Lawrence Chyall is currently enrolled at the University of California Berkeley where he is completing his baccalaureate studies in the Microbial Biology Department. He became interested in the biological sciences while working as a San Francisco maitre d'hotel and sommelier. His curiosity about the processes that govern life and his desire to help reduce human suffering inspired him to leave his career and enroll at San Francisco City College. After completing the prerequisite courses for the study of upper division undergraduate biology, Chyall undertook a summer internship at the Lawrence Berkeley National Laboratory. He has returned to LBNL for a second summer where he continues under the mentorship of Amy Kronenberg and Stacey Gauny. His scientific interests include radiation biology and carcinogenesis. He looks forward to graduate study in the life sciences and fulfilling his dream of serving others through science.

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Amy Kronenberg received her undergraduate degree in biology from Brown University where she did undergraduate research on Compton scatter radiography of bone. She then joined the Medical Physics group at Brookhaven National Laboratory starting with a DOE summer fellowship and continuing for several years thereafter working collaboratively with physicists and engineers to apply nuclear physics techniques to biological problems. She received her Sc.D. in Cancer Biology at the Harvard School of Public Health. Dr. Kronenberg joined the Lawrence Berkeley National Laboratory after completing her doctorate, where she has lead a vigorous research program for more than fifteen years. Her major research interests are the factors that regulate genomic stability, mechanisms of mutagenesis in human and rodent cells and tissues, and the connections between programmed cell death and DNA repair.

CHARACTERIZATION OF A TK6-BCL-X_L GLY-159-ALA HUMAN LYMPHOBLAST CLONE

LAWRENCE CHYALL, STACEY GAUNY, AND AMY KRONENBERG

ABSTRACT

TK6 cells are a well-characterized human B-lymphoblast cell line derived from WIL-2 cells. A derivative of the TK6 cell line that was stably transfected to express a mutated form of the anti-apoptotic protein Bcl-x_L (TK6-Bcl-x_L gly-159-ala clone #38) is compared with the parent cell line. Four parameters were evaluated for each cell line: growth under normal conditions, plating efficiency, and frequency of spontaneous mutation to 6-thioguanine resistance (hypoxanthine phosphoribosyl transferase locus) or trifluorothymidine resistance (thymidine kinase locus). We conclude that the mutated Bcl-x_L protein did not affect growth under normal conditions, plating efficiency or spontaneous mutation frequencies at the thymidine kinase (*TK*) locus. Results at the hypoxanthine phosphoribosyl transferase (*HPRT*) locus were inconclusive. A mutant fraction for TK6-Bcl-x_L gly-159-ala clone #38 cells exposed to 150cGy of 160kVp x-rays was also calculated. Exposure to x-irradiation increased the mutant fraction of TK6-Bcl-x_L gly-159-ala clone #38 cells.

INTRODUCTION

Shortly after Wilhelm Roentgen discovered the existence of ionizing radiation in the form of x-rays in 1895, the adverse effects of the "New Rays" became apparent. Radiation pioneers frequently suffered from epilations, erythemas, and painful desquamation as a result of occupational exposure to x-rays. The premature death of Clarence Dally (Thomas Edison's assistant in the manufacture of x-ray equipment) from lymphoma and the subsequent cancer deaths of other early radiation investigators led scientists to hypothesize that x-rays might be carcinogenic. It is now well known that exposure to ionizing radiation may lead to chromosomal damage, mutagenesis, and cancer. It has been thought that programmed cell death (apoptosis) is a means of limiting the number of mutations that can be accumulated by an organism and that dysregulation of apoptosis may be implicated in the multi-step process of carcinogenesis.

The intracellular protein Bcl-x_L helps limit apoptosis by titrating the concentration of the pro-apoptotic protein Bax through the formation of a heterodimer [1]. Cells overexpressing the protein Bcl-x_L better survive assaults on the genome that would otherwise lead to cell death. Cherbonnel-Lasserre *et al.* [2] found that human lymphoblastoid TK6 cells stably transfected with *pSFFV-neo-bcl-x_L* and overexpressing the Bcl-x_L protein survived x-irradiation in greater numbers with a significantly higher mutant fraction at an autosomal locus than TK6 cells transfected with the control vector, *pSFFV-neo*. It is, however, not currently understood if Bcl-x_L facilitates mutagenesis independently of its function in blocking apoptosis. Our group plans to investigate this question using a TK6-Bcl-x_L gly-159-ala clone that expresses a mutated form of Bcl-x_L protein (Bcl-x_L gly-159-ala) that cannot dimerize with Bax to inhibit the pro-apoptotic activity of Bax. The Bcl-x_L gly-159-ala protein is not expected to inhibit radiation-induced apoptosis, however it

may still lead to increased levels of radiation-induced mutations if the pro-mutagenic effect requires a portion of the protein that does not include amino acid 159.

