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ISBN 0-315-60596-0

UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

ACKNOWLEDGEMENTS

I extend my sincere gratitude to all those who have contributed to this thesis. My experiments were conducted at Atomic Energy of Canada Limited, Chalk River Nuclear Laboratories (CRNL), an excellent research facility. I thank CRNL and the Radiation Biology Branch for allowing me to utilize their resources and for providing financial support towards my graduate education. Special thanks to my two summer students Sheila Broughton and Yvonne Haas for expert technical assistance.

Although my co-supervisor Dr. Pearl Weinberger was overcome by serious illness during the course of this research, she nevertheless made significant contributions towards my success. I thank Dr. Weinberger for her support and wish her future health. Furthermore, I acknowledge the efforts of my graduate committee members and thank Drs. G.P. Raaphorst, D. Currie, and B. Hollebone for their time and effort.

Personal funding was provided through scholarships granted by the Natural Sciences and Engineering Research Council of Canada.

J.D. Knox was a fellow graduate student and friend. He has a unique character which has furnished many unforgettable memories.

I thank my co-supervisor Dr. Ron Mitchel who is an exceptional scientist and possessed all the qualities of a superb graduate supervisor. Ron Mitchel furnished exquisite guidance and for that I am in debt.

Finally, there was always my family and loving wife "Bugs".

Douglas R. Boreham

THE MECHANISM OF INDUCED RADIATION RESISTANCE

ABSTRACT

The present work investigated the mechanism of induced radiation resistance. Experiments were designed to identify the nature of lesions that induce this mechanism and the cellular components which regulated its expression. The investigation adopted the use of two well suited unicellular eukaryotes, the yeast *Saccharomyces cerevisiae* and the green alga *Chlamydomonas reinhardtii*. DNA recombinational repair capacity induced by DNA damage was believed to be the system that conferred resistance to killing by radiation in yeast. The nature of the radiation generated DNA lesions that induce this mechanism have been examined using the two different endpoints of resistance to cell killing and suppression chemical mutation.

This work has demonstrated that, in yeast, DNA lesions produced by low-LET (Linear Energy Transfer) γ-rays were more efficient at inducing radioresistance and mutation suppression than lesions produced by high-LET neutrons. It was inferred that DNA single strand breaks were important inducing lesions. Oxygen modifiable lesions and hydroxyl radicals (OH•) were also shown to be particularly efficient inducers of this repair mechanism. It was concluded that radiation induction of DNA repair depends not only on a variable induction response to different DNA lesions, but also on the availability of the induced repair system to deal with subsequent damage.

Heat shock was previously known to induced thermal tolerance and radiation resistance in yeast. In this thesis yeast mutants deficient in topoisomerase I, in topoisomerase II, or in DNA polymerase I were used to further investigate the possible involvement of these enzymes in the mechanism of induced thermal tolerance or radiation resistance. Results indicated that the actual systems that confer incresed resistance to heat or radiation were independent of either topoisomerase activity or DNA polymerase function but suggested, however, that topoisomerases may have a regulatory role during the signalling of these mechanisms. Experiments suggested that maintenance of correct DNA topology may prevent induction of the heat shock response, and that heat shock induction of a component of the full radiation resistance in yeast may be the consequence of topoisomerase I inactivation. DNA polymerase I did not seem to have a role in the thermal tolerance or radioresistance mechanism in yeast.

Previous literature cast doubt on the concept that DNA damage signals the radioresistance mechanism in *C. reinhardtii*. Also, heat shock-induced radioresistance (DNA repair and mutation suppression) was untested in *C. reinhardtii*. This work has demonstrated that synchronized G_1 or G_2 *C. reinhardtii* cells were neither radiosensitized nor did they become radioresistant in response to heat shock. However, synchronized G_1 or G_2 algal cells had a normal heat-induced thermotolerance response. Also, it was known that radiation, heat shock, or cycloheximide suppressed chemically induced mutations in yeast. Results from this work indicated that neither radiation, heat shock, nor cycloheximide suppressed chemical mutations in algae. This thesis demonstrates that the DNA repair capacity of *C. reinhardtii* was not altered by heat shock and in this organism

chemical mutations were not a consequence of an induced error-prone repair system.

In the present work it was shown that heat shock was capable of inducing only partial radioresistance in wild type yeast cells while radiation induced full radioreesistance. Dot blot analysis of the *RAD52* epistasis group revealed that heat shock increased expression of only *RAD52* transcripts whereas radiation increased expression of *RAD52, 54, and 57* therefore demonstrating differential induction of repair genes by heat shock or radiation. Yeast mutants deficient in recombinational repair (*rad52*) were radiosensitive and neither heat shock nor radiation exposure could induce radioresistance. Nonetheless these mutants exhibited enhanced expression of *RAD51, rad52, RAD54, 55, and 57* transcripts after either heat shock or radiation exposure. It was inferred that the inducible, non-functional *rad52* gene product was incapable of conferring radioresistance (repairing DNA damage) and, as a consequence, additional repair genes may have subsequently been induced in an unsuccessful attempt to repair the residual damage.

LE MECANISME DE L'INDUCTION DE RESISTANCE A LA RADIATION

L'ABSTRAIT

Ce travail a investigé le mécanisme de l'induction de résistance à la radiation. Les expériences ont été conçus pour identifier la nature des lésions qui incitent ce mécanisme et les constituantes cellulaire qui règlent son expression. L'investigation a adopté l'usage de deux eukaryotes unicellulaire très convenable, la levure Saccharomyces cerevisiae et l'algue verte Chlamydomonas reinhardtii. La capacité pour la réparation recombinational de l'ADN inciteé par le dommage dans l'ADN est le système qui accorde à la levure la résistance à la mort par la radiation. La nature de ces lésions d'ADN générées par la radiation, qui incitent ce mécanisme, a été examiné en utilisant deux limites différentes à la résistance de la mort cellulaire et à l'abilitée du système de réparation recombinational pour réprimer la mutation chimique.

Ce travail a demontré que, dans la levure, les lésions d'ADN produient par les rayons-γ de bas-TEL (Transfert d'Energie Lineaire) fût plus éfficace dans l'induction de radiorésistance et la suppression de mutation que les lésions produient par les neutrons de haut-TEL. Ceci amène a conclure que l'induction de réparation dans l'ADN par la radiation dépends sur une réponse d'induction variable, et sur la réparation du dommage resultant.

Dans ce thèse, les mutants de la levure défèctueux en topoisomerase I, en topoisomerase II ou en le polymerase d'ADN I ont été utilisés pour investiger la participation possible de ces enzymes dans le mécanisme de tolérance thermal ou de résistance a la radiation. Les expériences ont

suggérées que l'entretein de la topologie de l'ADN peut prévenir l'induction de la réponse à l'hyperthermie, et que l'induction de résistance à la radiation par l'hyperthermie dans la levure peut être une conséquence de l'inactivation de topoisomerase I. Le polymerase I d'ADN ne semblait pas avoir le même rôle dans la tolérance thermal ou dans le mécanisme de radiorésistance dans la levure.

La réparation d'ADN incitée par l'hyperthermie (radiorésistance et la suppression de mutation) n'a pas été mis à l'épreuve avec C. reinhardtii. Ce travail a demontre que les cellules de C. reinhardtii synchroniser en G1 ou G2 ne fût pas sensibiliser à la radiation ni résistant à la radiation par l'hyperthermie. Aussi, les résustats de ce travail ont démontrés que ni la radiation, ni l'hyperthermie, ni cycloheximide ont éliminés les mutations chimique dans l'algue. Ce thèse démontre que la capacité de l'ADN de C. reinhardtii pour réparer n'a pas été affecter par l'hyperthermie et dans cet organisme les mutations chimique ne fût pas une conséquence de l'induction du systeme de réparation defectueux.

L'analyse de "dot blot" du groupe de gène RAD52 a révelé que l'hyperthermie augmente l'expression des transcriptions RAD52 seulement tandis que la radiation augmente l'expression de RAD52, 54 et 57 donc expliquant l'induction différentiel des gènes de réparation par l'hyperthermie ou par la radiation.

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THE MECHANISM OF INDUCED RADIATION RESISTANCE

A.1 GENERAL INTRODUCTION

Most people fear radioactivity and associate it with the fallout of atomic bombs or disasters such as the explosion at the Chernobyl nuclear power station. In fact, radioactivity or ionizing radiation is present everywhere in the environment. All living organisms, including man, are continuously irradiated on a daily basis and have been since the evolution of life. Our bodies actually contain several different radionuclides thereby making us natural sources of radiation. It is reasonable to expect therefore, that living organisms must have evolved cellular defence mechanisms able to adequately cope with the effects of ionizing radiation (1,2).

Excessive exposure to ionizing radiation, as with most other natural substances, can be harmful to living organisms. Ionizing radiation is known to exert its harmful biological effects primarily as a consequence of interacting with, and structurally altering, DNA (3). Diverse types of DNA damage are produced including single strand breaks, various kinds of base damage including loss of a base, sugar damage, cross-linking of strands, strand to protein cross-links and double strand breaks (4). It is important to note however, that exposure to ionizing radiation in particular, produces no unique biological consequences. Rather, its action is to add to the frequency of occurrence of a wide variety of phenomena that naturally occur in living populations (5). In this investigation ionizing radiation was used as a tool to examine certain aspects of DNA repair.

The effects of ionizing radiation are difficult to assess since biological variation within different organisms alters the consequences of radiation damage. It is known that there is considerable variation between organisms and cells with respect to radiation resistance (5). Current data indicate that the risk of genetic damage from radiation is likely a function of cellular DNA repair capacity (6). Accordingly, it is probable that certain individuals will demonstrate variable responses to ionizing radiation. There are several known enzymatic repair systems that respond to ionizing radiation (7). It is interesting to consider the idea that stimulation or induction of DNA repair systems may confer cellular resistance to ionizing radiation. Therefore the possibility exisits that very low doses of ionizing radiation may not be harmful, it may even have a net benefit effect (8). This phenomenon is known as *hormesis*, and was first described in pharmacology as stimulation resulting from a low-level exposure to a substance that was toxic at high levels (7). The overall hypothesis of this research adopts this concept and postulates that resistance to harmful environmental agents can be induced by prior low doses of certain agents. Therefore, reduction in human susceptability or risk to DNA damage may be achieved by induction of cellular resistance mechanisms. Environment together with specific aspects of lifestyle probably influence the level of induced resistance expressed in each individual.

Within a cell there are several discrete DNA repair mechanisms capable of repairing the various types of DNA damage produced by ionizing radiation. The lethal effects of ionizing radiation, however, are very closely correlated with, and perhaps solely the consequence of unrepaired

DNA double strand breaks (9). The induction of resistance to the lethal effects of ionizing radiation is therefore considered to be an increase in a cell's capacity to repair double strand breaks (10). Currently, the only known DNA repair system capable of efficiently repairing DNA double strand breaks (DSB) is the recombinational repair mechanism (11). Accordingly, the investigation of inducible radiation resistance was thus a study of DNA recombinational repair. Therefore the three main objectives of this investigation were to distinguish the nature of the signal that induces DNA recombinational repair, to determine the cellular components that regulate this response, and to identify some of the genes that are expressed in this mechanism.

DNA recombinational repair involves strand exchange between chromosomes and is implicated in double-strand break repair (12). When a DSB occurs genetic information is typically lost. A duplicated source of genetic information exists within homologous chromosomes or sister chromatids. During DNA recombinational repair, this duplicate information can be used to repair the damaged DNA duplex. This repair process has a relatively low error frequency and has been called error-free repair (12).

One modern genetic strategy available to study cellular repair mechanisms is the exploitation of mutant organisms. This research has used two microorganisms well suited for genetic, biochemical and molecular investigations. The selection of these organisms was due in part to the simplicity of preparing large quantities of cells and in part to the powerful genetic and molecular techniques that have been developed for these microbes. This research has employed seven different strains of the budding brewers yeast *Saccharomyces cerevisiae* (Plate I, A and B) and two

strains of the unicellular green alga *Chlamydomonas reinhardtii (Plate I, C and D)*. The many virtues of using these organisms as experimental models have been reviewed (13-16).

Ionizing radiation is a common environmental stress and a cell's response to radiation exposure could be deemed a "stress response". Interestingly, in most organisms, various environmental stresses have been shown to elicit a common characteristic response known as the heat shock response or stress response (reviews; 17,18). It was noted that a prior sublethal heat shock caused a cell to become resistant to a second, normally lethal, heat exposure. Heat shock response has received considerable inquiry and is currently used as a powerful tool to study many aspects of gene regulation. Conveniently heat shock also induced radiation resistance in several types of cells including yeast (19,20). In this research heat shock response was utilized as another tool to investigate the mechanism of induced radiation resistance. At the same time, interesting aspects of heat shock response in yeast were resolved.

The presentation of the experimental results was arranged in four separate chapters. Chapter 1 reports experiments which explored the nature of DNA lesions that signal the induction of radioresistance (recombinational repair). Several techniques were used to modify the type of DNA damage introduced into the cell in order to determine how various types of lesions differentially induced the mechanism. The results

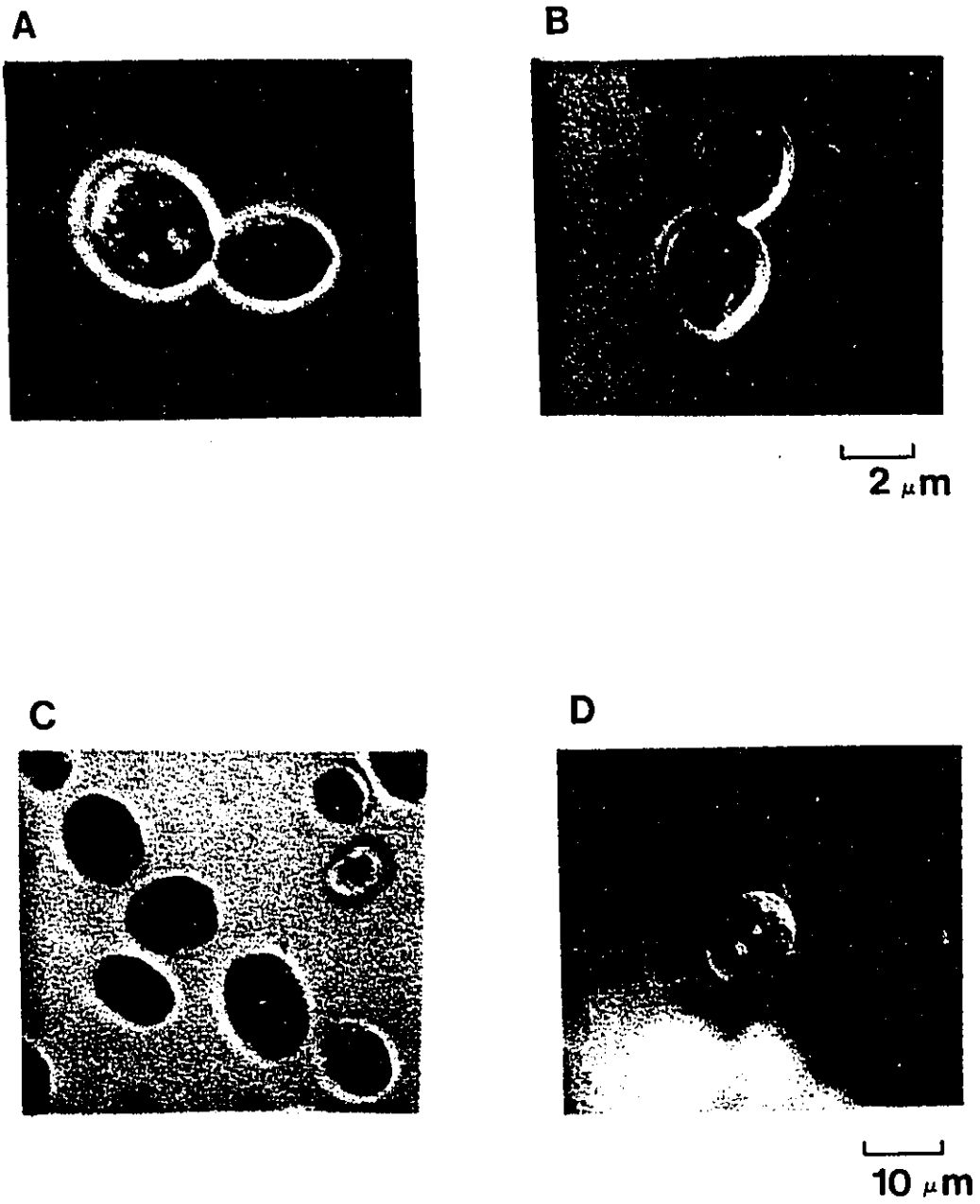

Plate I. Micrographs of *Saccharomyces cerevisiae* (A and B) and *Chlamydomonas reinhardtii* (C and D) taken with phase contrast (A), bright field (C), or Nomarski differential interference-contrast microscopy (B and D)(21).

suggested that perhaps perturbation to DNA tertiary structure was an important signal involved in regulating the expression of this mechanism.

In yeast, evidence indicates that error-prone DNA repair systems are present and these systems are inducible (22). The chemical mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) was shown to induce error-prone repair in yeast (23). MNNG is a mutagen and carcinogen that methylates DNA at a variety of sites including the N-7 of guanine, the N-3 of adenine, the phosphodiester backbone, and the O-6 of guanine (24). O^6-methylguanine (O^6-MG) is suggested to be the primary lesion responsible for the mutagenicity and carcinogenicity of this compound (25). O^6-MG lesions alter the hydrogen bonding properties of guanine thereby causing it to base pair during replication with thymine rather than cytosine, leading to GC to AT mutational transitions (25).

Ionizing radiation was shown to suppress mutation resulting from MNNG-induced error-prone repair. It was suggested that MNNG lesions are recognized by both the recombinational repair system and the inducible error-prone system (26). Ionizing radiation induced error-free repair and resulted in increased competition for the lesions, thereby reducing mutation. In this thesis suppression of MNNG-generated mutation has been used as an indicator of induced error-free repair (recombinational repair) in yeast (Chapter 1) and in algae (Chapter 3).

DNA topoisomerases are nuclear enzymes that control and regulate DNA tertiary structure or topology (references see Chapter 2). In Chapter 2, experiments were conducted using yeast mutants deficient in DNA topoisomerases to test if changes to normal DNA topological regulation would alter the radioresistance response. Specific differences between

the various topoisomerase mutants implied that changes to normal topology were involved in regulating the mechanism of radioresistance. The involvement of DNA polymerase I is also reported in this chapter and the experiments revealed that this repair enzyme has an insignificant role in this mechanism.

Chapter 3 reports the results of a comparison between particular features of yeast induced radioresistance and aspects of induced radioresistance in *C. reinhardtii*. The results demonstrated that significant differences exist between these two organisms which suggested that the mechanism of radioresistance might also be distinctive in other organisms.

In the final chapter, experiments are reported which attempted to identify certain genes associated with the induction of this response in yeast. Alteration to several different genes was known to vary the radiation sensitivity of yeast cells. It was suspected that these were repair genes and that high levels of expression would accompany cellular radioresistance. Chapter 4 reports that elevated expression of certain repair genes in wild type yeast is indeed concomitant with an increase in radioresistance. In a similar trial using recombinational repair deficient mutants, which do not demonstrate inducible radioresistance, differential gene expression was noted. The experiments in Chapter 4 produced significant information with respect to genetic regulation of the radioresistance mechanism. In fact, the results warrant further extensive molecular investigation.

In summary, this thesis addresses the mechanism of induced radioresistance by investigating the nature of the DNA lesions that induce

the response, the enzymes involved in regulating expression of the mechanism, and the genes responsible for conferring radioresistance. A comparison of inducible radioresistance between two distinct organisms was conducted, revealing significant evolutionary divergence. Important aspects of the heat shock response were also uncovered.

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CHAPTER ONE

DNA LESIONS THAT SIGNAL THE INDUCTION OF RADIORESISTANCE AND DNA REPAIR.

1.1 INTRODUCTION

Evidence is accumulating that both prokaryotic and eukaryotic organisms possess inducible repair systems which confer cellular resistance to DNA damaging agents (1-4). The yeast *Saccharomyces cerevisiae* is known to have several genetically discrete DNA repair processes (5). In yeast, it has been shown that specific genes are selectively induced in response to several types of DNA damaging agents (6-10). Of considerable interest are the genes responsive to ionizing radiation type damage and the genetic mechanism for radiation induced DNA repair and radioresistance. It was previously demonstrated that radioresistance in yeast, (due to recombinational repair capacity), is induced by exposure to a variety of stresses including γ-irradiation (11) and heat shock (12,13). In yeast, recombinational repair not only repairs radiation-induced DNA damage, it also successfully processes lesions produced by other chemical agents (4,14). Therefore induction of this repair system alters not only radioresistance but also sensitivity to chemical agents. The ability to regulate cellular DNA repair capacity supports the hypothesis that induced repair is a conserved evolutionary response intended to protect organisms from environmental damage.

The present work uses two distinct endpoints to examine the mechanism by which radiation-induced radioresistance (error-free recombinational repair) developes in yeast. From the standpoint of cell

killing, the induction of radiation resistance is a phenomenon whereby cells given a sublethal radiation exposure develop increased resistance to a subsequent normally lethal radiation dose. Presumably induction of the genes that confer resistance results from specific signals that recognize DNA damage (6-10). In light of the evidence that recombinational repair is essential for this mechanism (12), that DNA double strand breaks (DSB) contribute significantly to lethality (15,16), and that recombinational repair seems to be the only system capable of repairing DSBs (17), one might deduce that DSBs "signal" the induction mechanism (see Figure 1.1). Using this rationale radiation quality was varied in an attempt to alter the spectrum of DNA damage - in particular, the relative amount of DSBs - and assessed the degree of resistance that subsequently developed. Since the DSB/SSB ratio increases with LET (18, see Figure 1.2), it was postulated that high-LET neutrons would produce a stronger induction signal compared to the same dose of low-LET γ-rays. We found that this was not so.

Additionally, experiments were designed to examine the signalling efficiency of lesions produced by OH• at inducing the mechanism for radioresistance to cell killing , and present evidence that OH• derived lesions act as an induction signal. As well as cell killing by radiation as an endpoint, an alternative mutational endpoint was investigated. We monitored the ability of the induced recombinational repair system to compete for chemically (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG)) generated DNA lesions and to suppress mutation due to those chemical lesions (4). We again compared high- and low-LET radiation

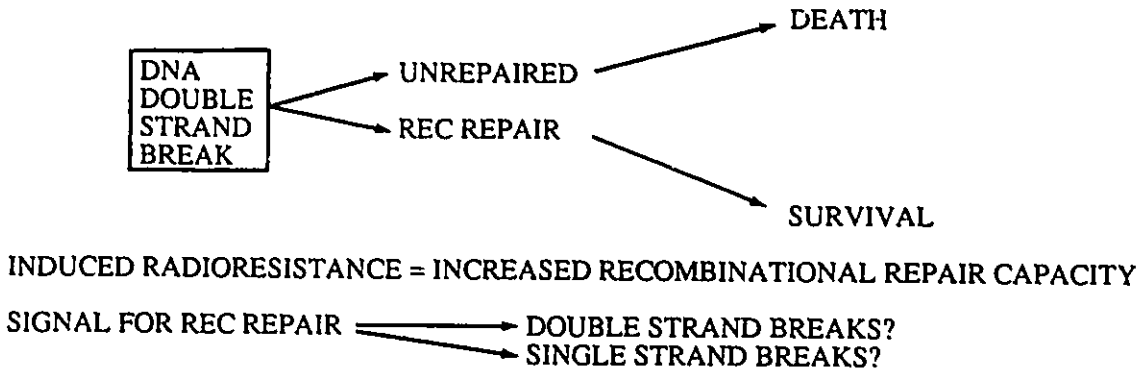

Figure 1.1. Unrepaired DNA double strand breaks (DSBs) are believed to be the critical lesions responsible for radiation induced cell killing. DNA recombinational repair is the only known system capable of repairing DNA DSBs. In yeast, induced radioresistance is considered to be a result of increased DNA recombinational repair capacity. Therefore it was postulated that DNA DSBs are the molecular lesions that signal the induction of recombinational repair and induce the radioresistance mechanism.

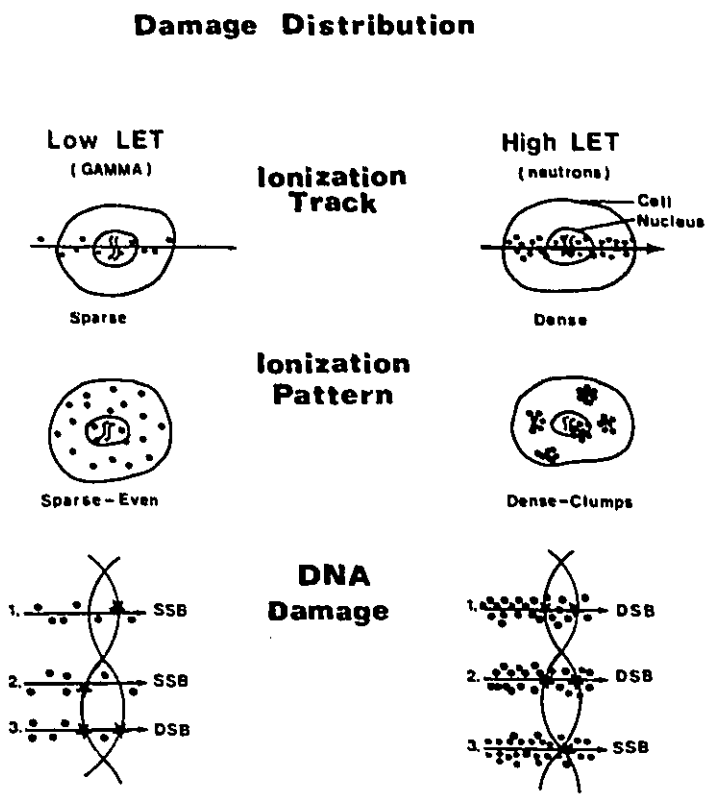

Figure 1.2. Ionizing radiation damage distribution within a typical cell. The ratio of DNA double strand breaks (DSB) to DNA single strand breaks (SSB) produced by ionizing radiation changes with radiation quality or linear energy transfer (LET). Low-LET radiation (γ-rays) produce a sparse ionization track with an evenly distributed ionization pattern throughout the cell. High-LET radiation (neutrons) produce a dense ionization track with a clustered or clumped ionization pattern. When radiation traverses the DNA molecule the probability of ionizing both strands and thereby producing a DSB is greatest with high-LET radiations. Therefore the DSB/SSB ratio increases with LET.

exposures for their induction efficiency, and arrived at the same conclusions as with the cell killing endpoint.

We have also compared the effectiveness of water derived OH• and of OH• produced by the reaction of e^-_{aq} with N_2O at inducing DNA repair capacity (see Figure 1.3), as measured by this alternative endpoint of mutation suppression (4). Interestingly, the ultimate level of induced repair capacity led to increased survival but did not directly correlate with increased mutation suppressibility. It was shown that while DNA lesions resulting from both types of OH• resulted in induced DNA repair capacity as measured by resistance to cell killing, DNA lesions produced from OH• via e^-_{aq}, impaired the ability of the repair system to process MNNG-generated lesions.

1.2 MATERIALS AND METHODS

1.2.1 Strains. The yeast *Saccharomyces cerevisiae* was used as the test organism in these experiments. All strains were diploid and homozygous for the *his1-7* mutation. The wild type cells were from the strain MS33 and were wild type for recombinational DNA repair (rec^+/rec^+). Strain MS32 was homozygous for *rad 52-1* which is defective in recombinational repair. Yeast strains were gifts from Dr. D.P. Morrison, AECL, Chalk River Nuclear Laboratories.

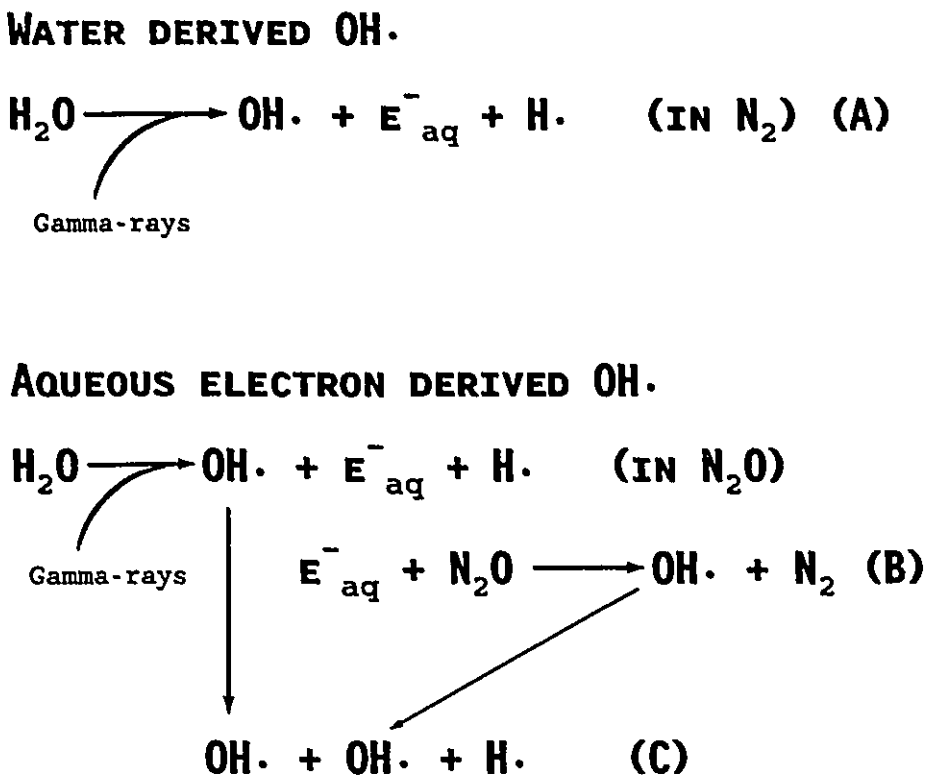

Figure 1.3. General summary of hydroxyl radical (OH•) production derived from the radiolytic decomposition of water. When cells are exposed to ionizing radiation, water absorbes most of the energy deposited within the cell. Under anaerobic conditions (in N_2) the three main water derived radicals produced by aqueous radiolytic decomposition are hydroxyl radicals (OH•), aqueous electrons (e^-_{aq}), and hydrogen atoms (H•) (Line A). In the presence of nitrous oxide (N_2O) an e^-_{aq} is converted into a OH• (Line B) and the end result is an increase in OH• at the expense of e^-_{aq} (Line C).

