J. RADIAT. RES., 43: SUPPL., S199–S203 (2002)

Radiation-induced Gene Expression in the Nematode *Caenorhabditis elegans*

GREGORY A. NELSON[1]*, TAMAKO A. JONES[1], AARON CHESNUT[1] and ANNA L. SMITH[1]

Nematode / Microarray / Gene expression / Radiation / High LET

We used the nematode *C. elegans* to characterize the genotoxic and cytotoxic effects of ionizing radiation in a simple animal model emphasizing the unique effects of charged particle radiation. Here we demonstrate by RT-PCR differential display and whole genome microarray hybridization experiments that gamma rays, accelerated protons and iron ions at the same physical dose lead to unique transcription profiles. 599 of 17871 genes analyzed (3.4%) showed differential expression 3 hrs after exposure to 3 Gy of radiation. 193 were up-regulated, 406 were down-regulated and 90% were affected only by a single species of radiation. A novel statistical clustering technique identified the regulatory relationships between the radiation-modulated genes and showed that genes affected by each radiation species were associated with unique regulatory clusters. This suggests that independent homeostatic mechanisms are activated in response to radiation exposure as a function of track structure or ionization density.

INTRODUCTION

The increasing use of charged particles in radiotherapy and the extension of manned space flight to longer tours of duty, where exposures to cosmic rays and protons set safety limits, have focused attention on the biological effects of densely ionizing radiation. Charged particle experiments show that patterns of DNA damage are more complex and more spatially clustered with high linear energy transfer (LET) radiation than with ionizing photons[1)]. This damage is repaired slower and less completely than from low LET radiation and leads to cell inactivation kinetics that are more linear and less sensitive to dose rate[2)]. At the tissue level, high LET radiation causes cells to remodel their microenvironment with different time courses and compositions than for low LET radiation[3)]. Thus, cells respond not only to the dose of absorbed energy but to the spatial features of the damage.

*Corresponding author: Phone: +1–909–558–8364
Fax: +1–909–558–0825
E-mail: gnelson@dominion.llumc.edu
[1] Loma Linda University Radiobiology Program 11175 Campus St., Chan Shun Pavilion A1010 Loma Linda, California 92354, USA

Gene transcription profiling can be used to describe the sets of genes and regulatory pathways employed by cells to respond to external stimuli. The patterns of mRNA transcript levels under specific conditions reflect the functional status of the cell or tissue. Thus, differential display and microarray methods have been employed to identify genes involved in cellular functions including stress and DNA damage responses. Transcription profiling of cells following irradiation has identified a number of genes that appear to be similarly programmed in different cells[4)]. These studies have all used low LET photons so little is known about the dependence of gene expression on radiation quality. We set about to determine how gene expression *in vivo* depends on radiation quality using the nematode *C. elegans* as a simple animal model.

The *C. elegans* genome sequence was completed in 1998[5)] and set the stage for a variety of bioinformatics investigations. 19,282 genes were identified and about 53% show significant homology to genes in other organisms. Previous investigations have described genes controlling sensitivity to radiation and chemical mutagens[6)] and have used RT-PCR differential display (RTPCR-DD) to identify genes responsive to reactive oxygen species[7)]. Most recently, Boulton *et al.*[8)] used a combination of bioinformatics methods to identify a set of 79 nematode genes defining a DNA damage response (DDR) homologous to humans and

yeast. These studies show how nematodes respond to environmental stresses using homeostatic mechanisms common to other eukaryotes.

Recently, microarray methods were applied to *C. elegans* for high throughput screening of genes regulating gender, gametogenesis, aging and stress responses[9]. These studies culminated in a tour-de-force investigation by Kim *et al.*[10] in which 553 microarray experiments sampling 17,661 genes (93% of genome) were integrated into a master gene expression map. A unique statistical clustering method (VxInsight™) translated all pair-wise correlations of gene expression levels under the 553 conditions into positions and distances in a gene expression space. We have used these methods to map gene expression following exposure to gamma rays, protons and accelerated iron ions, and show that gene expression is highly dependent on the quality or track structure of the ionizing radiation species.

MATERIALS AND METHODS

Nematode Culture

C. elegans wild type strain N2 was obtained from the *Caenorhabditis elegans* Genetics Center at the University of Minnesota. Worms were cultured under standard conditions on NGM agar plates seeded with *E. coli* strain OP50-1 as food[11]. Age-synchronized populations of first stage larvae (L1) were generated by a standard plate wash method[11]. L1 larvae were used immediately or allowed to grow to the fourth larval stage (L4). Both L1 and L4 stages were used for differential display experiments while only L4 animals were processed for microarrays. For gamma ray and proton exposures, worms were transferred to 100 or 150 mm NGM petri dishes and maintained at 20°C. For iron irradiation, worms were suspended in M9 buffer in T25 culture flasks and returned to plates post irradiation.

