Creation and Improvement of a Yeast RNR3-lacZ Genotoxicity Testing System

A thesis Submitted to the College of Graduate Studies and Research in Partial Fulfillment of the Requirements for the Degree of Master of Science in Toxicology Graduate Program University of Saskatchewan

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ABSTRACT

A variety of environmental toxicants can damage DNA and thereby produce congenital malformation and cancer. In order to evaluate the genotoxic effects of environmental agents, numerous genotoxicity testing systems have been developed. These tests either directly assess the genetic alterations (e.g., the Ames test) or indirectly measure the cellular response to DNA damage (e.g., SOS Chromotest).

With the knowledge obtained from studying molecular mechanisms of DNA damage and signal transduction in *Saccharomyces cerevisiae*, a sensitive and stable genotoxicity testing system was developed based on the induction of *a S. cerevisiae RNR3-lacZ* reporter gene expression in response to a broad range of DNA-damaging agents and agents that interfere with DNA synthesis. The tested agents include known carcinogenic and genotoxic agents, ranging from DNA alkylating agents, oxidative chemicals to ionizing radiation as well as some known non-genotoxic agents. All the tested known genotoxic agents were able to induce *RNR3-lacZ* expression at a sub-lethal dose. In particular, a potent colon carcinogen, 1, 2-dimethyl hydrazine, was not detected as a mutagen by a standard Ames test, but was able to induce *RNR3-lacZ* expression. In contrast, both non-mutagenic and non-genotoxic chemicals tested were unable to induce *RNR3-lacZ* expression. The sensitivity and inducibility of three well-characterized yeast DNA damage-inducible genes have been compared and it was found that *RNR3* is more sensitive than *RNR2* and *MAG1*. The effects of agent dose, post-treatment incubation time and cell growth stage on *RNR3-lacZ* expression were also determined and optimized. In order to create a stable and user-friendly testing system, the *RNR3-lacZ*...
cassette was integrated into the yeast genome to demonstrate that its inducibility is indistinguishable from that of the plasmid-based studies.

Although the sensitivity of RNR3-lacZ testing is comparable to that of the Ames test and SOS Chromotest, it was reasoned that the sensitivity might be further improved by using yeast strains defective in certain DNA repair pathways. Hence, several deletion mutant strains from different repair pathways were used as host strains for the RNR3-lacZ test. It was found that the magl null mutation specifically enhanced the sensitivity of RNR3-lacZ test in response to DNA alkylating agents such as methyl methanesulfonate and ethyl methanesulfonate, while the rad2 null mutation enhanced the sensitivity of this system in response to ultraviolet (UV) radiation and a UV mimetic agent 4-nitroquinoline 1-oxide. In the rad2 null mutant, RNR3-lacZ induction was also more sensitive to MMS than that in wild type strain at low dosed. In summary, it appears that the enhancement of RNR3-lacZ induction is agent and repair specific, although inactivation of the nucleotide excision repair pathway may affect a broad range of testing agents which can be incorporated into the RNR3-lacZ test.
ACKNOWLEDGEMENTS

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A loving thanks to my husband and my parents, who gave me unconditional love, understanding, help and encouragement all the time. My gratitude cannot be expressed in words.
To my family
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<tr>
<td>AAF</td>
<td>N-2-Acety-2-Aminofluorene</td>
</tr>
<tr>
<td>AP site</td>
<td>apurinic or apyrimidinic site</td>
</tr>
<tr>
<td>BER</td>
<td>base excision repair</td>
</tr>
<tr>
<td>β-gal</td>
<td>β-galactosidase</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>CAT</td>
<td>chloramphenicol acetyl transferase</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster cell</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>double distilled water</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>DSBs</td>
<td>double stranded breaks</td>
</tr>
<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>EMS</td>
<td>ethyl methanesulfonate</td>
</tr>
<tr>
<td>EtBr</td>
<td>ethidium bromide</td>
</tr>
<tr>
<td>FOA</td>
<td>5-fluoro-orotic acid</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
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<tr>
<td>his</td>
<td>histidine</td>
</tr>
<tr>
<td>HU</td>
<td>hydroxyurea</td>
</tr>
<tr>
<td>HNPCC</td>
<td>hereditary nonpolyposis colorectal cancer</td>
</tr>
<tr>
<td>HPRT</td>
<td>hypoxanthine-guanine phosphoribosyl transferase</td>
</tr>
<tr>
<td>HR</td>
<td>homologous recombination</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase pair</td>
</tr>
<tr>
<td>LUC</td>
<td>luciferase</td>
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<td>MMS</td>
<td>methyl methanesulfonate</td>
</tr>
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<td>MMR</td>
<td>mismatch repair</td>
</tr>
<tr>
<td>MNNG</td>
<td>N-methyl-N'-nitro-N-nitrosoguanidine</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>NHEJ</td>
<td>non-homologous end joining</td>
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<tr>
<td>NER</td>
<td>nucleotide excision repair</td>
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<tr>
<td>4-NQO</td>
<td>4-nitroquinoline</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>ONP</td>
<td>O-nitrophenol</td>
</tr>
<tr>
<td>ONPG</td>
<td>ortho-nitrophenyl-β-galactoside</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>PRR</td>
<td>postreplication repair</td>
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<td>RNR</td>
<td>ribonucleotide reductase</td>
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<tr>
<td>SD medium</td>
<td>synthetic dextrose minimal medium</td>
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<tr>
<td>S. cerevisiae</td>
<td>Saccharomyces cerevisiae</td>
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<td>SDMH</td>
<td>1,2- dimethylhydrazine</td>
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<td>SSBs</td>
<td>single strand breaks</td>
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<tr>
<td>ssDNA</td>
<td>single stranded DNA</td>
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<tr>
<td>TK</td>
<td>thymidine kinase</td>
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<tr>
<td>Ubc</td>
<td>ubiquitin-conjugating enzyme</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>UV</td>
<td>ultraviolet radiation</td>
</tr>
<tr>
<td>XP</td>
<td>Xeroderma Pigmentosum</td>
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<tr>
<td>XPRT</td>
<td>xanthine-guanine phosphoribosyl transferase</td>
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CHAPTER I: INTRODUCTION

1.1. DNA damage and environmental DNA damaging agents

A variety of toxicants are released into the general environment in large quantities and cause adverse health effects among people. Such effects are usually subclinical, except short-term responses that following acute exposure; however, cumulative damage also leads to chronic effects. Research indicates that a large proportion of human developmental diseases are caused by environmental agents that damage the genetic constitution of somatic cells. These agents, including chemicals, radiation, and viruses, are collectively referred to genotoxic agents. They can produce congenital malformation, cancer and a variety of more subtle effects on health and longevity.

Under normal conditions, four kinds of nitrogen bases (adenine, thymine, cytosine, and guanine) are contained in DNA molecules. Each base is held within the sugar-phosphate backbone of the polynucleotide chain through an N-glycosyl bond. DNA helix formation relies on the proper hydrogen bonding between complementary bases on opposite polynucleotide strands. Complementarity of bases matches cytosine with guanine via three hydrogen bonds, and adenine with thymine via two hydrogen bonds.

It is assumed that this macromolecule must be extraordinarily stable in order to maintain the high degree of fidelity. It is surprising to learn that the primary structure of DNA is, in fact, quite dynamic and subject to constant change. Besides large-scale changes such as chromosome rearrangement, translocation and transposition (Finnergan, 1990; Kleckner, 1990), DNA is also subject to alterations in the chemistry or the sequence of individual nucleotides (Lindahl, 1993; Ward, 1985; Singer and Grunberger, 1985).
1982). Many of these changes arise as a consequence of errors introduced during replication, recombination, and repair itself. Other base alterations arise from the inherent instability of specific chemical bonds that constitute the normal chemistry of nucleotides under physiological conditions of temperature and pH. Surprisingly, DNA reacts readily with a variety of chemical compounds and physical agents both in vivo and in vitro.

DNA damage refers to the modifications of the DNA molecular structure or strand breaks. The damage will block replication and increase the probability of incorporation of incorrect bases during replication. Different agents, according to their chemical or physical characteristics, cause different types of DNA damage. The phenotypic result of DNA damage may depend on the chromosomal locus at which it occurred, and on how the base sequence is affected. Some of the DNA damaging agents can be produced via cell metabolism, but most of them are present in the environment. For convenience, DNA damage can be classified into two major groups, referred to as endogenous DNA damage and exogenous DNA damage.

1.1.1. **Endogenous DNA damage**

DNA lesions can arise naturally through intracellular metabolism or the intrinsic instability of DNA. The chief source of DNA alterations arising during normal DNA metabolism is the mispairing of bases during DNA synthesis that results in base mismatches. Mismatches can arise from the synthetic events associated with a subset of nuclear DNA during repair and recombination. Generally, all of the bases in a genome must be replicated prior to cell division. However, under certain circumstances the
replication of DNA occurs on parental template strands carrying unrepaired noninstructional or misinstructional base damage, which can act as miscoding lesions.

Each of the common bases in DNA can spontaneously undergo a transient rearrangement of bonding to form a structural isomer of the base. Formation of an isomer of any base alters its base-pairing properties. If the base isomer persists during replication, DNA polymerase may incorporate incorrect bases opposite the modified base. If the incorporated base of the daughter strand remains, the lesion is fixed into a mutation.

Deamination of cytosine, adenine and guanine is also a major source of mutation. Deamination of cytosine, resulting in the formation of uracil, is the most common deamination reaction in the cell (Lindahl, 1993). The conversion of cytosine to uracil often results in a transition mutation, since adenine is likely incorporated opposite uracil. The base 5-methylcytosine, is a naturally occurring analogue of cytosine. The deamination of 5-methylcytosine causes the conversion from cytosine to thymine and therefore produces a transition mutation. The deamination of adenine, and guanine results in products of hypoxanthine and xanthine respectively. Hypoxanthine is a potentially mutagenic agent. Xanthine can arrest DNA replication and therefore cause cell death (Friedberg et al., 1995).

The loss of purines and pyrimidines from DNA has been most extensively studied at acidic pH. However, depurination and depyrimidination can also occur at appreciable rates at neutral or alkaline pH (Lindahl, 1993; Loeb and Preston, 1986). The chemical mechanism of hydrolytic DNA depurination at acidic pH is believed to be the same as that established for acid hydrolysis of deoxynuclosides. The mechanism of hydrolysis of
nucleosides at neutral and at alkaline pH is less well characterized. In *E. coli*, the rate of depurination is about one purine per cell per generation at 37 °C. For mammalian cells, in which genomes are much larger and replication times are longer, the loss of purine is estimated at the rate of 10,000 per cell per generation. Pyrimidine nucleosides are more stable than purine nucleosides with respect to the glycosylic linkage of the bases to deoxyribose. The mechanism of depyrimidination is the same as for depurination, but cytosine and thymine are lost at rates only 1/20 of that for adenine or guanine. This still results in the loss of hundreds of pyrimidines per mammalian cell per generation.

Attack by reactive oxygen species is another major source of spontaneous damage to DNA and other intracellular macromolecules such as proteins, lipids, and carbohydrates (Joenje, 1989; Ames and Gold, 1991). Reactive oxygen species are created during aerobic metabolism. The major intracellular source of oxygen radicals is probably leakage associated with the reduction of oxygen to water during mitochondrial respiration. These products include single oxygen, peroxide radicals (O₂⁻), hydrogen peroxide (H₂O₂), and hydroxyl radicals (·OH). They create single and double strand breaks, damage to the deoxyribose moiety, lethal abasic sites and various base modifications (Croteau & Bohr, 1997). The lesions induced by these reactive oxygen species overlap with those induced by ionizing radiation, since much of ionizing radiation-induced DNA damage is caused by reactive oxygen species.