1.2.2 Cell Growth. Cell cultures were grown at 23°C to mid-exponential phase (2-5 x 10^6 cells/ml) in liquid nutrient medium containing 0.1% yeast extract, 1% yeast nitrogen base with amino acids, 1% sodium succinate, and 2% glucose (called Yeast Extract/ Succinate medium or YES).

1.2.3 Radiation Survival. For irradiation wild type or *rad 52-1* strains were washed three times and suspended (at 0°C) in 20 mmol dm^{-3} (mM) sodium phosphate buffer, pH 7.0, at approximately 1 x10^7 cell/ml. These suspensions were equilibrated by bubbling the cell suspension at 0°C with N_2 (1 ppm O_2), O_2, or N_2O (Matheson ultrahigh purity) for 10 minutes before irradiation and bubbling was maintained during irradiation. Cells were irradiated with ^{60}Co gamma-rays (Gamma cell 220, Atomic Energy of Canada) at a dose rate of about 2 Gy/sec (Gy = Gray, an SI unit for absorbed dose). Survival was determined by plating suitable dilutions on solid agar (2%) plates containing 0.1% yeast extract, 1% yeast nitrogen base with amino acids, 1% sodium succinate, 2% peptone, 2% glucose (called Yeast/Peptone/Dextrose media or YPD), followed by growth at 23°C.

1.2.4 Radiation Induced Radiation Resistance. Cells were harvested, washed three times at 0°C in 20 mmol dm^{-3} (20mM) phosphate buffer (pH 7.0) and resuspended as described above. Nonlethal low-LET radiation inducing doses were delivered using ^{60}Co gamma rays (Gammacell 220 Atomic Energy of Canada Limited, 2 Gy/sec). High-LET 14 MeV neutron inducing doses were generated with a Texas Nuclear Corporation Model 9400 Neutron Generator at a dose rate of 1.0 Gy/min. Inducing doses were delivered to cell suspensions at 0°C after equilibration in either O_2, N_2 or N_2O by bubbling for ten minutes prior to and during the irradiation period. After receiving a predetermined inducing dose, cells were resuspended in YES

medium and incubated at 23°C for increasing time intervals. Survival to a 1.0 kGy gamma-ray test dose (delivered in oxygen as above) was used as a measure of radiation resistance. Survival to all treatments was determined by plating on YPD.

1.2.5 Mutation and Suppression of MNNG Induced Mutation by Radiation. High- and low-LET radiation-induced mutation was determined by measuring reversion to histidine independence. Mutant cells able to form colonies were counted after incubation at 23°C for 6 days on solid medium lacking histidine. Total survival after the various treatments was measured by scoring colony formation on YPD plates.

Chemical mutagenesis was performed at 23°C in 20 mmol dm^{-3} (mM) phosphate buffer (pH 7.0) at a cell density of 1 x 10^7 cells/ml. Cells were treated with 20 μg/ml of MNNG (Aldrich Chemical Co.) for 30 minutes subsequent to a radiation inducing treatment. Inducing doses consisted of a variety of radiation treatments under various radiobiological conditions. After MNNG exposure, cells were washed twice with 10% (w/v) sodium thiosulphate and twice with phosphate buffer, all at 0°C. Reversion to histidine independence and survival to treatments was determined as described above.

1.2.6 Hydroxyl Radical Scavenging. Cells equilibrated at 0°C in N_2 alone or N_2 plus 1M ethanol were given a 100 Gy inducing dose as previously described. Following the radiation the cells were washed three times at 0°C in 20 mM phosphate buffer to remove the ethanol and incubated in nutrient medium at 23°C. Induced radiation resistance was determined by a gamma-ray test dose as described above.

1.2.7 Data and Figures. All figures shown represent the combined results from at least three independent experiments. Standard error bars are shown except were the error is less than or equal to the symbol size. One-way analysis of variance and Student's T-tests were performed to determine the statistical significance between various treatments, and P-values are given.

1.3 RESULTS

1.3.1. Radiation Survival. Wild type MS33 yeast cells were irradiated with increasing doses of γ-rays in the presence of oxygen or in the presence of nitrogen. Cells were more sensitive to γ-rays when irradiated in oxygen compared to irradiation in nitrogen (Figure 1.4). This is a well known radiobiological oxygen effect. The term used to compare the variable radiation sensitivity of different cells under aerobic or anoxic irradiation conditions is the Oxygen Enhancement Ratio (OER). MS33 yeast cells had an OER of approximately 2.3 (see Figure 1.4).

1.3.2 Radiation-Induced Radioresistance; High-LET vs Low-LET. The quality of radiation and corresponding DNA lesions most efficient at inducing radioresistance was tested by comparing high-LET neutrons with low-LET γ-rays. At all inducing doses, cell survival was 100%. Figure 1.5A demonstrates that γ-rays (P=2.694E-05) and neutrons (P=.01222) induced radioresitance above control levels. By comparing equivalent radiation inducing doses in oxygen, gamma-rays seemed to be more efficient than neutrons (above 20 Gy) at inducing maximum radioresistance (P=.01964). In contrast, only a 10 Gy anoxic inducing dose of neutrons revealed a significant difference between equivalent doses of neutrons or

γ-rays at inducing radioresistance (P=1.5073E-02, Figure 1.5B). Control cultures tested immediately (without an incubation period) for induced radioresistance after exposure to γ-ray inducing doses were not significantly different (P=.4913, Figures 1.5A and 1.5B).

Figure 1.6 shows a similar experiment except induced cultures were incubated for 5 rather than 2 hours prior to the test dose. There did not appear to be an oxygen enhancement effect between radioresistance induced by γ-rays or neutrons under oxic (Figure 1.6A) or anoxic (Figure 1.6B) conditions (no significant difference between paired means).

It has been previously reported that the magnitude of induced radioresistance is proportional to the level of the inducing damage (11). For equivalent γ-ray inducing doses, induced radioresistance was greater in cells given oxic inducing doses compared to anoxic doses (11). The present work further substantiates a significant oxygen effect for the induction of radioresistance by low-LET γ-rays after 2 hours (P=3.6911E-02) and after 5 hours of incubation (P=4.0233E-02) (Figure 1.7). In contrast, there is no oxygen effect for the induction of radioresistance by high-LET neutrons (Figure 1.8A P=2.7701E-02), Figure 1.8B, P=4.0112E-02). This same lack of an oxygen effect for neutron induced radioresistance is also shown in Figure 1.9 which indicates that maximum radioresistance developed after 2 hours of incubation following a single neutron inducing dose and did not change significantly during an additional 3 hours of incubation (no significant difference between any values after 2 hours of incubation P=.9343). Figure 1.7 also shows that the maximum resistance induced by γ-rays in O_2 after 2 hours of incubation had declined by 5 hours.

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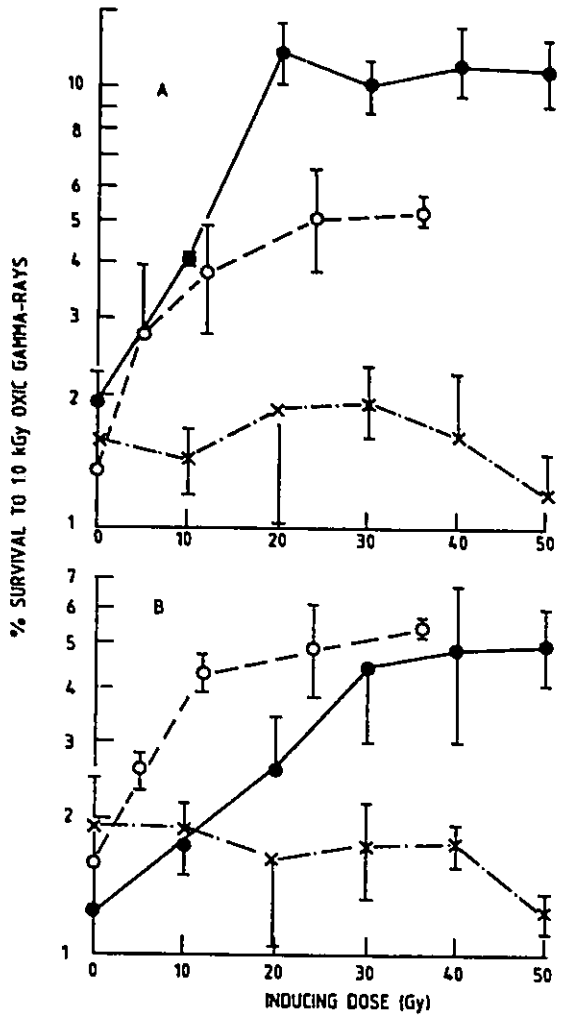

Figure 1.5. High- and low-LET radiation induced radioresistance in *Saccharomyces cerevisiae* exposed to either 14 MeV neutrons (o) or cobalt-60 gamma-rays (•). Radioresistance was measured as survival to a 1.0 kGy oxic gamma-ray test dose following two hours of incubation after oxic (A) or anoxic (B) inducing doses. Controls (X) were samples with no incubation time between the inducing dose and test dose. At all inducing doses, cell survival was 100%.

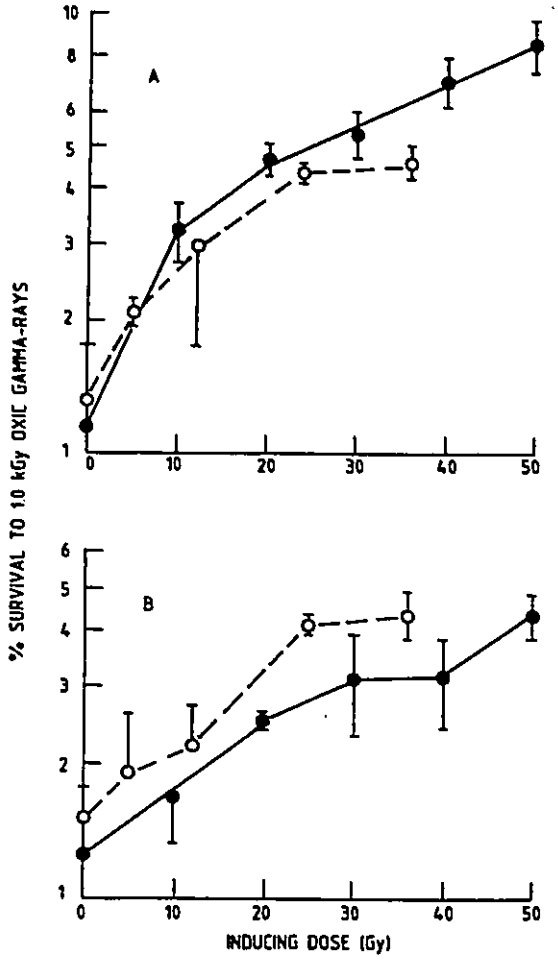

Figure 1.6. High- and low-LET radiation induced radioresistance in *Saccharomyces cerevisiae* exposed to either 14 MeV neutrons (o) or cobalt-60 gamma-rays (•). Radioresistance was measured as survival to a 1.0 kGy oxic gamma-ray test dose following five hours of incubation after oxic (A) or anoxic (B) inducing doses. At all inducing doses, cell survival was 100%.

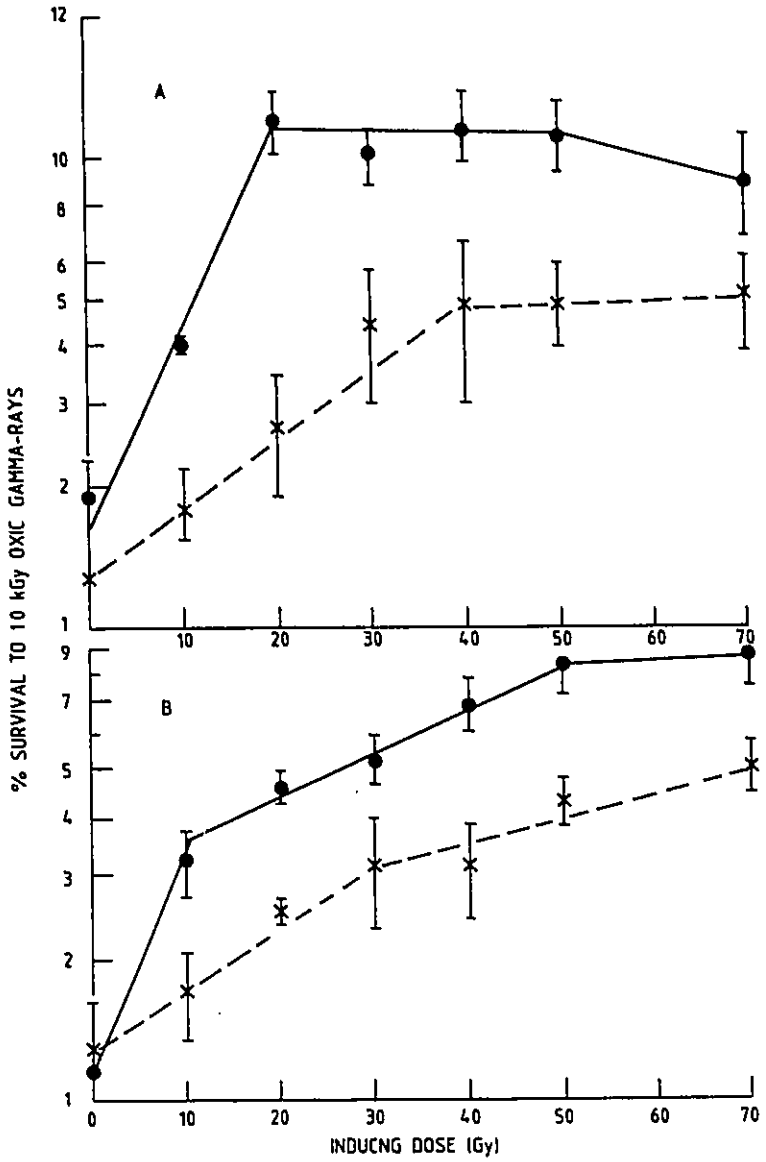

Figure 1.7. Oxygen effect for the induction of radioresistance in *Saccharomyces cerevisiae* by exposure to low-LET gamma-rays. Radioresistance was measured as survival to a 1.0kGy oxic gamma-ray test dose following two hours of incubation (A) or five hours of incubation (B). Gamma-ray inducing doses were delivered in the presence of oxygen (•) or in the presence of nitrogen (×). Data were replotted from Figures 1.5 and 1.6 except for 70 Gy inducing dose.

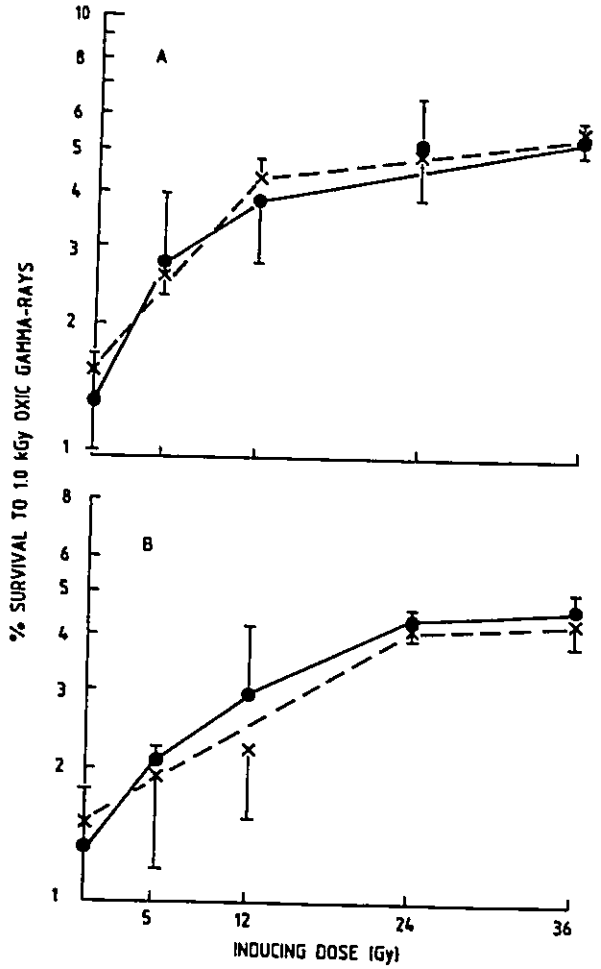

Figure 1.8. Lack of an oxygen effect for the induction of radioresistance in *Saccharomyces cerevisiae* by exposure to high-LET 14 MeV neutrons. Radioresistance was measured as survival to a 1.0 kGy oxic gamma-ray dose following two hours incubation (A) or five hours of incubation (B). Neutron inducing doses were delivered in the presence of oxygen (•) or in the presence of nitrogen (×). Data were replotted from Figures 1.5 and 1.6.

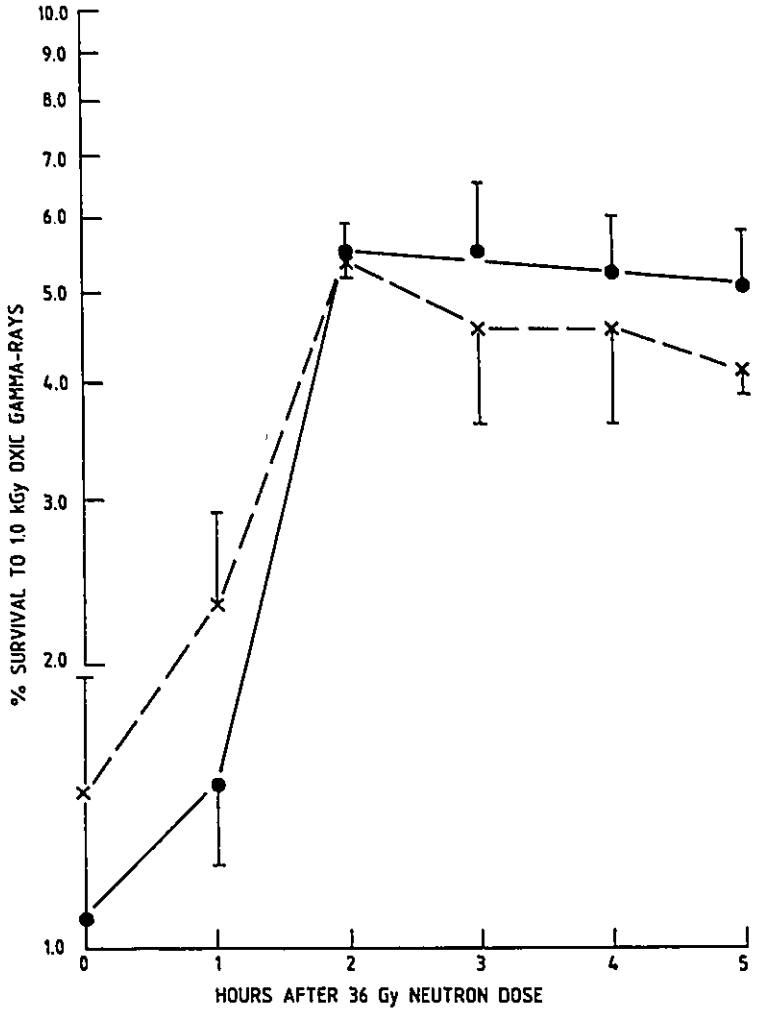

Figure 1.9. The time course of development of radioresistance in *Saccharomyces cerevisiae* following a single 36 Gy oxic (•) or anoxic (X) neutron inducing dose. Cell survival was 100% following the inducing dose.

1.3.2 Involvement of hydroxyl radicals. It was previously reported that recombination-proficient yeast cells showed increased sensitivity to killing by irradiation in the presence of N_2O as compared to N_2 (19). Using a different wild-type yeast (MS-33) than the strain previously used (19) the same result was verified (Figure 1.10). Mean survival values for cells irradiated in the presence of N_2O were significantly lower compared to cells irradiated in N_2 per unit dose (Figure 1.10, 1.5 kGy P=7.9131E-03, 2.0 kGy P=7.6778E-03, 3.0 kGy P=.7067, 4.0 kGy P=6.8105E-03). The isogenic recombinational repair mutant (MS-32) showed no enhanced lethality when irradiated in the presence of N_2O as compared to N_2 (Figure 1.11, means were not significantly different: 400 Gy P=5.8003E-02, 600 Gy P=.8661, 800 Gy P=.1704). It was suggested that lethal radiolytic free radical damage may be a function of existing enzymatic repair processes (19). Therefore it was postulated that specific DNA lesions might be responsible for variable induction of DNA repair. To test this idea we investigated the involvement of OH• damage in inducing radioresistance. Figure 1.12 demonstrates that cells given a single 100 Gy γ-ray inducing dose in the presence of N_2O became more radioresistant than cells induced in the presence of N_2 (Figure 1.12: 3, 4, 5 hours, P=1.2022E-02, 3.987E-02, 4.128E-02 respectively). Therefore, increasing the total yield of OH• per unit dose by substitution of OH• for e^-_{aq} increased the magnitude of the induced radioresistance response. Further evidence for the involvement of OH• damage as an induction signal is presented in Figure 1.13 where cells were radiation-induced in the presence of 1M ethanol (an efficient and selective OH• scavenger). For the same anoxic inducing dose, cells induced in the presence N_2 alone became more radioresistant

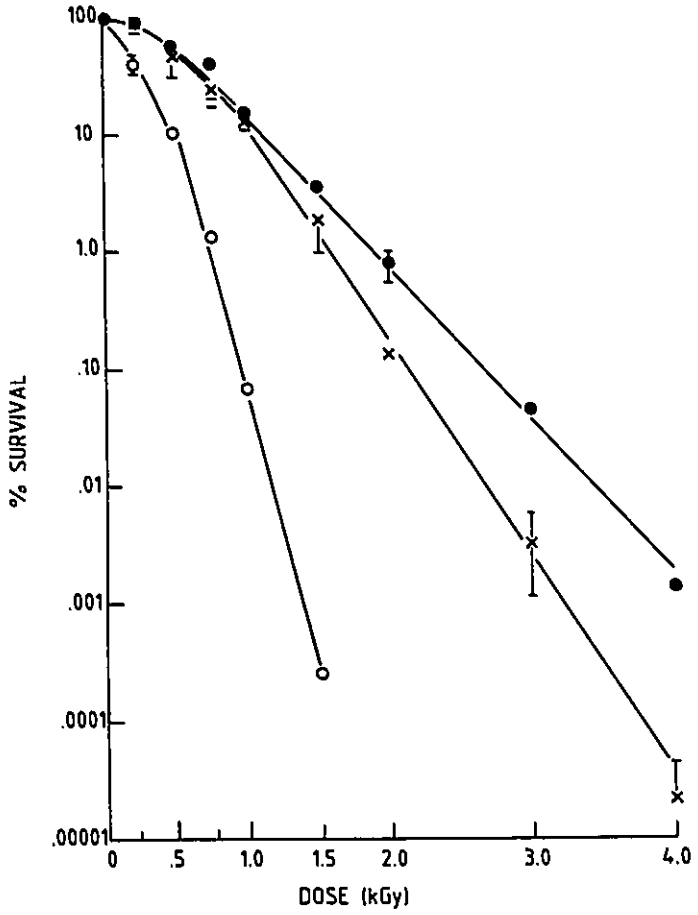

Figure 1.10. Survival of wild type *Saccharomyces cerevisiae* (strain MS33) to a single dose of ^{60}Co gamma-radiation in the presence of N_2 (•), N_2O (X) or O_2 (O).

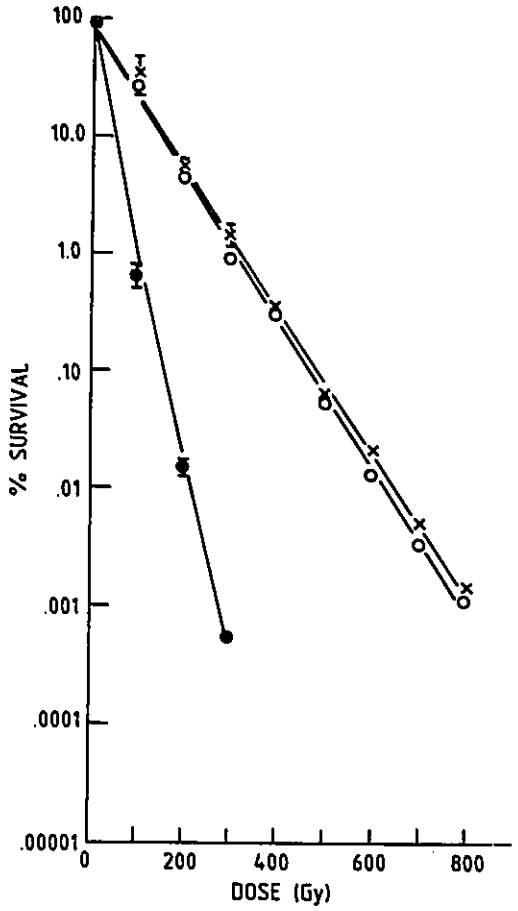

Figure 1.11. Survival of recombinational repair deficient *Saccharomyces cerevisiae* (strain MS32) following exposure to a single dose of ^{60}Co gamma-radiation in the presence of N_2 (X), N_2O (O), or O_2 (•). Strain MS32 is isogenic to strain MS33 (Figure 1.10).

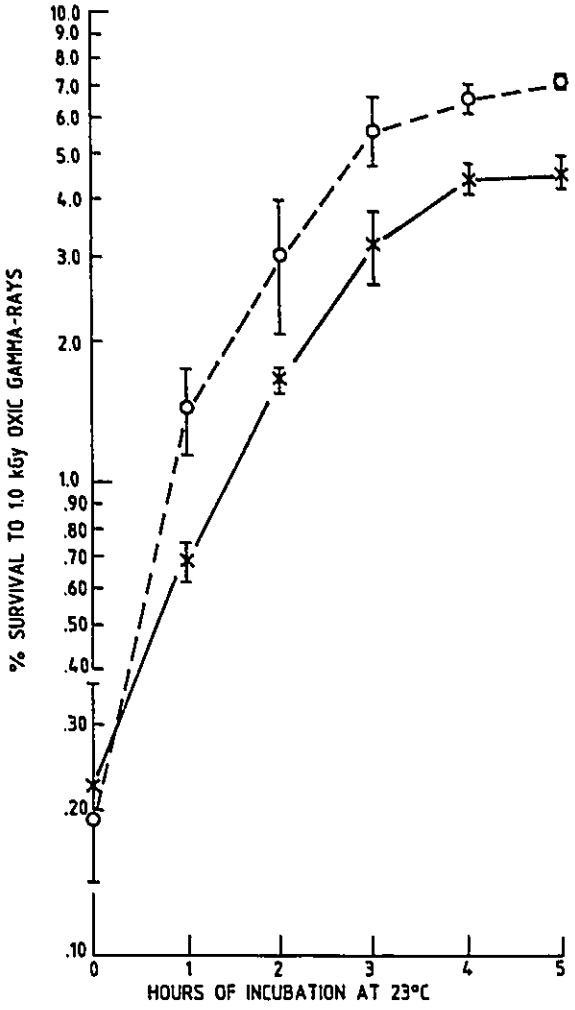

Figure 1.12. Gamma-ray induction of radioresistance in *Saccharomyces cerevisiae*. Cell were given a 100 Gy inducing dose in the presence of N_2 (X) or N_2O (O) and radioresistance was determined each hour, following the inducing dose, by measuring survival to a 1.0 kGy γ-ray test dose in O_2.

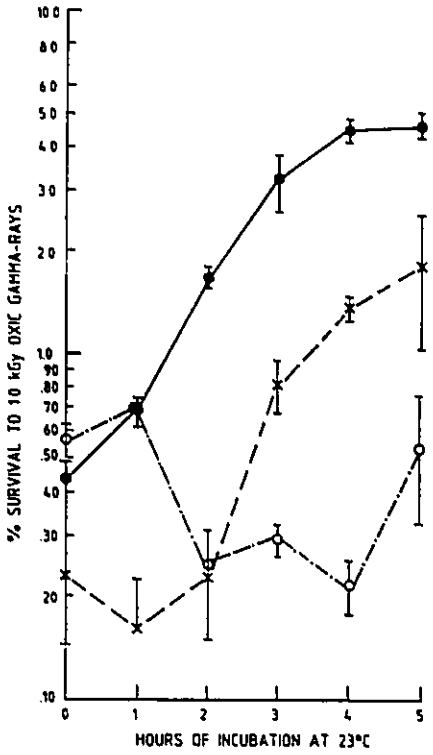

Figure 1.13. Gamma-ray induction of *Saccharomyces cerevisiae* radioresistance in the presence of a OH• scavenger (1M ethanol). Cells were induced with a 100 Gy γ-ray dose in the presence N_2 alone (•), or in N_2 with 1M ethanol (X). The development of radioresistance was monitored by measuring survival to a 1.0 kGy γ-ray test dose, in O_2, after incubation for various times under normal growth conditions. An uninduced control sample treated with 1M ethanol alone is also shown (o).

than cells induced in the presence of N_2 plus ethanol (Figure 1.13, means were significantly different after 3, 4, and 5 hours of incubation P=1.598E-02, 1.097E-02, and 2.009E-02 respectively). Under these experimental conditions 1M ethanol alone did not induce radioresistance (Figure 1.13, means were not significantly different P=.7765). The same experiment was also tested by radiation inducing cells in the presence of N_2O plus 1M ethanol. Under these conditions the overall level of radioresistance induced by radiation alone was not significantly reduced when cells were induced in the presence of 1M ethanol (Figure 1.14: 1, 2, 3, 4, 5 hours were P=.7808, .7995, .9087, .8721, .9291 respectively).