Irradiation Conditions

Three radiation sources provided γ-rays, protons and iron ions. An Eldorado ^{60}Co radiotherapy source delivered 3 Gy of gamma rays to worms at isocenter on the surface of petri dishes behind a 1 cm polystyrene build up layer at a dose rates of 0.25 Gy/minute (RTPCR-DD) or 1.51 Gy/minute (array). 250 MeV beams of protons from the Loma Linda University Proton Treatment Facility were directed vertically through blocks of polystyrene to "range shift" the protons to the end of their range. Worms at the surface of inverted petri dishes lay in a plane corresponding to the peak of the Bragg distribution. The energy vs. frequency distribution of the protons had a dose averaged lineal energy transfer of 6 keV/μm and a full width at half maximum energy spread of 3 keV/m,[12]. The 3 Gy exposure was calibrated according to ICRU 59 methods[13] and the dose rate was 2.0 Gy/min. Accelerated iron ion irradiations were performed at the Brookhaven National Laboratory Alternating Gradient Synchrotron accelerator. Beams of 1.08 GeV/u iron ions ($^{56}Fe^{26+}$) were directed to suspensions of worms in T25 flasks for 3 Gy exposures at 1.0 to 2.1 Gy/min. The track averaged LET of the particles was 148 keV/μm. Beam composition and dosimetry are described fully in Zeitlin *et al.*[14]. All control samples were sham irradiated and subjected to all handling procedures experienced by irradiated samples.

RNA Preparation and Quantitation

Following irradiation, worms were dispensed to seeded NGM plates and incubated at 20°C for 3 hr to allow the worms to respond to the radiation treatment. They were washed twice with ice-cold buffer, concentrated by centrifugation and frozen in liquid nitrogen for further processing. RNA was extracted with Trizol and further purified using Oligotex mRNA spin columns (Qiagen) according to manufacturer's instructions. The resulting RNA was DNase treated to remove any contaminating DNA. Differential display PCR amplifications, labeling and sequencing gels as well as sequencing were performed by Lark Technologies, Inc. (Houston, TX.) using the Genomyx Differential Display System with 12 anchored and 4 arbitrary primer pairs. Duplicate experimental gel lanes were compared by eye with control (sham-irradiated) lanes loaded with equal amounts of cDNA.

Purified mRNA for arrays was sent to the Stanford Microarray Database Consortium (SMD)[15] which performs hybridization services and database management to members. SMD performed cDNA synthesis, labeling with Cy3 and Cy5 dyes and hybridization of labeled DNA under standardized conditions against a custom glass array printed with coding sequences of 17,871 genes[16]. Fluorescence signals were read with a Gene Pix 4000 scanner (Axon Instruments) and processed with Gene Pix Pro 3.0 software to produce normalized data sets. SMD experiment identification numbers for the Jan. 9, 2002 hybridizations were 23871 through 23879 and all were performed using microarray print lot #529. Data analysis was performed with software tools posted at SMD web sites: http://genome-www.stanford.edu/microarray/ and http://cmgm.stanford.edu/~kimlab/topomap/vxinsight.htm. *C. elegans* gene annotations were obtained from http://www.wormbase.org with links to Proteome and NCBI.

RESULTS AND DISCUSSION

We characterized the transcription profiles of L1 and L4 stage worms 3 hr after irradiation with 3 Gy of ^{60}Co gamma rays, low energy protons and high-energy iron ions. The three irradiation conditions span a range of uniform versus highly structured energy deposition patterns at the cellular scale which we predict results in different patterns of gene expression despite the equivalence of energy deposition on a macroscopic scale in terms of absorbed dose. Differential display experiments identified 5040 amplified PCR products on gels. 222 differentially amplified sequences were identified by eye and sorted into expression pattern categories such as "up-regulated by iron ions only". The majority of products (76%) exhibited unique expression patterns associated with a single radiation type (Fig. 1).

To identify representative genes we sequenced 55 products selected from the different response categories and compared them to electronic databases. 50 products matched known worm sequences and 33 were in independent genes. The sampling using differential display was not uniform but was instructive. It suggested that about 2.6% of worm genes were differentially expressed at 3 hours following 3 Gy of radiation. Because of differential display's non-uniform sampling and inefficiency, we turned to DNA microarrays for a more comprehensive analysis.

