a ¹³⁷Cs γ -ray source. The dose rate was 1.84 Gy/min and homogeneity was better than $\pm 6.5\%$. The mice were then sacrificed at 24 or 48 hours post-exposure. Mice were housed in a pathogen-free barrier facility. All protocols were in accordance with USPHS guidelines and approved by the University of Rochester Committee on Animal Research.

2.2. DNA Extraction from Mouse Tissues

The small bowel and bone marrow tissues were collected and frozen immediately at -70°C until use. Total DNA was extracted from these tissues by standard proteolytic digestion followed by phenol/chloroform/isoamyl alcohol purification. Absorbance at 260 nm was used for quantification of the extracted DNA.

2.3. Real-time Quantitative PCR to Estimate mtDNA

The PCR primers for detecting the 12S rRNA gene of murine mitochondrial genome were designed on the basis of the GenBank nucleotide sequence (NC_006914, *Mus musculus domesticus* mitochondrion). The sequences for the primers were 5'-ACC GCG GTC ATA CGA TTA AC-3' (forward) and 5'-CCC AGT TTG GGT CTT AGC TG-3'

(reverse), producing a 177 bp amplicon. The primer sequences amplifying mouse nuclear 18S rRNA gene are 5'-CGC GGT TCT ATT TTG TTG GT-3' (forward) and 5'-AGT CGG CAT CGT TTA TGG TC-3' (reverse), which produce a 219 bp product. The real-time quantitative PCR was carried out using separate tubes for mtDNA and nDNA amplification. Each tube contained 30 ng of total DNA from the same extract as well as 20 μ l of reaction mixture consisting of 2 x iQTM SYBR Green Supermix (1 x final), and one pair of the primers directed against either mtDNA or nDNA, with each primer being 0.5 μ M. Each reaction was subjected to an initial denaturation of 5 min at 95°C followed by 40 amplification cycles of denaturation at 95°C for 30 s, 51°C to anneal for 20 s, and 1 min of extension at 72°C. A Bio-Rad i-cycler iQ real-time PCR detection system was employed to run the reactions. A conventional PCR was first run to develop the cycling conditions using the same primers. PCR assays were performed in duplicate or triplicate for each DNA sample. The cycle number (Ct) at which the fluorescent signal of a given reaction crossed the threshold value was used as a basis for quantification of mtDNA and nDNA copy numbers. The difference in Ct and the assumption of a doubling of DNA per cycle were used to calculate the mtDNA:nDNA ratio.

3. RESULTS AND DISCUSSION

3.1. Optimization of Real-Time Quantitative PCR

Conventional PCR was first performed to optimize the real-time PCR conditions. Duplicate PCR were run using the mtDNA and nDNA primers added to purified mouse small bowel DNA (Figure 1). As predicted, the size of the amplicon derived from the mtDNA was 177 bp, and from the nDNA was 219 bp. The band size and intensity were visualized with ethidium bromide after electrophoresis in 4% agarose gel; analysis indicated a high mtDNA:nDNA ratio.

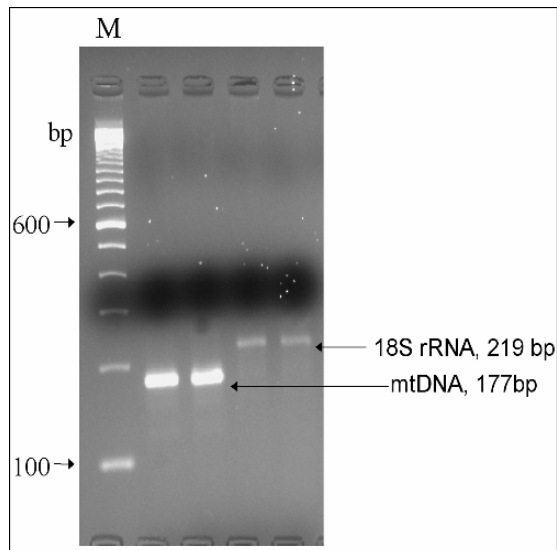


Figure 1. Conventional PCR for mtDNA and nDNA. PCR conditions were optimized to produce amplicons of the expected size and intensity.