1.3.3 *Mutation and Suppression of Mutation*. The mutation frequency (as measured by reversion to histidine independence) produced by high-LET neutrons or low-LET γ-rays, is shown in Figure 1.15. Under these experimental conditions there did not seem to be a difference between γ-rays and neutrons at producing mutations. The net mutation frequency produced by 20 μg/ml of MNNG in cells exposed to increasing doses of neutrons or γ-rays is shown in Figure 1.16. As described previously for γ-ray exposure, the cells show a dose dependent reduction in mutations generated by MNNG (4). For equivalent oxic doses the suppression of mutation was greater in cells induced with low-LET γ-rays than the high-LET neutrons (Figure 1.16: P=1.089E-03 at 2.4 Gy, P=1.008E-03 at 3.6 Gy).

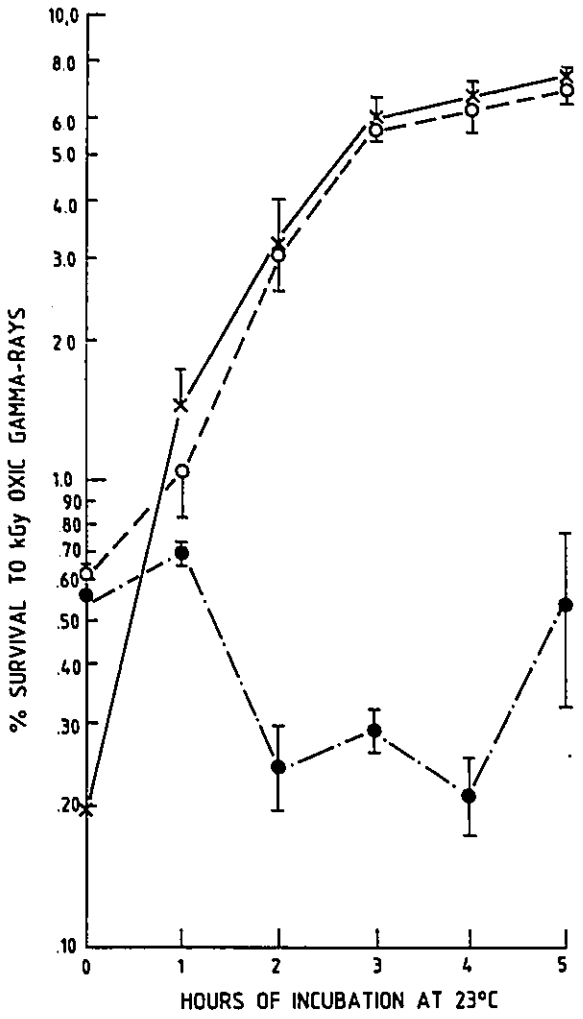

Figure 1.14. Gamma-ray induction of *Saccharomyces cerevisiae* radioresistance in the presence of a OH• scavenger (1M ethanol). Cells were induced with a 100 Gy γ-ray dose in the presence N_2O alone (×), or in N_2O with 1M ethanol (○). The development of radioresistance was monitored by measuring survival to a 1.0 kGy γ-ray test dose, in O_2, after incubation for various times under normal growth conditions. The uninduced control sample treated with 1M ethanol alone is again shown (•).

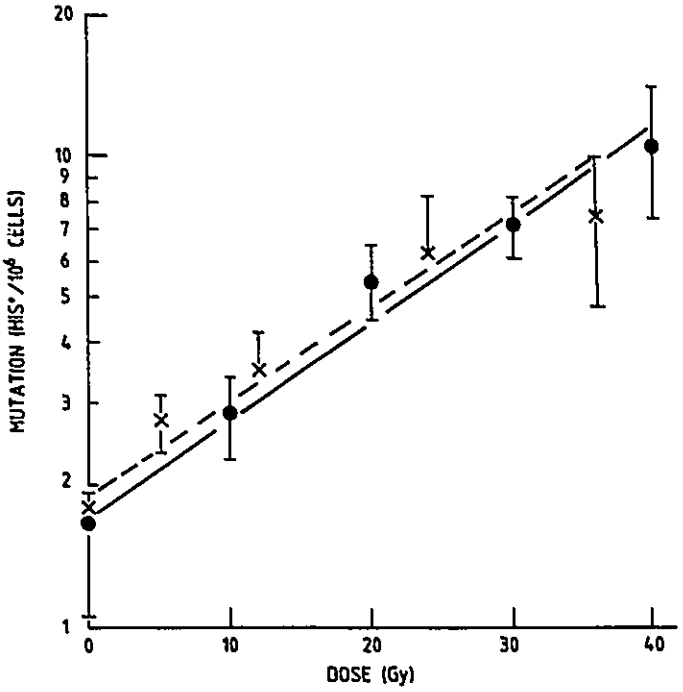

Figure 1.15. The observed mutation frequency in *Saccharomyces cerevisiae*, measured as reversion to histidine independence, following increasing single doses of ^{60}Co γ-rays (•) or 14 MeV neutrons (X), delivered in the presence of O_2. Cell survival was 100% at all doses.

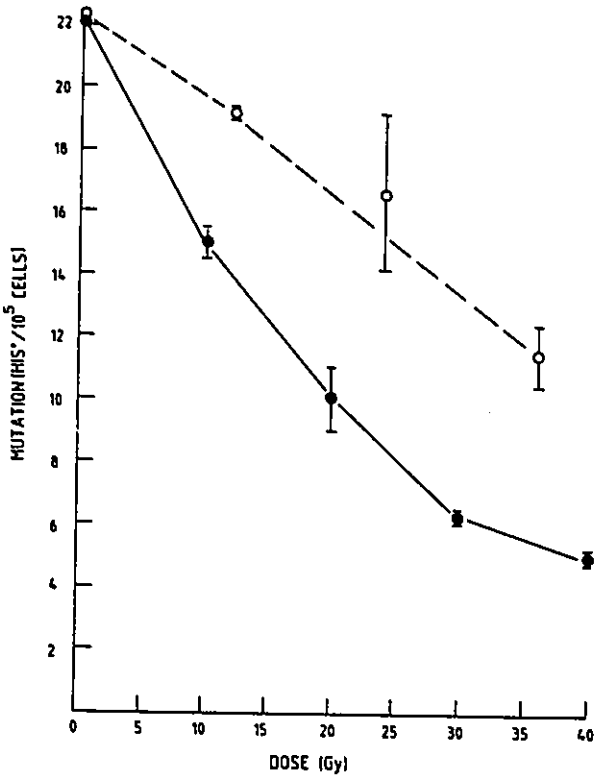

Figure 1.16. Radiation dose-dependent reduction in MNNG-generated mutations in yeast exposed to either γ-rays (•) or neutrons (o) (in O_2) The cells were treated with MNNG (20 μg/ml, 30 minutes) immediately after radiation exposure. These data have been corrected for mutations generated by the radiation alone (see Figure 1.15).

It was concluded therefore, that DNA lesions produced by low-LET irradiation induce error-free recombinational repair more efficiently than lesions from high-LET irradiation.

There is a radiobiological oxygen effect for γ-ray induced mutation (4). Here results showed that there was a greater mutation frequency above 50 Gy when cells were γ-irradiated in O_2 as compared with N_2 (Figure 1.17, P=1.205E-02 at 50 Gy, P=1.191E-02 at 70 Gy, P=1.112E-02 at 100 Gy). Cells irradiated in N_2O did not show a significantly greater rate of mutation as compared with cells irradiated in N_2 at non-lethal doses (Figure 1.18), also as previously noted (20).

It was additionally reported that suppression of MNNG-generated mutation by radiation also demonstrates a radiobiological oxygen effect (4). This result is confirmed in the present work and Figure 1.18 shows that for the same γ-ray dose, cells induced in the presence of O_2 suppressed MNNG-generated mutations to a greater extent than cells induced in the presence of N_2 (P=1.299E-03 at 100Gy). Also shown in Figure 1.18 is the surprising new result that cells induced with γ-rays in the presence of N_2O were less effective at suppressing MNNG mutations compared with cells given the same inducing dose in N_2 (all doses above 20 Gy are significantly different with a level of significance greater than P=1.00E-03).

1.4 DISCUSSION

Low-LET radiations like γ-rays characteristically produce a different pattern and distribution of DNA ionizations than those of high-LET radiations like neutrons (21-25). In general it is believed that low-LET irradiation produces a higher ratio of DNA SSBs to DSBs per unit dose

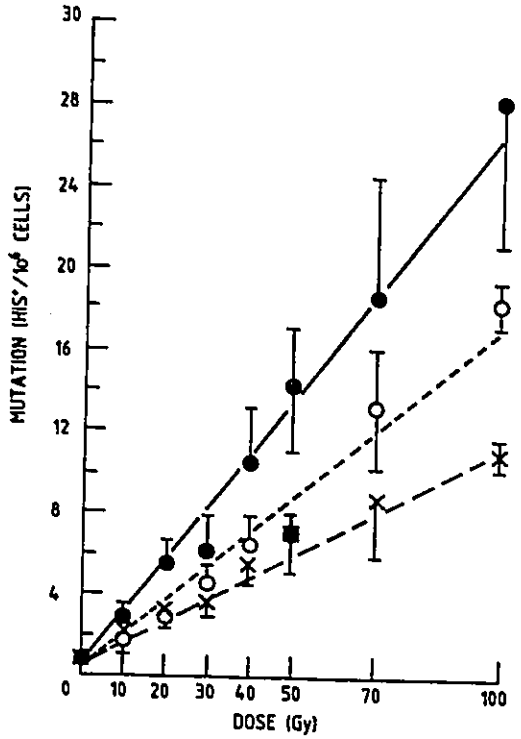

Figure 1.17. Gamma-radiation generated mutations in *Saccharomyces cerevisiae* measured by reversion to histidine independence. Cells were exposed to single increasing doses in O_2 (•), N_2 (X), or N_2O (o).

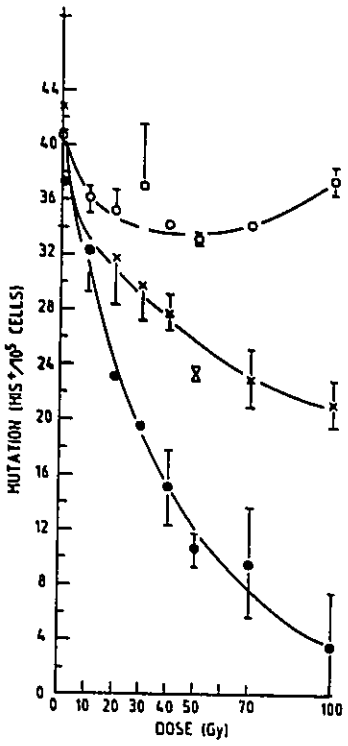

Figure 1.18. Radiation suppression of MNNG-generated mutations in yeast cells γ-irradiated with single doses in O_2 (•), N_2 (X), or N_2O (o). The cells were treated with MNNG (20 μg/ml, 30 minutes) immediately after radiation exposure. These data have been corrected for mutations generated by radiation alone (see Figure 1.17).

than high-LET irradiation (18,26-28, see Figure 1.13). It was shown here that low-LET γ-rays produce oxygen modifiable DNA lesions that are significantly more efficient at inducing recombinational repair per unit dose (as measured by either resistance to cell killing, Figure 1.5, or competition for MNNG lesions, Figure 1.16), than those lesions produced by high-LET neutrons. It was concluded that DNA SSBs are important lesions that signal the induction of the repair mechanism and are more important than DSBs (Figure 1.19). A possible explanation for this phenomenon may be that the relatively large number of DNA SSBs produce more alteration in DNA topology than the relatively few DSBs and this subsequently produces a proportional inducing response. Many genes have been shown to be regulated by DNA supercoiling (29-32) and perhaps this repair mechanism has evolved to be regulated in response to DNA topological perturbation. This rationale inspired the research on DNA topoisomerases reported in Chapter 2 of this thesis.

It was shown (Figure 1.7) that low-LET radiation induction of radioresistance to cell killing demonstrates an oxygen effect. Cells induced with γ-rays in the presence of O_2 became more radioresistant than cells induced with the same γ-ray dose in the presence of N_2 (Figure 1.7), indicating that O_2 modifiable lesions from low-LET radiation are efficient inducing lesions. Here the results showed that there was little observable oxygen effect for high-LET radiation-induced radioresistance (Figures 1.6 and 1.8). Because the induction of recombinational repair is a response that is proportional to damage (11), the type of initial damage produced by high-LET neutrons under oxic or anoxic conditions might be biologically similar as an inducing signal. This apparent reduced oxygen enhancement ratio (OER) for high-LET radiation to induce

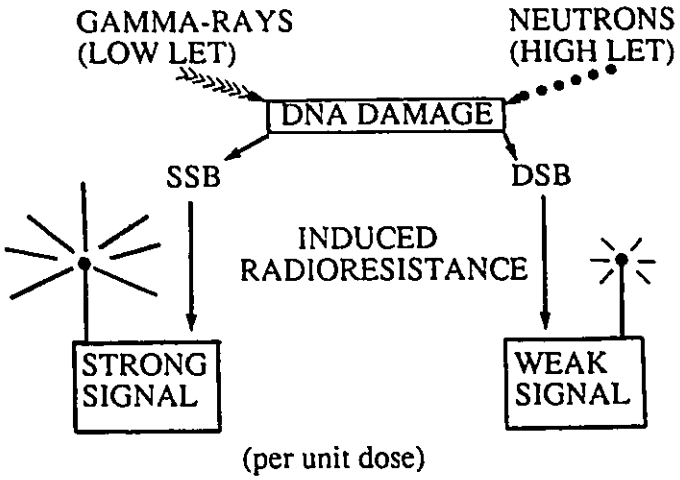

Figure 1.19. DNA single strand breaks (SSBs) generated by low-LET gamma-rays produced a strong induction signal for the radioresistance mechanism. DNA double strand breaks generated by high-LET neutrons produced a weak induction signal.

radioresistance may thus be analogous to the results normally observed with other endpoints like lethality or gene conversion (33,34). Figures 1.7 and 1.8 show that low-LET radiation O_2 modified lesions produce a transient maximum radioresistance (after 2 hours of incubation) whereas high-LET radiation seemed to induced a constant maximum radioresistance (up to 5 hours). This probably reflects a difference in the repairability of the respective inducing lesions; high-LET lesions being more difficult to repair (27) and therefore producing a persistent induction signal.

The importance of OH• damage as a signalling lesion for the induction of the repair mechanism was investigated. The results demonstrated that cells given the same inducing dose in N_2O became more radioresistant than those cells induced in N_2 (Figure 1.12). This was not surprising since this repair mechanism responds proportionally to the initial amount of DNA damage and there was presumably more damage per unit dose produced in N_2O (see figure 1.10 survival curves). It was concluded therefore, that OH• type damage is an important inducing lesion in this mechanism.

Many authors have demonstrated that ethanol will successfully scavenge cellular OH• (35-38). The significance of OH• damage at inducing radioresistance was examined by testing the inverse response. That is, removal of OH• by ethanol scavenging during the radiation-inducing dose should decrease the amount of DNA damage and therefore the level of induced radioresistance. The results support this postulate and demonstrate that OH• scavenging decreases the level of induced resistance (Figure 1.13) further supporting the conclusion that OH• produce significant inducing DNA lesions. It was suspected that the lack of

reduction of induced radioresistance demonstrated when cells were induced in N_2O (Figure 1.14) was a result of insufficient scavenging by ethanol since OH• yield is increased under these radiochemical conditions.

Wild type yeast cells and some bacteria cells are radiosensitized for killing when irradiated in N_2O compared to cells irradiated in N_2 (19,39,40, Figure 1.10). This radiosensitization has been attributed to the increase in OH• generated from e^-_{aq} via N_2O. Here an isogenic recombinational repair deficient yeast mutant did not seem to be radiosensitized by γ-irradiation in N_2O (Figure 1.11), apparently unlike the parental wild type (Figure 1.10). Recently, Ewing and Guilfoil (41) have reported the lack of sensitization by N_2O in several radiation sensitive *E. coli* strains. It is clear that an increase in OH• derived from e^-_{aq} only sensitizes cells that possess a functional recombinational repair system, suggesting that repair or attempted repair of these lesions can be a lethal response. DNA lesions produced from e^-_{aq} in N_2O may be chemically distinct (42) and here the evidence suggests that these lesions may have a unique biological characteristic at the level of DNA repair. Our data suggest that the chemical nature of the DNA damage formed under N_2O conditions is unusual and seemingly more difficult to repair. Possibly the location of the e^-_{aq} before conversion to OH• is an important consideration. Little is known about migration of aqueous electrons within the nucleus of a cell. It is possible that polar e^-_{aq} could accumulate in soluble fractions of the chromatin or nuclear membranes. Subsequent conversion of e^-_{aq} to OH• via N_2O would then produce areas of significant OH• damage (including closely associated regions of DNA). This concept could implicate site specific OH• attack and corresponding DNA topological perturbation (43). Alternatively, an altered type of DNA

damage produced by those OH• derived from e^-_{aq} (in N_2O) may alter DNA tertiary structure such that repair of these lesions becomes hindered. Therefore, attempting to repair these unique lesions could result in lethal events in repair competent cells as observed (Figure 1.10). It is possible that when these lesions are processed by the recombinational DNA repair mechanism they retard the repair system and create a situation whereby the cells become less repair competent. This explanation is consistent with the lack of effect seen in recombinational repair deficient mutants (Figure 1.11) where the absence of this repair system precludes its modulation. This postulate has important ramifications if the recombinational repair system has significant repair functions for non-radiation lesions as is further discussed below.

The third approach to investigate the mechanism of induced repair was to assess the capacity of yeast cells to repair chemically-induced mutagenic DNA lesions. It was previously reported that a γ-radiation exposure will induce recombinational repair (rec repair) (19) and rec repair can eliminate mutagenic lesions produced by the mutagen MNNG, consequently suppressing the mutagenic effects of a fixed dose of MNNG (4). Therefore, under these conditions, a decrease in mutation frequency may be indicative of an increase in rec repair capacity. Using this test as an endpoint for induced rec repair, and based on the observations shown in Figure 1.5, it was postulated that neutrons would induce less rec repair than γ-rays and therefore neutron generated lesions would be less efficient at signalling the suppression of MNNG-induced mutations. The results supported this hypothesis and again suggested that low-LET radiation damage may signal the induction of the rec repair mechanism more efficiently than high-LET radiations (Figure 1.16).

The actual suppression of MNNG-generated mutations is considered to be a competition response (4). In yeast, MNNG is believed to induce an error-prone DNA repair system which is responsible for generating DNA mutations (14). Therefore a fixed dose of MNNG will induce a prescribed level of error-prone repair which would be responsible for a given frequency of mutations. Radiation-induced error-free repair is capable of repairing MNNG-generated mutations and thus an increase in radiation-induced error-free repair capacity will decrease the overall observed mutation frequency. It was postulated that variable competition between DNA repair systems would alter the biological consequences of an MNNG exposure. This work has supported this contention and demonstrated that low-LET γ-radiation was more efficient than high-LET neutrons at suppressing MNNG-induced mutations (see Figures 1.16 and 1.20).

The result that cells induced by γ-radiation in the presence of N_2O seemingly could not suppress MNNG mutations was unexpected (Figure 1.18). Since cells irradiated in N_2O apparently acquired greater rec repair capacity than cells irradiated in N_2, as measured by increased resistance to killing (Figure 1.12), it was anticipated that cells induced with γ-rays in the presence of N_2O would suppress MNNG mutations to a greater extent, per unit dose, than cells induced in the presence of N_2. Upon reflection, it may be possible that the observed lack of suppressibility of MNNG-generated mutations exhibited by cells induced with γ-irradiation in the presence of N_2O (Figure 1.18) further supports the hypothesis that OH• generated via N_2O produce unusual DNA lesions (42) that retard the normal function of rec repair. This apparent delay/impairment/retardation of the recombinational repair mechanism may be a contributing factor in the biological increase in radiosensitivity to killing observed when wild-

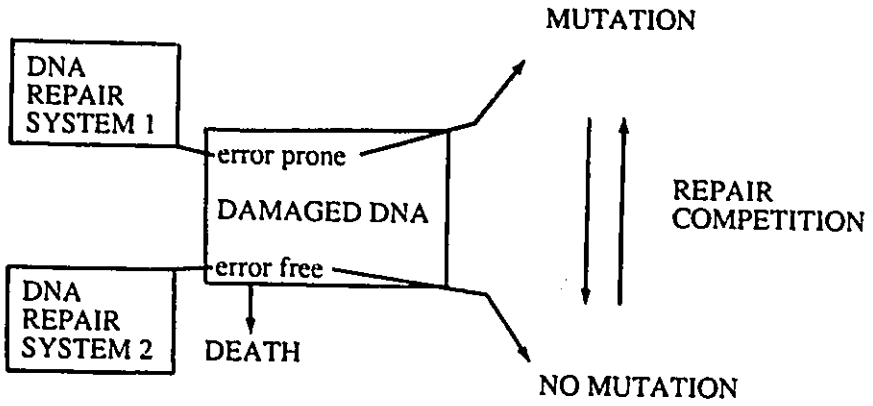

Figure 1.20. A model describing the suppression of MNNG-generated mutations by ionizing radiation. MNNG is a DNA alkylating agent which induces an error-prone DNA repair system in yeast. When MNNG-damaged DNA is processed via this induced error-prone repair system (system 1) mutations result. Ionizing radiation also damages DNA yet it induces an error-free DNA repair system (system 2) which produces few mutations. Induced error-free repair not only repairs radiation-induced damage but it can also repair MNNG-induced damage. When both system 1 and system 2 are activated they compete to repair all the DNA lesions. Increasing doses of radiation induce system 2 to a higher repair capacity and thus more DNA lesions are processed via the error-free repair pathway. Therefore, there is an observed supression in the frequency of MNNG-induced mutations (Figures 1.16 and 1.18). Results show that error-free repair is induced to a greater capacity by gamma-rays compared to the same dose of neutrons, again supporting the contention that DNA SSBs are important signalling lesions in this mechanism.

type cells were γ-irradiated in the presence of N_2O (Figure 1.10). In the presence of N_2, lesions produced by a given dose of γ-rays are repaired at a particular rate and fidelity, and cells exhibit a characteristic survival curve (Figure 1.10). In the presence of N_2O, some of the types of DNA lesions produced by the same dose (but via e^-_{aq} derived OH•) may be more difficult or impossible to repair (42), decreasing the overall repair rate and fidelity, and leading to the observed phenotypic radiosensitization (Figure 1.10). Conversely, the apparent radiosensitization by N_2O seen in rec repair proficient cells is absent in recombinational repair deficient mutants (Figure 1.11) since the rate and fidelity of rec repair cannot be changed in a cell were that repair is already absent for genetic reasons.

In conclusion, this chapter attempts to show that DNA SSBs are important rec repair inducing lesions and that oxygen modifiable lesions may be particularly efficient inducing signals. It was also shown that hydroxyl radical damage may be recognized by the cell as a signal for the induction of the rec repair mechanism and the system probably responds specifically to these types of DNA lesions. It is suggested that OH• derived from e^-_{aq} (in N_2O) produce DNA lesions functionally different from OH• derived directly from H_2O and these different types of lesions somehow, in a manner we do not yet understand, render normal recombinational repair less efficient. The alteration to the rate and/or fidelity of repair may be related to the chemical nature and topological perturbation of the DNA produced under this radiochemical condition. The attempted repair of these lesions perturbs repair efficiency, leading to increased cell lethality and to the observed inability of the inhibited rec repair system to process MNNG-derived lesions when repair competent cells are irradiated

in the presence of N_2O. In summary, radiation-induction of radioresistance in yeast seemingly not only depends upon a variable cellular response to different inducing DNA lesions, but also on the availability of the induced recombinational repair system to deal with subsequent damage. Inducing type DNA damage which also concurrently retards the induced repair system can have a net null or negative effect on resistance to a second exposure.

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CHAPTER TWO

THE INVOLVEMENT OF TOPOISOMERASES AND DNA POLYMERASE I IN THE MECHANISM OF INDUCED THERMAL AND RADIATION RESISTANCE.

2.1 INTRODUCTION

Various environmental stresses have been shown to induce a set of genes which elicit a characteristic response known as the heat shock or stress response (see reviews; 1,2,3). The heat shock response occurs in animals (4), plants (5), lower eukaryotes including yeast (6), and prokaryotes (7). The fundamental response was initially observed in *Drosophila* (8) and demonstrated that cells exposed to a sublethal heating became resistant to a subsequent, normally lethal heat shock. The mechanism of induced thermal tolerance has been further characterized (9) and thermal tolerance is also induced by a variety of other stresses including ethanol (10,11), nutrient depletion (12) and ionizing radiation (13). Neither the nature of the stress induced signal nor the mechanism by which a cell is subsequently able to increase its heat resistance is known.

In yeast, heat shock not only induces thermal tolerance but U.V. resistance (14) as well as ionizing radiation resistance (12; resulting from the induction of recombinational repair [15]). Conversely, ionizing radiation will induce thermal tolerance (13), radiation resistance (16), mutagen resistance (17,18) and resistance to radiation induced chromosomal aberrations (19).

Radiation or heat may exert cellular effects directly by damaging the molecular nature of DNA (13) or indirectly by interacting with DNA

metabolic enzymes such as DNA polymerases (20,21) or DNA topoisomerases (22,23,24). There are two major types of topoisomerases, I and II. They have been well characterized in yeast (25-27) and in humans (28). DNA topoisomerases are nuclear enzymes which maintain and modify DNA topology. In general, DNA topoisomerases alter the topology of DNA by a concerted cleavage and resealing of a single strand (topoisomerase type I) or double strand (topoisomerase type II) of DNA (29). They are also involved in many other critical cellular processes including: DNA replication (30), transcription (31), recombination (32), and repair (24). DNA polymerase I, which is the yeast analog of mammalian DNA polymerase α (33), is also a nuclear enzyme believed to be involved in DNA replication and repair (34). The yeast DNA polymerase I gene has been characterized (35) and the mutant used in this study has been previously described (34).

The work presented here tested the influence of DNA topology, as maintained by topoisomerases, and the role of DNA polymerase I on the mechanism of inducible thermal and radiation resistance.

2.2 MATERIAL AND METHODS

2.2.1 Strains. The organism used in these experiments was the yeast *Saccharomyces cerevisiae*. The topoisomerase mutants and parental wild type were gifts of Dr. R. Sternglanz (State University of New York at Stony Brook) and the DNA polymerase I strain and parental wild type were obtained from Dr. M. Budd (California Institute of Technology). The genotypes of the yeast strains are listed in Table 1. The topoisomerase II and DNA polymerase I strains were temperature sensitive mutants with

a non-permissive temperature of 37°C. The temperature sensitive mutant phenotypes were verified by growing strains at the non-permissive temperature. The *top2 (topoisomerase II deficient)*, *top1top2 (topoisomerase I and II deficient)*, and *pol1* (polymerase I deficient) phenotypes were lethal at 37°C and therefore these mutants did not grow.

2.2.2 Preparation of Cells. The yeast strains were grown at 25°C to mid log phase (2-5x10^6 cells/ml) in liquid medium containing 0.1% yeast extract, 1% yeast nitrogen base with amino acids, 1% sodium succinate, and 2% glucose (YES medium). Cell samples to be tested for heat or radiation resistance were washed three times in 20 mmol dm^{-3} (20 mM) sodium phosphate buffer (pH 7.0) at 0°C. The cells were resuspended in a small volume of the same buffer and were passed through a 25 gauge hypodermic needle five times to break up cell clumps. Following treatment, survival was determined by plating suitable cell dilutions on YPD agar plates (1% yeast extract, 2% peptone, 2% glucose, solidified with 2% agar) followed by growth at 25°C.

2.2.3 Thermal Tolerance. Thermal tolerance was determined by placing a number of 2.5 ml cell aliquots (2x10^6 cells/ml in YES medium at 0°C in 16x125 mm glass test tubes) into a 52°C water bath. Every 30 seconds for a total period of 10 minutes, an aliquot was removed, rapidly recooled to 0°C, and plated for survival as described above.