TK6 is a well characterized human cell line derived from WIL-2 cells with a known mutation that disables one copy of the thymidine kinase (*TK*) gene located on chromosome 17 [3, 4]. The other (active) copy of the *TK* gene is a useful genetic locus to study mutations at a model heterozygous autosomal locus. Cells that survive growth in medium containing trifluorothymidine (TFT) have been shown by others to be deficient in thymidine kinase [4]. Additionally, TK6 is a male cell line that is hemizygous for the hypoxanthine phosphoribosyl transferase gene (*HPRT*) on the X chromosome, providing another useful locus to study mutations. It has been shown by others that cells deficient in the HPRT protein survive growth in medium containing 6-thioguanine (6-TG) [4].

This paper reports on the growth properties and spontaneous mutant frequencies at the *TK* and *HPRT* loci of TK6-Bcl-x_L gly-159-ala clone #38 cells and compares them to similar data acquired for the parent TK6 cell line. In addition, the *TK* mutant fraction of TK6-Bcl-x_L gly-159-ala clone #38 cells exposed to 150 cGy of 160 kVp x-rays is compared to the *TK* mutant fraction of non-irradiated cells from the same cell line.

MATERIALS AND METHODS

Cells and cell culture conditions

The TK6-Bcl-x_L gly-159-ala clone #38 cell line was established in the Kronenberg laboratory from the parent TK6 cell line by stably transfecting TK6 cells with *pSFFV-neo-bcl-x_L-gly-159-ala* (kindly provided by S. Korsmeyer of the Dana Farber Cancer Institute and G. Nunez of the University of Michigan). Each cell line was grown in 100ml suspension cultures using T-175 culture flasks and RPMI 1640 medium + L-glutamine supplemented with 10% heat-inactivated horse serum and 50units/ml penicillin + 50µg/ml streptomycin (all from Gibco Laboratories, Grand Island, NY). Cultures were maintained at 37˚C in a humidified 5% CO_2 atmosphere. Cells were counted Monday through Friday using a Coulter Counter and diluted as necessary to a density of either 2 X 10^4 cells/ml (Fridays) or 4 X 10^5 cells/ml (Monday through Thursday) to keep them in exponential growth.

Growth curve

Growth curves were set up for TK6 and TK6-Bcl-x_L gly-159-ala #38 cells. Measurements of the cell density of 100ml cultures, each set up with an initial density of 1.0 X 10^4 cells/ml, were made over a six-day period. To avoid nutrient starvation or hypoxia, cells were diluted as necessary to 4.0 X 10^5 cells/ml. The growth of the two cells lines was compared by graphing cell density as a function of time on a semi-log plot.

To assess the effect on growth rate when TK6 cells are maintained at higher densities, a parallel TK6 culture was established at a density of 3.1 X 10^5 cells/ml after 3 days of standard growth under the conditions described above. This culture was not diluted further during the 6-day observation period. Cell density was measured on day 6 and growth was compared with the TK6 cells maintained in exponential growth throughout the experiment.

Plating efficiency

Plating efficiency (PE) is defined as the probability that a single cell will form a colony. The plating efficiency of each cell line was determined using a method of limiting dilution in 96-well plates [5]. Cells were diluted to a final density of 5 cells/ml and 200µl of cell suspension was seeded in each well using an 8-channel pipettor. This corresponds to delivering an average of one cell per well. Plates were incubated at 37˚C in a humidified 5% CO_2 atmosphere for 11 days. Following incubation, plates were visually inspected for the presence of macroscopic colonies. Wells without any apparent colony growth were counted as negative. Wells with evidence of growth may contain one or more individual colonies that are difficult to discriminate and were counted as positive for growth. Plating efficiency was calculated using the formula:

$$PE = -\ln \frac{\chi_0}{\eta_0}$$

χ_0 = number of negative wells
η_0 = number of total wells

Cleansing the population for pre-existing TK and HPRT mutants

The spontaneous mutant fraction (MF) at either the *TK* or *HPRT* locus was determined according to previously described methods [4, 5]. Cells without functional TK or HPRT proteins ($TK^{-/-}$ or $HPRT^-$ cells) were removed from the population by treating the culture for two days with normal growth medium supplemented with CHAT (10µM deoxycytidine, 200µM hypoxanthine, 0.2µM aminopterin, and 17.5µM thymidine, all from Sigma Chemical, St. Louis, MO). Following two days of treatment, the cells were spun out of the CHAT medium at 1000rpm for 5 minutes and re-suspended in standard medium supplemented with THC (17.5µM thymidine, 200µM hypoxanthine, and 10µM deoxycytidine). Cells were maintained in exponential growth for two additional days.

Selection for spontaneous HPRT-deficient mutants

Two days after re-suspension in medium supplemented with THC, cell cultures were diluted to 2 X 10^5 cells/ml and treated with 0.5µg/ml 6-thioguanine (6-TG, Sigma Chemical, St. Louis, MO). Cells were seeded in 96-well plates in 200µl of cell suspension per well, corresponding to 40,000 cells/well. Plates were incubated for 11 days under the conditions described above and inspected for the presence of macroscopic colonies.

Selection for spontaneous TK-deficient mutants

Cell cultures were diluted to 2 X 10^5 cells/ml following two days of growth in medium supplemented with THC. The cell cultures were treated with 2.0µg/ml triflurothymidine (TFT, Sigma

Chemical, St. Louis, MO) and seeded in 96-well plates at a density of 40,000 cells/well. To determine the plating efficiency, an aliquot of each culture was seeded in 96-well plates at low density in the absence of TFT. Following 11 days incubation under the above described conditions, plates were examined for the presence of macroscopic early-arising mutant colonies. Cells were re-fed with TFT by adding 20µl of growth medium supplemented with triflurothymidine (22.0µg/ml) to each well. Plates were returned to the incubator for an additional 7 days. On day 18 the plates were re-examined for the presence of late-arising *TK*-mutant colonies.

Selection for radiation-induced TK-deficient mutants in TK6-Bcl-x_L gly-159-ala clone #38 cells

Cell cultures were diluted to a density of 4 X 10^5 cells/ml one day after re-suspension in THC-supplemented medium. On the following day, samples were exposed to 150 cGy of x-rays using a 160 kVp Pantak Mk II x-ray generator. Unirradiated control cultures were maintained in parallel. Following irradiation, cultures were split to a density of 5 X 10^5 cells/ml and returned to the incubator for three days to allow expression of the *TK* mutant phenotype. Cells were split daily to prevent overgrowth. Cells were treated with TFT, plated, and scored for early-arising and late-arising mutant colonies as described above.

Calculation of mutant fraction

The mutant fraction (MF) at a given locus is defined as the ratio of mutants detected at a given time. The MF was calculated using previously described methods [5]. Microtiter plates were inspected for the presence of macroscopic colonies and the number of wells without apparent colonies was recorded. MF was calculated using the following formula:

$$MF = \frac{-\ln(\chi_s/\eta_s)}{-\ln(\chi_0/\eta_0)} \bullet \frac{(total\ cells/well\ of\ non-selective\ PE\ plates)}{(total\ cells/well\ of\ selective\ mutation\ plates)}$$

χ_0 = number of empty wells in non-selective conditions
η_0 = total number of wells in non-selective conditions
χ_s = number of empty wells in non-selective conditions
η_s = total number of wells in non-selective conditions

RESULTS

Growth curve

Figure 1 shows growth curves for the TK6 and TK6-Bcl-x_L gly-159-ala clone #38 cell lines. Each cell line grew exponentially with a doubling time of approximately 15 hours when maintained at a density of between 1 X 10^4 cells/ml and 1.2 X 10^6 cells/ml. The growth curve of TK6 cells grown without dilution is displayed on Figure 2. After six days, the total number of cells of this culture was 2.34 X 10^6 cells/ml compared with 1.19 X 10^7 cells/ml for the culture maintained under standard conditions, indicating that the culture maintained at a higher density grew at a slower rate.