1.1.2. Environmental DNA damaging agents

The environment outside a cell or organism contains many natural and artificial
DNA damaging agents. According to their sources, they can be classified as physical or chemical agents.

1. 1. 2. 1. Physical agents that damage DNA

Physical DNA damaging agents are generally referred to as ionizing irradiation and UV irradiation.

1. 1. 2. 1. 1. Ionizing irradiations

Ionizing radiation has been a source of naturally occurring physical damage to the DNA of living organisms since the beginning of biological evolution. However, various man-made therapeutic, diagnostic, or occupational sources of exposure to ionizing radiation are of far greater importance. In 1926, Muller exposed fruit flies to X rays and showed for the first time that ionizing radiation produces mutations within individual genes as well as gross chromosomal aberrations. Ionizing radiation can randomly cause damage to all cellular components and induces a variety of DNA lesions. Radiation damage to DNA is ascribed to both direct and indirect effects. Direct effects result from the direct interaction of the radiation energy with DNA. Indirect effects result from the interaction of DNA with reactive species formed by the radiation. The lesions created by ionizing radiation include base damage, sugar damage and strand breaks. It has been estimated that damage to sugar residues in DNA may be less than damage to bases. However, such damage is of biological importance primarily because of the strand breakage that might result. Ionizing radiation also induces strand breakage directly, and most of the lethal effects of ionizing radiation can be attributed to these lesions
1. 1. 2. 1. 2. Ultraviolet irradiation

Ultraviolet light is highly relevant biologically. Living organisms have had to contend with the genotoxic effects of solar UV radiation since the beginning of evolution of life on this planet. It has been suggested that unattenuated UV radiation during the pre-Phanarazoic aeons might have precluded the development of terrestrial life. At present, the exposure of cells to UV radiation is probably the best studied and most extensively used model system for exploring the biological consequences of DNA damage, its repair and tolerance. The UV radiation spectrum has been subdivided into three wavelength bands designated UV-A (400 to 320 nm), UV-B (320-290 nm), and UV-C (290-100nm). Solar UV radiation consists mainly of UV-A and UV-B, so they have more relevance to human health. Most of UV-C is absorbed by the atmospheric ozone layer. When DNA is exposed to radiation at wavelengths approaching its absorption maximum (~260 nm), adjacent pyrimidines become covalently linked by the formation of a four-membered ring structure, called cyclobutane dipyrimidine or pyrimidine dimer. Some photoproducts such as pyrimidine-pyrimidine (6-4) lesions are also present in UV-irradiated DNA. These photoproducts introduce a major distortion in the double helical structure of DNA. Other photoproducts in DNA include pyrimidine hydrates, thymine glycols, but they are not very common (Friedberg et al. 1995).

1. 1. 2. 2. Chemical agents that damage DNA

Chemical substances that induce mutation occur in widely divergent chemical groups, ranging from simple compounds such as formaldehyde to complex ones such as alkaloids. Many of these chemicals are carcinogenic. Exposure to chemicals is
ubiquitous in industrial societies. It has been estimated, for example, that more than 50,000 chemicals are in common use in the United States. Most of these compounds have not been tested for mutagenicity; of those that have been, about 20 percent produced mutations in the Ames test. The total exposure to mutagenic chemicals is thus unknown, but it undoubtedly presents a definite public health risk.

1.1.2.1. Alkylating agents

Alkylating agents are electrophilic compounds with affinity for nucleophilic centres in organic macromolecules. These include a wide variety of chemicals, many of which are proven or suspected carcinogens. There are two types of alkylating agents: monofunctional and bifunctional. The former has a single reactive group and thus interacts covalently with single nucleophilic centres in DNA. Bifunctional alkylating agents have two reactive groups. Each molecule is potentially able to react with two sites in DNA. There are numerous potential reaction sites that can be alkylated within a DNA strand. For monoalkylating agents, these sites include: \( N^1 \), \( N^3 \), \( N^6 \), \( N^7 \) in adenine; \( N^1 \), \( N^2 \), \( N^3 \), \( N^7 \), and \( O^6 \) guanine; \( N^2 \), \( O^2 \), and \( O^4 \) in thymine; and \( N^3 \), \( N^4 \), and \( O^2 \) in cytosine.

Alkylation of oxygen in phosphodiester linkages results in the formation of phosphotriesters (Singer, 1986). Base modification by alkylation weakens the N-glycosylic bond. Hence treatment with many alkylating agents leads to depurination/depyrimidination and the appearance of alkali-labile abasic sites (Friedberg et al., 1995).

Bifunctional alkylating agents can react with two different nucleophilic centres in DNA. If the two sites are on opposite polynucleotide strands, interstrand DNA cross-
links occur. If these sites are situated on the same polynucleotide chain of a DNA duplex, the reaction product is referred to as an intrastand cross-link. Interstrand DNA-links represent an important class of chemical damage to DNA, since they prevent DNA strand separation and hence can constitute complete blocks to DNA replication and transcription. Nitrogen mustard, mitomycin and various platinum derivatives are famous cross-linking agents (Lawley and Phillips, 1996). In addition, UV radiation at 254 nm and ionizing radiation can result in the formation of intermolecular DNA cross-links as well as DNA-protein cross links as minor products of DNA damage (Friedberg et al., 1995).

1. 1. 2. 2. Base analogs

Generally, the biologically relevant base damage does not originate from the direct reaction of chemical agents with DNA itself, but from the incorporation of damaged deoxynucleotides during replication of damaged templates. Among the most extensively studied base analogs are the halogenated uracil derivatives, 5-bromouracil, 5-fluorouracil, and 5-iodouracil. All of them are thymine analogs that can result in mutations when present in template DNA undergoing DNA replication (Miller, 1983; 1985; 1992). The adenine analog, 2-aminopurine is also mutagenic (Rotilio, 1986; Sowers et al., 1987).

1. 1. 2. 3. Chemicals that need to be metabolized to active forms

A variety of relatively nonpolar (and hence chemically unreactive) compounds can be metabolized to more reactive forms in the affected cells. Many of these compounds are potent mutagens and carcinogens. It is now known that the metabolic activation of
these compounds is catalyzed by specific metabolizing enzymes. The biological function of these enzyme systems is to protect cells against cytotoxic effects by converting potentially toxic nonpolar chemicals to water-soluble excretable forms. However, some of the reaction products become activated to electrophilic forms that are particularly reactive with nucleophilic centres in organic macromolecules such as DNA. The best-studied activating enzymes are the cytochrome P-450 system (Ullrich, 1977). Other examples involved in activating genotoxic chemicals include microsomal and cytoplasmic glutathione-S-transferases, sulfotransferase, acetyltransferases, UDP-glucuronosyltransferases, and adenosylating as well as methylating enzymes.

*N-2-Acetyl-2-Aminofluorene* (AAF) belongs to a class of chemicals known as aromatic amines. After being catalyzed by cytochrome P-450 and cytosolic enzymes, it becomes a highly reactive alkylating agent (Friedberg, 1995).

Aflatoxin, especially Aflatoxin B1, is one of the most potent liver carcinogens. It is oxidized by the mixed function oxygenases of the P-450 system in liver cells, and gives rise to aflatoxin B1-8, 9-epoxide as the major product. Certain guanine residues in double-stranded DNA are preferentially attacked by this reactive electrophilic epoxide (Friedberg, 1995).

4-Nitroquinoline 1-Oxide (4-NQO) is often referred to as a “UV radiation mimic” agent because it produces bulky adducts. The repair of these bulky adducts by nucleotide excision repair (NER) is similar to the repair of cyclobutane pyrimidine dimer and (6-4) photoproducts. Seryl-tRNA-synthetase is known involved in the activation of 4-NQO.

Benzo(a)pyrene is a very potent carcinogenic polycyclic aromatic hydrocarbon that has a remarkable correlation with cancer of the scrotum. Unmodified benzo(a)pyrene is
an unreactive nonpolar compound. It is now known that components of the P-450 system
known as arylhydrocarbon hydroxylases can metabolize benzo(a)pyrene to excretable
phenols and dihydrodiols. Some of the metabolites are electrophilic epoxides that can
damage DNA.

Glutathione is a major intracellular sulfhydryl component. It is strongly
nucleophilic and reacts with electrophilies. Glutathione conjugation is, in general,
considered to be a detoxification reaction. However, it can be potentially genotoxic when
it involves the activation of carcinogenic agents such as N-nitrosamines (Singer and
Grunberger, 1983) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) (Kistler et al.,
1986).

1.2. Cellular response to DNA damage

When cells are exposed to conditions that cause DNA damage, they activate
numerous repair mechanisms that result in the restoration of the DNA sequence to its
original condition. The repair mechanism involves various overlapping repair pathways.
However, if the cell is exposed to conditions that result in severe DNA damage and/or
conditions that interfere with chromosomal replication, it co-ordinately activates the
expression of a large number of diverse unlinked genes involved in DNA repair and
error-prone DNA replication. This coordinated activation of diverse metabolic functions
in response to DNA damage has been called the SOS response in prokaryotes. Similar
mechanisms have also been found in eukaryotic cells. Cellular responses that do not
include the removal of the primary damage to the genome are considered DNA damage
tolerant rather than DNA repair mechanisms. Many DNA tolerance mechanisms result in
permanent mutations in the genome. The ability of cells to tolerate DNA damage is biologically as important as their ability to repair such damage.

1.2.1. **DNA repair**

DNA repair is involved in processes that minimize cell killing, mutations, replication errors, persistence of DNA damage and genomic instability. Abnormalities in these processes have been implicated in cancer and aging. The robust DNA repair systems are necessary for enhanced survival and reduced mutagenesis of cells. DNA repair includes direct reversal of DNA damage, DNA excision repair, DNA recombinational repair and post-replication repair pathways.

1.2.1.1. **Direct reversal of DNA damage**

The direct reversal DNA repair pathway removes the lesion without additional alteration to the native DNA molecules. This type of DNA repair is often performed by a single enzyme that only recognizes the specific lesions. The repair is usually just a single-step reaction that restores the DNA structure to its normal state.

As indicated in section 1.1.2.1, cyclobutane pyrimidine dimer and pyrimidine (6-4) pyrimidine photoproducts are the two major classes of cytotoxic, mutagenic and carcinogenic DNA photoproducts produced by UV light irradiation of cells. The dimer is lethal to the cell if it is not removed before replication (Berends et al., 1961). Direct reversal of DNA repair pathway which removes pyrimidine dimers (Nairn et al., 1989; Sancar, 1990) and (6-4) photoproduct (Todo et al., 1993) have been reported, respectively.
Another example of direct reversal of DNA damage is found in the repair of alkylation damage. Certain alkylating lesions can be removed by the alkyltransferase family. Alkyltransferases have been isolated in bacteria, yeast and mammalian cells. They can directly remove the alkyl group from DNA molecules by accepting the alkyl group at a cysteine residue in their active sites (Pegg and Byers, 1992). For example, the O\(^6\)-alkylguanine DNA alkyltransferase (Mgt1) in \textit{S. cerevisiae} can specifically remove the methyl group from O\(^6\)-methylguanine and O\(^4\)-methylthymine lesions (Xiao et al., 1991).