2.2.4 Induction by Heat Shock. Prepared cells, resuspended (2x10^6 cells/ml) in fresh YES medium at 25°C were heat shocked by an abrupt transfer of the culture (50 ml in a 500ml flask) to a shaking water bath at 37°C. At progressively increasing time intervals, 5 ml aliquots were removed and rapidly cooled to 0°C. At these temperatures and for the

Table I. Yeast strains used in this study

Strain	Genotype
RS-188 haploid wild type	MATa/ade 2-1/ura 3-1/his 3-11/trp 1-1/ leu 2-3,112/can 1.
RS-190 haploid Topoisomerase I minus	Same as RS-188 plus top 1-8
[1]RS-191 haploid Topoisomerase II minus	MAT a/ade 2-1/ura 3-1/his 3-11/trp 1-1/ leu 2-3, 112/top 2-1 (T.S)
[1]RS-192 haploid Topoisomerase I and II minus	Same as RS-191 plus top 1-8.
488 haploid wild type	Matα/leu2/ura3-52/his1-7/can1.
[2]488m haploid DNA polymerase I minus	Same as 488 except pol 1-17 (T.S).

[1]temperature sensitive topoisomerase II mutation with a non-permissive temperature of 37°C.

[2]temperature sensitive DNA polymerase I mutation with a non-permissive temperature of 37°C.

times used in these experiments, all strains showed 100% survival to this heat shock. The heat shocked cells were tested for thermal tolerance and radiation resistance. Thermal tolerance was determined by measuring survival to a 4 minute 52°C thermal test dose. Radiation resistance was measured by exposing buffer washed heat shocked cells, under oxic conditions, to a 1.5 kGy gamma-ray test dose.

2.2.5 Induction by Radiation Exposure. Cell suspensions at 0°C in phosphate buffer were equilibrated for 10 minutes by bubbling 0_2 through

the suspensions prior to and during the exposure. Cobalt-60 gamma irradiations were performed in a Gamma-Cell 220 (Atomic Energy of Canada Limited) at a dose rate of about 2 Gy/sec. Samples were removed at various doses and survival was determined by plating as described above. Radiation-induced radioresistance and thermal tolerance was tested after a 0.5 kGy gamma-ray exposure at 0°C. The irradiated cells were resuspended in fresh YES medium at $2x10^6$ cells/ml and incubated at 25°C (or 37°C where noted). Samples were removed thereafter every hour for 5 hours, washed three times in cold buffer, and resuspended in the same buffer. A 1.0 kGy gamma-ray test dose was used to test for induced radioresistance and a 4 minute 52°C heat shock was used to test for thermal tolerance. Cells were plated as previously described.

2.2.6 Data and figures. Each experiment was repeated at least three times from independent cultures. All figures shown represent the combined results of all the experiments. Standard error bars are shown except were the error is less than or equal to the symbol size. One-way analysis of variance and Student's T-tests were performed to determine the statistical significance between the various treatments. P-values are given where results are considered important.

2.3 RESULTS

2.3.1 Thermal and Radiation Resistance. The development of thermal tolerance in cells exposed to lethal temperatures has been known for some time (36) and is observed as the "flattening" or "leveling" of thermal survival curves. Considerable evidence indicates that such flattening is

characteristic of this response and not due to a heat resistant cell subpopulation (37-39). Yeast thermal survival curves also demonstrate a flattening (13), indicating that they also become thermally tolerant in response to a heat shock at lethal temperatures. We have used this characteristic to examine the sensitivity of topoisomerase and DNA polymerase mutant strains for induction of thermotolerance at lethal temperatures (Figure 2.1). By this test topoisomerase mutants and their parental wild type showed induction of thermal tolerance at a lethal temperature of 52°C (Figure 2.1A). Wild type and the *top1* mutant showed similar induction kinetics for this acquisition of thermal tolerance. In comparison, *top2* and the *top1 top2* double mutant demonstrated more rapid induction kinetics. After 6 minutes *top1 top2* cells seemed to be more tolerant to 52°C when compared to wild type and *top1* strains (P=1.023E-02 at 8 min., P=1.0952E-03 at 10 min.). Figure 2.1B shows the thermal survival curves of a DNA polymerase I deficient strain. The DNA polymerase I mutant and isogenic wild type both exhibited similar killing kinetics when subjected to a 52°C heat shock (means of the paired samples were not significantly different P=.8106). Wild type survival curves shown in Figure 2.1A and 2.1B may have been slightly different and probably reflects phenotypic variation.

Figures 2.2 and 2.3 show the survival of topoisomerase strains in oxygen and in nitrogen respectively to increasing doses of gamma-rays. Under these experimental conditions the temperature sensitive *top2* phenotype is not expressed. At higher doses *top1* and *top1 top2* mutants were more radioresistant than wild type and *top2* strains. At doses of 1.5 kGy in oxygen (Figure 2.2) and 3.0 kGy in nitrogen (Figure 2.3) *top1* cells

were significantly more radioresistant than wild type and *top2* cells (P=1.238E-02 at 1.5 kGy in O_2 and P=1.978E-02 at 1.0 kGy in N_2). Insert A on Figure 2.2 and insert B on Figure 2.3 show survival at low doses where all strains had similar survival curves, as would be expected in haploid G_1 cells which lack the duplicated genome required for recombinational repair (15).

2.3.2 Heat Induced Thermal Tolerance. Cells actively growing at 25°C were given an abrupt heat shock at a non-lethal temperature of 37°C. Figure 2.4 shows the induction of thermal tolerance in all strains. A heat shock of 37°C induced thermal tolerance to similar levels and with similar kinetics in all topoisomerase deficient and parental wild type cells (Figure 2.4A), and the absence of either or both topoisomerase activities did not seem to make a difference in the development of thermal tolerance compared to wild type cells. After 30 minutes of heat shock, maximum thermal tolerance was apparently reached in all strains; no further change seemed to occurred from 60-120 min (data not shown). Figure 2.4B shows that DNA polymerase I deficiency also did not alter the development of thermal tolerance.

2.3.3 Heat Induced Radiation Resistance. Figure 2.5A shows that a heat shock at 37°C induced radiation resistance in wild type and *top2* cells. This result demonstrates that topoisomerase II activity does not seem to be essential for the heat inducible radiation resistance mechanism. The *top1* and the *top1 top2* mutants did not appear to undergo heat induced radiation resistance; they were already radioresistant and

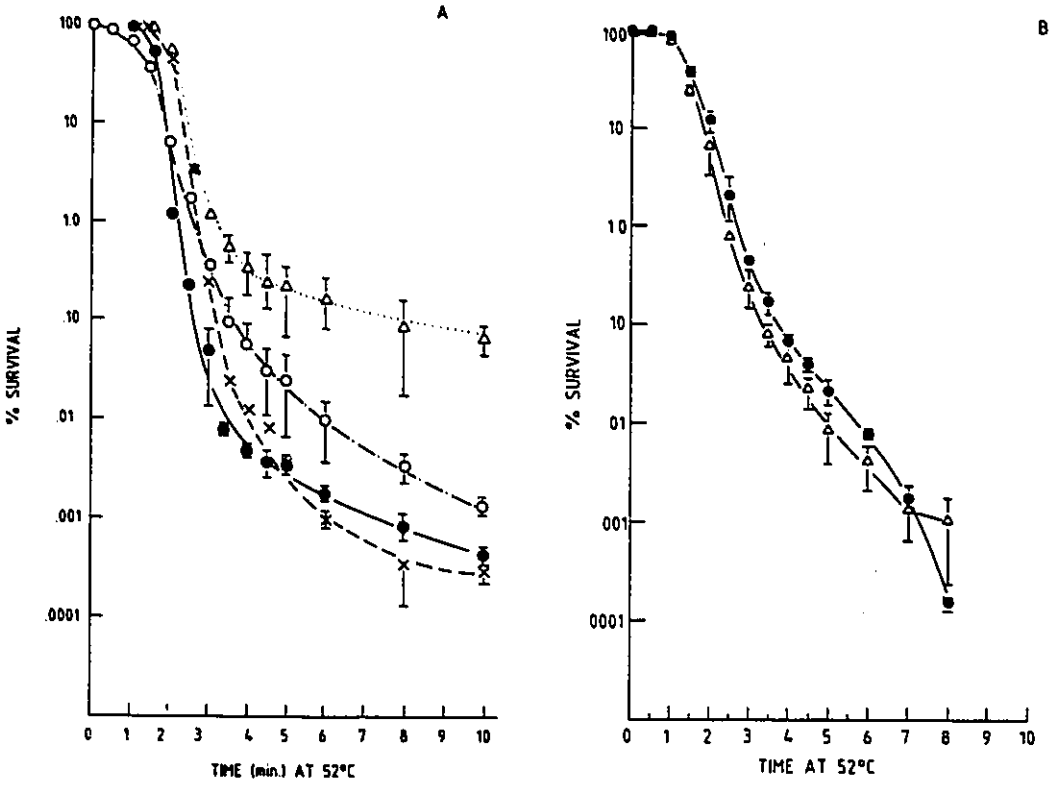

Figure 2.1. Survival of *Saccharomyces cerevisiae* to a 52°C 4 minute heat shock. (A) *S. cerevisiae* strains: wild type, •; *top1*, ×; *top2*, ○; *top1 top2*, Δ. (B) *S. cerevisiae* strains: wild type, •; *pol I-17*, Δ.

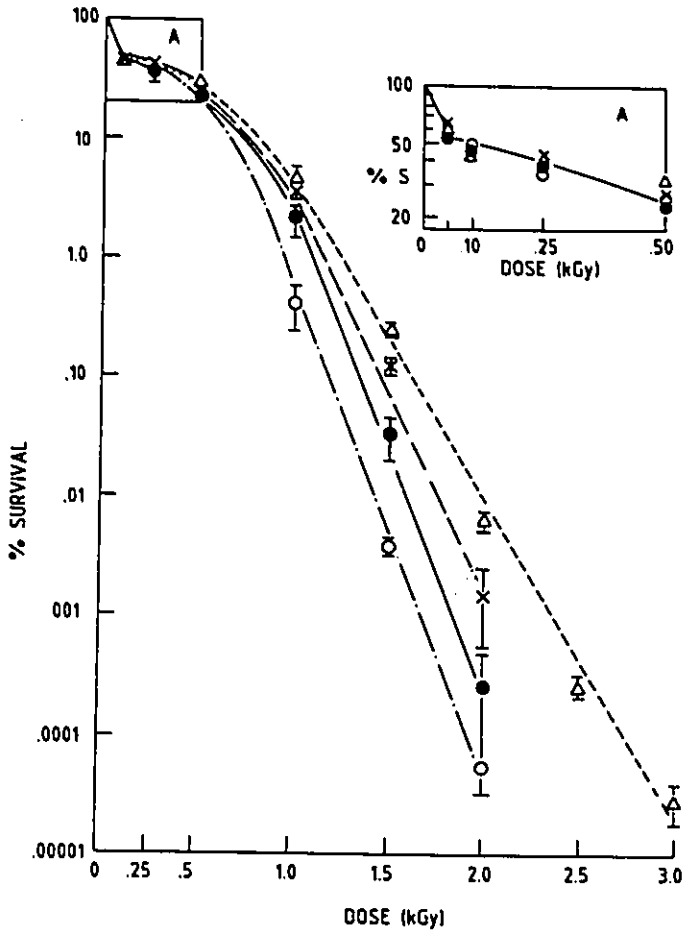

Figure 2.2. Survival of *Saccharomyces cerevisiae* topoisomerase strains and parental wild type after gamma irradiation in oxygen: wild type, •; *top1*, ×; *top2*, ∘; *top1 top2*, Δ. Insert A shows survival of the same strains at low doses.

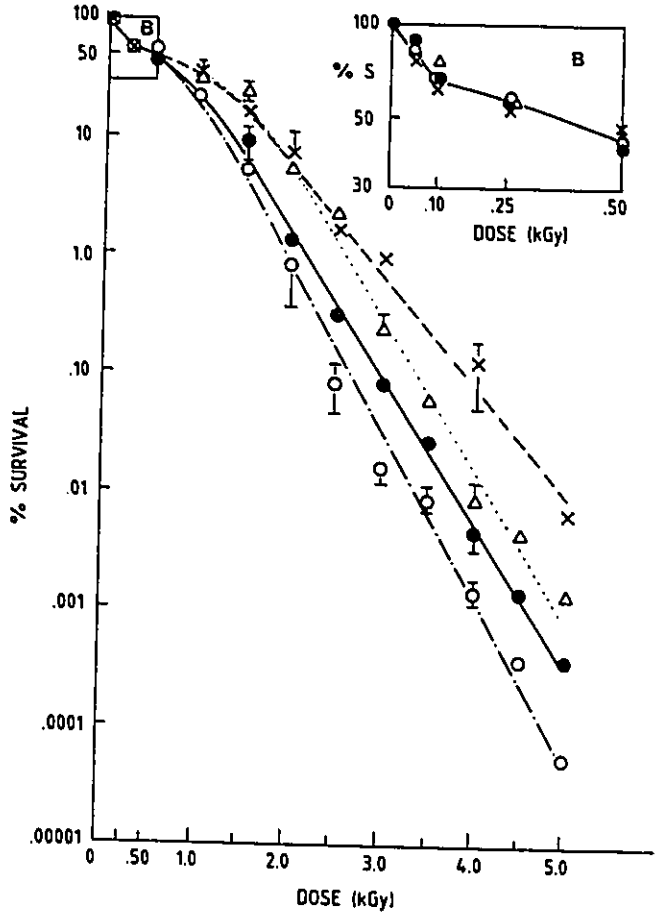

Figure 2.3. Survival of *Saccharomyces cerevisiae* topoisomerase strains and parental wild type after gamma irradiation in nitrogen: wild type, •; *top1*, ×; *top2*, ○; *top1 top2*, Δ. Insert B shows survival of the same strains at low doses.

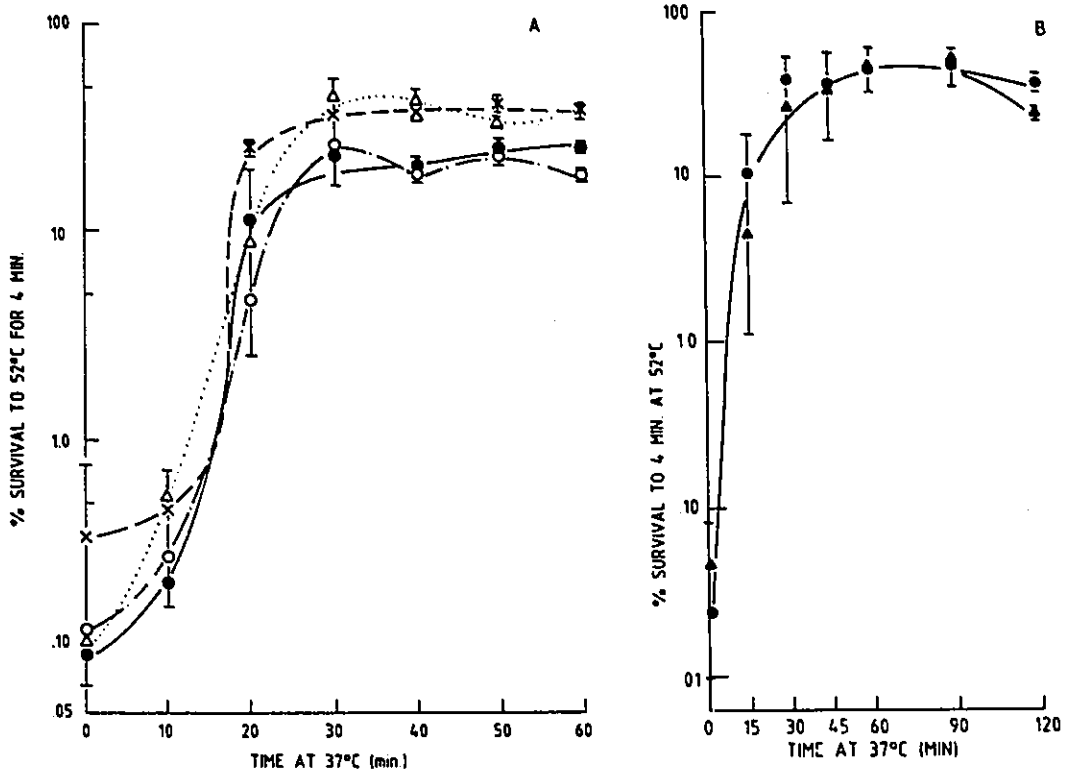

Figure 2.4. Heat shock induction of thermal tolerance. (A) Resistance of topoisomerase strains and parental wild type to a 4 minute 52°C thermal test dose following a sublethal heat shock of 37°C: wild type, •; *topI*, ×; *top2*, ○; *topI top2*, Δ. (B) Survival of DNA polymerase I strain and parental wild type to the same conditions: wild type, •; *pol I-17*, ▲.

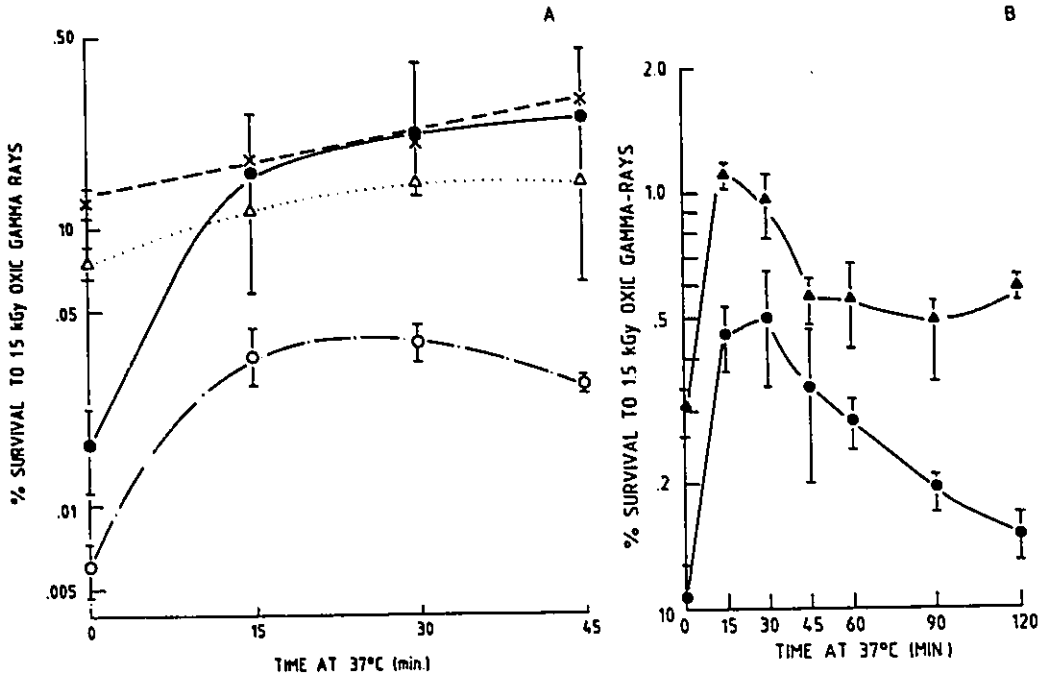

Figure 2.5. Heat shock induction of radiation resistance. (A) Survival of topoisomerase strains and parental wild type to a 1.5 kGy oxic gamma-ray test dose following a heat shock of 37°C: wild type, •; *topI*, ×; *topII*, ○; *topI topII*, Δ. (B) Survival of DNA polymerase I strain and parental wild type to the same conditions: wild type, •; *polA1*, ▲.

could not be further induced by heat. The absence of topoisomerase I conferred radioresistance to a level comparable to that of heat shocked wild type cells.

Figure 2.5B shows that a 37°C heat shock seemingly induced a transient radioresistance in both the DNA polymerase I deficient strain and its parental wild type. Induction was rapid and maximum resistance was reached after approximately 15-30 minutes, a result similar to that seen with the *top2* mutant (Figure 2.5A). The DNA polymerase I deficient phenotype may have had a slightly higher constitutive radioresistance compared to wild type cells. The data presented (combined from three experiments) in Figure 2.5B were obtained by using cultures derived from a single clone for each strain.

It has been previously suggested that different clones show the same relative results, but absolute values are somewhat variable (12). It was suspected that the slightly higher observed constitutive radioresistance of *pol 1-17* mutants may have been phenotypic variation between the clones used. In any case, the results demonstrate that the absence of polymerase I activity apparently does not prevent the induction of radioresistance by heat.

2.3.4 Radiation Induced Thermal Tolerance. The possible induction of thermal tolerance by radiation was determined by inducing cells with gamma-rays (0.5 kGy in oxygen) then incubating them at 25°C followed by a thermal test dose of 52°C for 4 minutes. The *top2*, *top1 top2* and *pol 1-17* cells were not used since their respective mutations are not expressed at normal (25°C) incubation temperatures. Figure 2.6A shows that radiation did induce thermal tolerance in both the wild type and the *top1* strains.

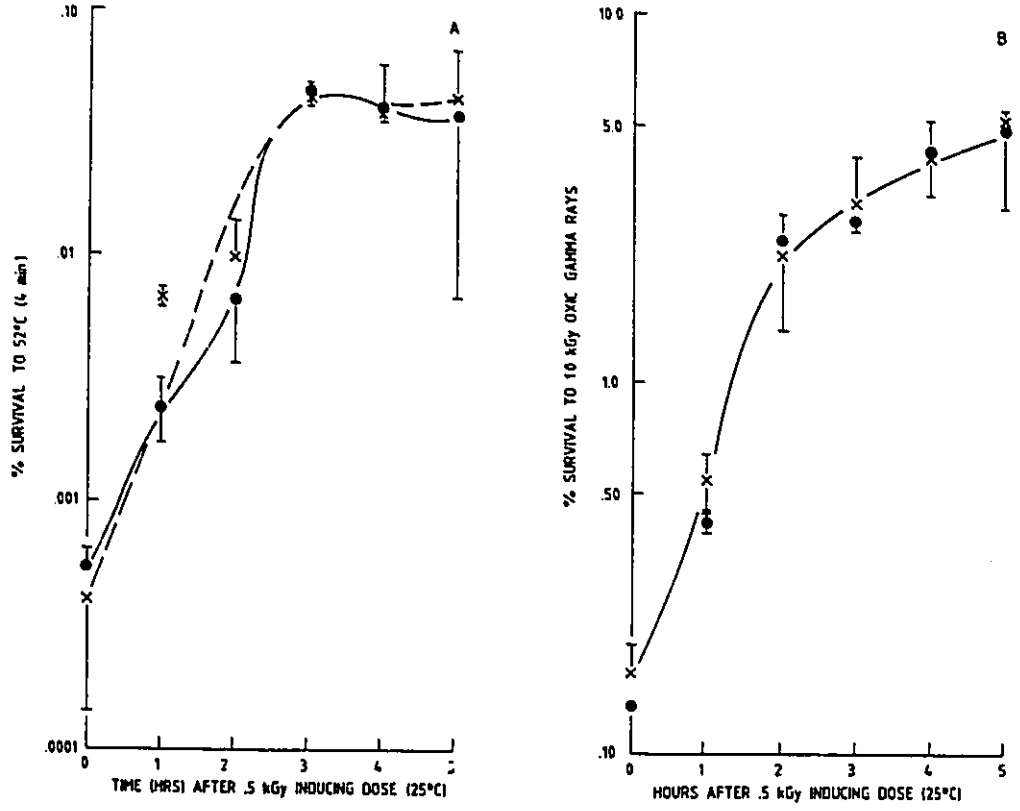

Figure 2.6. The induction of thermal tolerance (A) and radiation resistance (B) following a 0.5 kGy gamma-ray inducing dose in oxygen. Survival was determined following incubation under normal growth conditions at 25°C for increasing times: wild type, •; topl ×.

The two strains showed similar increased thermal tolerance following the gamma-ray inducing dose, reaching a maximum after approximately 3 hours of incubation (no significant difference between means for 3, 4, 5 hours with P=.9412). The data indicate functional topoisomerase I activity is not required for radiation induced thermal tolerance.

2.3.5 Radiation Induced Radiation Resistance. Figure 2.6B shows radiation induced radioresistance in wild type and *topI* cells. Both wild type and *topI* cells became radioresistant following a radiation inducing dose (all values are significantly different compared to the 0 hour control, P=1.453E-02). The data demonstrate that functional topoisomerase I activity was not required for the induction of radioresistance by radiation.

2.3.6 Combined Radiation and Heat Induced Thermal and Radiation Resistance. The induction of thermal tolerance in topoisomerase deficient strains by an exposure to gamma radiation (0.5 kGy oxic) and incubation at heat shock temperature (37°C, non-permissive for topoisomerase II activity) is shown in Figure 2.7A. Together heat and radiation resulted in induction of similar levels of thermal tolerance with similar kinetics in all strains. Maximum thermal tolerance was apparently reached after about 30 minutes of incubation. This result shows that radiation prior to heat shock did not seem to alter the kinetics or maximum level of induced thermal tolerance in any strain. Figure 2.7B demonstrates that combined radiation and heat similarly induced radiation resistance in all strains. Again, there did not seem to be a difference in the induction of mutant strains compared to wild type cells. This result should be compared to that shown in Figure 2.5A, where heat shock alone failed to induce further radiation resistance in the already radioresistant topoisomerase I deficient strains. However, radiation alone or together

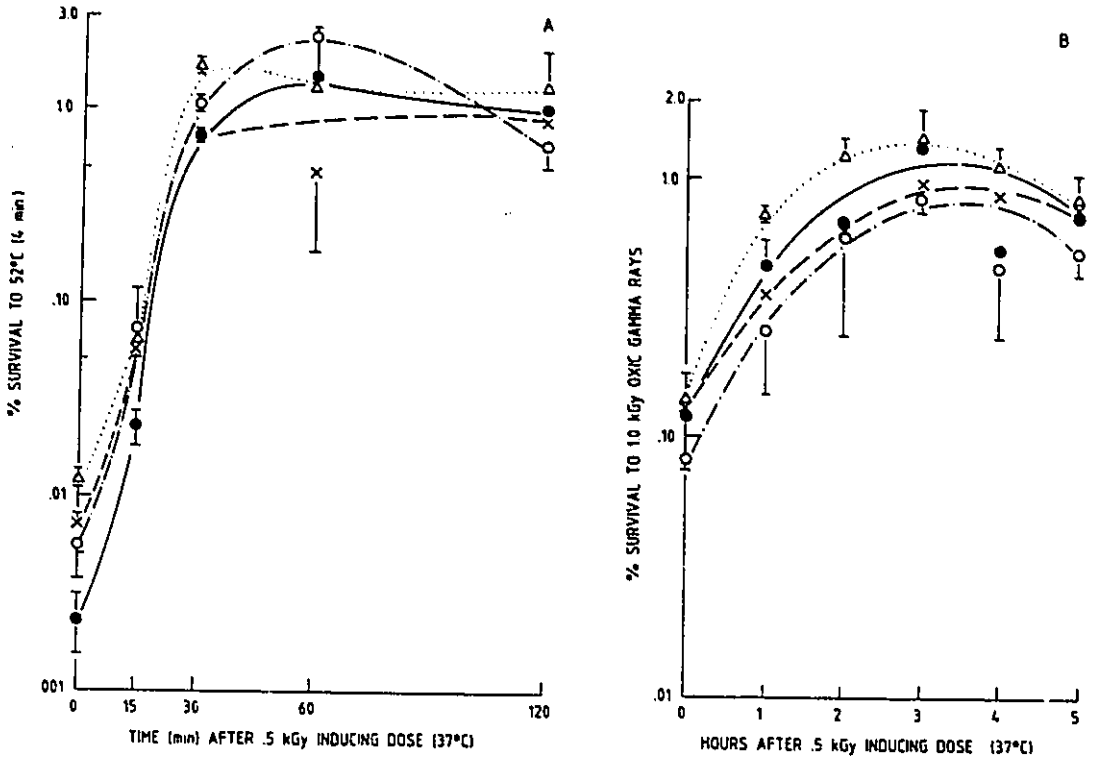

Figure 2.7. The induction of thermal tolerance (A) and radiation resistance (B) following a 0.5 kGy gamma-ray inducing dose in oxygen. Survival was determined following incubation at a temperature (37°C) which was both heat shock and non-permissive for *top 2*: wild type, •; *top1*, ×; *top2*, ∘; *top1 top2*, Δ.

with heat could induce additional radiation resistance in these *top1* and *top1 top2* strains (Figures 2.6B and 2.7B). Therefore, the absence of topoisomerase I activity (*top1*) resulted in a partial radioresistance similar to the maximum "heat-inducible" radioresistance response observed in wild type cells (Figure 2.5A), but less than the maximum "radiation-inducible" radioresistance response observed in the wild type and *top1* cells (Figure 2.6B and 2.7B). In summary, *top1* mutants which already exhibited this partial "heat-inducible" radiation resistance component, became additionally radioresistant in response to radiation alone (Figure 2.6B) or in combination with heat shock (Figure 2.7B). Radiation induced a maximal radioresistance response in all strains whereas a heat shock or topoisomerase I deficiency seemingly induced only a partial radioresistance response.

There was generally 100% cell survival to all the previously described inducing treatments (Figure 2.8). Viability was determined by plating appropriate dilutions and scoring colony forming ability. All yeast topoisomerase strains incubated for 120 minutes at the heat shock temperature of 37°C remained viable throughout the treatment (Figure 2.8A). Yeast strains given a 0.5 kGy γ-ray inducing dose in oxygen and incubated at 23°C for five hours showed no apparent decrease in cell survival (Figure 2.8B). The combination of heat shock (37°C) and γ-rays (0.5 kGy in oxygen) may have slightly decreased cell viability after 120 minutes of incubation (Figure 2.8C). The data shown were taken from a single representative experiment.