For analysis of array expression patterns, we established the following criteria for spot quality and biological significance that was satisfied in at least two of three hybridization replicates. The signal strength criterion was: spot median brightness in both the irradiated (Cy3) and control (Cy5) channels was 300 fluorescence intensity units in the GenePix scanner. The signal to noise criterion required that 95% of spot pixels had intensities ≥ (local background intensity + two standard deviations) in both fluorescence channels. The biological significance level was set at 2-fold change relative to controls. That is, the transcription ratio of experiment to control for each significantly regulated gene was 2 for up-regulated genes or ½ for down-regulated genes.

599 of 17871 genes analyzed (3.4%) showed differential expression 3 hrs after exposure to 3 Gy of radiation. 193 were up-regulated, 406 were down-regulated and 90% were affected by a single species of radiation only. Fig. 2 summarizes the distribution of these genes by regulatory pattern as a Venn diagram. These results confirm the observations with differential display that most genes are specifically regulated as a function of radiation type. To determine the functional and regulatory relationships between the radiation-modulated genes we employed the clustering analysis method based on VxInsight software. In this method, the difference in expression ratios of gene pairs is depicted as a "Euclidean distance" in a gene expression space. Genes that are close to each other respond similarly in terms of direction and magnitude of expression. Stuart Kim *et al.*[10] have used the VxInsight method to develop a topological map of nematode gene expression that depicts the consensus positions of 17871 genes after normalizing the results from 553 array experiments that varied age, gender, growth conditions, genotype and external stresses such as heat shock and hyperoxia. The map illustrates that there are 44 major gene expression clusters (mountains) and Kim *et al.*[10] have verified that the mountains represent biologically significant groups of genes. For example, heat shock genes are 337-fold enriched in mountain 36 vs. random distribution in gene space.

Genes whose transcription levels responded significantly to radiation exposure were located in the topological map. Positions of radiation-regulated genes were highly ordered and mapped to definite clusters that were unique for each

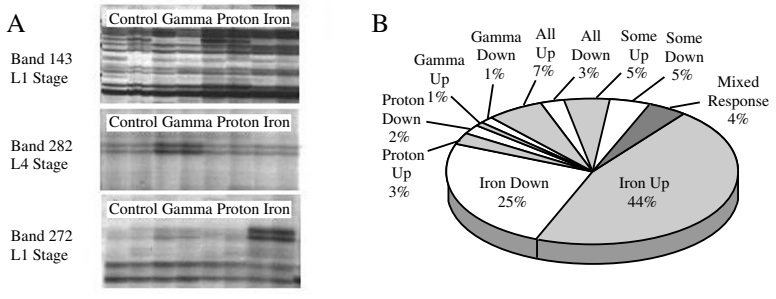

Fig. 1. Three examples of differentially expressed PCR products (A) and the distribution of 222 products by response category (B). In A the duplicate gel lanes of amplified products Band 143 and Band 272 from L1 stage worms and Band 282 from L4 stage worms are shown for RNA isolated 3 hours after sham treatment or 3 Gy exposures to gamma rays, protons or iron ions. In B the pie chart illustrates the proportions of the differentially expressed products responding to single or multiple radiation exposure conditions.

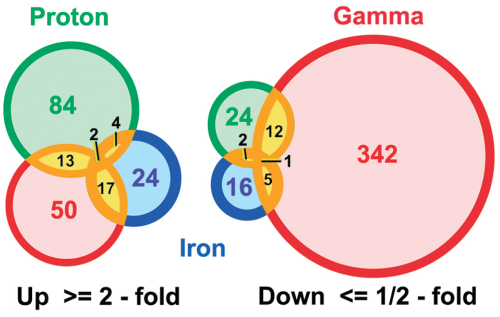

Fig. 2, left. Fig. 3, right.

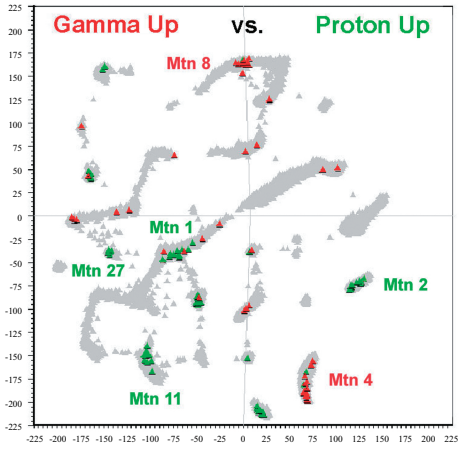

Fig. 2. Venn diagram showing the proportion of genes whose transcription levels increased or decreased by a factor of 2 or more as a function of radiation type. Gold regions refer to genes regulated by two or more radiation types.