1.2.1.2. DNA excision repair

DNA excision repair consists of at least three separate pathways: base excision repair (BER), NER and the mismatch repair pathway (MMR, Friedberg et al., 1995). These pathways remove DNA lesions by excising the damaged sequence, followed by replacement with undamaged nucleotide sequence.

1.2.1.2.1. Base excision repair

All BER is initiated by the action of a specific class of DNA enzymes called DNA glycosylases. The glycosylase recognizes and binds to the damaged site, then mediates the cleavage of the damaged base from the sugar backbone. The resulting abasic site is incised exclusively by AP endonucleases. The one-nucleotide gap is filled by DNA polymerase and sealed by DNA ligase (Wallace, 1994; Sancar, 1994; Seeberg, 1995). This process is called short-patch BER. An alternative BER pathway, which is called long-patch BER, has been discovered in eukaryotes that involves the replacement of...
more than a single nucleotide (about 7 nucleotides). In long patch BER, DNA polymerase fills in the missing base and continues to displace the existing strand. The resulting flap-like structure is excised by Fen1/ Rad27, and the strand break is ligated together (Wilson and Thompson, 1997).

Defects in BER have been shown to result in hypersensitivity to alkylating agents, oxidative agents, ionizing radiation and endogenous damage (Wallace, 1994). This suggests that BER is an important pathway for protecting cells from mutagenic and lethal damage.

1.2.1.2.2. Nucleotide excision repair

The NER pathway primarily repairs DNA damage resulting from UV, 4-NQO and cross-linking agents. All these DNA damaging agents cause helix-distorting lethal lesions. The following steps are involved in NER: lesion recognition, strand separation between damaged and undamaged strands, excision of the damaged sequence and polymerization of DNA to replace the excised sequence (Friedberg et al., 1995).

There are two classes of NER: transcription-coupled NER and global genome NER. Studies carried out with E. coli, yeast and mammalian cells demonstrate that NER preferentially repairs actively transcribed genes (Bohr and Kober, 1985; Mellon and Hanawalt, 1989; Smerdon and Thoma, 1990). The preferential repair of active genes is accounted by the fast repair of the transcribed strand. In contrast, NER also can recognize and repair lesions throughout the genome, but this random process occurs slowly.
Lacking of XP genes and therefore, a defective in NER pathway results in a human hereditary disease known as Xeroderma Pigmentosum (XP; Cleaver, 1968). One of the most consistent cellular phenotypes of XP cells is an increased sensitivity to killing following exposure to a wide variety of DNA damaging agents, including UV radiation. Patients with XP syndrome have an extremely high incidence of premalignant actinic keratoses, as well as benign and malignant skin tumors (Friedberg et al., 1995).

1.2.1.2.3. Mismatch repair pathway

Three different components contribute to the overall fidelity of DNA replication: first is the polymerization reaction itself; second is the proofreading by a 3'→5' exonuclease activity; finally, the postreplication mismatch repair. Without the intervention of any cellular factors, the error frequency during DNA replication is about $10^{-1}→10^{-2}$ in *E. coli* (Loeb and Kunkel, 1981). The combined effects of base selection and proofreading of newly inserted nucleotides by certain DNA polymerases, together with the enhancement of DNA synthesis reduce the error efficiency by 3-6 orders of magnitude. Mismatch repair increases the error frequency to approximately $10^{-10}$ (Kunkel and Loeb, 1981).

Heritable mutations in a MMR gene lead to a so-called mutator phenotype, that is a very high susceptibility of the cell or organism to mutations (Loeb, 1994). It is known that hereditary nonpolyposis colorectal cancer (HNPCC) is resulted by defects in MMR gene *hMSH2* or *hMLH1*. A striking feature of HNPCC is instability of simple repeated sequences (Fischer et al., 1992; Parsons et al., 1995).
1. 2. 1. 3. Recombination repair

The induction of double strand breaks (DSBs) in DNA by exposure to DNA damaging agents, or as intermediates in normal cellular processes, constitutes a severe threat for the integrity of the genome. If not properly repaired, DSBs may result in chromosomal aberrations, which, in turn, can lead to cell death or to uncontrolled cell growth. To maintain the integrity of the genome, multiple pathways for the repair of DSBs have evolved during evolution: homologous recombination (HR), non-homologous end joining (NHEJ) and single-strand annealing (SSA). Homologous recombination has the potential to lead to accurate repair of DSBs, whereas NHEJ and SSA are essentially mutagenic. Homologous recombination is the common way for repair DSB in *S. cerevisiae*, but in higher eukaryotes, both HR and NHEJ are important. In *S. cerevisiae*, the *RAD52* epistasis group genes are responsible for recombination repair. This group includes *RAD50, RAD51, RAD52, RAD54, RAD55, RAD57, RAD59, RDH54/TID1, MRE11*, and *XRS2* (Friedberg et al., 1995). *S. cerevisiae* is extremely efficient at catalyzing homologous recombination. It involves interactions between two DNA molecules sharing substantial homology, and includes both reciprocal and nonreciprocal information exchange. Reciprocal exchange does not alter the content of the genome but rearranges genetic linkage patterns. Nonreciprocal exchange results in a net gain or loss of functional information. When a DSB occurs, it is resected by 5' to 3' exonucleases to expose single-stranded tails with 3' termini. This single stranded tail invades an uncut homologous duplex and promotes repair synthesis followed by branch migration to produce two Holliday junctions. The Holliday junctions are then resolved to the normal structure of DNA strand by Holliday junction resolvases (Friedberg et al., 1995). This
progress ensures that all missing sequence is replaced by replicating from the homologous duplex molecule sequence (Shinohara and Ogawa, 1995). Rad51 (homologous to E. coli RecA) assembles on the 3'-ended single-stranded DNA and, in concert with Rad54, stimulates strand invasion at homologous sequence and facilitates the initiation of DNA synthesis (Paques and Haber, 1999). Other proteins, such as Rad52, Rad55 and Rad57, are believed to assist the functions of Rad51 and Rad54 (Sung, 1997).

Although RAD52 group genes share the property of conferring resistance to ionizing radiation, they show considerable variation in assays for DSB repair and recombination. RAD50, XRS2, and MRE11 form a subgroup with similar properties. Their complex is nucleolytic and plays a role in nonhomologous end rejoining/filling (Moore and Haber, 1996).

1. 2. 1. 4. Post-replication repair

DNA post-replication repair (PRR) is defined as an activity to convert DNA damage-induced single-stranded gaps into large molecular weight DNA without actually removing the replication-blocking lesions. Hence, PRR is actually a damage tolerance pathway. In bacteria such as E. coli, this activity requires RecA and the RecA-mediated SOS response and is accomplished by recombination and mutagenic translesion DNA synthesis. In the yeast S. cerevisiae, PRR is centrally controlled by RAD6 and RAD18, whose products form a stable complex with single-stranded DNA-binding, ATPase and ubiquitin-conjugating activities. Post-replication repair can be further divided into translesion DNA synthesis and error-free modes. Mutagenic translesion bypass is due to the mutagenic DNA polymerases, such as Polζ and Polη in yeast; hRev3, XPV, Polı and
Polk in human; UmuCD and DinB in *E. coli* (Baynton and Fuchs, 2000; Goodman and Tippin, 2000; Johnson et al., 2000). In the yeast *S. cerevisiae*, error-free PRR requires Rad6, Rad18, Mms2, Ubc13, Rad5, Pol30 (PCNA) and Pol13 (Polδ). The PRR processes appear to be highly conserved within eukaryotes, from yeast to human (Broomfield et al., 2001).

1. 2. 2. **Cell cycle checkpoints and DNA damage**

DNA lesions can be fixed in the genome of a cell in the form of a mutation, that is, a heritable alteration that can be corrected only by elimination of the cell. Genetic stability is controlled by a number of interrelated and overlapping cellular functions, the most important of which are the various DNA replication, repair and recombination pathways. A major component of cellular defense against DNA damage are cell cycle checkpoints, which are monitoring systems for DNA damage that temporarily halt replication until the lesions are repaired (Carr, 1996). These mechanisms are thought to prevent the replication of damaged templates and the segregation of broken chromosomes. The DNA damage checkpoint pathway is well conserved from yeast to humans. The following genes: *RAD9, RAD17, RAD24, RAD53, CHK1, DCD1, DCD2, MEC3, POL2*, and *MEC1* have been identified in the yeast *S. cerevisiae*. When mutated, these genes inactivate certain checkpoint controls (Elledge, 1996; Paulovich et al., 1997), among which, *RAD9, 17, 24* and *MEC3* are required to activate the DNA damage checkpoint when cells are in G1 or G2 phase (Hartell and Weinert, 1989; Siede et al., 1993; Weinert and Hartwell, 1994); *POL2* is required to sense replication blocks when cells are in S phase (Navas et al., 1995); whereas *MEC1* (Paulovich and Hartwell, 1995)
and *RAD53* (Allen et al., 1994) appear to form a downstream signal transduction cascade required for all three cell cycle stages.

In mammalian cells, two important examples of cell cycle checkpoint components are the tumor suppressor genes *p53* and *ATM*. The *p53* gene is activated by DNA damage to subsequently induce the p21 cdk inhibitor, which mediates growth arrest (Hartwell and Kastan, 1994). The *p53* gene product is also considered to play a key role in apoptosis pathways (Lowe et al., 1993). The *ATM* protein might be an upstream component of a radiation-induced signal transduction pathway which is activated by DNA damage and involves the recruitment of *p53* (Savitsky *et al.*., 1995), and perhaps other checkpoint proteins (Carr, 1996). The *ATM* protein shares the phoshoinositide 3-kinase (PI-3 kinase) domain with several large proteins in yeast, *Drosophila*, and mammals (Keith and Schreiber, 1995). There is also significant homology between *ATM* and the yeast cell cycle checkpoint gene, *MEC1*.

### 1. 2. 3. Changes in gene expression after DNA damage

In response to DNA damage, both prokaryotic and eukaryotic cells activate stress responses that result in specific alterations in patterns of gene expression and active inhibition of cell division.

The SOS regulatory network of *E. coli* is a stress response that is well understood. Exposure to a variety of DNA-damaging agents induces the expression of approximately 30-40 genes through a common mode of transcriptional de-repression controlled by the *recA* and *lexA* gene products. As a consequence of DNA damage or interference with DNA synthesis, single-stranded DNA accumulates and activates RecA molecules.
Activated RecA potentiates the autoproteolytic cleavage of the LexA repressor that binds within the operators of SOS-inducible genes that mediate a number of functions related to DNA metabolism and cell division. Similar stress responses have been found in S. cerevisiae and mammalian cells although it is much more complex than that in E. coli.

The transcription of a number of yeast genes has been demonstrated to accumulate after exposure to DNA-damaging agents, and at least some of them function directly in DNA repair. Some regulatory genes that control the transcriptional response to DNA damage have been identified. Interestingly, some of these regulatory genes are also functional in cell-cycle checkpoint controls that couple cell-cycle progression to the status of genome duplication and integrity. Interfering with DNA replication activates the S-phase checkpoint that prevents entry into mitosis with unreplicated DNA. DNA lesions encountered outside of S phase activate additional checkpoint pathways that delay cell-cycle progression prior to the G1/S and G2/M transitions, presumably to prevent the replication or segregation of damaged chromosomes.