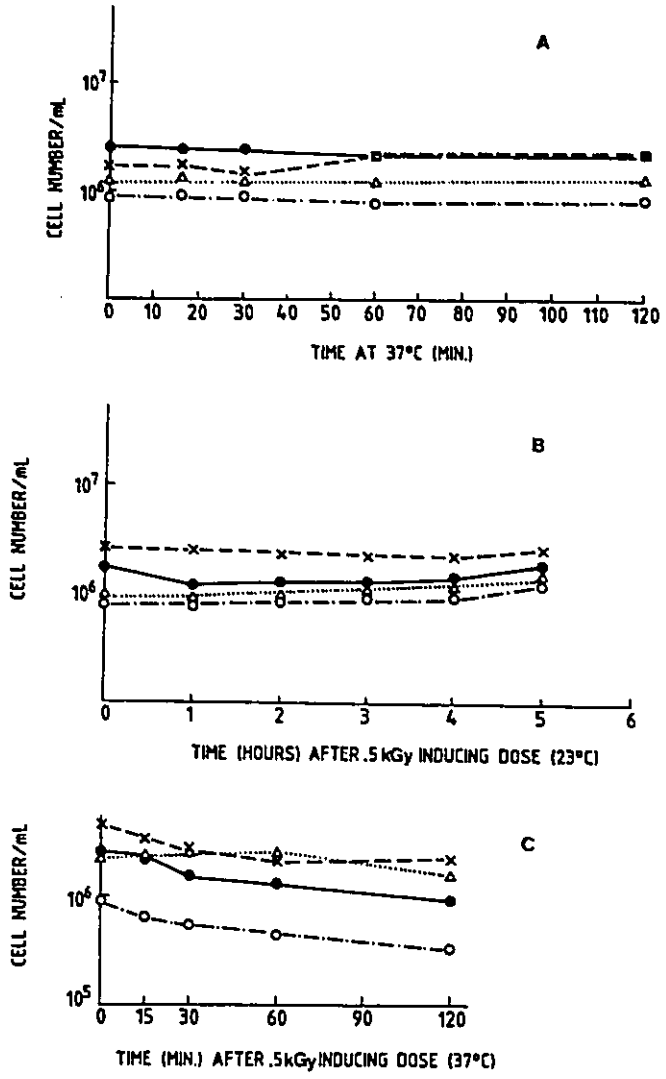

Figure 2.8. Survival of topoisomerase yeast strains to the various inducing treatments. (A) Heat shock at 37°C for 120 minutes. (B) A 0.5 kGy γ-ray inducing dose in oxygen and incubation at 23°C for 5 hours. (C) A 0.5 kGy γ-ray inducing dose in oxygen combined with incubation at 37°C for 120 minutes: wild type, •; *top1*, ×; *top2*, ○; *top1 top2*, Δ.

2.4 DISCUSSION

It was previously demonstrated that DNA damage serves as a signal for the induction of thermal resistance (as well as radiation resistance) in yeast, and postulated that alterations at the level of DNA may act in all cells as the signal for this ubiquitous response (13). DNA damage however, does not appear to serve universally as a signal for the induction of thermotolerance. Mammalian cells for example are not induced to thermal tolerance by DNA damage caused by ionizing radiation and yet are fully capable of induction of thermal tolerance by a heat shock (40). Given the absolute evolutionary conservation of this stress response, however, it would seem likely that the signal which controls the response would also be tightly conserved, even if there may have been evolutionary changes with regard to the number of genes which respond. In the experiments presented here a more general version of the original DNA damage hypothesis was investigated (13, 16). We have tested idea that perturbations to normal DNA supercoiling, which could be induced by either heat (41) or radiation (42), might alter normal gene expression and serve as a regulatory element during stress response. Yeast topoisomerase mutants were useful tools to test this hypothesis, since these enzymes in wild type cells maintain normal DNA topology by regulation of DNA supercoiling.

It has been proposed that transcribing genes are located within topoisomerase II constrained DNA chromatin loops (43) and that DNA topoisomerase I is concentrated on the transcribing regions but not on the non-transcribed flanking sequences (43). This relationship between topoisomerases and transcribing genes is consistent with a possible

regulatory association. Indeed there are many examples that demonstrate certain genes are responsive to DNA supercoiling and topoisomerase activity. Negative supercoiling induces unwinding of DNA duplex at various genomic sites including specific promoter regions (44) and several examples demonstrate gene regulation by DNA supercoiling. Some diverse cases include: transcriptional regulation of nitrogen fixation genes (45), chloroplast promoter regulation (46), and regulation of some prokaryotic metabolic operons (47). Also, while some genes are regulated by DNA supercoiling other genes may have simply evolved to function only under certain DNA supercoil tensions (47). Here experiments showed that the reduction in cellular control of supercoiling in the *top 2* and *top 1 top 2* mutants resulted in a more rapid thermal tolerance induction in response to a severe heat shock (Figure 2.1A). These results suggest that heat shock may be disrupting normal DNA supercoiling and in doing so the heat shock response is induced and thermal tolerance is conferred. The maintenance of normal DNA topology by topoisomerase activity may "down regulate" or repress the response. In the absence of any type of topoisomerase activity, as in the *top1 top2* mutants (Figure 2.1A), the cells ability to maintain normal DNA supercoiling during a heat challenge is severely impaired. The resulting rapid heat-induced supercoiling perturbation would predict increased sensitivity for induction of thermal tolerance in such cells, as was observed (Figure 2.1A).

Although the signal that controls the induction of thermal tolerance may be dependent upon DNA supercoiling, it appears that once activated the mechanism of thermal tolerance becomes independent of topoisomerase I and II activity. Heat (Figure 2.4A) or radiation (Figure 2.6A) induce thermal tolerance in all mutant and wild type cells. Han *et al.*, (22) reported that inhibition of topoisomerase II activity with novobiocin (also a DNA

gyrase inhibitor in *E. coli*) blocked transcription of *Drosophila* heat shock genes. Their results suggest that topoisomerase II activity is necessary for the normal induction of heat shock response. Here the results specifically demonstrate that yeast mutants deficient in topoisomerase II still possess a normal heat-inducible heat shock response.

As previously reported (12,16), it was observed that either heat shock (Figure 2.5A) or radiation (Figure 2.6B) induced radiation resistance in wild type cells. However, the absence of topoisomerase I activity conferred constitutive radioresistance compared to wild type cells (Figures 2.2 and 2.3), and heat shock could not further induce radiation resistance (Figure 2.5A). Nonetheless both topoisomerase I deficient cells and heat-induced radioresistant cells could still be induced to higher radioresistance by an additional radiation inducing dose (Figure 2.7B). These results suggest that the inducible DNA repair mechanism responds differentially to radiation and heat. Heat shock induces a partial response and the same response is signalled by topoisomerase I deficiency. The observed radioresistance in topoisomerase I deficient cells presumably reflects a greater DNA recombinational repair capacity (15) which implies a stronger induction signal. In essence, the type of signal is such that it confers radiation resistance with the same magnitude as the maximum observed partial response for heat shocked wild type cells. In this instance, with respect to radiation resistance, the response mechanism perceives a topoisomerase I deficiency in the same manner as a heat shock. A plausible explanation may be that topoisomerase I activity is stimulated in regions of heat shock gene activity. Subsequently, heat shock may inhibit or inactivate topoisomerase I function such that single strand breaks are created (similar to

topoisomerase I inhibitors such as camptothecin). This could initiate the partial induction of the radiation resistance mechanism.

Radiation induced additional radiation resistance in both topoisomerase I deficient cells and heat-induced radioresistant cells (compare Figures 2.6B and 2.7B and note that the initial level of resistance (0 Hours) is already at the maximum "heat-inducible" radioresistance observed in Figure 2.5A). If induction results from perturbations in DNA supercoiling, this result implies that the perturbation resulting from the lack of topoisomerase activity is insufficient to fully induce the recombinational DNA repair genes which respond to heat and radiation in yeast (15,16). Additional perturbation in the form of DNA damage was able to fully induce this response. It was previously shown that the level of induced resistance is proportional to the radiation dose; *ie.* proportional to DNA damage (16). This result is consistent with the idea that the inducing signal generated by a heat shock is a secondary consequence of topoisomerase I inactivation and subsequent perturbation to DNA supercoiling. It is also consistent with the hypothesis that the actual inducing signal for the induction of radiation resistance is likewise a disruption to normal DNA topology. Ueshima *et al.*, (48) recently reported that an *E. coli* gene encoding the heat shock sigma subunit of RNA polymerase is specifically activated by DNA supercoiling. They suggest that the activation by increased temperature is due to local DNA unwinding. The actual activation of heat shock genes also causes some perturbation to the nucleosome structure of chromatin (49). Coincidentally, the physical extent of the DNA perturbation was identified to be in regions of topoisomerase I activity. Therefore, heat induces transcriptional activation of heat shock genes which necessitates concomitant associated topoisomerase I activity.

Again, this would presumably generate abnormal DNA topology and cause the subsequent induction of repair genes. Heat-induced activation of repair genes may therefore be achievable only in localized areas of gene activity. Therefore, partial activation of radioresistance in topoisomerase I deficient mutants may be explained by a similar type of chromatin disruption. Similarly, the lack of induction of radiation resistance by a heat shock in mammalian cells may in part be due to the absence of repair genes in areas of active genes where heat shock could disrupt topoisomerase I activity and subsequently activate the repair genes.

Because of the universality of the heat shock response it is unlikely that topoisomerase II plays a major role in this response. Cellular levels of topoisomerase II vary considerably depending upon cell cycle (50), cell proliferation (51,52), nutrient condition (53), and even among the cell lines (54,55). Here the results indicated that the absence of topoisomerase II activity did not prevent or alter induction of thermal or radiation resistance by heat or radiation. On the other hand, cellular levels of topoisomerase I are invariably more constant (53,56) which further supports the concept that topoisomerase I could be a probable target for the ubiquitous heat shock response.

DNA polymerase α has been identified in several repair processes including damage induced by UV radiation (57) or chemicals (58). Heating cells before x-irradiation has been shown to inhibit repair of DNA damage in mammalian cells. DNA polymerase α has also been proposed as a possible hyperthermic inactivation target (20,59). Heat shock and the induction of thermotolerance in mammalian cells has not revealed any alteration in DNA polymerase α activity (20,60) but the possibility exists that DNA polymerase α could be a component of the inducible DNA repair mechanism

(61). Kampinga *et al.* (62) have recently reported that heat inactivation of DNA polymerase α is not the general cause for hyperthermic repair inhibition in HeLa cells. In this thesis the experiments demonstrated that DNA polymerase I (the yeast analogue of mammalian DNA polymerase α) is not a target responsible for thermal killing in yeast (Figure 2.1B). Furthermore, the mechanism of induced thermal tolerance or radiation resistance is independent of DNA polymerase I activity (Figures 2.4B, 2.5B). It is concluded therefore that DNA polymerase I is not a necessary inducible component of the radioresistance or thermal tolerance mechanism in yeast. The thesis results also corroborate the conclusions of Budd *et al.* (33) demonstrating that DNA polymerase I is not an essential enzymatic component involved in the repair of radiation-induced DNA damage.

In summary, it is suggested that induction of thermal tolerance and radiation resistance is controlled by alterations in DNA topology (see model in Figure 2.9). Evidence is presented that under heat stress, the absence of topoisomerase I and II activity causes a cell to increase its thermal tolerance sooner than a wild type cell. It is postulated that maintenance of normal DNA topology suppresses activation of certain stress-inducible genes, and the loss of supercoiling control in the topoisomerase mutants results in their increased sensitivity to induction by a heat stress. The actual system that confers thermal tolerance or radiation resistance is independent of topoisomerase I and II function. However, topoisomerase I deficiency or heat shock will induce comparable radiation resistance responses. These radioresistant cells can be further induced by an additional radiation exposure. It was postulated that heat shock indirectly induces partial radiation resistance due to disruption of topoisomerase I activity and its corresponding loss of local control of supercoiling. Radiation induced DNA damage may then result in further

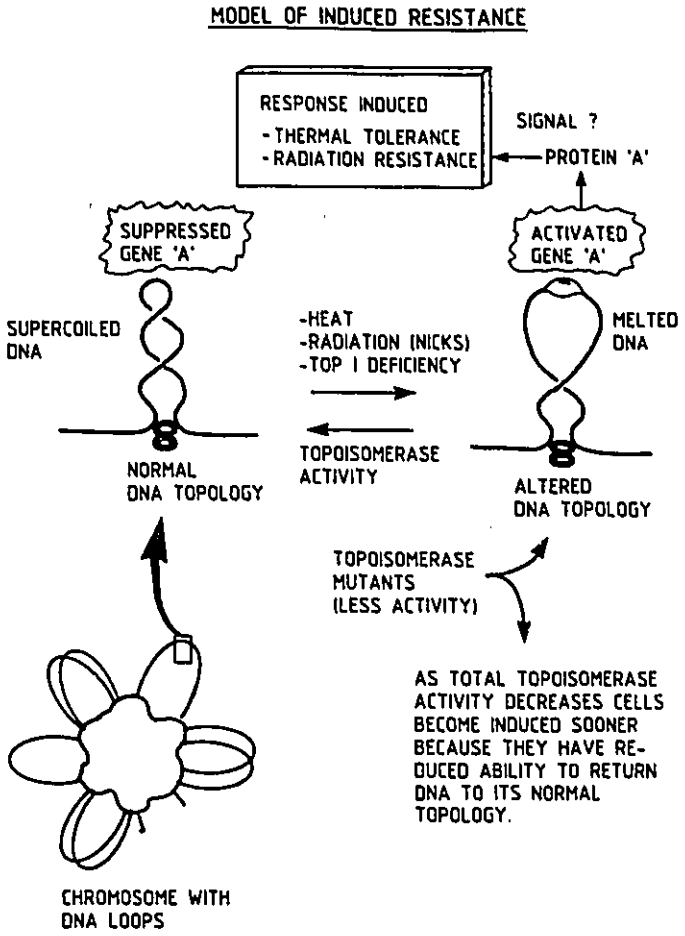

Figure 2.9. Model demonstrating that the mechanism of induced thermal tolerance and radiation resistance could be regulated by perturbation to DNA topology. Supercoiled DNA is packaged into several higher orders of structure. DNA wraps around histone proteins to form nucleosomes. Nucleosomes are bundled into regular repetitive arrays known as chromatin fibres which are further organized into looped domains that project from the backbone of the chromosome (63). Topoisomerases control DNA topology and maintain normal supercoiling. Genes involved in the mechanisms of thermal tolerance and radiation resistance are suppressed under normal supercoil tension. Heat, ionizing radiation, or topoisomerase I deficiency generates localized changes in DNA topology that causes the supercoiled domain to unwind. This melts the DNA double helix, activates a particular gene, and produces a protein that may regulate the respective mechanism.

supercoiling changes and further induction of radiation resistance. It was concluded that DNA polymerase I is not involved in the signalling nor does it participate in the mechanism of induced thermal tolerance and radiation resistance in yeast. Finally, it is noted that this thesis hypothesis suggesting environmentally generated changes in DNA supercoiling as the signal for inducible stress responses meets the criteria for a very basic or primitive type of gene control which could have evolved very early in simple organisms and which could have been evolutionarily conserved in a diversity of phyla.

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CHAPTER THREE

EFFECT OF HEAT SHOCK OR RADIATION ON DNA REPAIR IN GREEN ALGAE.

3.1 INTRODUCTION

The unicellular microorganisms *Saccharomyces cerevisiae* and *Chlamydomonas reinhardtii* have been used to examine certain aspects of DNA repair. In this chapter, inducible DNA recombinational repair processes observed in *S. cerevisiae* (Chapter 1) were compared with those of *C. reinhardtii*, to assess the universality of this mechanism. The decision to compare *C. reinhardtii* was inspired, in part, by previous reports in the literature suggesting that this organism exhibited unusual radiobiological behaviour (1).

The fate of an irradiated cell depends principally upon the dose delivered, the presence or absence of oxygen, and the cell's genetically determined competence for DNA repair (2). As mentioned (Chapters 1 and 2), DNA recombinational repair is believed to be the major inducible repair pathway which confers radioresistance to yeast cells. In yeast, there is a proportional relationship between the level of induced radioresistance and the amount of initial inducing damage (2). Yeast cells sublethally irradiated in oxygen subsequently became more radioresistant than cells irradiated with the same dose under anoxic conditions. Thus, a report by Bryant (1) demonstrating radiation-induced radioresistance of *C. reinhardtii* but suggesting a lack of an oxygen effect for this response was curious, contradictory to results observed

in yeast, and cast doubt on the universality of this mechanism (3, Chapter 1).

Initially, it was proposed that the lack of an oxygen effect for the induction of radioresistance of *C. reinhardtii* was an artifact of experimental protocol (different radiation source, insufficient gassing conditions, variation in plating protocol, etc.). Therefore, Bryant's (1) experiments were repeated using the same experimental parameters except adopting the procedures used for yeast experiments (3).

In yeast, heat shock was also shown to induce DNA recombinational repair and confer radioresistance (Chapter 2). In mammalian cells however, heat shock inhibits certain aspects of DNA repair and therefore sensitizes cells to radiation (5). It was unknown how heat would affect DNA repair and radioresistance in *C. reinhardtii*. Since *C. reinhardtii* previously demonstrated inducible radioresistance similar to yeast (6) but unlike mammalian cells (7), it was postulated that *C. reinhardtii* would respond in a manner analogous to yeast (also a unicellualr eukaryotic organism) and become radioresistant in response to heat shock. Interestingly, the results demonstrated a response in *C. reinhardtii* dissimilar to results observed in other organisms.

N-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) is a chemical mutagen and is known to produce mutations in a variety of organisms (8-10). Treatment of cells with nonlethal doses of MNNG results in the induction of a set of enzymes which repair the DNA lesions generated by this compound. In bacteria and mammalian cells O^6-MG lesions are repaired by a specific inducible transferase protein which transfers the methyl group to a specific cysteine residue of the protein resulting in inactivation

of the protein (11,12). This inducible DNA repair response in called the adaptive response. Both *C. reinhardtii* and *S. cerevisiae* apparently lack methyltransferase and the adaptive response (9,10). Although yeast lack an adaptive response and a methyltransferase activity for repair of MNNG-induced mutation, it was shown that they were capable of repairing O^6-MG lesions via the recombinational DNA repair pathway (4). As previously reported (3,4) and confirmed in Chapter 1, radiation-induced recombinational repair and the coinciding suppression of MNNG-generated mutations clearly demonstrated an oxygen effect in yeast. Therefore, it was postulated that radiation-induced suppression of MNNG-generated mutation could be used as an alternative endpoint to demonstrate an oxygen effect for the induction of radioresistance in *C. reinhardtii*. The experimental results did not support this postulate.

Finally, as previously demonstrated in yeast (4,13,and Chapter 1), DNA repair induced by heat shock also conferred resistance to MNNG-generated mutations. Frost and Small (9) have concluded that *C. reinhardtii* lacked a mechanism to repair MNNG-induced mutations. Here it was postulated that, like yeast, heat shock would induce DNA repair capable of repairing MNNG-generated mutations in *C. reinhardtii*. This postulate was also rejected and the results corroborated the conclusions of Frost and Small (9).

3.2 MATERIALS AND METHODS

3.2.1 Growth of Chlamydomonas. The organisms used in the following experiments were two strains of the unicellular green alga *Chlamydomonas reinhardtii*. Strain CC-137 (mating type plus) was a wild type cell whereas CC-85 was a nicotinamide requiring mutant (*Nic-7*, mating type plus). Both cultures were obtained as gifts from Dr.E. Harris at the Chlamydomonas Genetics Centre, Duke University. Both algal cultures were grown in tris-acetate-phosphate medium (TAP) (14) supplemented with 0.75 μg/ml of nicotinamide. Cells were grown at 21°C and maintained at a cell density of 1×10^6 cells/ml. To maintain unsynchronized algal populations, cultures received 8 hours of light over a 24 hour period. Cultures were synchronized by a 12 hour light and dark cycle as previously described (15). Cell synchrony was verified by sizing cells with a Coulter Counter. Solid TAP media plates were prepared by adding 15 g/l of highly purified agar (Difco) to the liquid growth media.

3.2.2 Radiation Survival and Induced Radioresistance. Freshly grown *Chlamydomonas* cultures were harvested by centrifugation and the cells were washed three times in 20 mmol dm^{-3} (20 mM) phosphate buffer, pH 7.0, at 0°C. Cells were resuspended (1×10^6 cells/ml) in buffer and equilibrated by bubbling the suspension with either nitrogen or oxygen (Matheson ultrahigh purity) in the dark for 15 minutes before and during irradiation. Cells were irradiated with ^{60}Co γ-rays (Gamma cell 220, Atomic Energy of Canada) at a dose rate of about 2 Gy/sec. Radioresistance was induced using two different techniques.

The first method involved irradiating algal cells with increasing doses of γ-rays in the presence of oxygen or in the presence of nitrogen. The cells were then resuspended in TAP media and incubated in the light under normal growth conditions. After two or four hours of incubation, induced radioresistance was determined by measuring cell survival to a single 150 Gy γ-ray test dose delivered in the presence of oxygen.

The second method consisted of inducing cells with a single 150 Gy γ-ray dose delivered in the presence of oxygen or in the presence of nitrogen, allowing a four hour incubation period under growth conditions, and then subsequently administering increasing doses of γ-rays delivered under oxic conditions. Survival was determined by scoring colony formation on TAP plates after 4-5 days of incubation in continuous light. It should be noted that considerable care was taken to ensure that the algal cells were truely anoxic. Viable photosynthetic algal cells produce oxygen in the presence of light. Prior to irradiation, cell culture tubes were wrapped in foil, room lighting was switched off, and cultures were bubbled with the appropriate gas prior to (15 min.) and during the irradiation.

3.2.3 Thermal Tolerance. Thermal tolerance was determined by placing a number of 2.5 ml cell aliquots ($2x10^6$ cells/ml in TAP medium at 0°C in 16x125 mm glass test tubes) into a 45°C water bath. Every minute for a total period of 7 minutes, an aliquot was removed, rapidly recooled to 0°C, and plated for survival as described above.

3.2.4. Heat shock induced thermotolerance and radiation resistance. Experiments were conducted with either unsynchronized or synchronized algal cultures. Cells in G_1 or G_2 phase of the cell cycle were harvested

and resuspended in fresh 21°C TAP nutrient media. Algal cells (50 ml) were placed in a 500 ml flask and abruptly transferred to a shaking water bath at 37°C. Aliquots were removed at appropriate times and were tested for induced thermotolerance and radioresistance. Thermotolerance of heat shocked cells was assessed by measuring survival to a seven minute, 45°C thermal test dose whereas radioresistance was determined by measuring survival to a single 150 Gy γ-ray test dose delivered in the presence of oxygen. Unsynchronized algal cultures were heat shocked (37°C, 30 min.) and tested for induced radioresistance by subsequently irradiating the cells with increasing doses of γ-rays (in N_2).

3.2.6. Survival and MNNG-induced mutation. Unsynchronized algal cells (CC-85) were washed three times in 20 mmol dm^{-3} (20 mM) phosphate buffer and pelleted. A stock solution of MNNG was freshly made by dissolving MNNG in 20 mmol dm^{-3} (20 mM) phosphate buffer with the aid of a warm water sonicator. Appropriate amounts of MNNG were added to the cell pellets and the suspensions were incubated at 23°C for 30 minutes in the light. The MNNG was then inactivated by diluting the cell suspension with 10 ml of 10% sodium thiosulfate followed by centrifugation. The cell pellet was washed three times in 0°C buffer and resuspended at a density of approximately 1 x 10^7 cells/ml. Treated samples (1 ml) were plated directly onto TAP plates lacking nicotinamide to determine the number of revertants to nicotinamide independence, a measure of mutation. Separate aliquots were appropriately diluted and plated on TAP plates containing nicotinamide to determine survival to the MNNG treatment.

3.2.7. Heat shock and cycloheximide suppression of MNNG-induced mutations. A single algal culture (CC-85) was divided into two flasks.

Cycloheximide (100 μg/ml) was added to one flask and the flask was incubated at 23°C in the light. The second flask was heat shocked in a hot water bath (37°C) in the light. At increasing time intervals, aliquots from each flask were removed and immediately cooled to 0°C. The aliquots were all treated with MNNG (20 μg/ml) and reversion to nicotinamide independence was determined as previously described.

3.2.8. Radiation suppression of MNNG-induced mutation. Radiation-induced suppression of MNNG mutation was attempted using the identical technique previously reported in yeast (4). Unsynchronized algal cells (CC-85) were washed and resuspended in buffer at 0°C as previously described. Cells were then equilibrated in either N_2 or O_2, exposed to γ-rays, and aliquots were removed at increasing doses. Each irradiated sample was held at 0°C before and after the MNNG treatment. MNNG treatment and mutation assessment was conducted as previously described. Radiation treatments did not produce mutation as determined by reversion to NIC+.

3.2.9. Data and Figures. Each experiment was repeated at least three times from independent cultures. All figures shown represent the combined results of all the experiments. Standard error bars are shown except were the error is less than or equal to the symbol size. One-way analysis of variance and Student's T-tests were performed to determine the level of significance between various treatments. P-values are given where values are considered experimentally relevant.

3.3 RESULTS

3.3.1 Radiation survival and induced radioresistance. Unsynchronized algal cells were more sensitive to the lethal effects of γ-radiation in the presence of O_2 compared to cells irradiated in the presence of N_2. *C. reinhardtii* strains CC-137 and CC-85 clearly demonstrated a radiobiological oxygen effect for survival to γ-rays (Figures 3.1 and 3.2). In Figures 3.1 and 3.2 the paired means (O_2 with N_2) above 100 Gy are all signficantly different (P<1.0E-03). In Figure 3.3 paired values above 40 Gy were all significantly different (P<1.0E-03). Wild type algal cells had an oxygen enhancement ratio (OER) of approximately 2.5. However, these same unsynchronized algal cells lacked an oxygen effect for the induction of radioresistance by γ-rays.

In radiobiological terms, an oxygen effect refers to an enhanced response observed in the presence of oxygen. Therefore it was postulated that cells given inducing doses in oxygen would become more radioresistant compared to cells given the same inducing doses in nitrogen. Cells induced with increasing doses of γ-rays in oxygen did not become more radioresistant than cells given the same inducing dose in the presence of nitrogen, following two hours of incubation (Figure 3.4). Even after four hours of incubation, cells radiation-induced in the presence of oxygen did not acquire greater maximum radioresistance than cells induced in the presence of nitrogen (Figure 3.5). The hypothesis that algal cells would demonstrate a radiobiological oxygen effect for radiation-induced

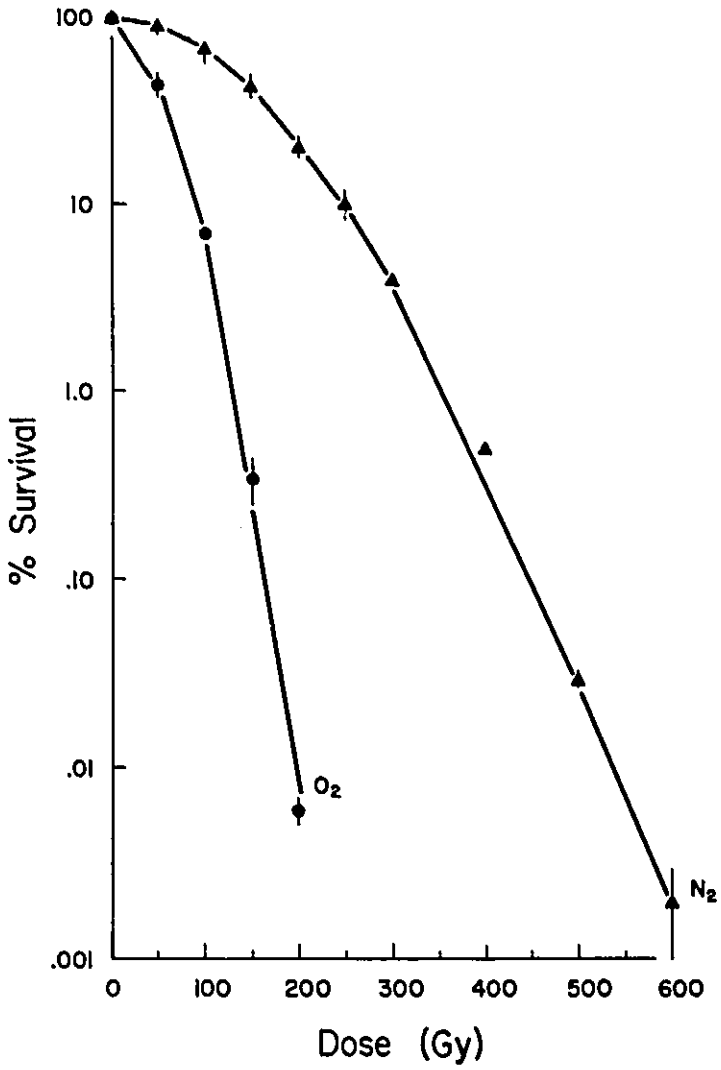

Figure 3.1. Survival of unsynchronized *Chlamydomonas reinhardtii* (wild type CC-137) to γ-rays in the presence of O_2 (•), or N_2 (▲).