Fig. 3. VxInsight gene expression topology map with proton and gamma ray up-regulated genes highlighted in color. Selected "mountains" enriched for up-regulated genes are labeled according to reference 10.

radiation type. Fig. 3 compares the distribution of gamma and proton up-regulated genes on a two-dimensional version of the topological map where gene positions are indicated by triangle symbols and clusters are seen as overlapping shaded areas. Proton regulated genes are highly enriched in mountains 1, 2, 11 and 27 while gamma-regulated genes are enriched in mountains 4 and 8. Note that each mountain contains genes from many different functional categories that together represent a regulatory cluster. We compared the distribution of genes regulated by gamma, proton and iron with those identified in our differential display experiments and the DNA damage response set identified by Boulton *et al.*[8]. Fig. 4 shows the distribution of genes against the ten mountains most enriched for radiation up-regulated genes. A different pattern is obtained for down-regulated genes.

The distribution clearly illustrates: 1) expression profiles are unique for each radiation species, 2) DDR genes co-locate in gene expression space with radiation-induced genes, and 3) genes identified by differential display and arrays also co-locate, providing an independent confirmation of the array results. Current efforts are directed at understanding the time course and dose responses of radiation-regulated gene expression and the biological roles of the genes identified in this study; the results will form the basis of future reports.

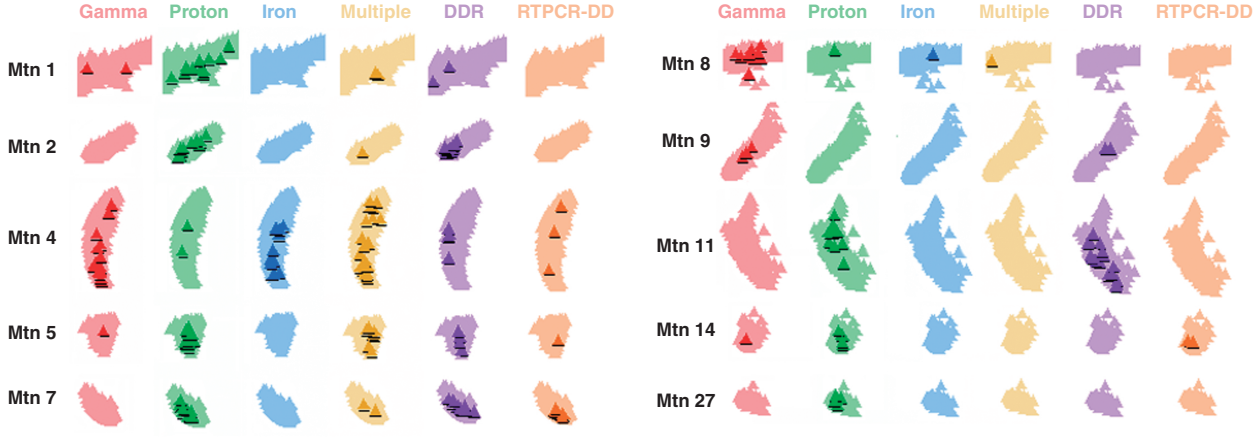

Fig. 4. Distribution of genes in ten mountains showing statistical enrichment. Gamma, proton and iron represent genes up-regulated by a single radiation species. Multiple represents genes up-regulated by 2 or more radiation species. DDR represents genes identified in ref. 8 as participating in the worm DNA damage response. RTPCR-DD indicates radiation-modulated genes identified in differential display experiments.

CONCLUSIONS

The identity and regulatory relationships of nematode genes modulated *in vivo* by exposure to ionizing radiation is highly dependent on the quality of radiation to which the animals were exposed. Radiation regulated genes map to distinct expression clusters in which they are highly enriched and co-locate with important DNA damage response genes. These conclusions are supported by two independent methods - differential display and microarray hybridization.

ACKNOWLEDGEMENTS

We thank Stuart Kim, Kyle Duke and Jubin Ryu for their help in array hybridizations by the Stanford Microarray Database Consortium. Nematode strains used in this work were provided by the *Caenorhabditis* Genetics Center, which is funded by the NIH National Center for Research Resources (NCRR). This work has been funded by NASA under Cooperative Research Agreements NCC9-79, NCC9-118 and NCC9-149.