1.2.3.1. DNA damage inducible genes in S. cerevisiae

The number of genes induced by DNA damage in S. cerevisiae is very large. In a recent genome scale study (Jelinsky and Samson, 1999), as many as 325 genes showed at least a 4-fold increase at the transcription level after treatment with the alkylating agent MMS. This represents about 5% of the yeast genome. These genes can be divided into several classes, some of them function directly in the repair of damaged DNA, and some others function primarily in nucleotide metabolism, DNA synthesis and protein turnover.
1. DNA damage inducible genes that function directly in DNA repair

Within the NER pathway, *RAD2*, *RAD27*, and *RAD23* are induced approximately fivefold by UV irradiation (Madaua and Prakash, 1986; Robinson et al., 1986; Jones et al., 1990). *RAD2* encodes an endonuclease and is required for the incision step of NER. *RAD7* and *RAD23* appear to fulfill accessory or regulatory roles in NER biochemistry. In addition to UV irradiation, *RAD2* also responds to other DNA damaging agents such as 4-NQO, γ irradiation, bleomycin, nitrogen mustard, mitomycin, MMS and cyclohexamide (Robinson et al. 1986).

Within the recombinational repair pathway, *RAD51* and *RAD54* are DNA damage inducible. Both of them also function in normal recombination. Their expression can be induced 5-10 fold by X ray irradiation and MMS (Cole et al., 1987; Basile et al., 1992).

Within the PRR pathway, *RAD6* encodes a ubiquitin-conjugating enzyme (Ubc) that is required in response to UV irradiation. *RAD18* encodes a protein with DNA binding domains that is capable of binding single-stranded DNA. Research (Bailly et al., 1994) indicated that Rad6 and Rad18 form a heterodimer whose function is similar to that of RecA in *E. coli*. Rad18 binds single-stranded DNA formed at a stalled replication fork and targets Rad6 to the site of damage. Both *RAD6* and *RAD18* can be induced to several folds by UV irradiation (Madura et al., 1990; Jones and Prakash, 1991). Rad30 is a newly discovered DNA polymerase (Pol11) and is placed in the error-free arm of PRR (Johnson et al., 1999). It is also UV inducible (McDonald et al., 1997).

*MAGI* encodes a 3'-methyladenosine DNA glycosylase specifically involved in the repair of alkylated bases (Chen et al., 1989). It is induced not only by alkylating agents
like MMS and MNNG, but also by UV irradiation and 4-NQO (Chen et al., 1990; Chen and Samson, 1991).

*PHR1* encodes the yeast photoreactivating enzyme that catalyzes the light-dependent repair of pyrimidine dimer in DNA and stimulates NER activity in the dark. *PHR1* is specific for the repair of pyrimidine dimer, but is induced by a variety of DNA-damaging agents by chemically modifying DNA or causing DSBs (Sebastian et al., 1990).

The mechanism of why and how these genes are induced is still not completely understood. Some genes can be induced by a variety of DNA damaging agents. For example, generally, UV damage does not need 3-methyladenine DNA glycosylase for the repair, but *MAG1* is also induced by UV irradiation. It has been postulated that the promiscuous inducibility of genes occurs either because the sensory/signalling system for inducible repair genes can recognize several forms of DNA damage, or because different forms of DNA damage are converted into discrete form or intermediate that acts as a damage signal (Nickoloff and Hoekstra, 1998).

1. 2. 3. 1. 2. **DNA damage inducible genes without direct function in DNA repair**

There are a number of other inducible genes that do not function directly in the above repair pathways. Most of them function in the metabolism of nucleic acids or proteins. For example, *CDC8* encodes thymidylate kinase, *CDC9* encodes DNA ligase, *HIS3* and *HIS4* function in histidine metabolism. The best studied gene family is the *RNR* gene family.
1.2.3.1.2.1. **RNR gene family and ribonucleotide reductase (RNR)**

The RNR family consists of RNR1, RNR2, RNR3 and RNR4. They encode ribonucleotide reductase (RNR) which catalyzes the first and rate-limiting step in the production of deoxyribonucleotides. The reaction is of the following form:

\[ \text{NTP} + \text{reductant-(SH)} \rightarrow \text{dNTP} + \text{reductant-(S-S)} \]

Deoxyribonucleotide levels are critical to many cellular functions. During the DNA synthetic phase of the cell cycle, their levels must be sufficient to replicate the genome. Furthermore, their relative ratios must be balanced to ensure high-fidelity DNA replication. Unbalanced nucleotide pools lead to enhanced misincorporation and mutation frequency. Deoxyribonucleotide levels are also important for DNA repair processes. All of excision repair, recombination repair and PRR require DNA synthesis and are thought to be impaired in the absence of sufficient nucleotide levels.

Ribonucleotide reductase is an enzyme with \( \alpha_2\beta_2 \) structure. RNR1 and RNR3 encode the alternative large subunit Rl. RNR1 encodes \( \alpha_1 \) and RNR3 encodes \( \alpha' \). Normally, Rl is a dimer of \( \alpha_1 \) with a monomeric molecular mass of 90 kDa (Caras et al., 1985). Each monomer contains two distinct binding sites for deoxynucleoside triphosphates which act as allosteric regulators of the enzymatic activity. One site controls the substrate specificity of RNR enzyme and is responsible for maintaining balanced nucleotide pools, thus facilitating optimal fidelity of DNA replication. The other monitors the ATP/dATP ratio and modulates the overall activity of the enzyme, presumably to ensure that sufficient dNTPs are produced for DNA synthesis without depleting ribonucleotides needed for RNA synthesis. RNR2 and RNR4 encode the small subunit R2 which contains a binuclear ferric iron centre and a tyrosyl free radical that is
essential for activity. \textit{RNR1}, \textit{RNR2}, and \textit{RNR4} are essential for mitotic growth while \textit{RNR3} is not. It is assumed that under normal vegetative conditions, only \textit{RNR1}, \textit{RNR2} and \textit{RNR4} are expressed, and thus the ribonucleotide reductase in the cell is $\alpha_2\beta\beta'$. However, in the presence of DNA damage, \textit{RNR3} is induced, producing other forms of the enzyme (Huang and Elledge, 1997).

The expression of \textit{RNR1} and \textit{RNR2} is increased in response to UV light, the UV mimetic compound, 4-NQO, bleomycin, and MMS. They can also be induced by an inhibitor of ribonucleotide reductase - hydroxyurea (HU) which functions by quenching the free radical on the small subunit, thus inhibiting the activity of RNR enzyme (reviewed by Elledge et al., 1992). The induction of \textit{RNR3} can reach 50-100 fold by MMS and 4-NQO and 50-200 fold by UV irradiation (Yagle and McEntee, 1990; Elledge and Davis, 1990). Why can \textit{RNR3} be induced to such high levels? Since its function and regulatory mechanisms are highly conserved, it is hypothesized that \textit{RNR3} must provide a selective advantage, but so far the nature of this advantage remains unclear.

\textbf{1. 2. 3. 1. 2. 2. Regulation of \textit{RNR3} gene expression}

Both expression and activity of ribonucleotide reductase are highly regulated. The \textit{RNR} genes are cell cycle-regulated and inducible by DNA damaging agents in all organisms examined, including yeast (Elledge and Davis, 1987; Hurd, et al., 1987; Elledge and Davis, 1990) and human (Hurta and Wright, 1992). In addition to high levels of expression in S phase, \textit{RNR} genes are further up-regulated when DNA synthesis is blocked.
The expression of *RNR3* is under the negative regulation by Crt1 which is homologous to the mammalian RFX family of DNA binding proteins that can bind to a conserved DNA sequence, the “X box”. The X box sequences have been identified in the promoter of *RNR3* and other *RNR* genes (Huang et al., 1998). It is a highly conserved DNA sequence of 13 nucleotides firstly found in the promoter of all MHC class II genes (Reith et al., 1990). One strong X box and two weak boxes are found in the *RNR3* promoter. Under normal conditions, Crt1 binds to the X box and recruits the Ssn6-Tup1 repression complex to the promoter region, thus repressing the transcription of *RNR3*. Two models have been proposed to explain the repression by the Tup1-Ssn6 complex. The first model suggests that the complex may inhibit transcription through direct interaction with one or more of these transcription factors. In the other hypothesis, the Ssn6-Tup1 complex exerts repression through modulation of chromatin structure. The activation of the Mec1-Rad53-Dun1 pathway involves inactivation of Crt1 by phosphorylation. As Crt1 becomes phosphorylated in response to the replication blocks and DNA damage, it loses its ability to bind to X-box elements and Ssn6-Tup1 complex is released. This leads to transcriptional induction of *RNR3* (Huang et al., 1998).

1.3. Current short-term genotoxicity testing systems

Many chemical and physical methodologies have been developed to meet the greater demand to detect environmental pollutants. Some of them require analytical equipment such as gas or high-pressure liquid chromatography, mass and atomic absorption spectrometry. Although they are powerful, accurate and sensitive, they are expensive and need to be run in specialized laboratories. More importantly, they fail to
provide data as to the bioavailability of a pollutant, the effects of pollutant on living systems, and the potential interaction (synergistic/antagonistic) among different toxicants. To overcome these disadvantages of chemical and physical methods, a variety of bioassays have been developed.

Genotoxicity testing is a key element for safety evaluation of food, drugs, as well as environmental health. Genotoxicity tests are \textit{in vitro} and \textit{in vivo} tests designed to detect compounds that induce genetic damage. A large number of genotoxicity tests are presently available for use in hazard evaluation, including: (1) tests that directly assess the genetic alterations (gene mutations and chromosomal effects), and (2) indirect genotoxicity tests that respond to DNA damage known to lead to these alterations. The latter category of tests may assess either DNA damage (e. g., DNA adducts or DNA strand breakage) or cellular responses to DNA damage (e. g., unscheduled DNA synthesis).

1. 3. 1. \textbf{Tests that directly assess genetic alterations}

Some experiments are designed to detect the alterations on DNA and chromosomes. The most commonly used tests are listed below.

1. 3. 1. 1. \textbf{Bacterial reverse mutation test: the Ames test}

It is difficult, time consuming and expensive to directly assay potential carcinogens by testing their ability to form tumors in animals. However, in addition to causing tumors in animal cells, most carcinogens are mutagens. Based upon this insight, Ames and colleagues (Ames et al., 1973a; b) developed a simple, indirect assay for potential
carcinogens. The assay is based upon the reversion of mutations in the histidine (his) operon in the bacterium *Salmonella typhimurium*. The his operon encodes enzymes required for the biosynthesis of the amino acid histidine. Strains with mutations in the his operon are histidine auxotrophs and are unable to grow in the absence of histidine. Revertants that restore the His\(^+\) phenotype will grow on minimal medium plates without histidine. This provides a simple, sensitive selection for revertants of his mutants.

The his mutants are mixed with the potential mutagen and then plated on minimal medium with a very small amount of histidine. The concentration of histidine used is limiting, so that after the cells go through several cell divisions, the histidine is used up and the auxotrophs stop growing. However, if the potential mutagen induces His\(^+\) revertants during the initial few cell divisions, each of the resulting revertants will continue to divide and form a colony. The number of colonies produced is proportional to how efficiently the mutagen reverts the original mutation. For example, if chemical A produces a higher frequency of reversion than the control, it is considered a mutagen and likely a carcinogen. If chemical B does not produce a higher frequency of reversion than the control and if this result is obtained for each type of mutation tested, then the results suggest that B is not a mutagen (Ames et al., 1973a; b).

Several different types of his mutants are used to test for different classes of mutagens. For example, frameshift mutagens will revert a frameshift mutation in his, etc. The *S. typhimurium* his mutant strains used have three additional properties that make them more sensitive to mutagens (McCann et al., 1975). First, they have an rfa mutation that makes the outer membrane more permeable to large molecules. Second, they have a mutation in the *uvrB* gene to eliminate NER. Third, they carry the plasmid pKM101.
which increases error-prone PRR of DNA damage. Thus, reversion of his mutations in these strains provides a sensitive test for a broad spectrum of mutagens.