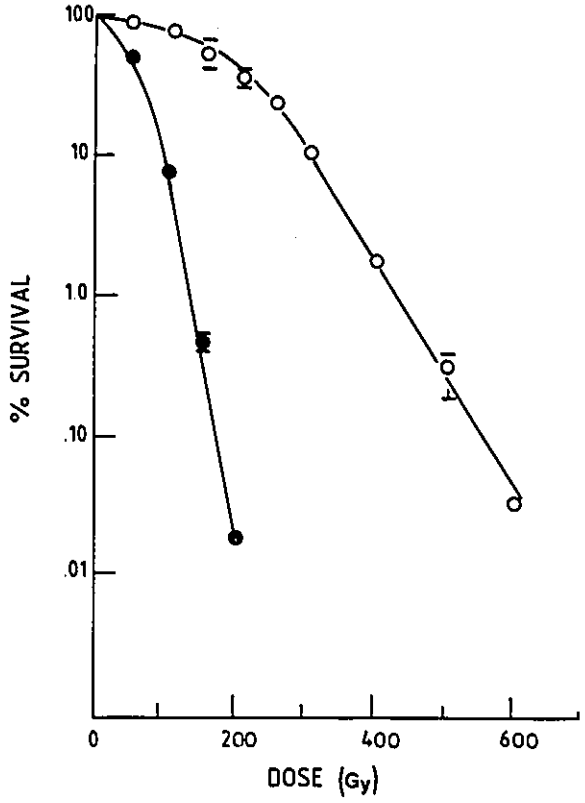

Figure 3.2. Survival of unsynchronized *Chlamydomonas reinhardtii* (nicotinamide requiring mutant CC-85) to γ-rays in the presence of O_2 (•), or N_2 (o).

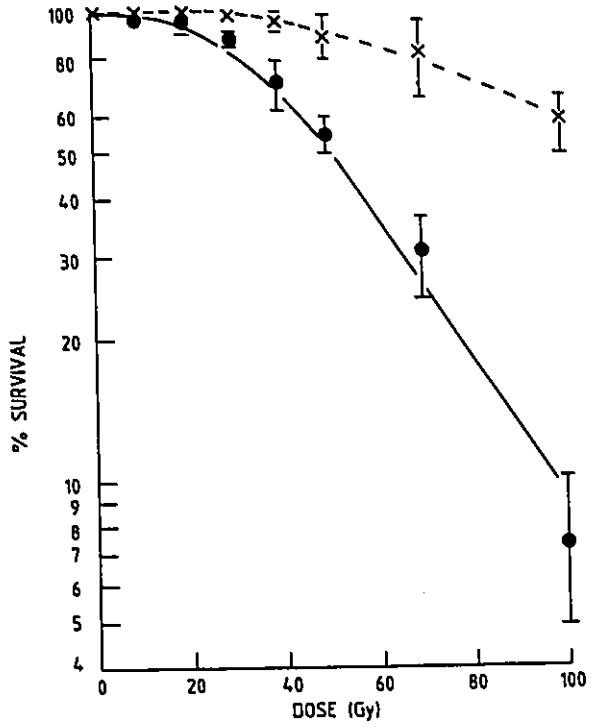

Figure 3.3. Survival of unsynchronized *Chlamydomonas reinhardtii* (wild type CC-137) to low doses of γ-rays in the presence of O_2 (•), or N_2 (X).

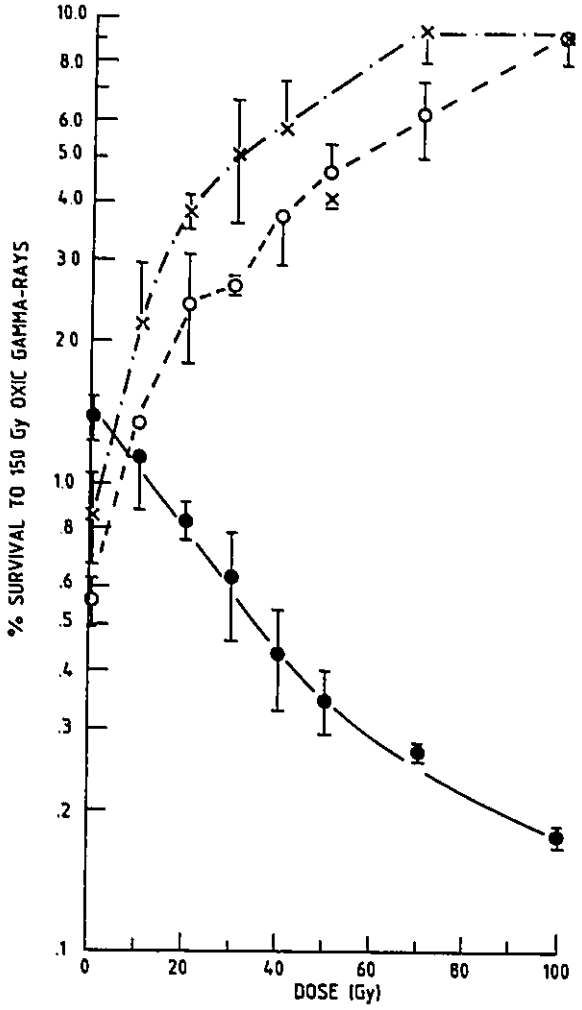

Figure 3.4. Radiation-induced radioresistance in unsynchronized Chlamydomonas reinhardtii (CC-137). Increasing γ-ray inducing doses were delivered in the presence of O_2 (o) or N_2 (×) and cells were incubated for two hours. Induced radioresistance was measured as survival to a single 150 Gy γ-ray test dose delivered in the presence of oxygen. Controls were given inducing doses in the presence of N_2 (•) and immediately tested for radioresistance.

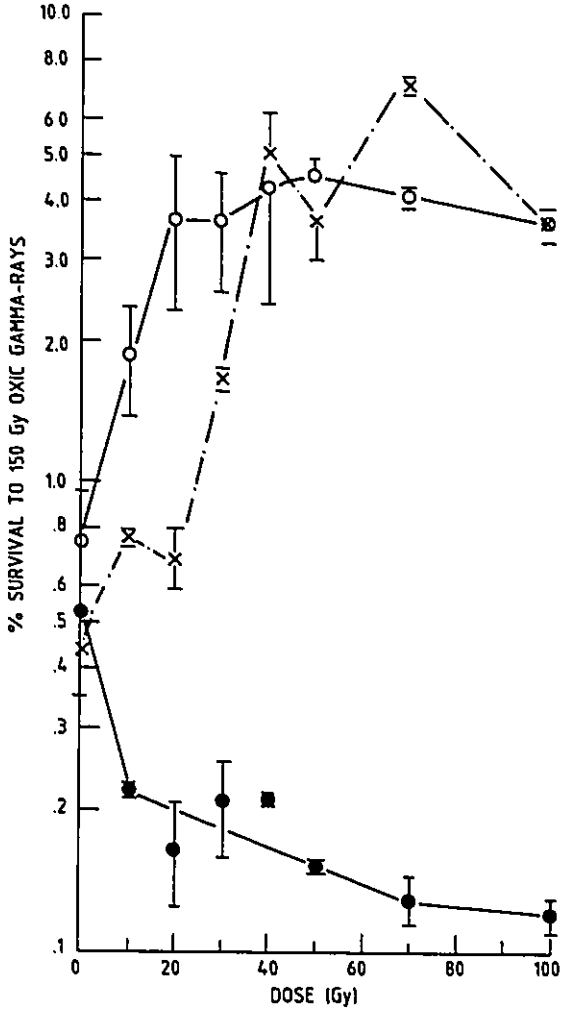

Figure 3.5. Radiation-induced radioresistance in unsynchronized Chlamydomonas reinhardtii (CC-137). Increasing γ-ray inducing doses were delivered in the presence of O_2 (o) or N_2 (×) and cells were incubated for four hours. Induced radioresistance was measured as survival to a single 150 Gy γ-ray test dose delivered in the presence of oxygen. Controls were given inducing doses in the presence of N_2 (•) and immediately tested for radioresistance.

radioresistance was rejected. At maximum induced radioresistance (100 Gy) there was no significant difference between cells induced in the presence of O_2 compared to cells induced in the presence of N_2 (Figure 3.4 and 3.5, P=1.123E-03 and 1.329-02 respectively). Control cells given γ-rays in the presence of N_2 and immediately tested for resistance did not become radioresistant and seemingly became slightly radiosensitive (Figure 3.4 and 3.5). Survival of the control cultures decreased with increasing γ-ray dose.

Figure 3.6 also demonstrates the lack of an oxygen effect for the induction of radioresistance in unsynchronized algal cells. Cells were first given a single 150 Gy γ-ray inducing dose either in the presence of oxygen or nitrogen and allowed to incubate for four hours under normal growth conditions. Subsequent to incubation, survival to increasing test doses of γ-rays in the presence of O_2 showed that cells given a prior oxic γ-ray inducing dose did not become more radioresistant than those given a prior anoxic inducing dose. It seems that cells given radiation-inducing doses in the presence of nitrogen were significantly more radioresistant (Figure 3.6: 250 Gy P=1.834-03, 300 Gy P=4.701E-02). Survival of control cells to γ-rays in O_2 is also shown in Figure 3.6. It is important to note that 150 Gy is a high radiation inducing dose with lethal effects when delivered in the presence of oxygen. Nonetheless, the experimental protocol was designed to repeat previously reported observations (1).

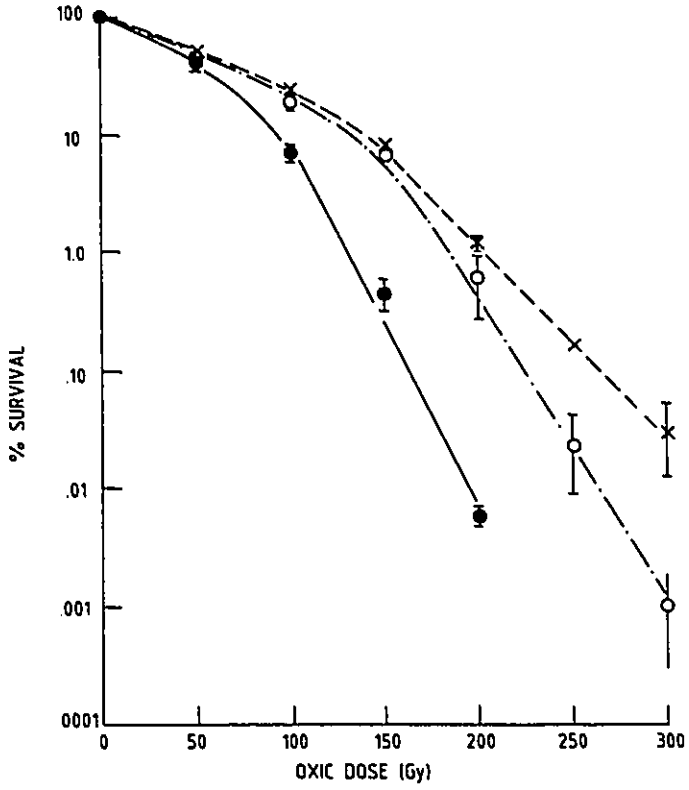

Figure 3.6. Radiation-induced radioresistance in unsynchronized *Chlamydomonas reinhardtii* cells (CC-137). Cells were given a single 150 Gy γ-ray inducing dose in the presence of O_2 (∘) or N_2 (×) and incubated for four hours under normal growth conditions. Radioresistance was measured as survival to increasing doses of γ-rays delivered in the presence of oxygen. Control cell survival to γ-rays in O_2 is shown (•).

A similar experiment is depicted in Figure 3.7 except that synchronized G_1 cells (4 hours into the light cycle) were given a single 50 Gy γ-ray inducing dose in the presence of O_2 or N_2. Cell synchrony was verified by measuring cell size (coulter counter) and morphology (light microscopy). Induced cells were then incubated for three hours (based on preliminary experiments) under normal growth conditions. There was no significant difference in the level of induced radioresistance between cells given either oxic or anoxic inducing doses (no difference between paired means P<1.0E-02). Figure 3.7 also demonstrates that radioresistance in control cells did not change throughout the three hour incubation period (mean survival values from two independant experiments are plotted on graph).

3.3.2. The effect of heat shock on radioresistance. The sensitivity of unsynchronized algal cells to a 45°C heat shock is shown in Figure 3.8. Heat shock (30 min., 37°C) did not seem to sensitized nor did it induce resistance to radiation in unsynchronized wild type *C. reinhardtii* cells (Figure 3.9). There appears to be a slightly greater radioresistance at 400 Gy and 500 Gy in heat shocked cells however the means are not significantly different (P=.8529).

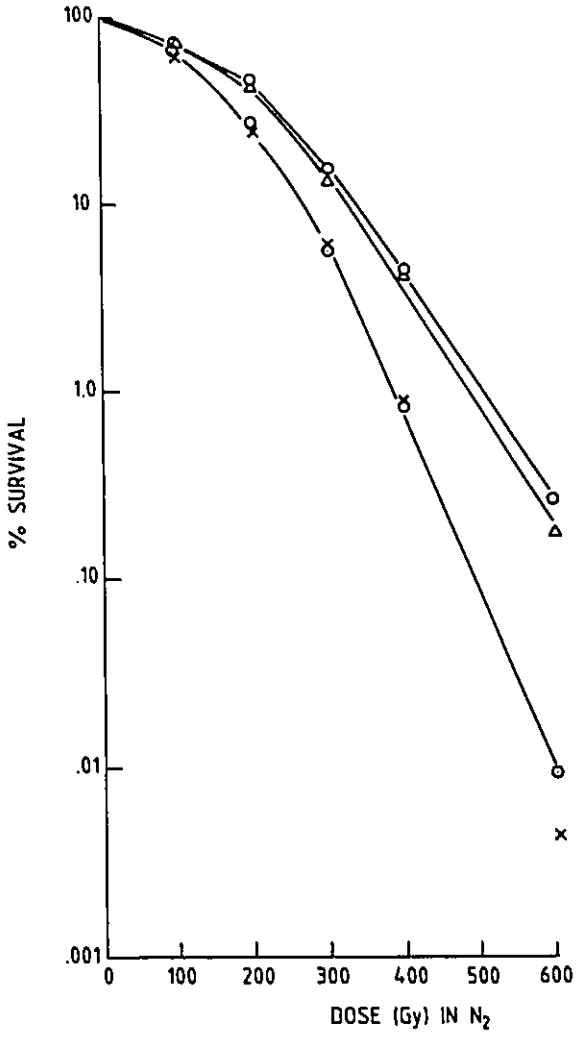

Figure 3.7. Radiation-induced radioresistance in synchronized G_1 *Chlamydomonas reinhardtii* cells. Cells were given a single 50 Gy inducing dose in the presence of either O_2 (o) or N_2 (Δ) and were incubated for 3 hours under normal growth conditions. Radioresistance was measured as survival to increasing doses of γ-rays delivered in the presence of N_2. Survival of control cells before (o) and after 3 hours (×) of incubation is shown.

The same result was observed in synchronized algal cultures. Heat shock did not alter the radioresistance of either G_1 cells (Figure 3.10A, all radiation survival means not significantly different P=.8991) or G_2 cells (Figure 3.10B, all radiation survival means not significantly different P=.7910). Initial survival to the radiation or thermal test doses were about the same for G_1 and G_2 cells (Figures 3.10 A and B). Synchronized cells demonstrated a normal heat shock response and became thermal tolerant in response to a 37°C heating. Maximum thermal tolerance was reached after 30 minutes at 37°C in both G_1 (Figure 3.10A) and G_2 (Figure 3.10B) cells. Therefore algal cells became thermal tolerant yet radioresistance was unaffected by heat shock (Figures 3.10A and 3.10B). These responses were independent of cell cycle progression.

3.3.3. Survival and MNNG-generated mutations. Survival and mutation (reversion of CC-85 algal cells to nicotinamide independence, NIC+) was measured after treating cells with increasing concentrations of MNNG (30 minute treatment). Figure 3.11 shows that concentrations of MNNG above 5 μg/ml are lethal to algal cells. However, an increase in mutation frequency was seen above 5 μg/ml of MNNG (Figure 3.12). A maximum mutation rate (reversion to NIC+) was observed at approximately 40 μg/ml of MNNG (Figure 3.12) while survival to the same dose was less than 30% (Figure 3.11).

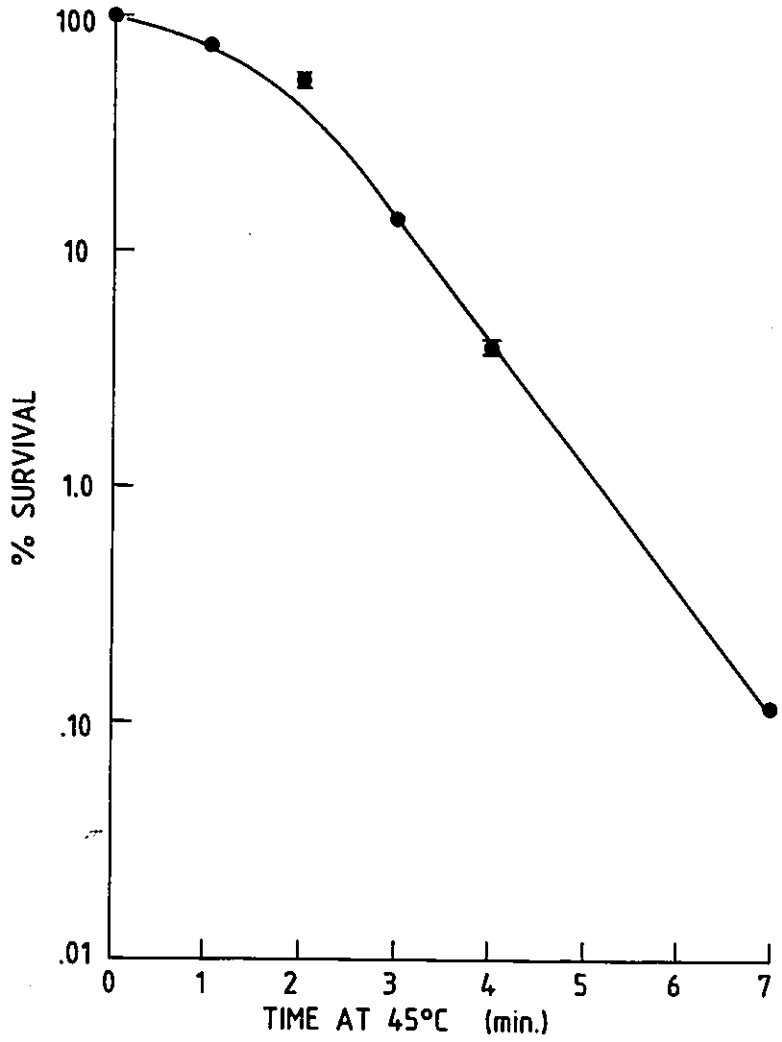

Figure 3.8. Survival of unsynchronized wild type (CC-137) *Chlamydomonas reinhardtii* cells to a thermal challenge of 45°C.

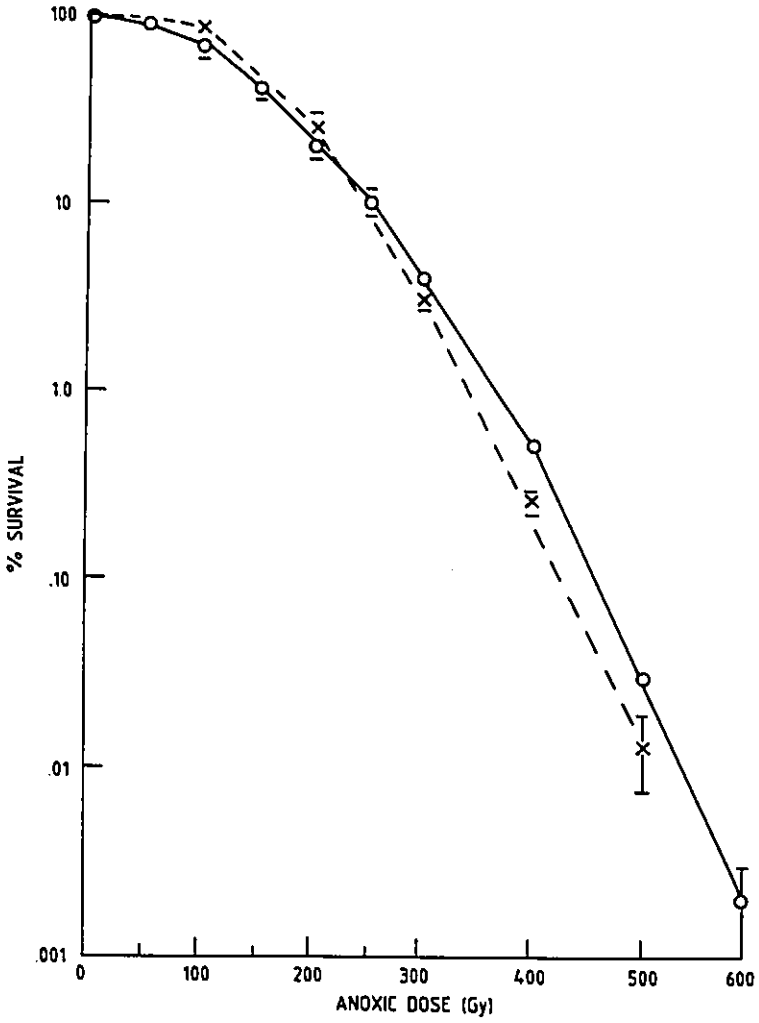

Figure 3.9. The effect of heat shock (37°C for 30 min.) on the radioresistance of unsynchronized wild type (CC-137) *Chlamydomonas reinhardtii* cells. Survival to γ-rays delivered in the presence of N_2 was determined for control (×) and heat shocked (o) cells.

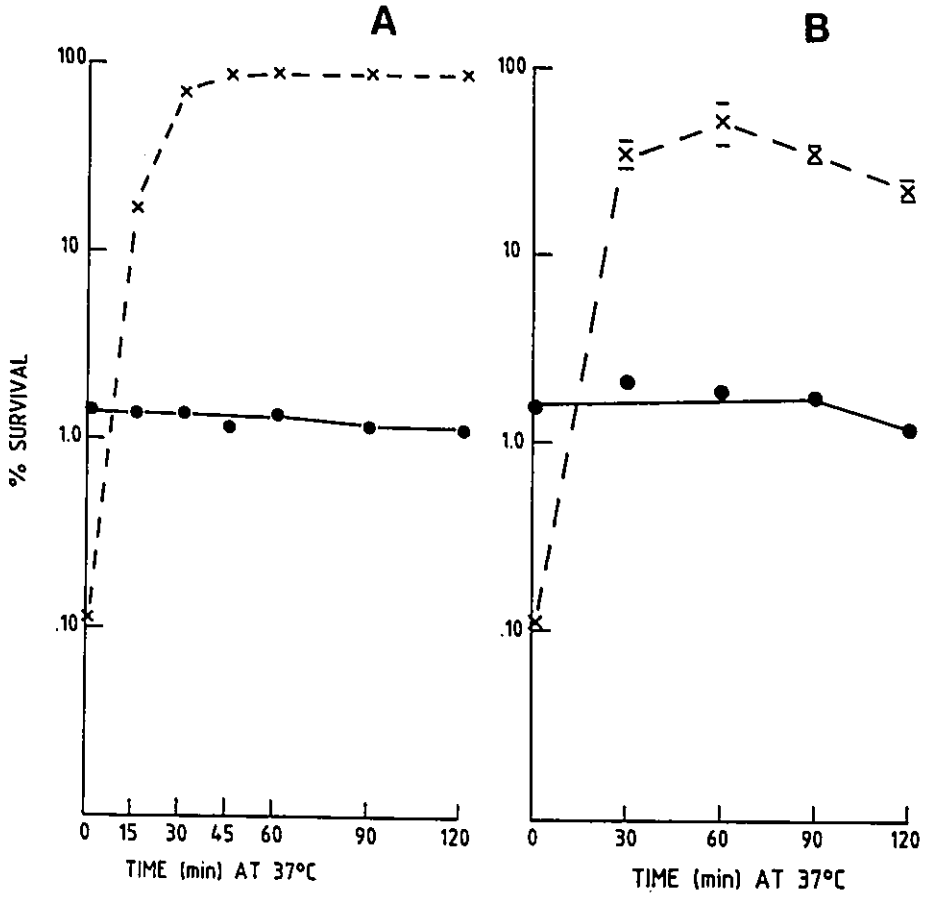

Figure 3.10. Heat shock-induced thermal tolerance and radioresistance of synchronized G_1 (A) or G_2 (B) *Chlamydomonas reinhardtii* cells. Thermal tolerance (×) was measured as survival to a 7 minute 45°C thermal test dose whereas radioresistance (•) was measured as survival to a single 150 Gy γ-ray test dose delivered in the presence of O_2.

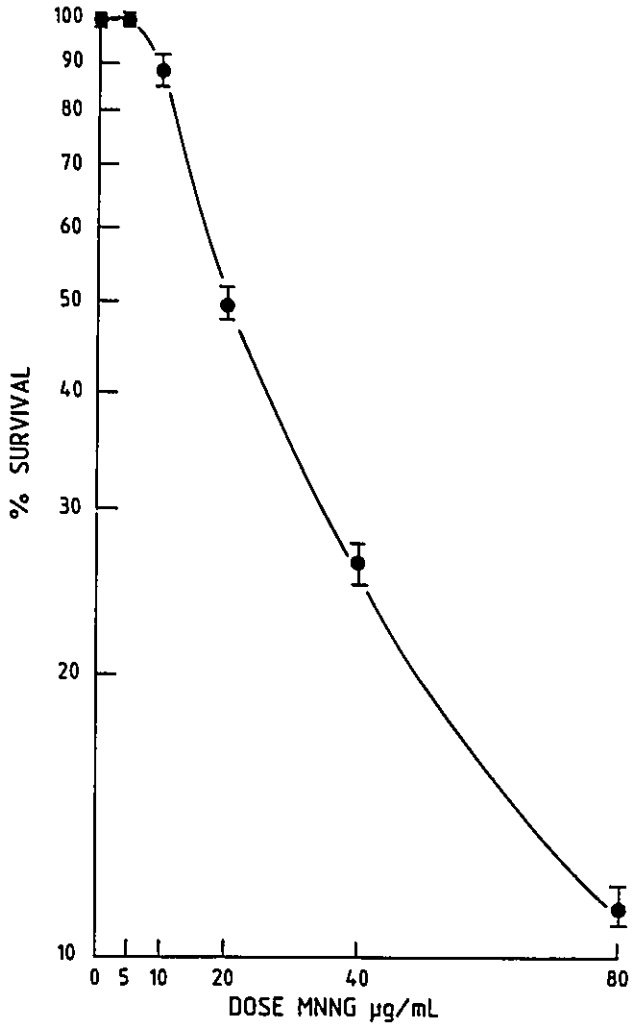

Figure 3.11. Survival of unsynchronized *Chlamydomonas reinhardtii* cells (strain CC-85) to increasing doses of MNNG.

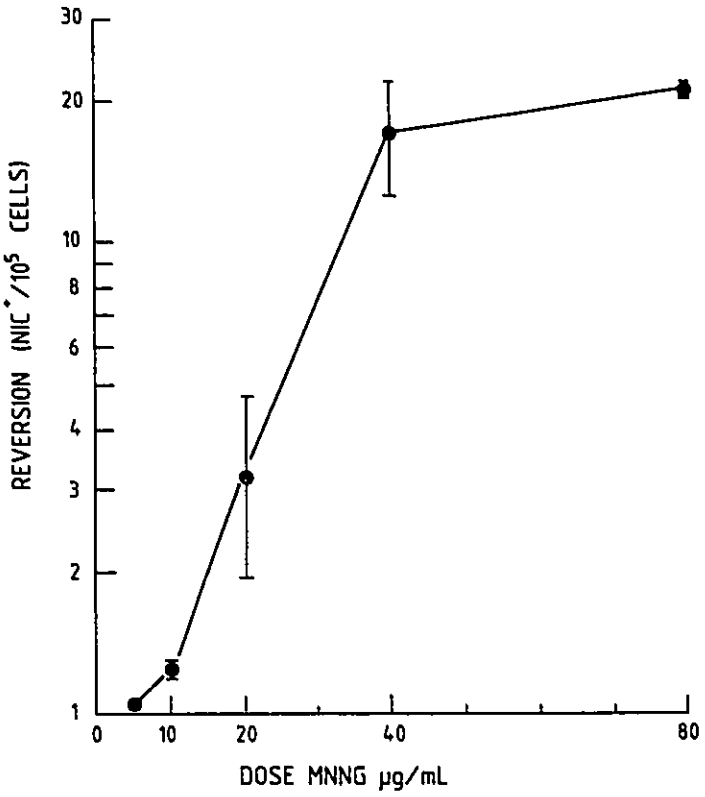

Figure 3.12. MNNG-generated mutations of unsynchronized *Chlamydomonas reinhardtii* cells (strain CC-85). Mutation frequency per surviving cell was measured as reversion to nicotinamide independence.

3.3.4 The effect of heat shock on MNNG-generated mutations. There did not seem to be an effect of protein synthesis inhibition (100 μg/ml of cycloheximide) on MNNG-generated mutations (reversion to NIC+) in unsynchronized algal cells (Figure 3.13, P=.7999). Heat shock (37°C) showed only a small effect (significant increase at 15 min. P=5.583E-03 and 30 min. P=4.433E-03). Protein synthesis inhibition did not significantly alter the number of mutations generated by MNNG even after two hours of inhibition prior to MNNG exposure (P=.8210). Algal cells heat shocked at 37°C were slightly more mutable during the first 30 minutes of heat shock but the overall mutation frequency did not change significantly after 2 hours of heat shock (Figure 3.13, P=.8191). The results indicate that MNNG-generated mutations were not dependent upon protein synthesis and heat shock response did not alter cellular mutagenicity.

3.3.5 The effect of radiation on MNNG-generated mutations. Unsynchronized algal cells (Figure 3.14) were given increasing sublethal doses of γ-rays in the presence of either N_2 or O_2, followed by treatment with MNNG. These radiation-induced cells showed neither a reduction nor an increase in the frequency of MNNG-generated mutations (reversion to NIC+). The means of all the samples were not significantly from each other at the 95% confidence level.