REFERENCES

1. Ward, J. F. (1994) The complexity of DNA damage. Relevance to biological consequences. Int. J. Radiat. Biol. **66**: 427–432.
2. Antonelli, F., Bettega, D., Calzolari, P., Cherubini, R., Vecchia, M. D., Durante, M., Favaretto, S., Grossi, G., Marchesini, R., Pugliese, M., Scampoli, P., Simone, G., Sorrentino, E., Tabocchini, M. A., Tallone, L. and Tiveron, P. (2001) Inactivation of human cells exposed to fractionated doses of low energy protons: Relationship between cell sensitivity and recovery efficiency. J. Radiat. Res. **42**: 347–359.
3. Barcellos-Hoff, M. H. (1993) Radiation-induced transforming growth factor beta and subsequent extracellular matrix reorganization in murine mammary gland. Cancer Res. **53**: 3880–3886.
4. Chan, J., Chen, L.-K., Chang, J.-F., Ting, H.-M., Goy, C., Chen, J.-L., Hwang, J.-J., Chen, F.-D., Chen, D. J. and Ngo, F. (2001). Differential gene expression in a DNA double-strand-break repair mutant *xrs-5* defective in Ku80: Analysis by cDNA microarray. J. Radiat. Res. **42**: 371–385.
5. The *C. elegans* Sequencing Consortium. (1998) Genome sequence of the nematode *C. elegans*: a platform for investigating biology. Science **282**: 2012–2018.
6. Hartman, P. and Nelson, G. A. (1997) Processing of DNA Damage in the Nematode *Caenorhabditis elegans*. Chapter 24. *In:* DNA Damage and Repair. Biochemistry, Genetics, and Cell Biology. Eds., Hoekstra, M. F.& Nickoloff, J. A.. Humana Press, Inc., Totowa, N. J.: pp. 557–576.
7. Tawe, W., Eschbach, M.-L., Walter, R., and Henkle-Dührsen, K. (1998) Identification of stress-responsive genes in C*aenorhabditis elegans* using RT-PCR differential display. Nucleic Acids Res. **26**: 1621–1627.
8. Boulton, S., Gartner, A., Reboul, J., Vagio, P., Dyson, N., Hill, D. and Vidal, M. (2002) Combined functional genomic maps of the *C. elegans* DNA damage response. Science **295**: 127–131.
9. Jiang, M., Ryu, J., Kiraly, M., Duke, K., Reinke, V. and Kim, S. (2001) Genome-wide analysis of developmental and sex-regulated gene expression profiles in *C. elegans*. P.N.A.S. (USA) **98**: 218–223.
10. Kim, S. K., Lund, J., Kiraly, M., Duke, K., Jiang, M., Stuart, J., Eizinger, A., Wylie, B. and Davidson, G. S. (2001) A gene expression map for *Caenorhabditis elegans*. Science **293**: 2087–2092.
11. Epstein, H. and Shakes, D. (1995) *Caenorhabditis elegans*: Modern biological analysis of an organism. Methods in Cell Biology Vol. 48. Wilson, L. and Matsudaira, P., eds. Academic Press, San Diego
12. Coutrakon, G., Cortese, J., Ghebremedhin, A., Hubbard, J., Johanning, J., Koss, P., Maudsley, G., Slater, C. and Zuccarelli, C. (1997) Microdosimetry spectra of the Loma Linda proton beam and relative biological effectiveness comparisons. Med. Phys. **24**: 1499–1506.
13. ICRP. (1998) Clinical proton dosimetry part 1: Beam production, beam delivery and measurement of absorbed dose. International Commission on Radiation Units and Measurements, Bethesda, MD.
14. Zeitlin, C., Heilbronn, L. and Miller, J. (1998) Detailed characterization of the 1087 MeV/nucleon iron-56 beam used for radiobiology at the Alternating Gradient Synchrotron. Radiat. Res. **149**: 560–569.
15. Sherlock, G., Hernandez-Boussard, T., Kasarskis, A., Binkley, G., Matese, J., Dwight, S., Kaloper, M., Weng, S., Jin, H., Ball, C., Eisen, M., Spellman, P., Brown, P., Botstein, D. and Cherry, J. M. (2001) The Stanford Microarray Database. Nucleic Acids Research **29**: 152–155.
16. Reinke, V., Smith, H. E., Nance, J., Van Doren, C., Begley, R., Jones, S. J. M., Davis, E. B., Scherer, S., Ward, S. and Kim, S. (2000) A Global profile of germline gene expression in *C. elegans*. Molecular Cell **6**: 605–616.

Received on May 31, 2002
Revision on October 18, 2002
Accepted on November 1, 2002