As indicated above, some chemicals (called pro-mutagens) are not mutagenic unless metabolized to more active derivatives. To test for such pro-mutagens, an extract of rat liver enzymes (S9 extract) is included in the reversion assay (McCann et al., 1975).

The bacterial reverse mutation test is commonly employed as an initial screen for genotoxic activity and, in particular, for the point mutation-inducing activity. An extensive database has demonstrated that many chemicals that are positive in this test also are genotoxic in other tests. There are examples of mutagenic agents that are not detected by this test; reasons for this shortcoming can be ascribed to the specific nature of the endpoint detected, differences in metabolic activation, or differences in bioavailability. In addition, the prokaryotic cells utilized in bacterial reverse mutation test differ from mammalian cells in such factors as uptake, metabolism, chromosome structure and DNA repair processes. Tests conducted in vitro generally require the use of an exogenous source of metabolic activation. In vitro metabolic activation systems cannot mimic entirely the mammalian in vivo conditions such as metabolized activation of some pro-mutagens. The test therefore does not provide direct information on the mutagenic and carcinogenic potency of a substance in mammals. For these cases, usually other in vitro mammalian cell tests should be performed using different cell types and different endpoints, i.e., gene mutation and chromosomal damage. However, it is still important to perform the bacterial reverse mutation test.
1. 3. 1. 2. **In vitro mouse lymphoma thymidine kinase+/- gene mutation assay**

An *in vitro* mammalian cell gene mutation test can be used to detect gene alterations induced by chemical substances. While there are a number of cell lines that can be used, the L5178Y TK+/-3. 7. 2C mouse lymphoma cell line using the thymidine kinase (TK) gene is the cell line and assay of choice. The mouse lymphoma assay (MLA) was chosen because of a body of research indicating that many types of genetic alterations are detected. The assay detects mutations known to be important in the etiology of cancer and other human genetically mediated illnesses. There is evidence that the assay detects gene mutations (point mutations) and chromosomal events (deletions, translocations, mitotic recombination/gene conversion and aneuploidy) (Hozier, et al., 1981; Moore, et al., 1985; Sawyer, et al., 1989; Applegate et al., 1990). The efficiency of detection of all of these mutational events is still under investigation.

There are some other *in vitro* mammalian gene mutation assays. For example, the assays using either Chinese hamster cell lines (CHO; Carver et al., 1983; Tindall et al., 1986) or human lymphoblastoid cells (Crespi et al. 1985) have been reported. In these cell lines the most commonly used genetic endpoints measure mutation at either the hypoxanthine-guanine phosphoribosyl transferase (HPRT), a transgene of xanthine-guanine phosphoribosyl transferase (XPRT), or TK. The TK, HPRT and XPRT mutation tests detect different spectra of genetic events. The autosomal location of TK allows for the detection of genetic events that are not detected at the HPRT locus on the X-chromosome (Moore et al., 1989).

The various mutation assays are capable of detecting different spectra of genetic damage. Thus, it is not expected that a chemical will give uniformly positive or negative
results in the various assays. In particular, the bacterial *Salmonella* assay detects only point and other very small-scale gene mutations. Furthermore, the *in vitro* mammalian assays using the HPRT locus are unable to detect chemicals that do not induce point mutations (Moore et al., 1989).

1.3.1.3. **Mammalian erythrocyte micronucleus test**

The purpose of the micronucleus test is to identify substances that cause cytogenetic damage that result in the formation of micronuclei containing lagging chromosome fragments or whole chromosomes. Micronuclei are cytoplasmic chromatin-containing bodies formed when acentric chromosome fragments or chromosomes lagging during anaphase and fail to become incorporated into daughter cell nuclei during cell division. Because genetic damage that results in chromosome breaks, structurally abnormal chromosomes, or spindle abnormalities leads to micronucleus formation, the incidence of micronuclei serves as an index of these types of damage. It has been established that essentially all agents that cause double strand chromosome breaks induce micronuclei. Since the detection of micronuclei is much faster and less technically demanding, and the micronuclei arise from two important types of genetic damage (clastogenesis and spindle disruption), the micronucleus assay has been widely used to screen for chemicals that cause these types of damage (Kirsch-Volders et al., 1997)

The mammalian erythrocyte micronucleus assay is widely used. This *in vivo* micronucleus test is employed for the detection of damage induced by the test substance to the chromosomes or the mitotic apparatus of erythroblasts by analysis of erythrocytes sampled in bone marrow and/or peripheral blood cells of animals, usually rodents. When
a bone marrow erythroblast develops into a polychromatic erythrocyte, the main nucleus is extruded; micronuclei that have been formed may remain behind in the otherwise enucleated cytoplasm. Visualization of micronuclei is facilitated in these cells using specific staining techniques and by the occurrence of cells a main nucleus. An increase in the frequency of micronucleated polychromatic erythrocytes in treated animals is an indication of induced chromosome damage.

This mammalian in vivo micronucleus test is especially relevant to assessing mutagenic hazards in that it allows consideration of factors of in vivo metabolism, pharmacokinetics and DNA-repair processes, although these may vary among species, among tissues and among genetic endpoints. An in vivo assay is also useful for further investigation of a mutagenic effect detected by an in vitro system. However, if there is evidence that the test substance, or a reactive metabolite, will not reach the target tissue, it is not appropriate to use this test.

1.3.2. Genotoxicity tests that assess cellular responses to DNA damage

1.3.2.1. SOS Chromotest

In the early 1980’s, the SOS Chromotest was described (Quillardet et al., 1982), in which a lacZ gene is placed downstream of the promoter of one of the SOS genes such as sfiA to allow a simple and direct colorimetric assay of the SOS response to DNA damage. In view of its simplicity and its rapid response, the SOS Chromotest was proposed as a complementary or alternative test to the Ames test. The assay was shown to detect genotoxins inactive in the Ames test and to allow the identification of false positives (Quillardet and Hofnung, 1993). Several other SOS genes were subsequently employed
to construct similar systems. In "umu-lacZ" test (Oda et al., 1985), the test organism S. typhimurium TA1535 has an excision repair deficiency (uvrB), increased membrane permeability (rfa), and a natural deletion of the lac operon. The test allows chemicals that may be too toxic for the standard Ames test to be assayed. Vollmer et al. (1997) described a sensor system in which DNA damage-inducible promoters of recA, uvrA, alkA genes from E. coli were fused to luxABCDE of Vibrio fischeri. The regulation of recA and uvrA, which are a part of the bacterial SOS DNA repair system, is lexA dependent. Gene alkA encodes 3-mA-DNA glycosylase II. The alkA defective mutant strains are very sensitive to alkylating agents (Friedberg et al., 1995). The recA, uvrA, alkA fusion system exhibited the most prominent and sensitive responses to mitomycin C, H2O2, MNNG, ethidium bromide (EtBr) or UV irradiation.

1.3.2.2. Common reporters in genotoxicity assays

To enable a cell to function as a biosensor, two elements are needed: a sensing element and a reporter element. The sensing element senses the presence of the target damaging agents, and turns on the reporter gene to emit a detectable signal. A good reporter should be readily detectable. Just as the specificity of the final construct depends upon the proper selection of the sensing promoter, the sensitivity and degree of resolution of the detection depend to a large extent upon the proper choice of the reporter. Several commonly used reporters are discussed below.

1.3.2.2.1. Bacterial β-galactosidase

β-galactosidase catalyzes the hydrolysis of colorless galactosides to yield colored
products. The enzyme is encoded by $\text{lacZ}$ of $E.\ coli$ and can be used in prokaryotic as well as in eukaryotic cells. The normal biological substrate for $\beta$-galactosidase is lactose. Under laboratory conditions, ortho-nitrophenyl-$\beta$-galactoside (ONPG) is usually used as a substrate. It is colorless and composed of galactose bonded to nitrophenol. $\beta$-galactosidase will recognize this bond as a substrate and cleave this molecule to produce galactose and O-nitrophenol (ONP) which is also colorless at neutral or acid pH, but in an alkaline solution it is bright yellow. The absorption of o-nitrophenolate can be measured at 420 nm. If ONPG is available in excess, then the amount of O-nitrophenolate produced is proportional to the amount of enzyme ($\beta$-galactosidase) present. The reaction is stopped when pH increases to 11 (with Na$_2$CO$_3$), which inactivates $\beta$-galactosidase. Since ONP is a product of $\beta$-galactosidase activity, the spectrophotometric measurements may be used as an assay for the enzyme. Fluorescent assays have been developed for $\beta$-galactosidase by using other substrates, such as 4-methylumbelliferone, or fluorescein digalactoside.

1.3.2.2.2. Bacterial luciferases (LUC)

Luciferase is the protein that makes fireflies glow in the dark. It is found only in the gut of fireflies and several other marine organisms. Luciferases catalyze the obligatory aerobic oxidation of a reduced flavin mononucleotide and a long chain aldehyde to flavin mononucleotide and the corresponding carboxylic acid, with light emission at around 490 nm. The reaction is:

$$\text{ATP} + \text{luciferin substrate} + O_2 \rightarrow \text{AMP} + \text{oxylucifer} + \text{LIGHT}$$
Luciferase is encoded by \textit{luxA} and \textit{luxB} of the \textit{lux} operon, and the synthesis enzymes for the aldehyde are encoded by \textit{luxCDE}. In constructs where only \textit{luxAB} is present, the aldehyde has to be added externally (Kohler et al., 2000). The great advantage of using luciferase as a reporter gene is that it is extremely sensitive. There is no background because luciferase is not found in mammalian cells. However, luciferase does require the luciferin substrate for activity. It also requires expensive equipment such as a luminometer or scintillation counter to detect the signal.

1.3.2.2.3. **Green fluorescent protein (GFP)**

Green fluorescent protein, GFP, is a spontaneously fluorescent protein isolated from coelenterates, such as the Pacific jellyfish, \textit{Aequoria victoria}. Its role is to transduce, by energy transfer, the blue chemiluminescence of another protein, aequorin, into green fluorescent light. The molecular cloning of \textit{GFP} cDNA and the demonstration that \textit{GFP} can be expressed as a functional transgene have opened exciting new avenues of investigation in cell, developmental and molecular biology. Fluorescent GFP has been expressed in bacteria (Chalfie et al., 1994), yeast (Kahana et al., 1995), slime mold, plants, drosophila, zebrafish, and in mammalian cells (Prasher, 1995). It can function as a protein tag, as it tolerates N- and C-terminal fusion to a broad variety of proteins, many of which have been shown to retain native function (Cubitt et al., 1995). The advantage of GFP is that it requires no substrate; it constantly emits green light. The drawback is that the emission is not particularly strong. Therefore one must use a very strong promoter to increase expression of GFP so that one can see it within the cell. In addition, it is difficult to quantify.
1.3.2.2.4. Chloramphenicol acetyl transferase (CAT)

The oldest reporter assay relies upon the chloramphenicol acetyl transferase (CAT) gene. The CAT enzyme covalently modifies chloramphenicol by adding an acetyl group. This reporter gene assay utilizes a proprietary colorimetric system. As the solution changes from yellow to red in wells containing CAT enzyme, the absorbance is read in a standard plate reader at 475 - 500 nm. The CAT enzyme activity is determined from the standard curve. The advantage for using the CAT gene is that it is not found in mammalian cells. Thus, there is no background. However, the CAT assay is not as sensitive as the luciferase assay. It is also rather expensive, time consuming, and radioactive isotopes are needed for some CAT systems.