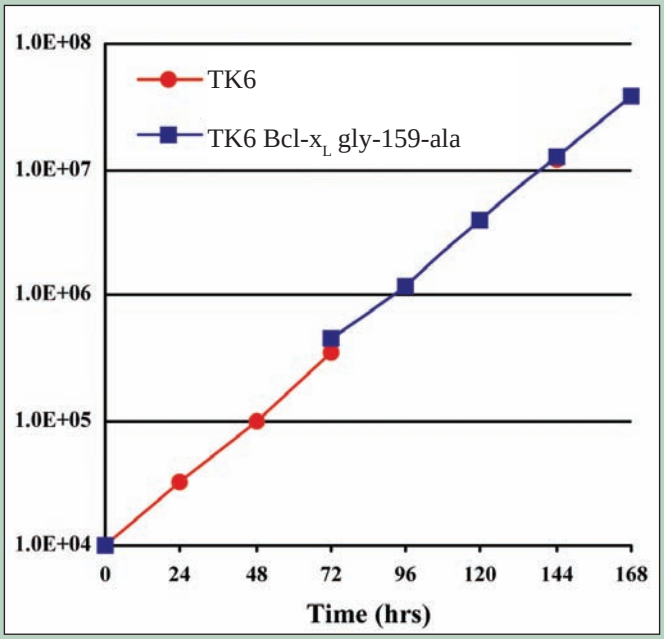

Figure 1. Growth rate comparison of TK6 cells and TK6 Bcl-x_L gly-159-ala clone #38 cells.

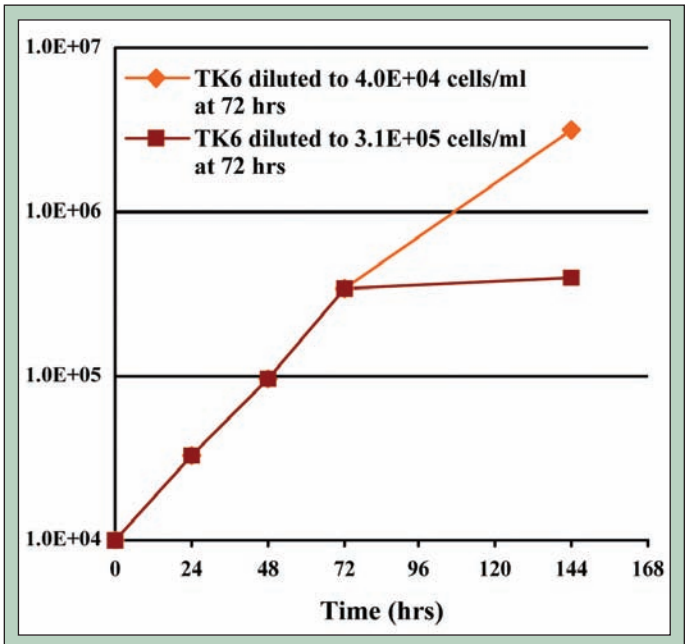

Figure 2. Growth rate comparison of TK6 cells maintained at normal density and high density.

Plating efficiency

Plating efficiency (PE) data for each cell line is given in Table 1. TK6 cells were found to have a mean PE of 62.8% (±10.2%). TK6-Bcl-x_L gly-159-ala #38 cells displayed a mean PE of 72.5% (±3.7%). A two tailed t-test (t= 1.84, p=0.116) showed no difference between the means.

TK6			TK6-pSFFV- Bcl-x_L gly-159-ala clone #38		
Sample	no. of plates	PE %	Sample	no. of plates	PE %
A	2	47.8	A	2	74.7
B	2	67.3	B	2	68.3
C	2	70.3	C	2	74.7
D	4	65.8			
mean PE = 62.8% ± 10.2%			mean PE = 72.5% ± 3.7%		
t= 1.84, p= 0.116, two tailed t-test					

Table 1. Plating efficiencies of TK6 cells and TK6 Bcl-x_L gly-159-ala clone #38 cells.

Spontaneous HPRT-deficient mutants

No mutant colonies were observed among the cells plated in the presence of the selective agent 6-TG. A significant amount of debris was observed in all of the wells prohibiting the scoring the wells as either positive or negative. The experiment did not produce useful results.