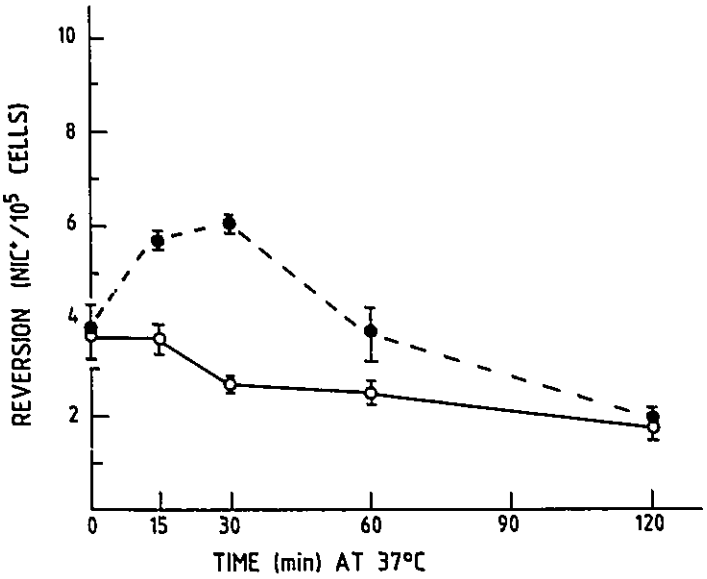

Figure 3.13. The effect of heat shock or cycloheximide on MNNG-generated mutations of *Chlamydomonas reinhardtii (strain CC-85)*. Cells were heat shocked at 37°C for increasing time intervals prior to MNNG treatment (•). Cells were treated with 100 μg/ml of cycloheximide for increasing time intervals before and during a MNNG treatment (o). Cells were treated with 20 μg/ml of MNNG for 30 minutes at 23°C. Mutation frequency was measured as reversion to nicotinamide independence.

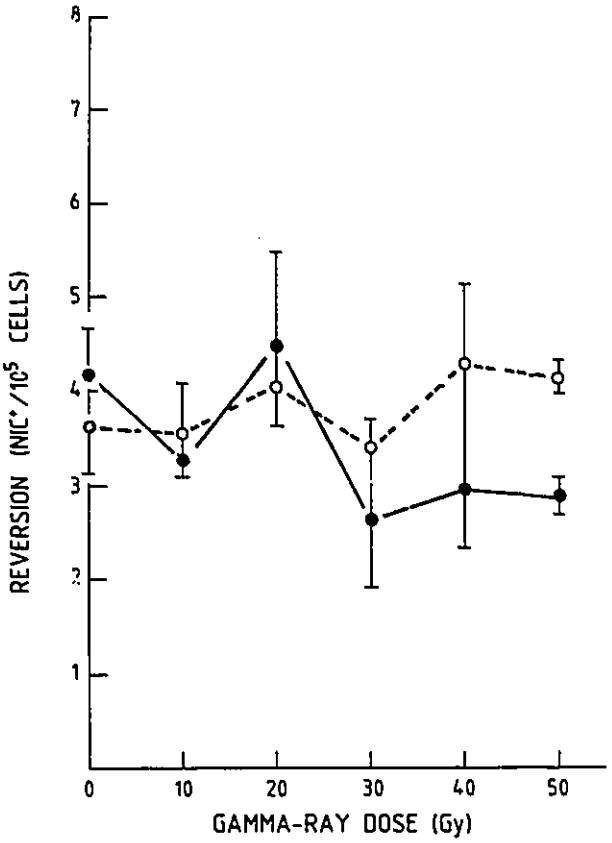

Figure 3.14. The effect of radiation on MNNG-generated mutations of *Chlamydomonas reinhardtii* cells (strain CC-85). Cells were given increasing doses of γ-rays delivered in the presence of either N_2 (•) or O_2 (○), and cells were then treated with 20 μg/ml of MNNG for 30 minutes at 23°C. Mutation was measured as reversion to nicotinamide independence.

3.4 DISCUSSION

DNA repair and radiosensitivity of *Chlamydomonas reinhardtii* has been extensively investigated. Ultrastructural changes induced by X-rays and changes in radiosensitivity during the cell cycle of vegetative algal growth have been described (16,17). The induction of radioresistance by split doses of radiation is well known in *C. reinhardtii* (1) and was shown to be dependent upon protein synthesis (6). Algal radioresistance was shown to vary depending upon radiation dose rate (18), radiation quality, and cell ploidy (19). These radiobiological characteristics of *C. reinhardtii* are consistent with those observed for *S. cerevisiae* (20). Like yeast (21), algal cells demonstrated an oxygen effect for cell killing by γ-radiation (Figures 3.1-3.3). Presumably γ-irradiation in the presence of oxygen produced extremely damaging and lethal DNA lesions. These oxygen modifiable lesions however, were inefficient inducers of algal radioresistance (Figures 3.4-3.7), unlike the case with yeast (3, Chapter 1). The present algal experiments could not demonstrate an radiobiological oxygen effect for the induction of radioresistance. The observation that cells radiation-induced in N_2 may have developed greater radioresistance than cells induced in O_2 (Figure 3.6) is not understood.

Heat shock induces thermal tolerance in a wide variety of cells including, *S. cerevisiae* (Chapter 2) and *C. reinhardtii* (24, Figure 3.10). Here, it was shown that the induction of thermal tolerance in *C. reinhardtii* is independent of cell cycle (Figure 3.10). Cells which were in G_1 or G_2 of the cell cycle became equally thermal tolerant. More

importantly, it was previously shown (25, Chapter 2) that heat shock also induced radioresistance in wild type *S. cerevisiae*. Mammalian cells in tissue culture exhibit enhanced radiosensitivity when heat shocked (27). Here it was postulated that algal cells would respond to heat shock and radiation in a manner analogous yeast cells (unicellular eukaryote). Surprisingly, evidence suggested that algal cells were neither sensitized nor did they become resistant to radiation subsequent to a heat shock. This was also independent of cell cycle (Figure 3.10). The acquisition of radioresistance is therefore not a component of heat shock response in *C. reinhardtii*. Furthermore, the constitutive DNA repair capacity of algal cells seemed to have thermal stability since heat shock did not alter radiosensitivity in these cells (Figure 3.9 and 3.10). This may have evolutionary relevance because algal cells are routinely subjected to rapid changes in water temperature and perhaps they would otherwise be excessively susceptible to DNA damage.

Yeast mutations produced by MNNG are a consequence of induced error-prone DNA repair (4). Therefore treatment with the protein synthesis inhibitor cycloheximide prevented an MNNG-exposure from becoming mutagenic (13). Heat shock also prevented error-prone repair by a similar inhibition of protein synthesis (13). The lack of suppression of MNNG-generated mutation by cycloheximide treatment or heat shock in *C. reinhardtii* (Figure 3.13) suggests that MNNG mutagenesis may not be a consequence of inducible error-prone repair (Figure 3.13). Radiation induced DNA repair could not repair MNNG damage (Figure 3.14) implying that unlike yeast, the radiation repair system of *C. reinhardtii* has limited DNA repair and/or recognition capabilities. MNNG-induced

mutagenesis seems to be an independent molecular process in *C. reinhardtii*. Here the results corroborate the conclusions of Frost and Small (9) suggesting that *C. reinhardtii* lacks the ability to repair DNA O^6-MG lesions.

In this report, the induction of radioresistance was assumed to be a result of increased recombinational repair capacity as in yeast (Chapters 1 and 2). The essential characteristic of this repair process is the requirement for a duplicate genome. Haploid G_1 cells do not have a duplicated nuclear genome and therefore are presumably incapable of this type of repair process. Using this criteria, haploid G_1 cells would be radiation sensitive (see Chapter 2), lack a shouldered radiation survival curve, and could not be induced for radiation resistance. Oddly, haploid G_1 algal cells had a shouldered radiation survival curve (19,28, Figure 3.7), and were capable of radiation-induced radioresistance (19, Figure 3.7). Hence G_1 algal cells were responding in a manner analogous to cells competent for recombinational repair (ie. G_2 haploids or diploid cells). Therefore it may be concluded that induced radioresistance in *C. reinhardtii* is either not a consequence of recombinational repair or that the recombinational repair mechanism has some unique characteristics.

If we assume that radiation resistance does involve a recombinational repair process, then haploid G_1 algal cells were responding functionally as either G_2 haploid or diploid cells with respect to radiation repair, suggesting the possible involvement of some duplicate genome. Perhaps the chloroplast genome has an essential role in recombinational repair for this organism. *C. reinhardtii* has a single large chloroplast that occupies about 40% of the cell and contributes

about 14% of the total DNA in vegetative cells (22,23). There is evidence suggesting that nuclear DNA repair processes may be separate from those of chloroplast DNA repair processes in *C. reinhardtii* (26). It is also probable, however, that induced radioresistance to killing is a combined function of nuclear and chloroplast DNA repair capacity. Recombinational repair could be a concerted effort between the chloroplast and nuclear genomes in *C. reinhardtii*. If the chloroplast is involved in the mechanism of radioresistance, then the experiments examining the nuclear endpoints of oxygen effect or mutation may have been inappropriate. Defining the role of the chloroplast genome in the mechanisms of recombinational repair and radioresistance, will require additional investigations.

In summary the results in this chapter have demonstrated that radiation did not induce DNA repair capable of repairing MNNG-generated mutations in algae. MNNG mutagenesis was not suppressed by heat shock or cycloheximide and therefore it is unlikely a consequence of inducible error-prone repair, possibly resulting from a lack of repair , or a constitutive error prone repair system. Algal cells were shown to have a normal heat-induced thermal tolerance response yet heat shock neither induces radioresistance nor does it radiosensitize these cells. Radioresistance in *C. reinhardtii* is part of a separate inducible repair mechanism functioning independently from the heat shock response. Unlike mammalian cells, the algal repair mechanism has thermal stability since heat shock does not radiosensitize cells. Finally, an oxygen effect for the induction of radioresistance by γ-rays in *C. reinhardtii* could not be demonstrated. The type of radiation damage that signalled the radioresistance mechanism did not seem to be modified by oxygen. In

conclusion, algal cells apparently have unique DNA repair characteristics distinctive from yeast, indicating that the radioresistance mechanism may not have strong evolutionary conservation amoung various phyla.

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CHAPTER 4

RADIORESISTANCE AND THE INDUCTION OF YEAST RAD GENES BY HEAT SHOCK OR GAMMA-RAYS.

4.0 INTRODUCTION

Single cell organisms often exist in environments that may change rapidly, and some of the enzymes that are needed presently may become useless or even counterproductive soon afterwards. Similarly, cells may suddenly require an enzyme whose presence was previously unnecessary. There are a variety of genes in *S. cerevisiae* which display increased transcription after treatment of cells with various DNA damaging agents (1). More recently it was demonstrated that that two of these DNA damage responsive genes were also transcriptionally responsive to heat shock (2). Many genes function at highly variable rates, so the corresponding mRNA is made at appreciable levels only when the genes receive the appropriate signal. When a signal is received from an external source and the corresponding genes are expressed, it is known as "inducible transcriptional control" (3). In this chapter, inducible transcriptional control of several DNA repair genes was investigated in DNA repair competent and DNA repair deficient yeast strains.

A systematic nomenclature for radiation-sensitive yeast mutants was established at the International Conference on Yeast Genetics held at Atomic Energy of Canada Limited, Chalk River, Ontario, in 1970 .

It was decided that all mutants abnormally sensitive to killing by radiation should be designated as *rad*, with identifying locus and allele numbers. Locus numbers greater than 50 designate genes which primarily affect sensitivity to ionizing radiation. X-ray sensitive mutants were established from loci *RAD50* through *RAD57* (4) and are known as the *RAD52* epistasis group (5). Among the *RAD52* group, the *rad51*, *rad52* and *rad54* mutants are extremely sensitive to ionizing radiation and are defective in double strand break repair (6,7). The *rad50*, *rad53*, *rad55*, *rad56*, and *rad57* mutants are also classified in the *RAD52* epistasis group, although they are less sensitive to ionizing radiation than the *rad51*, *rad52*, and *rad54* mutants (8).

Among the mutations producing defects in recombination and double strand break repair, *rad52* has been investigated most extensively. The primary structure of the gene has been characterized (9) and its function of recombination has recently been reexamined (10). In this thesis, we have utilized a *rad52* mutant (defective in recombinational repair) and its parental wild type to ascertain the involvement of several *RAD* genes during the induction of radioresistance in yeast. The expression of *RAD51*, *52*, *54*, *55*, and *57* genes was monitored by dot blot analysis in wild type and *rad52* mutants following a heat shock or radiation inducing dose.

4.2 MATERIALS AND METHODS

4.2.1 Strains. The yeast *Saccharomyces cerevisiae* was used as the test organism in these experiments. Both strains were diploid and homozygous for the *his1-7* mutation. The wild type cells were from the strain MS33 and were wild type for recombinational DNA repair (rec^+/rec^+). Strain MS32, the isogenic mutant of MS33, was homozygous for *rad52-1* and was defective in recombinational repair (rec^-/rec^-). Yeast strains were gifts from Dr. D.P. Morrison, AECL, Chalk River, Ontario, Canada.

4.2.2. Preparation of Cells. The yeast strains were grown at 23°C to mid log phase ($2\text{-}5 \times 10^6$ cells/ml) in liquid medium containing 0.1% yeast extract, 1% yeast nitrogen base with amino acids, 1% sodium succinate, and 2% glucose (YES medium). Cell samples to be tested for heat or radiation resistance were washed three times in 20 mmol dm^{-3} (20 mM) sodium phosphate buffer (pH 7.0) at 0°C. Survival to the various treatments was determined by plating suitable cell dilutions on YPD agar plates (1% yeast extract, 2% peptone, 2% glucose, solidified with 2% agar) followed by growth at 23°C.

4.2.3 Thermal Tolerance. Thermal tolerance was determined by placing a number of 2.5 ml cell aliquots (2×10^6 cells/ml in YES medium at 0°C in 16x125 mm glass test tubes) into a 52°C water bath. Every 30 seconds for a total period of 5 minutes, an aliquot was removed, rapidly recooled to 0°C, and plated for survival as described above.

4.2.4 Heat Induced Thermal Tolerance. Prepared cells from both strains were resuspended (2×10^6 cells/ml) in fresh YES medium at 23°C and

were heat shocked by an abrupt transfer of the culture (50 ml in a 500 ml flask) to a shaking water bath at 37°C. At progressively increasing time intervals, 5 ml aliquots were removed and rapidly cooled to 0°C. At these temperatures and for the times used in these experiments, both strains showed 100% survival to this heat shock. The heat shocked cells were tested for thermal tolerance and radiation resistance. Thermal tolerance was determined by measuring survival to a 15 minute 52°C thermal test dose. Radiation resistance was measured by exposing buffer washed heat shocked cells, under oxic conditions, to a gamma-ray test dose. Wild type cells received a 1.0 kGy γ-ray test dose whereas the radiation sensitive MS32 strain received a 200 Gy test dose.

4.2.5 Plasmids. *E. coli* strains containing selected plasmids (YEp13) were obtained from Dr. P. Unrau, Atomic Energy of Canada Limited, Chalk River Nuclear Laboratories. The plasmid constructs contained yeast DNA repair genes from the *RAD52* epistasis group. YEp13 plasmids contained yeast chromosomal DNA, 2μ DNA and *pBr322*. The appropriate *RAD52* epistasis genes had been inserted at the *SalI* restriction site of pBr322. Figure 4.1 shows the plasmid constructs with their respective *RAD* gene inserts and corresponding sizes. The plasmids were grown, amplified and extracted as previously described (12).

4.2.6 Plasmid Identification. Purified plasmid DNA was identified by restriction nuclease digestion and gel electrophoresis. Whole plasmids were electrophoresed together with plasmids that were fragmented by *SalI*

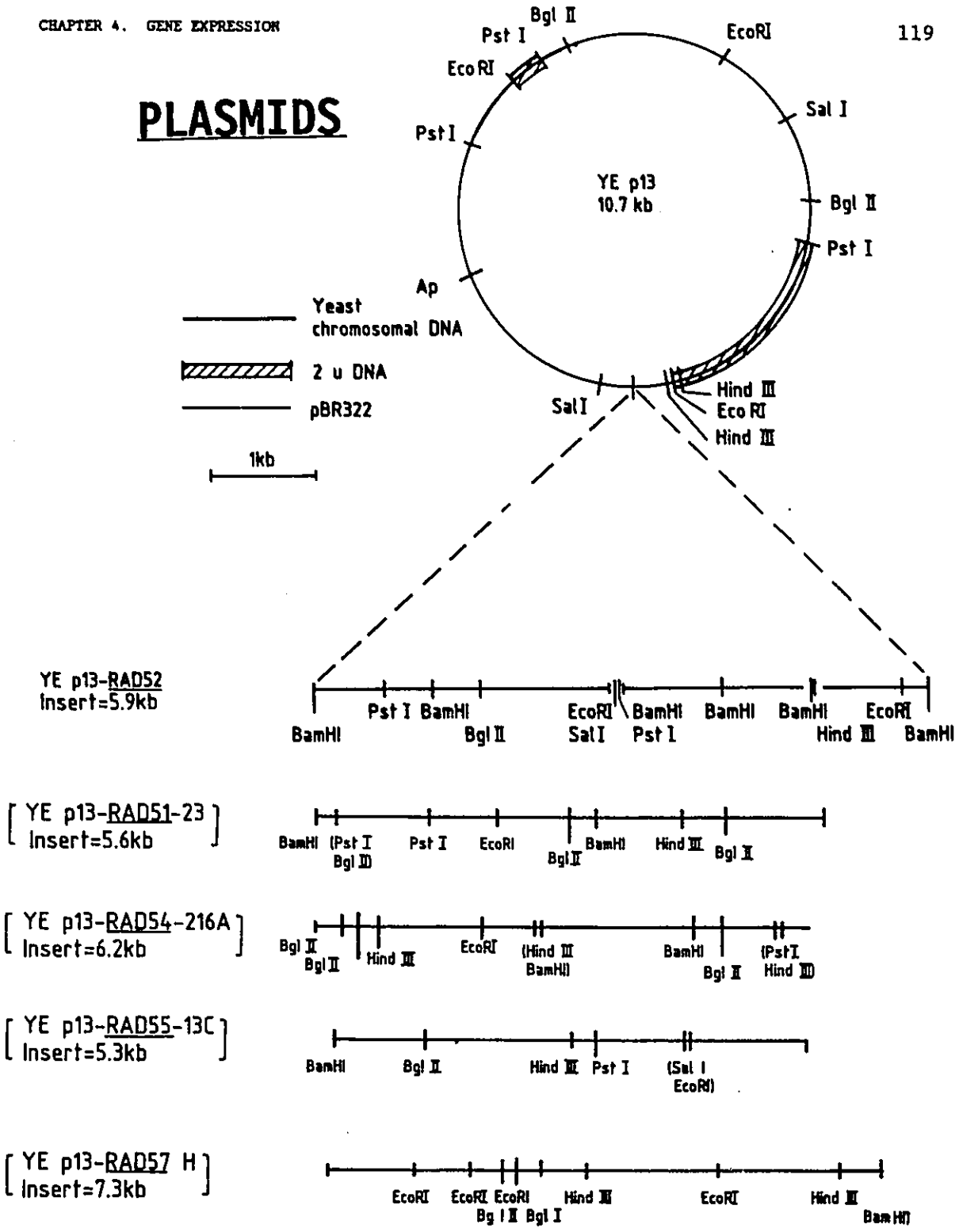


Figure 4.1. Restriction map of the YEp13 plasmids containing the various *RAD52* epistasis genes. Insert diagrams reflect insert sizes.

restriction nuclease digestion (Bethesda Research Laboratories). Fragments generated by *SalI* digestion were examined according to previously described methodology (13) and plasmids were verified by their restriction fragment gel band patterns.

4.2.7 Blot Hybridization. A nitrocellulose membrane filter was prepared with a fixed pattern of appropriate plasmid DNA. Plasmid dots were allocated with a filtration manifold (Minifold I, Schleicher and Schuell). Three individual dots of varying plasmid DNA concentrations (20, 10, and 5 μg) were prepared for each plasmid. Plasmid DNA was fixed to the nitrocellulose by baking the filter at 80°C for 2 hours under vacuum.

4.2.8 Isolation of RNA. Total cytoplasmic RNA was isolated from cultured cells according to procedures described elsewhere (1) with the following modifications. Yeast cells ($2x10^8$ cells) were washed in 20 mmol dm^{-3} (20 mM) phosphate buffer, pelleted, and frozen at -70°C in dry ice. The pellet was thawed with 1 ml of 4 M guanidine thiocyanate and passed through a 26 gauge syringe five times. An equal volume of 250 μm glass beads were added along with 80 μl of 10% sodium dodecyl sulfate. The mixture was vortexed for 10 minutes and an additional 1 ml of guanidine thiocyanate was added.

Total RNA was extracted by adding an equal volume of phenol, chloroform, isoamyl alcohol (25:24:1). The two phases were separated by a brief centrifugation and the aqueous phase was removed and re-extracted. The RNA was precipitated with ethanol at -20°C overnight, pelleted by centrifugation (10,000 x g in an Eppendorf centrifuge 5412) at 0°C and resuspended in a minimal volume of diethyl pyrocarbonate-treated water.

Messenger RNA (mRNA) was extracted and purified using oligo (dt)-cellulose Type 7 (Pharmacia LKB Biotechnology) as per manufacturers instructions.

4.2.9 Radiolabelling of cDNA. A radiolabelled complementary DNA (cDNA) library of total mRNA (2 μg) was synthesized using phosphorous-32 (^{32}PdCTP) (Dupont New Research Products) and a reverse transcriptase kit (Bethesda Research Laboratories, Life Technologies Inc.) according to manufacturers directions.

4.2.10 Blot Hybridization. Radiolabelled cDNA was hybridized to the nitrocellulose plasmid dots as previously described (14). Hybridization solution was adjusted to $1x10^7$ Bq cm^{-3} and hybridization was conducted at 37°C for 24 hours. Dot blots were washed, wrapped in plastic, and exposed to X-ray film, using an intensifier screen, for 7 days. Dot densities were visually quantified to determine relative abundance of each cDNA, indicative of the respective level of mRNA expression for each gene (ie. the specific plasmid gene insert). Strain MS32 had low constitutive expression of all *RAD52* epistasis genes and therefore the activity of the cDNA hybridization solution was increased 2-fold and X-ray film exposure time was increased by 2 days for the rec$^-$ strain (Figure 4.9). Figure 4.2 summarizes the methods used to determine the level of mRNA expression for each plasmid gene. Figure 4.2 uses the *RAD52* gene as an example, however, all genes were examined using the same technique.

4.2.11 Data and Figures. Each figure curve represents the mean of at least three experiments and each experiment was performed in quadruplicate. The confidence interval for each point reflects inter-experimental variation. Plasmid verification gels were tested once while

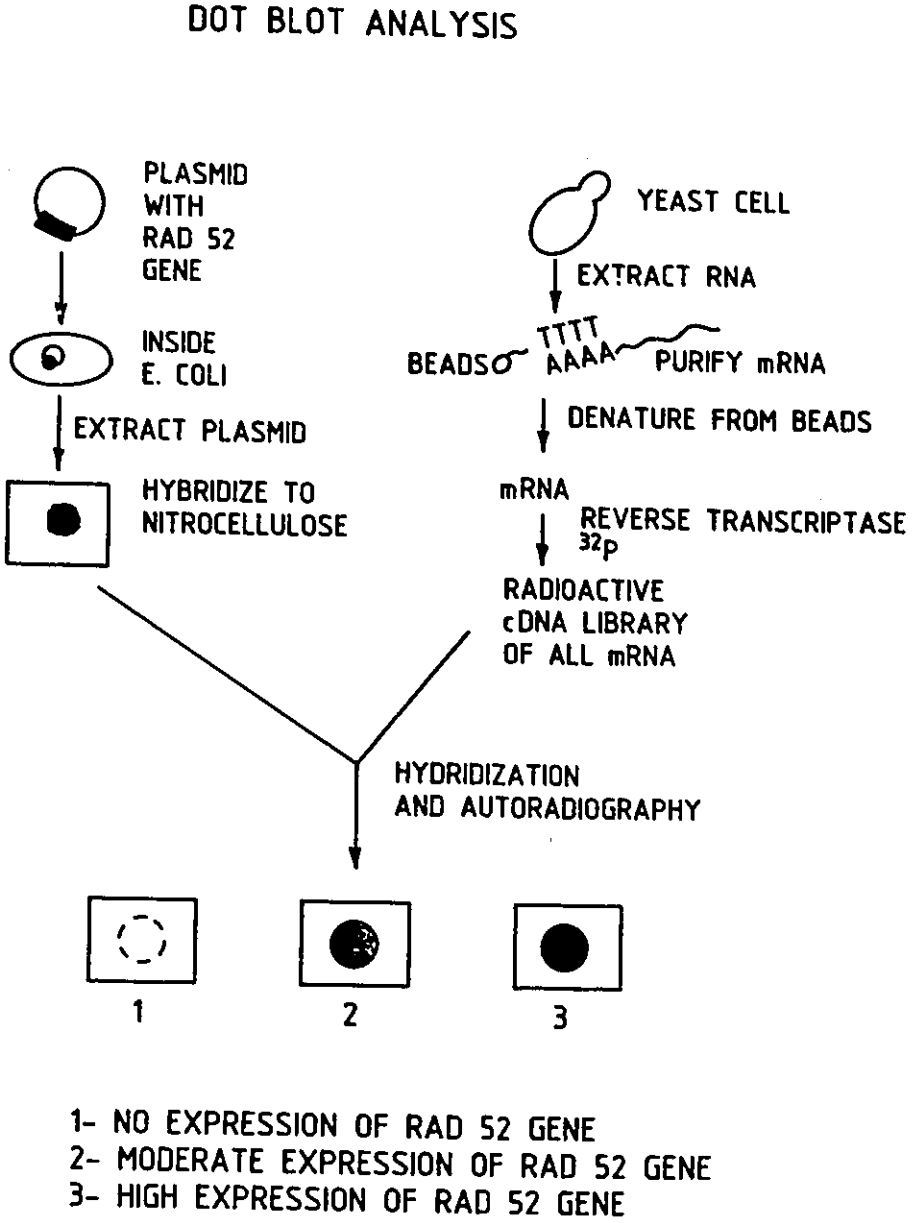


Figure 4.2. Summary of dot blot procedures used to determine the level of gene expression in yeast cells. A plasmid containing the gene sequence under investigation was extracted from an *E. coli* host. The plasmids were purified and fixed to nitrocellulose. Yeast cells were treated with either heat shock or γ-rays. Total expression of mRNA was determined by synthesizing a radiolabelled complementary DNA (cDNA) library. The cDNA library was hybridized to the nitrocellulose dot blot containing the respective plasmid insert. The amount of radiolabelled cDNA hybridized to the dots was quantified with autoradiography. The intensity of the dot represented the level of mRNA expression for the respective gene. The figure uses RAD52 as an example.

the dot blots shown were representative blots taken from two independent experiments. One-way analysis of variance was performed to determine the statistical significance of various treatments. P-values are given where values are considered significant.

4.3 RESULTS

4.3.1 Thermal Survival. Wild type yeast cells appeared to be more sensitive to the lethal effects of a 52°C heat-shock compared to the rec$^-$ strain (Figure 4.3, after 1 minute of heat shock all paired means are significantly different with P<1.0E-03). Rec$^-$ cells were about 100 fold more thermal tolerant to a 4 minute 52°C heat shock compared to wild type cells.

4.3.2 Heat Induced Thermal Tolerance. A heat shock of 38°C induced thermal tolerance in both rec- and parental wild type cells. The maximum level of thermal tolerance was achieved in rec$^-$ cells after 60 minutes (Figure 4.4). Rec$^-$ cells demonstrated a 1000 fold increase in thermal tolerance whereas wild type cells exhibited only a 100 fold increase.

4.3.3 Heat Induced Radiation Resistance. Figure 4.5 shows that a heat shock of 38°C induced radiation resistance in wild type yeast cells but did not induce radioresistance in rec$^-$ cells. The absence of functional *RAD52* gene product prevented the acquisition of increased radiation resistance. Maximum radiation resistance was observed in wild type cells within about 15 minutes of heat shock (there is no significant difference between any mean value after 15 minutes, P=1.593E-02).

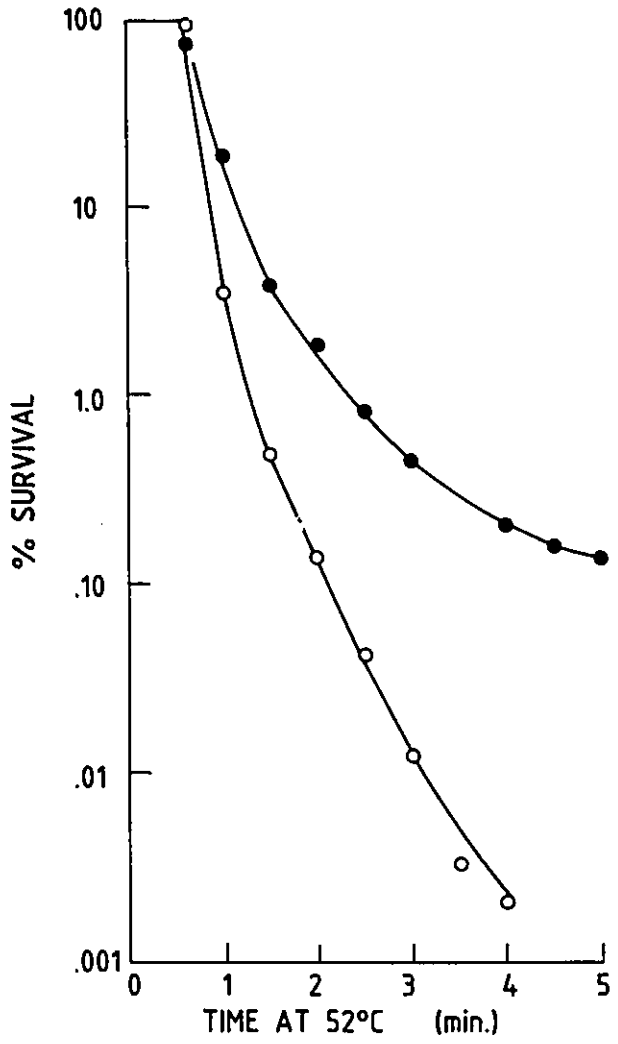


Figure 4.3. Survival of *Saccharomyces cerevisiae* to a 52°C heat shock: Wild type, o; recombinational repair deficient (*rad52*), •.