The comparison of the above four reporters are summarized in Table 1-1. For a short-term genotoxicity test, reporters with the following characteristics are preferred: high sensitivity, wide dynamic ranges of response, ease of assay, inexpensive to assay and environmentally safe.

1.4. Development of a yeast-based RNR3-lacZ genotoxicity testing system

Because of their simplicity, the Ames test and SOS Chromotests have become routine genotoxicity testing systems for environmental safety evaluation. Both of these systems are based on bacterial cells. Regarding the differences between eukaryotic and prokaryotic cells in metabolism, DNA damage responses and other physiological aspects, it is possible that an agent may be genotoxic for bacterial cells, but not for mammalian cells, and vice versa. The fact that some mammalian
Table 1-1: Comparison of commonly used β-gal, CAT, LUC and GFP reporters

<table>
<thead>
<tr>
<th></th>
<th>β-gal</th>
<th>LUC</th>
<th>GFP</th>
<th>CAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity (molecules of substrates)</td>
<td>3 x 10^8 (ONPG) 900-1 (Fluorogenic substrates)</td>
<td>6x10^4~6x10^7</td>
<td>less sensitive than CAT, luciferase or β-gal</td>
<td>6x10^6~6x10^10</td>
</tr>
<tr>
<td>Substrate required</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Equipment required</td>
<td>Spectrometer*</td>
<td>luminometer / scintillation counter</td>
<td>fluorescent microscope</td>
<td>spectrometer</td>
</tr>
<tr>
<td>Degradation of product</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

- Color filter assay.
- β-gal: β-galactosidase; LUC: luciferase; GFP: green fluorescent protein; CAT: chloramphenicol acetyl transferase.
genotoxic and carcinogenic agents cannot be detected in bacterial systems reflects these
differences to some extent. 1,2-dimethylhydrazine is one example. As a well-known
potent carcinogen, it cannot be detected as a bacterial mutagen in standard Ames test
(Lijinsky et al., 1985).

1.4.1. Advantages of yeast as host organism

The characteristics of the yeast *S. cerevisiae* have made it an ideal tool in scientific
research and as such, it has been used extensively. There are essentially four major
reasons to use yeast. First, although it is eukaryote, it shares the advantages of bacteria
with regard to rapid growth, ease of manipulation and growth on a variety of carbon
sources. Second, because it is eukaryotic organism, the metabolism and DNA damage-
induced responses likely resemble those of human responses. This is very important
when using a biosensor to reveal potential hazards to other eukaryotes such as humans.
As was mentioned above, prokaryotic cells differ from eukaryotic cells in such factors as
uptake, metabolism, chromosome structure and DNA repair processes. So the bacterial
tests do not provide direct information on the mutagenic and carcinogenic potency of a
substance in mammals. Third, among microorganisms, yeast is particularly robust with a
wide physicochemical tolerance. These qualities are desirable in whole cell biosensors
exposed to the real world. Yeast has a wide tolerance to pH, freezing temperature and
osmolarity which ensures it can be used under field conditions. Finally, with regards to
*S. cerevisiae*, it is one of the best-understood and most-readily manipulated organisms. It
is the first eukaryotic organism whose entire genome sequence was determined (Dujon et
al., 1997; Bowman et al., 1997). The DNA damage response mechanism is well studied
compared to other eukaryotes. The wealth of knowledge with respect to yeast biology will enable us to modify the test yeast cells and alter the physiological and biological responses to environmental stress.

1.4.2. Rationale of the present RNR3-lacZ genotoxicity testing system

The rationale of RNR3-lacZ system is based on the induction of the DNA damage inducible gene RNR3 in response to DNA damaging agents. The model of RNR3 induction is reminiscent of the SOS response of E. coli. Hence, employment of the RNR3 gene promoter as a sensing element of genotoxicity testing system is actually comparable to that of SOS Chromotest. The maximum degree of RNR3 induction, according to previous data in our laboratory (Zhu and Xiao, 1998) and other laboratories (Ruby and Szostak, 1985; Elledge and Davis, 1990; Yagle and McEntee, 1990), is much higher than that of other yeast genes examined. More importantly, it seems that RNR3 can respond to much broader spectrum of DNA damaging agents than bacterial cells.

In this study, a lacZ gene was placed downstream of the RNR3 promoter. When treated with DNA damaging agents, the RNR3 gene will be induced, initiating the expression of lacZ and produce β-galactosidase. By measuring the cellular level of induced β-galactosidase before and after treatment, it can easily be determined whether an agent is genotoxic.

1.5. Objectives of the present study

The objectives of this study were:
(1) to develop a eukaryotic genotoxicity testing system based on the budding yeast *S. cerevisiae*;

(2) to test its responses to different DNA damaging agents;

(3) to improve the sensitivity of this system to different types of DNA damaging agents by altering host yeast strains.
CHAPTER II: MATERIALS AND METHODS

2.1. Cell culture and manipulation

2.1.1. Yeast strains and cell culture

The yeast strains used in this study are listed in Table 2-1. Haploid S. cerevisiae strain DBY747 was used as the wild type host strain of the present RNR3-lacZ system.

YPD is a standard, complex medium composed of 1% Bacto-yeast extract, 2% Bacto-peptone, 2% glucose, and 2% agar (if making YPD plates). Synthetic Dextrose minimal medium (SD medium) is used for selective growth of yeast auxotrophs. It contains 0.67% yeast nitrogen base (without amino acids), 2% glucose, 2% agar (if making SD plates), supplemented with necessary amino acids and bases. If a necessary nutritional supplement (X) is absent in the media, it is denoted as SD-X. Yeast cells were grown at 30 °C either in rich YPD medium or in SD medium supplemented with appropriate amino acids and bases.

For long-term storage, yeast cells were grown on plates (rich or minimal media) at 30 °C. After 2-3 days growth, the yeast cells were removed from the plate with a sterile toothpick, and re-suspended into 1.0 ml of sterile 15% (v/v) glycerol. For yeast cells grown in liquid culture, after overnight growth in appropriate media, 0.7 ml of cells were added into 0.3 ml of 50% glycerol. The cells are then stored at -70 °C.

The cell titer is determined by the absorption at 600 nm on a NovaspecII visible spectrometer (Pharmacia).
Table 2-1. Yeast strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>DBY747</td>
<td>$MAT\alpha\ his3-11\ leu2-3,\ 112\ trp1-289\ ura3-52$</td>
<td>Dr. D. Botstein</td>
</tr>
<tr>
<td>WXY9573</td>
<td>DBY747 with $rad2\Delta::TRP1$</td>
<td>Xiao et al., (1998)</td>
</tr>
<tr>
<td>WXY9376</td>
<td>DBY747 with $rad6\Delta::LEU2$</td>
<td>Xiao et al., (1996a)</td>
</tr>
<tr>
<td>WXY9326</td>
<td>DBY747 with $rad18\Delta::TRP1$</td>
<td>Xiao et al., (1996a)</td>
</tr>
<tr>
<td>WXY9221</td>
<td>DBY747 with $rad50\Delta::HisG-URA3-hisG$</td>
<td>Xiao et al., (1996a)</td>
</tr>
<tr>
<td>WXY9387</td>
<td>DBY747 with $rad52\Delta::LEU2$</td>
<td>Xiao et al., (1996a)</td>
</tr>
<tr>
<td>WXY814</td>
<td>DBY747 with $apn1::HIS3\ apn2::LEU2$</td>
<td>Xiao et al., (2001)</td>
</tr>
<tr>
<td>JC8901</td>
<td>DBY747 with $mag1\Delta::HisG-URA3-HisG$</td>
<td>Chen and Samson (1991)</td>
</tr>
<tr>
<td>WXY788</td>
<td>DBY747 with $mag1\Delta::HisG\ apn1::HIS3$ apn2::LEU2</td>
<td>Xiao et al., (2001)</td>
</tr>
</tbody>
</table>
2.1.2. Yeast transformation

DBY747 cells carrying plasmids containing *RNR3-lacZ*, *RNR2-lacZ*, or *MAG1-lacZ* were created by transforming yeast cells with pZZ2, pZZ18, or pWX1254, respectively.

Yeast cells were transformed using a method derived from Ito et al (1983) and Hill et al (1991). A 2 ml culture of *S. cerevisiae* was grown overnight at 30 °C in rich media. The next day, the 2 ml culture was subcultured into 3 ml of fresh media, and allowed to grow at 30 °C until it reached a mid-logarithmic phase of growth. The yeast cells were collected by centrifugation, washed with a LiOAc solution (0.1 M LiOAc, 10 mM Tris HCl (pH 8.0), 1 mM EDTA) and resuspended in 1 ml of LiOAc. For each transformation, 100 μl of cells from the 1ml suspension was mixed with 4 μl of ssDNA (single stranded salmon sperm DNA) and 1-5 μl of transforming DNA in a 1.5 ml centrifuge tube. After a 5-minute incubation at room temperature, 280 μl of PEG₄₀₀₀ solution (50% polyethylene glycol (MW=4000) in LiOAc solution) was added and the contents mixed by inverting 4-6 times. After the transformation mixture was incubated for 45 minutes at 30 °C, 39 μl of DMSO was added, followed by a 5-minute heat shock in 42 °C waterbath. After the heat shock treatment, the transformation mixture was washed with double distilled water (ddH₂O), and then resuspended in 0.1 ml of ddH₂O. The resuspended cells are plated onto the appropriate minimal media.

2.1.3. Yeast genomic and plasmid DNA isolation

The protocol for the extraction of yeast DNA was developed by Hoffman and Winston (1987). Yeast cells from liquid culture were collected by centrifugation and resuspended in 200 μl of extraction buffer [2% TritonX-100, 1% SDS, 0.1 M NaCl, 1
mM EDTA, 0.01 M Tris HCl (pH 8.0)]. 0.1 ml of phenol, 0.1 ml of chloroform and 0.3 g of acid-washed glass beads were added to the cell mixture. The tube was then vortexed for 3 minutes (2 minutes for isolating plasmid DNA) at top speed. After a 5-minute centrifugation, the top aqueous layer was transferred to a clean tube, and 2 volumes of 95% ethanol was added to precipitate the DNA. After 30 minutes at −20 °C, the DNA sample was centrifuged for 15 minutes, dried and resuspended in 200 μl of ddH2O (10 mM Tris HCl, 1 mM EDTA, pH 8.0), and then treated with 5 μl of RNase (10 mg/ml stock) at 37 °C for 10 minutes. The DNA was precipitated in 2 volumes of 100% ethanol with 8 μl of 5 M NaCl and resuspended in 50 μl of ddH2O.