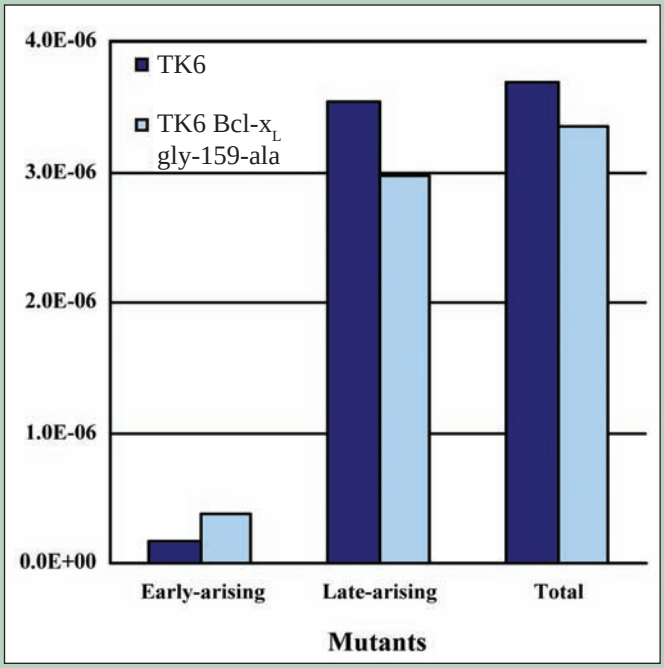

Figure 3. Comparison of spontaneous mutant fractions at the *TK* locus in TK6 cells and TK6 Bcl-x_L gly-159-ala clone #38 cells.

Spontaneous TK-deficient mutants

Figure 3 displays the TK mutant fraction in untreated TK6 and TK6-Bcl-x_L gly-159-ala #38 cells. The mutant fraction for early-arising TK-deficient mutants was 1.78 X 10^{-7} for TK6 cells and 3.71 X 10^{-7} for TK6-Bcl-x_L gly-159-ala #38 cells. TK6 cells had a late-arising mutant fraction of 3.54 X 10^{-6} compared with 2.97 X 10^{-6} for TK6-Bcl-x_L gly-159-ala #38 cells. The total mutant fraction (combined early-arising and late-arising) for TK6 cells was 3.69 X 10^{-6} and the total mutant fraction for TK6-Bcl-x_L gly-159-ala #38 cells was 3.34 X 10^{-6}. TK6-Bcl-x_L gly-159-ala #38 cells had a similar

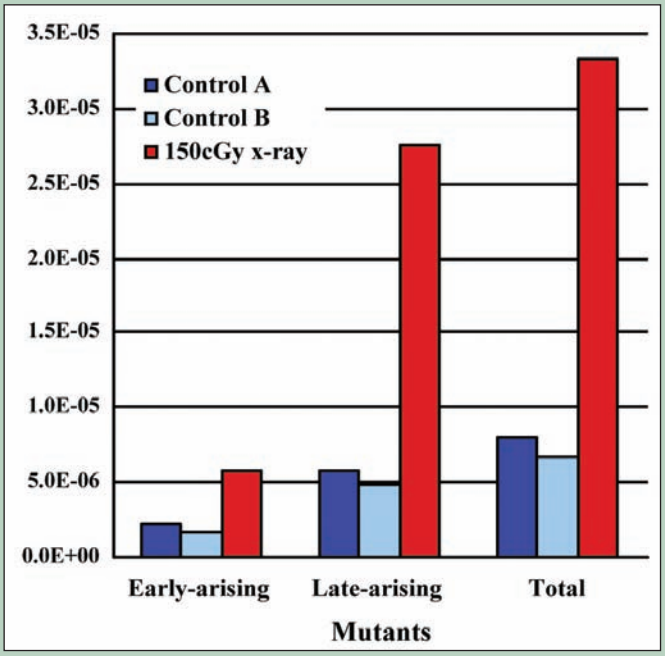

Figure 4. Radiation induces mutations at the *TK* locus in TK6 Bcl-x_L gly-159-ala cells.

MF to TK6 cells, with a similar proportion of early-arising and late-arising mutant colonies. No statistical analysis can be done since these data represent the results of a single experiment.

Radiation-induced TK-deficient mutants

Figure 4 summarizes the radiation-induced mutant fraction at the *TK* locus for TK6-Bcl-x_L gly-159-ala #38 cells. The early-arising MF for TK6-Bcl-x_L gly-159-ala #38 cells exposed to 150cGy of 160 kVp x-rays was 5.85 X 10^{-6}. Two unirradiated control cultures had early arising MF's of 2.15 X 10^{-6} and 1.75 X 10^{-6} respectively. The late-arising TK MF was 2.75 X 10^{-5} for irradiated cells, while the values for the two control cultures were 5.86 X 10^{-6} and 4.88 X 10^{-6}. Total MF (combined early-arising and late-arising) for irradiated cells was 3.34 X 10^{-5}. One control culture had a total MF of 8.01 X 10^{-6} while the second control culture had a total MF of 6.63 X 10^{-6}. Late-arising mutant colonies compromised the bulk of the observed mutants and x-irradiation increased the mutant fraction approximately five-fold above the control levels.