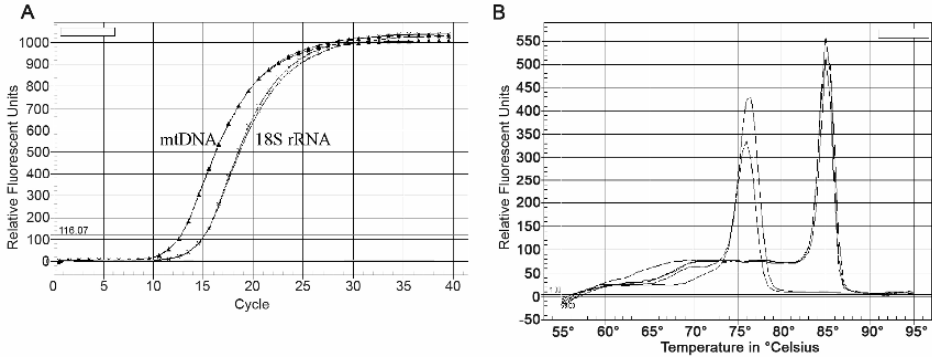


Figure 2. Optimized real-time quantitative PCR. **A.** Sample amplification curves obtained using the real-time quantitative PCR system for mtDNA and nDNA genes. **B.** Sample melt-curve analysis of mtDNA and nDNA products from a SYBR Green I assay.

The real-time PCR amplification plot in Figure 2A shows the typical two phases, an exponential phase followed by a plateau phase. A threshold is set during the early exponential phase, as shown. The lower number of cycles needed to reach the threshold level indicates a greater copy number of the mtDNA compared with the nDNA. The 18S rRNA gene is present in multiple copies in the nuclear genome, and therefore the ratios obtained by this assay are not meant to be equivalent to the average copies of mtDNA per cell.⁸ Melt curves are shown in Figure 2B. The expected single, narrow peaks indicate that the mtDNA and nDNA products are sufficiently pure and specific. Taken together, the data confirm that the real-time PCR conditions are satisfactorily optimized and can be used to determine relative changes in copy numbers of the respective transcripts.

3.2. Dose-Dependent Increase of mtDNA Copy Number in Small Bowel

The small bowel mucosa is a rapidly renewing tissue. Within a few days of radiation exposure, there is complete replacement of the entire luminal epithelium.⁹ Thus loss of mtDNA could seriously impact recovery given the potential for hemi-depletion with each cell division. It was therefore interesting to see how mtDNA might respond to low dose radiation. To test this, radiation doses that have no obvious histological effects were used. The small bowel tissues were collected from mice 24 hr after 0 Gy, 2 Gy, or 4 Gy irradiation. The total DNA was extracted from each sample and subjected to real-time PCR. Each cycle leads to almost a doubling in DNA copies, and thus the difference between cycle numbers measured at the threshold (designated β) can be used to calculate the relative ratio of mtDNA:nDNA using the equation: 2^{β} . At 24 hours after irradiation, we observed an increase in the mtDNA:nDNA ratio compared with non-irradiated tissues (Figures 3 and 4A). Tissues irradiated to 4 Gy indicated a clear increase in copy ratio compared with 2 Gy, consistent with a dose response. This result is also consistent with a compensatory replication of mtDNA to replace lost or damaged mtDNA. Other explanations include a lowering of the copy number of chromosomes per cell by redistribution of the cell cycle; however, this is very unlikely since the proportion of cells in S-phase at 24 hrs after irradiation is stable or moderately increased, which would lead to a decrease rather than the observed increase in mtDNA:nDNA ratio.