2.1.4. β-Galactosidase activity assay

For yeast cells carrying autonomously replicating plasmids, the β-gal assay was performed as described (Xiao et al., 1993; Zhu and Xiao, 1998). Briefly, 0.5 ml of an overnight culture of yeast cells was used to inoculate 2.5 ml of fresh medium. After a 2-hour incubation, when cell density reached an OD600 of approximately 0.2-0.3, cells were treated with test chemicals or exposed to radiation and returned to incubation for another 4 hours or as specified. 1 ml of culture was used to determine the cell density at OD600. The cells from the remaining 2 ml of culture were collected by centrifugation and used for the β-gal assay. The cells were resuspended in 1 ml of buffer Z (60 mM Na2HPO4•7H2O, 40 mM NaH2PO4•H2O, 10 mM KCl, 1 mM MgSO4•7H2O, 40 mM β-mercaptoethanol, pH 7.0), permeabilized by adding 50 μl of 0.1% SDS and 50 μl of chloroform and vortexed at top speed for 10 seconds. The reaction was initiated by adding 200 μl of 4 mg/ml ONPG and incubating at 30 °C for 20 minutes. The reaction
was stopped by adding 500 μl of 1 M Na₂CO₃. The tube was centrifuged and the OD₄₂₀ value of the supernatant was measured to determine the β-gal activity using the following equation:

\[
β\text{-gal activity} = \frac{1000 \times (OD_{420\text{nm}})}{\text{reaction time (min)} \times \text{culture volume (ml)} \times OD_{600\text{nm}}}.\]

The β-gal activity is expressed in Miller units (Guarente, 1983). The value of OD₄₂₀ and OD₆₀₀ were determined on a Novaspec II visible spectrometer from Pharmacia company. The value of fold induction was calculated as a ratio of β-gal activity of the cells with and without treatment in the same experiment. The results were the average of at least three independent experiments with standard deviation calculated by Microsoft Excel2000.

For cells carrying an integrated \textit{RNR3-lacZ} cassette in the genome, non-selective YPD medium was used during incubation. 100 μl of overnight culture was inoculated into 2.9 ml of fresh YPD at a cell titer of approximately 0.1 at OD₆₀₀. Cells were immediately treated with test chemical or exposed to radiation, and incubated for another 4 hours. The remaining steps were as described in the previous paragraph.

2.1.5. Chemicals and special media

All the agents and their mechanisms of function are listed in Table. 2-1. Genotoxic chemicals used in this study were MMS, EMS, SDMH, H₂O₂, 4-NQO, HU, 5-fluorouracil, phleomycin, MNNG and EtBr. Non-genotoxic chemicals were L-canavanine and tetracycline (hydrochloride tetracycline). All the above chemicals were purchased from Sigma-Aldrich.

The 1.0 mg/ml MNNG stock solution was made in 100 mM acetate buffer (pH 5.0), aliquoted, frozen and thawed only once. Tetracycline was dissolved in ethanol.
Table. 2-2: Agents used in the this study and their mechanisms of function

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Functional mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O₂</td>
<td>Oxidative damage to DNA</td>
</tr>
<tr>
<td>4-NQO</td>
<td>Cause bulky adducts, UV radiation mimic, oxidative</td>
</tr>
<tr>
<td>HU</td>
<td>Inhibition of DNA replication (inhibitor of ribonucleotide reductase)</td>
</tr>
<tr>
<td>MMS</td>
<td>Alkylating agent</td>
</tr>
<tr>
<td>EMS</td>
<td>Alkylating agent</td>
</tr>
<tr>
<td>EtBr</td>
<td>Intercalating agent</td>
</tr>
<tr>
<td>SDMH</td>
<td>Alkylating agent</td>
</tr>
<tr>
<td>MNNG</td>
<td>Alkylating agent</td>
</tr>
<tr>
<td>Phleomycin</td>
<td>Cleavage of DNA</td>
</tr>
<tr>
<td>5-Fluorouracil</td>
<td>Base analog</td>
</tr>
<tr>
<td>UV light</td>
<td>Photoproducts in DNA, DNA cross-linking and strand breaks</td>
</tr>
<tr>
<td>γ ray</td>
<td>Base, sugar damage and strand break</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>Inhibit protein synthesis in prokaryotes (interacts with 30S subunit)</td>
</tr>
<tr>
<td>Canavanine</td>
<td>Non-specific inhibition of arginine decarboxylase activity</td>
</tr>
</tbody>
</table>

Note: 4-NQO: 4-nitroquioline; HU: hydroxyurea; MMS: methyl methanesulfonate; EMS: ethyl methanesulfonate; EtBr: ethidium bromide; SDMH: 1,2-dimethyl hydrazine; MNNG:N-methyl-N'-nitro-N-nitrosoguanidine; UV: ultraviolet light; Tetracycline: hydrochloride tetracycline
and stored at −20 °C. All the other chemicals were freshly made in sterile ddH₂O.

5-Fluoro-orotic acid (FOA) is toxic to yeast cells with a functional URA3 (Boeke et al., 1987). The gene product of URA3 metabolizes FOA to a toxic intermediate. Fluoro-orotic acid was purchased from US Biologicals (Swampscott, MA), and is stored as an aqueous solution at −20 °C. FOA plates were prepared as follows: A mixture of 1.34% bacto-yeast nitrogen base, 0.4% drop-out mix (-Ura), 4% glucose, 100 μg/ml uracil, 0.2% 5-FOA in 500 ml of ddH₂O was filter sterilized. The above solution was combined with 500 ml of an autoclaved 4% bacto-agar, then pour into petri dishes.

2.1.6. Chemical and physical agents treatment

The chemical agent was added to the cells after 2 hours incubation. After treatment, cells were incubated for another 4 hours. Yeast cells were precipitated by centrifugation, washed twice with sterile ddH₂O, and resuspended in buffer Z for the β-gal assay.

For UV treatment, cells were collected by centrifugation and resuspended in 200 μl sterile ddH₂O, plated on YPD plate and exposed to 254 nm UV light in a UV cross-linker (Fisher model FB-UVXL-1000) or UV lamp (UVP model UVGL-25 at 40 μW/cm²) at given doses in the dark to prevent photoreactivation. The cells were washed, resuspended in 3 ml of liquid YPD/SD-Ura and incubated at 30 °C in the dark for another 4 hours prior to the β-gal assay.

For γ ray irradiation, the cells were collected by centrifugation and resuspended in 1 ml of ddH₂O in an eppendorf tube. The tubes containing yeast cells were exposed to a
\(^{60}\)Co \(\gamma\) ray source, and the yeast cells were diluted into SD-Ura medium and incubated at 30 °C for another 4 hours prior to the \(\beta\)-gal assay.

2.1.7. Cell killing

The cell killing test was used to determine the cell survival rates after treatment by chemical and physical agents. Chemical induced liquid killing was performed as previously described (Xiao et al., 1996). In order to maintain the transformed pZZ2 plasmids, cells were grown overnight in SD-Ura medium. 500 \(\mu\)l of overnight culture was subcultured into 5 ml of fresh medium the next morning and allowed to grow until mid-logarithmic growth was achieved. 1 ml of culture was removed, diluted \(10^{5-6}\) and plated on SD-Ura plates. These cells represent the untreated control cells. At this time, chemical agent was added to the remaining culture, to the highest concentration as indicated in each experiment. After 4 hours of incubation, 1 ml of cells were removed, washed twice with sterile water, diluted to an appropriate concentration and plated in duplicate on SD-Ura plates. Plates were incubated for 3 days at 30 °C.

For UV killing, cells were plated at different dilutions and then exposed to 254 nm UV light, either in a UV crosslinker (FB-UVXL-1000, FisherBiotech company) or with a UV lamp (UVGL-58, UVP company) at specified doses. Cells were plated in duplicate on YPD to score cell survival, and the plates were incubated at 30 °C for 3 days. Irradiation and the subsequent incubation of the cells were performed in the dark, to prevent photoreactivation.
For γ irradiation killing, cells were collected by centrifugation, resuspended in ddH$_2$O, and exposed to a $^{60}$Co γ-ray source in a centrifugation tube. The cells were plated on appropriate media. The plates were incubated for 3 days at 30 °C before counting the colonies.

2.2. Molecular biology techniques

2.2.1. Bacterial culture and storage

The E. coli strain commonly used for bacterial transformation was DH10B (Gibico/BRL, Grand Island, NY USA). Transformed strains were stored on LB plates (1% Bacto-tryptone, 0.5% Bacto-yeast extract, 0.5% NaCl and 1.2 % Agar) containing 50 μg/ml of Ampicillin, since all of the vectors used contained the bla marker gene. For long-term storage of transformed cells, cells were grown overnight in 900 μl of LB + Amp; the following day 100 μl of DMSO was added and the cells were immediately placed in a −70 °C freezer.

2.2.2. Preparation of competent cells

For transformation by electroporation, E. coli cells were prepared as suggested in the BioRad E. coli Pulser manual. One litre of culture was incubated until an OD$_{600nm}$ of 0.6 was reached. The culture was centrifuged at 3500 rpm in a Beckman GSA rotor and the pellet was resuspended in 500 ml of 10% sterile glycerol. The centrifugation was repeated 4 times, with each pellet resuspended in a reduced volume; the last pellet was
resuspended in 4 ml of cold, sterile 10% glycerol. The cells were aliquoted into 1.5 ml eppendorf tubes to a volume of 25 μl, and were quickly placed in the −70 °C freezer for storage.

2.2.3. Bacterial transformation

All bacterial transformations in this study used the electroporation method. The DNA to be transformed was added to competent E. coli cells. After a brief incubation on ice, the cell mixture was transferred to a chilled electroporation cuvette (BioRad), where the cells were exposed to a voltage of 1.8 kV (for cuvettes with 0.1 mm width) using the E. coli Pulser (BioRad). 280 μl of SOC medium was added to the cuvette after electroporation. The cell mixture was transferred to a 1.5 ml eppendorf tube for a 45-minute incubation at 37 °C. Following the incubation, the cells were plated on LB + Amp plates and incubated at 37 °C overnight.

2.2.4. Plasmid DNA isolation (Mini-prep)

Plasmid amplification and isolation was performed according to the method of Maniatis et al. (1982). Cells were inoculated into 1 ml of LB + Amp liquid media, and grown overnight at 37 °C. Cells were collected by centrifugation, the supernatant removed, and the pellet was resuspended in 350 μl of STET (8% sucrose, 0.5% Triton X-100, 50 mM EDTA pH 8.0, 10 mM Tris-HCl pH 8.0). After adding 20 μl of lysozyme (10 mg/ml; Sigma, St Louis MI), the mixture was quickly put in a boiling water-bath for 30-40 seconds, followed by centrifugation for 10 minutes. The pellet was removed with
a toothpick, and 8 μl of 5 M NaCl and 2 volumes of 95% ethanol were added to precipitate the DNA. The DNA was then resuspended in ddH₂O.

2.2.5. **Agarose gel electrophoresis**

For analysis of plasmid and genomic DNA, 0.8% agarose gel was used in this study. Gels were prepared in 1 x TAE buffer (40 mM Tris-acetate, 2 mM Na₂EDTA). To monitor the migration of the DNA, a sucrose dye (30% sucrose, 0.15% bromophenol blue, 0.1M EDTA, pH 8.0) was added to the samples. Bacterial phage λ DNA was digested with HindIII, yielding DNA bands at 23.130, 9.416, 6.557, 4.361, 2.322, 2.027, and 0.564 kb. These bands were used to determine the size of the linear DNA of interest. Electrophoresis was performed in 1 x TAE buffer using Fisher Biotech electrophoresis systems (Fisher Scientific, Pittsburgh, PA USA). The gel was stained in diluted EtBr solution (diluted from a 10 mg/ml stock solution) to visualize the DNA and photographed by UVP BioDoc-It™ system for a permanent record.

2.2.6. **Isolation of DNA fragment from agarose gel**

The method of DNA fragment isolation from agarose gel was adapted from Wang and Rossman (1994). After enzyme digestion, the sample was electrophoresed through 0.8% agarose gel and stained with EtBr. The band of interest was identified and cut out of the gel under UV-illuminator. A 0.5 ml microcentrifuge tube was pierced at the bottom, and packed with chopped cheesecloth. The gel slice containing the DNA fragment was placed into the prepared tube, which was placed into another 1.5 ml tube,
left it in the \(-70 \, ^\circ C\) freezer for 30 min and spun for 10 min at top speed. The flow through was treated with the same volume of phenol/chloroform (1:1) extraction and precipitated by ethanol.