DISCUSSION AND CONCLUSIONS

TK6-Bcl-x_L gly-159-ala clone #38 cells behaved similarly to the parent TK6 cell line when cultured under normal conditions. Growth curves indicated that the two cell lines grew exponentially when diluted to optimal concentrations. It is noteworthy that growth of the parallel culture of TK6 cells slowed when the cell density exceeded 1 X 10^6 cells/ml. This may have been the result of hypoxia, nutrient depletion, an accumulation of waste products, or a combination of these events. It is reasonable to anticipate that TK6-Bcl-x_L gly-159-ala clone #38 cells would also grow non-exponentially when maintained in an over-dense environment, given the similarity of growth patterns of the TK6-Bcl-x_L gly-159-ala clone #38 cells to

the parent TK6 cell line. No statistically significant difference was found in the plating efficiencies of the two cell lines. A single cell from either cell line is, therefore, equally likely to form a colony.

The inconclusive findings for the selection for HPRT-deficient mutants may be attributable to two possible causes. One possibility is that the presence of an unusually high amount of debris (arising from dead cells) in wells obscured any mutant colonies that formed. A second possibility is failure of the selective agent 6-TG. An insufficient concentration of active 6-TG would have enabled $HPRT^+$ cells to survive 6-TG treatment, potentially resulting in the observed debris. Based on the clear results obtained from the plates treated with TFT, we do not believe that the high background on the 6-TG containing plates is due to failure of the aminopterin "pre-cleaning" procedure. Aminopterin blocks the *de novo* synthesis of thymidine by competitively binding with dihydrofolate reductase, thus inhibiting the reduction of dihydrofolate to tetrahydrofolate (a molecule required for the methylation of deoxyuridate to thymidine and many other one carbon transfer reactions). Cells treated with aminopterin cannot synthesize DNA by the *de novo* pathway. Only cells producing functional salvage pathway proteins (i.e. cells with at least one functional copy of the *TK* and *HPRT* genes) will be able to synthesize DNA and live in the presence of aminopterin. Thus, if the aminopterin had failed then we would expect to have seen a high background in both the 6-TG containing plates and the TFT containing plates.

Spontaneous mutant fractions for TK-deficient mutants were similar in both cell lines. In addition to similar total mutant fractions, both cell lines displayed a similar pattern of distribution between early-arising and late-arising colonies (the majority of mutant colonies were of the late-arising type for both cell lines). The frequency of spontaneous mutations at the *TK* locus of the TK6 cells does not appear to have been changed by transfecting the parent cell line with the *pSFFV-Bcl-x_L gly-159-ala* plasmid. TK6-Bcl-x_L gly-159-ala clone #38 cells were mutated by x-rays, displaying an elevated mutant fraction at the *TK* locus when exposed to 150cGy of x-rays. This data is consistent with earlier results noted by the Kronenberg group.

In summary, the growth under normal conditions and plating efficiency of the TK6-pSFFV-Bcl-x_L gly-159-ala clone #38 mirrors the parent TK6 cell line. Both cell lines undergo spontaneous mutation at the *TK* locus with a similar frequency. No conclusions could be reached about the spontaneous mutant fraction at the *HPRT* locus. Exposure of the TK6-pSFFV-Bcl-x_L gly-159-ala clone #38 cells to x-rays led to an increase in the TK mutant fraction as compared to the unirradiated controls.

ACKNOWLEDGEMENTS

This work was supported by the Director, Office of Science, Office of Science and Workforce Development, of the U.S. Department of Energy under contract No. DE-AC02-05CH11231. The patient and kind mentorship of Amy Kronenberg, Stacey Gauny, and all of the scientists and staff at the Kronenberg lab (Xing Kwoh, Caren Oto and Hiroko Sudo) is gratefully acknowledged. Additionally, heartfelt thanks is offered to the Lawrence Berkeley Laboratory Center for Science and Engineering Education program staff and to Philip Jardim of San Francisco City College.

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