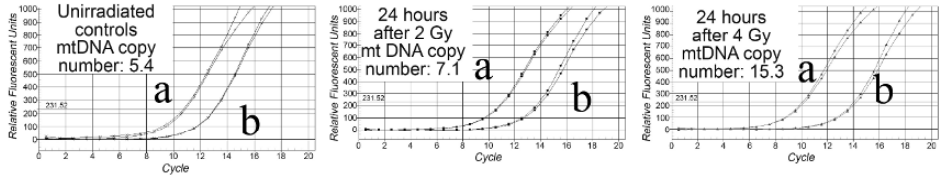


Figure 3. Representative real-time PCR of mtDNA and nDNA extracted from small bowel of mice 24 hours after 2 or 4 Gy TBI. **(a)** Denotes change in relative copy number of mtDNA; **(b)** Denotes change of relative copy number of nDNA.

3.3. Dose- and Time-Dependent Increase in mtDNA Copy Number in Bone Marrow

Bone marrow is probably the most sensitive organ to radiation. Within a few days of TBI, the bone marrow has only a few microscopically detectable hematopoietic cells, yet within two or three weeks of sublethal exposure, the bone marrow repopulates. This extraordinary cellular replication must include a matching mtDNA replacement. Damage to bone marrow mtDNA could otherwise lead to devastating replication-related hemi-depletion. To examine for changes in mtDNA of the bone marrow following irradiation, animals were given sublethal TBI (4 Gy) or near LD_{50/30} doses (7 Gy). Marrow was harvested at 24 or 48 hours after exposure and analyzed as above. Again the copy ratio of mtDNA in bone marrow of mice increased after 4 Gy.

As in the previous small bowel studies, the amplification of mtDNA seems to be dose dependent; here, 7 Gy produced a higher mtDNA:nDNA ratio. The ratio seems to increase further at 48 hrs compared with 24 hrs (Figure 4B).

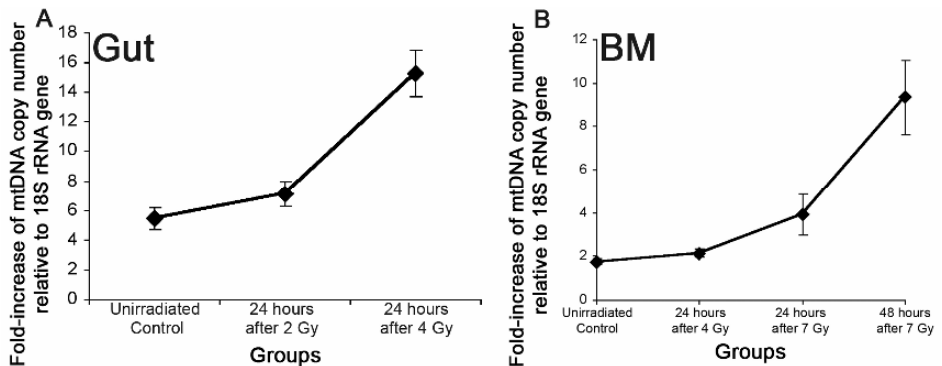


Figure 4. A: Increased mtDNA in the irradiated gut is dose- and time-dependent. Mice were exposed to 2 or 4 Gy TBI. The small bowel was harvested at 24 hours. **B:** Increased mtDNA in the irradiated bone marrow is dose- and time-dependent. Mice were exposed to 4 or 7 Gy TBI. The bone marrow was harvested at either 24 or 48 hours. Values are mean \pm 1 SD.

While the nDNA is a traditional standard to normalize the number of cells, radiation can cause cell cycle synchrony. However, we did not observe any change in either the S or the G2 phase, and the expected increase in nDNA, if synchrony occurred, would have lowered, not raised, the mtDNA/nDNA ratio.

4. CONCLUSIONS

We report a set of primers and procedures that can be used to measure relative copy number of mtDNA to nDNA, and which can be used on DNA purified from murine tissues. Sequence modification would make this technology applicable to other animal tissues. Further, we have demonstrated that small bowel and bone marrow collected from irradiated mice have an increase in mtDNA copy number after irradiation when measured at 24 or 48 hours and that the copy number may increase with radiation dose. These results are consistent with our previous hypothesis that some radiation effects are due to damage of mitochondrial DNA offset in part by adaptive replication of mtDNA.^{1, 10}

5. ACKNOWLEDGEMENTS

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