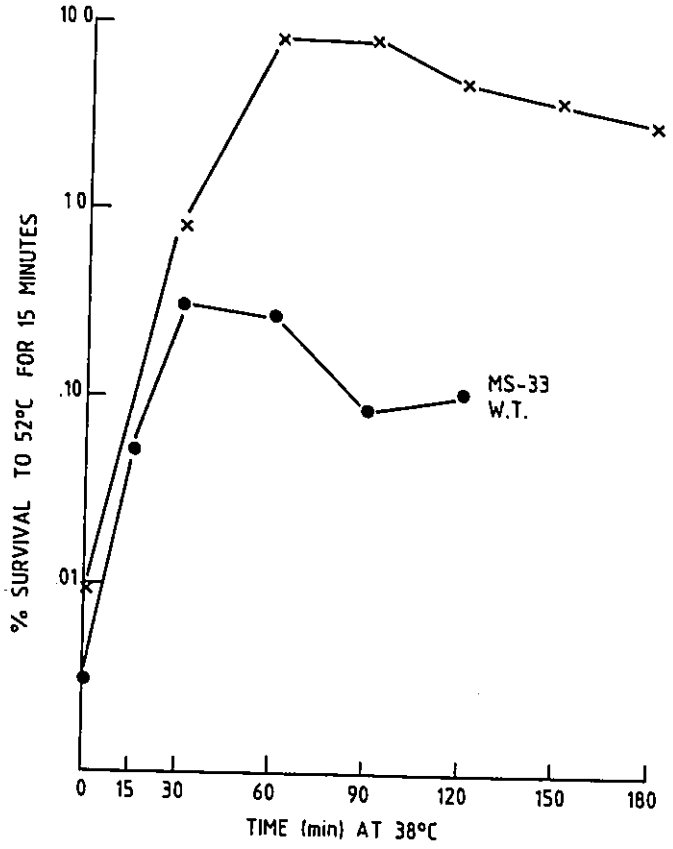


Figure 4.4. Heat shock induction of thermal tolerance. Thermal tolerance of *Saccharomyces cerevisiae* recombinational repair deficient mutants and parental wild type to a 15 minute 52°C thermal test dose following a heat shock of 38°C: wild type, •; recombinational repair deficient mutant (*rad52*), X.

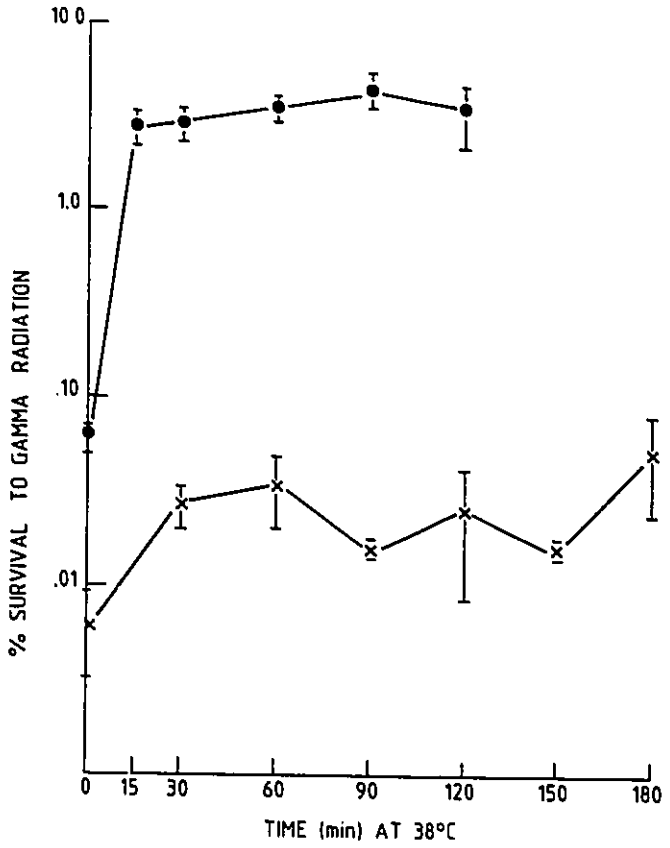
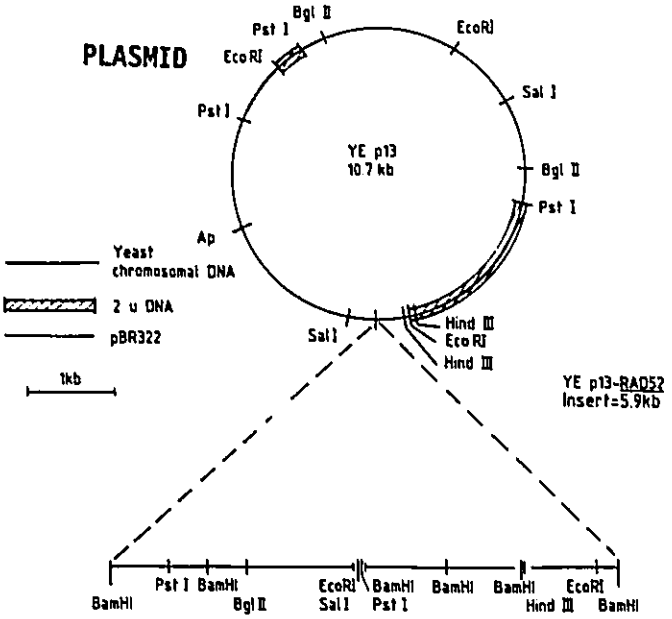
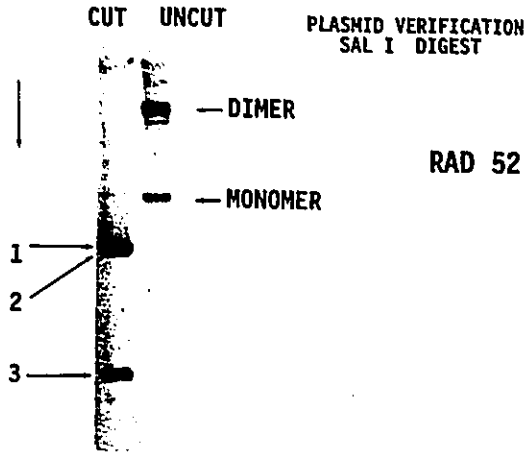


Figure 4.5. Heat shock induction of radiation resistance. Survival of *Saccharomyces cerevisiae* recombinational repair deficient mutant (*rad52*) and parental wild type to a 1.0 kGy oxic γ-ray test dose following a heat shock of 37°C: wild type, •; recombinational repair deficient mutant (*rad52*), X.





1) SAL I - SAL I INSERT CUT PLUS 1/2 INSERT (3886 B + 3200 B = 6800 B).
2) VECTOR (6814 B).
3) SAL I - SAL I INSERT (3300 B).

Figure 4.6. Restriction map of YEp13 plasmid containing the *RAD52* gene insert (5.9 kb). Verification of correct plasmid insert by *SalI* digest is shown. Uncut plasmid is shown in right hand lane whereas *SalI* plasmid fragments are in right hand lane. Three fragment bands are identified by numbers 1-3.

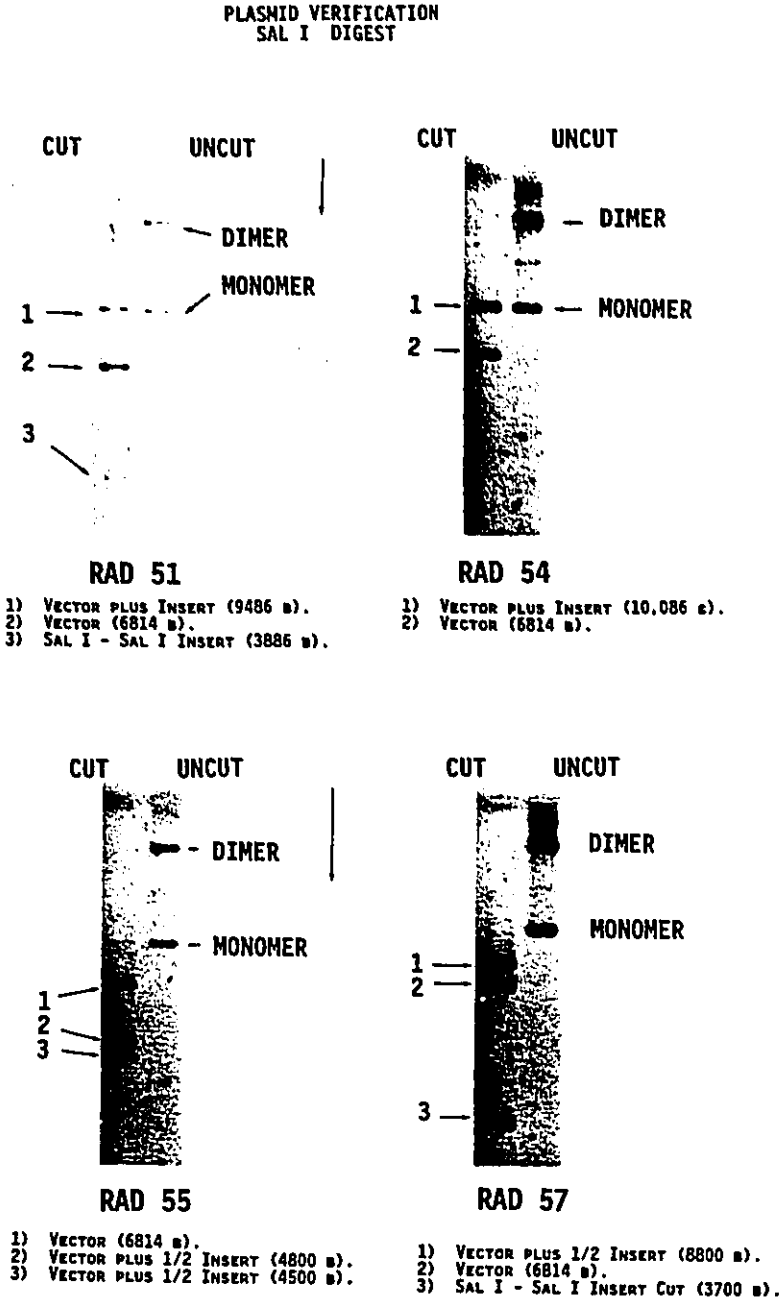


Figure 4.7. Verification of YEp13 plasmids with *RAD52* epistasis gene inserts by gel electrophoresis and SalI digestion. Right hand lanes are uncut plasmids whereas left lanes are *SalI* digestion fragments. Fragments are numbered and band identification is given.

4.3.4 Plasmid Verification. Figures 4.6 and 4.7 show the gel electrophoresis of normal and restriction nuclease digested plasmids. The untreated plasmids existed in the dimer and monomer form of covalently closed circular (CCC) DNA. Upon *SalI* restriction digest, several linear fragments of the plasmid were produced. Figure 4.6 shows that three fragment bands appeared when *YEp13-Rad52* was digested. Based on restriction map analysis, band 1 was identified as the plasmid vector with about half of the *Rad52* insert remaining (6800 kb). Band 2 was identified as the plasmid vector alone (6814 kb) while Band 3 was identified as the *Rad52* insert fragment. Figure 4.7 shows similar gels of the remaining *RAD* plasmids. All plasmids were identified as carrying the correct inserts subsequent to purification.

4.3.5 Heat Shock Induced Gene Expression. Yeast cells were given a 30 minute 37°C heat shock. Figure 4.8 shows the dot blots for wild type and rec$^-$ yeast cells. The constitutive expression of *RAD51, 52, 54, 55* and *57* was greater in wild type cells compared to rec$^-$ cells. A 30 minute heat shock of 37°C increased the expression of *RAD51, rad52, RAD54, 55* and *57* in rec$^-$ cells but only RAD52 expression increased in wild type cells (Figure 4.8).

4.3.6 Radiation Induced Gene Expression. Yeast cells were exposed to a 50 Gy γ-ray dose delivered in the presence of oxygen and incubated for 2 hours under normal growth conditions. In wild type cells, the expression of *RAD51, 55* and *57* increased slightly whereas *RAD52* and *54* gene expression increased to a greater extent (Figure 4.9). In the rec$^-$ cells, there was a significant increase in expression of *RAD51, rad52, RAD54, 55* and 57. The constitutive *RAD* gene expression of rec$^-$ cells

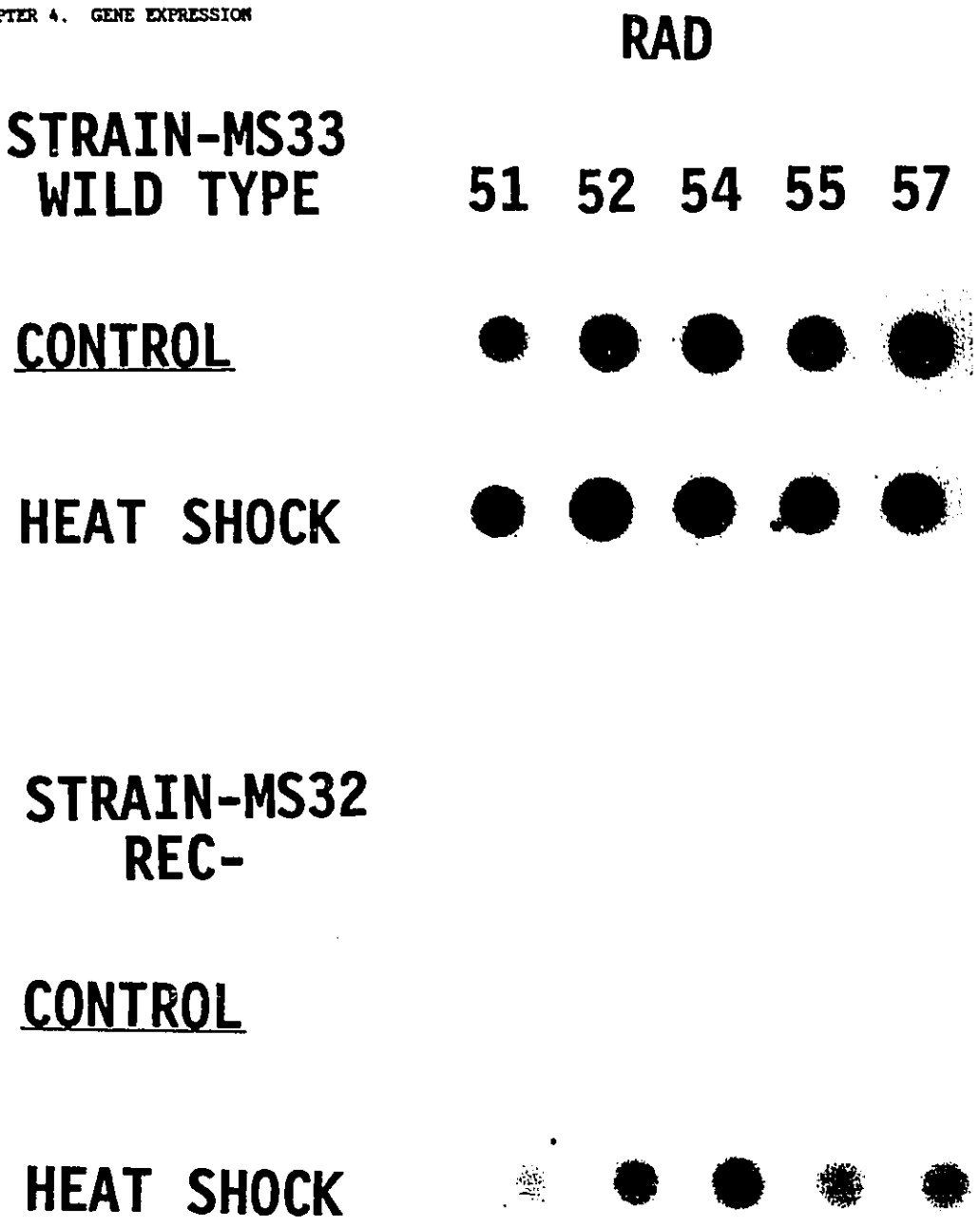


Figure 4.8. Messenger RNA dot blot hybridization of various *RAD52* epistasis genes (*RAD51*, *52*, *54*, *55* and *57*) following a 37°C, 30 minute heat shock in wild type and recombinational repair deficient mutants (*rad52*) in *Saccharomyces cerevisiae*. The dots represent 20 μg of plasmid DNA per dot.

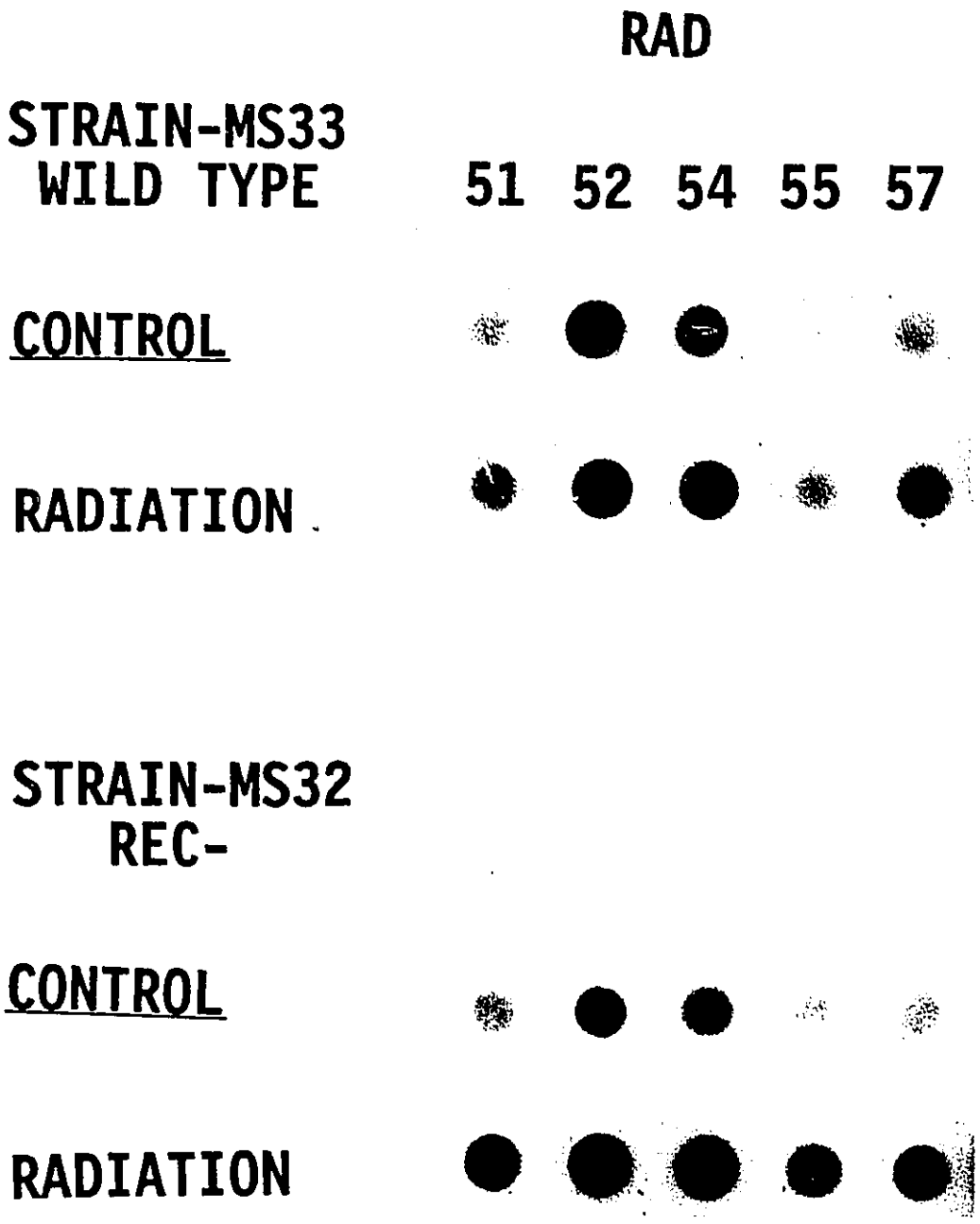


Figure 4.9. Messenger RNA dot blot hybridization of various *RAD52* epistasis genes (*RAD51*, *52*, *54*, *55* and *57*) following a 50 Gy gamma-ray exposure delivered in the presence of oxygen and 2 hours of incubation under normal growth conditions. Wild type cells and recombinational repair deficient mutants (*rad52*) of *Saccharomyces cerevisiae* are shown. The three dots represent 20 μg of plasmid DNA per dot.

depicted in Figure 4.9 was apparently greater than the constitutive *RAD* gene expression in rec$^-$ cells shown in Figure 4.8 because the total activity of the hybridization solution was adjusted (see Methods 4.2.5). Radiation exposure caused greater *RAD* gene expression in wild type cells compared to heat shocked wild type cells. In rec$^-$ cells, *RAD* gene expression increased equally in response to either heat shock or γ-irradiation.

4.4 DISCUSSION

The *RAD52* epistatis groups represent a conveient way of examining the genetic complexity of cellular responses to certain types of DNA damaging agents in yeast. The *RAD52* epistatis group of genes is thought to reflect the existence of a recombinational repair pathway in yeast (4). Accordingly, the induction of radiation resistance (recombinational repair) may be accompanied by increased expression of some or all of the *RAD52* genes (5). In fact, there were previous indications that the *RAD52* gene may be very weakly inducible by DNA damaging agents (15).

The recombinational repair deficient (rec$^-$) mutants were thermal tolerant compared to wild type cells (Figure 4.3). This suggests that the *rad52* gene defect may cause sufficient cellular stress to constitutively induce a partial heat shock response in these mutants. The fact that rec$^-$ mutants were constitutively thermal tolerant supports the contention (11) that DNA perturbation serves as a regulatory signal for the inducible thermal tolerance response. It is probable therefore that this mutation results in substantial DNA disruption. Results demonstrating that rec$^-$

yeast cells have low constitutive expression of all *RAD* genes (Figure 4.8) and that they are induced to thermal tolerance by heat shock (Figure 4.4) indicates that the *RAD52* gene product is not a component of the thermal tolerance mechanism. Heat shock did induce expression of *RAD52* in wild type cells (Figure 4.8) as well as functional radioresistance and therefore the *RAD52* gene may be considered a heat shock gene.

Heat shocked wild type yeast cells became radioresistant whereas heat shocked rec$^-$ mutants did not become radioresistant (Figure 4.5). This suggests that functional *RAD52* gene product is essential to confer radioresistance and therefore must be a component of the mechanism. Only *RAD52* was induced in thermal tolerant heat-induced radioresistant wild type cells whereas thermal tolerant heat-induced rec$^-$ cells displayed induced expression of all the *RAD* genes, and yet remained radiosensitive (Figures 4.5 and 4.8). This suggests that the *RAD52* gene product may have an indirect regulatory role in this mechanism since the *rad52* gene defect resulted in the additional expression of the other *RAD* genes. The heat-induced expression of all the *RAD* genes may imply that the lack of repair capability due to the *rad52* deficiency elicits a response that induces the additional genes. Perhaps the residual unrepaired DNA damage resulting from *rad52* deficiency generates an "all out effort" by the cell to repair the damaged DNA. Accordingly, the other secondary *RAD* repair genes become expressed.

The extreme constitutive radiosensitivity observed in rec$^-$ mutants (see Chapter 1, Figure 1.11) is due to the *rad52* defect. The corresponding lack of recombinational repair capacity therefore sensitizes cells to DNA damage. It has been previously demonstrated that

reestablishment of functional RAD52 activity by transforming yeast cells with plasmid containing *RAD52* inserts restores wild type radiation resistance (16). This suggests that the primary basis for radiation resistance is functional *RAD52* gene product. The results obtained in these experiments suggest that an indirect secondary basis for radiosensitization may be a consequence of the other *RAD* genes under-expression (Figure 4.8), again implying that the *RAD52* has a regulatory role in the mechanism of radioresistance.

In chapter 2, it was concluded that heat shock only induced a partial radiation resistance whereas radiation induced a maximum radioresistance in wild type cells. Here coincidentally, heat shock only induced *RAD52* (Figure 4.8) but radiation induced *RAD52* plus *RAD54* and *RAD57* (Figure 4.9) in wild type cells. This result implies that induction of all three *RAD* genes is necessary for maximum induced radioresistance. Like heat shock, radiation also induced all *RAD* genes in rec$^-$ cells. The differential expression of *RAD* genes between heat shocked and irradiated cells supports the postulate that two distinctive mechanisms confer respective resistances. However, either stress induces reciprocal resistance suggesting a common signal for the separate mechanisms. The evidence would suggest the common signal is a perturbation in the DNA, either damage or changes in DNA topology.

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THESIS SUMMARY

The mechanism of induced radiation resistance was investigated. Radioresistance is an inducible cellular response which protects the cell from DNA damage by increasing DNA repair capacity. The system is signalled by or responds to specific types of DNA damage. In yeast, it was shown that DNA lesions produced by low-LET γ-rays were more efficient at inducing radioresistance to killing than lesions produced by high-LET neutrons, providing new evidence that DNA single strand breaks are more important at inducing the radioresistance mechanism than DNA double strand breaks. Cells induced to killing radioresistance with high-LET radiation did not suppress *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG)-generated mutations as well as cells induced with low-LET radiation, supporting the thesis conclusion that the type of DNA damage produced by low-LET radiation is a better inducer of radioresistance. This investigation has further demonstrated that radiation-induction of radioresistance in yeast depends not only on a variable induction response to different DNA lesions, but also on the availability of the induced recombinational repair system to deal with subsequent damage.

In yeast, the actual systems that confer resistance to heat or radiation were independent of either topoisomerase activity or DNA polymerase I function but topoisomerases may have a regulatory role during the signalling of these mechanisms. The results indicated that maintenance of correct DNA topology prevents induction of the heat shock response, and that heat shock induction of a component of the full

radiation resistance in yeast may be the consequence of topoisomerase I inactivation.

RAD52 was an essential component of the radioresistance mechanism in yeast. Non-functional *rad52* gene product was incapable of conferring radioresistance (repairing DNA damage) and, as a consequence, additional repair genes subsequently became induced in an unsuccessful attempt to repair the residual damage. Evidence presented in this thesis suggested that *RAD52* may have an indirect regulatory role in the radioresistance mechanism in yeast.

It was demonstrated that DNA repair of ionizing radiation damage in *C. reinhardtii* was not altered by heat shock and was independent of cell cycle. In this organism the mutations produced by MNNG were apparently not a consequence of an induced error-prone repair system. Results from algal experiments indicated that the radioresistance mechanism may not be highly conserved throughout the different phyla.

APPENDIX 1

LIST OF ABBREVIATIONS AND DEFINITIONS:

DSB: Double Strand Break - Both strands of DNA are broken.

Epistatis Group - a family of genes with related functions.

Error-Free Repair - Repair of DNA damage with a very low error frequency. Recombinational repair is suggested to be error-free. Radiation induces error-free repair in yeast.

Error-Prone Repair - Repair of DNA damage with a high error frequency. This type of repair results in a high rate of mutation. MNNG induces error-prone repair in yeast.

Gy: Gray - SI unit of absorbed dose. Equal to the amount of energy absorbed in joules per kilogram (J/Kg) of mass. 1 Gy = 100 rad (rad is the old unit of absorbed dose).

HIS+: Histidine Synthesis Proficient - A cell capable of synthesizing histidine from nutient precursors.

HIS-: Histidine Synthesis Deficient - A cell that must be directly supplemented with histidine.

LET: Linear Energy Transfer - The amount of energy deposited by radiation per unit length of travel in an absorber. Expressed as kiloelectron volts pre micrometer (KeV/μm).

MeV: Megaelectron volts - ie. 14 MeV neutrons.

MNNG: *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine - A DNA methylating agent which is a known mutagen and carcinogen.

OER: Oxygen Enhancement Ratio - The ability of oxygen to potentiate radiation response is called the oxygen effect, which is expressed in terms of the OER. OER = Dose under anoxic condition to produce an effect divided by dose under oxygenated condition to produce same effect.

REC Repair: Recombinational Repair - A DNA repair process whereby lost genetic information (including DSB) is repaired by utilizing information from homologous chromosomes or sister chromatids.

REC+: Recombinational Repair Proficient - A cell capable of repairing damaged DNA by rec repair.

REC-: Recombinational Repair Deficient.

SSB: Single Strand Break - a single strand of DNA is broken.

YPD: Yeast extract/Peptone/Dextrose - growth media for yeast.

YES: Yeast Extract/Succinate - growth media for yeast.