2.2.7. Ligation

To ligate a DNA fragment into a vector plasmid, approximately 0.5 \(\mu g\) of DNA insert was combined with 0.2 \(\mu g\) of plasmid vector. 1 \(\mu l\) of T4 DNA ligase (Gibco/BRL) and 4 \(\mu l\) of T4 DNA ligase buffer (5 X stock solution) were added to the DNA, resulting in a total volume of 20 \(\mu l\). Ligation was performed overnight at 16 \(^\circ C\) to promote the stable association of cohesive DNA ends.

2.2.8. Plasmids and plasmid construction

Plasmids pZZ2 and pZZ18 (Zhou and Elledge, 1992) were obtained from Dr. S. Elledge (Baylor College of Medicine, Houston, USA) and utilized to create the \(RNR3\)-\(lacZ\) and \(RNR2\)-\(lacZ\) testing systems, respectively. Plasmid pWX1254, constructed in our laboratory (Zhu and Xiao, 1998), was used to create the \(MAG1\)-\(lacZ\) testing system. Plasmid M4366 (Voth et al., 2001) was obtained from Dr. D. Stillman (University of Utah, Salt Lake City, USA) and was used to integrate the \(RNR3\)-\(lacZ\) fragment into the yeast genome. Table 2-2 summarizes all the plasmids used in this study.

In order to create a stable testing system, the \(RNR3\)-\(lacZ\) cassette was integrated into the yeast host genome. Plasmid pZZ2 was digested with \(NsiI\) and \(EcoRI\) and a fragment containing the 0.88 kb \(RNR3\) promoter and the full length of \(lacZ\) was isolated.
and inserted into PastI and EcoRI digested plasmid pBluescript, then digested the constructed plasmid with BamHI to obtain RNR3-lacZ fragment and ligated the fragment into BamHI digested M4366 so that the RNR3-lacZ cassette together with a hisG-URA3-hisG cassette were flanked by 5' and 3' HO sequences with NotI sites on each side.

M4366 carrying the RNR3-lacZ fragment was digested with NotI and transformed into yeast cells. Through homologous recombination between the HO-hisG-URA3-hisG-RNR3-lacZ-HO cassette and the yeast genome, the RNR3-lacZ fragment was integrated into the host genome at the HO locus (Fig. 2-1). Positive clones were selected by assaying β-gal activity and the hisG-URA3 pop-out derivatives were obtained by selection on a plate containing 5-FOA plates. The resulting strain was expected to contain only the RNR3-lacZ cassette plus one copy of hisG replacing the inactive HO gene.

2.2.9. Radioactive labeling of DNA fragments

The 0.9 kb HO fragment from plasmid M4366 and 2.0 kb of lacZ fragment from pZZ2 were used as RNR3 probe and lacZ probe (Fig. 2-1), respectively, for Southern analysis.

The DNA was labelled using the Random Primer Labeling kit from Gibco/BRL. As stated in the manufacturer’s instructions, 25 ng of DNA was placed in a boiling water bath to denature the DNA, followed by immediate storage on ice. The DNA was mixed with the random primers buffer mixture (0.67 M HEPES, 0.17 M Tris-HCl, 17 mM MgCl2, 33 mM 2-mercaptoethanol, 1.33 mg/ml BSA, 18 OD260 units/ml oligo
deoxyribonucleotide primers (hexamers), pH 6.8), dNTP's, 50μCi of [32P]αdCTP, and the Klenow fragment of *E. coli* DNA polymerase I, and incubated for more than 2 hours at 25 °C. Stop buffer was then added to stop the reaction; the DNA was precipitated by ethanol and re-suspended with ddH2O.

Table 2-3: Plasmids used in this study, their genotypes and sources.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant characteristics</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pZZ2</td>
<td>YCp, <em>URA3</em>, <em>RNR3-lacZ</em></td>
<td>Zhou and Elledge (1992)</td>
</tr>
<tr>
<td>pZZ18</td>
<td>YE, <em>HIS3</em>, <em>RNR2-lacZ</em></td>
<td>Zhou and Elledge (1992)</td>
</tr>
<tr>
<td>pBluescript SK(-)</td>
<td>Phagemid vector</td>
<td>Stratagene, CA, USA</td>
</tr>
</tbody>
</table>
Fig. 2-1: Integration of the RNR3-lacZ cassette into the HO locus. A: RNR3-lacZ cassette was inserted into the plasmid M4366, flanked by a hisG-URA3-hisG cassette and a fragment from the right end of HO gene. After being linearized by NotI, the plasmid was transformed into yeast cells. Homologous recombination occurred between HO-L, HO-R and the chromosomal HO gene. B: The hisG-URA3-hisG-RNR3-lacZ cassette was integrated into genomic HO locus. C: URA3 marker was popped out from the genome through homologous recombination between hisG tandem repeats D: A 0.9 kb fragment from the left end of the HO gene and a 2.0 kb lacZ gene fragment were used as probes for Southern hybridization.
2.2.10. **Southern hybridization**

In order to confirm the genomic structure of the RNR3-lacZ integrated DBY747 strain, Southern hybridization was carried using a 0.9 kb HO fragment (Voth et al., 2001) and a 2.0 kb lacZ fragment as probes (Zhou and Elledge, 1992).

After digestion of the genomic DNA with BgII, the DNA fragments were separated on a 0.8% agarose gel. The gel was then treated in solutions of 1) 0.25 M HCl for 10 minutes for depurination; 2) 0.4 M NaOH/0.6 M NaCl for 30 minutes for denaturation, and 3) 1.5 M NaCl/0.5 M Tris-HCl (pH 7.5) for 30 minutes for neutralization. The DNA was transferred from the gel to a nylon-based membrane (GeneScreen plus membrane, DuPont) using 10x SSC (3M NaCl, 0.3 M tri-sodium citrate, pH 7.0) overnight.

The following day, the membrane was placed into a hybridization bottle with 5 ml of pre-hybridization solution [2x SSC, 10% dextran sulphate, 5x Denhardt’s solution (50X stock: 10 g Ficoll, 10 g polyvinylpyrrolidone, 10 g bovine serum albumin; add ddH2O to 500 ml total volume), 50% formamide, and 1% SDS] and incubated at 42 °C in the hybridization oven for at least 2 hours. Before hybridization, 50 µl of boiled ssDNA DNA and 60 µl of probe were added to the prehybridization solution. The membrane was then incubated with the probe overnight at 42 °C. The membrane was washed twice for 5 minutes at room temperature in 2x SSC/0.1% SDS and washed twice at 65 °C in 0.2x SSC/0.1% SDS. The membrane was then placed with X-ray film at -70 °C and developed.
CHAPTER III: CONSTRUCTION AND EVALUATION OF THE RNR3-lacZ SYSTEM

3.1. Introduction

One of the major concerns in the use of a bacterial short-term testing systems is that the genomic structure, genetic regulation and metabolism of bacteria are rather different from eukaryotic cells. Hence, in this study, a sensitive and stable genotoxicity testing system using S. cerevisiae was developed. The rationale is similar to that of SOS Chromotest assay except that it is carried out in a yeast system. The RNR3 promoter is used as a sensing promoter to detect environmental damage to DNA; lacZ is fused to the RNR3 promoter as a reporter. The fused RNR3-lacZ cassette can be introduced into the host cell either as a plasmid or integrated into the yeast chromosome. The lacZ gene was chosen as a reporter gene for the following reasons. First, it is very easily detected and does not require special expensive equipment. This is a critical point for future field use of this system. Second, as a common reporter gene to study gene expression, comparable data can be found in various publications, so that we can compare our results to other previous data and test their reliability. Third, there is no β-gal background since the lacZ gene is not found in yeast cells.

3.2. Results

3.2.1. RNR3 is a more suitable reporter than other DNA damage-inducible genes

An ideal sensor gene would respond to a relatively low dose of damage treatment and have a high level of induction. Three such candidate genes, RNR2, RNR3, and MAG1
were compared for their inducibility by MMS in this study. As seen in Fig. 3-1, all three genes can be induced by MMS. However, the induction of RNR3-lacZ and RNR2-lacZ peaked at 0.02% whereas that of MAG1-lacZ reached a maximum expression after 0.05% MMS treatment. The maximum expression of RNR3-lacZ increased 53-fold while that of RNR2-lacZ and MAG1-lacZ increased 19 and 6.6-fold, respectively. These results are consistent with previous studies utilizing both Northern hybridization and reporter gene analyses (Hurd et al., 1987; Elledge and Davis, 1989; Hurd and Roberts, 1989; Elledge and Davis, 1990; Zhou and Elledge, 1992; Xiao et al., 1993) as well as using different DNA damaging agents including MMS, UV, γ-ray and 4-NQO. A large number of yeast DNA damage inducible genes whose induction profiles are similar to MAG1 have been analyzed, including DDI1 (Liu and Xiao, 1997), UBC13 (Brusky et al., 2000), PHR1, GGT2, RAD2 and RAD51 (unpublished work of Dr. Y Zhu), and their fold induction is comparable to or less than that of MAG1. Therefore, it is concluded that RNR3-lacZ appears to be the best candidate among yeast DNA damage inducible genes studied to date.

3.2.2. Induction of the RNR3-lacZ system by different test agents

Genotoxic agents were selected to represent a broad range of DNA damaging agents that may or may not have a direct effect on mutagenesis (Table. 2-1), including DNA alkylating agents (MMS, EMS, MNNG and SDMH), an oxidative agent (H₂O₂), UV and a UV mimetic agent (4-NQO), ionizing radiation (γ-ray), a base analog (5-fluorouracil) and an agent (phleomycin) that causes strand breaks. This test also included a ribonucleotide reductase inhibitor (HU) that interferes with DNA synthesis. The
Fig. 3-1. Different expression level of RNR3-lacZ, RNR2-lacZ and MAG1-lacZ after MMS treatment. DBY747 cells carrying pZZ2 (RNR3-lacZ, □), pZZ18 (RNR2-lacZ, ●) or pWX1255 (MAG1-lacZ, o) were incubated in SD minimal medium and treated with various doses of MMS for 4 hours before β-gal activity assay. The results are the average of three independent experiments and are expressed as fold of induction relative to their respective untreated cells.
mechanistic effects of these agents are well known (Friedberg et al., 1995). As seen in Fig. 3-1 and 3-2, and summarized in Table 3-1, all 11 tested genotoxic chemicals can induce the RNR3-lacZ system to varying degrees. The sensitivity is comparable to or higher than the published SOS Chromotests values. For example, the threshold doses (at which the β-gal activity increased by 2-fold) for MMS, EMS, HU and H₂O₂ in the sfiA-lacZ system are about 0.10 mM, 10 mM, 10 mM and 1 mM, respectively (von der Hude et al., 1988), whereas the corresponding doses for the RNR3-lacZ test are 0.15 mM, 4 mM, 5 mM and 0.4 mM, respectively (Fig. 3-1 and 3-2). In this study, a well-known colon carcinogen, 1,2-dimethylhydrazine (SDMH) was studied. It was undetectable as a bacterial mutagen by the Ames test (McCann et al., 1975; Lijinsky et al., 1985) and SOS Chromotests. Interestingly, the RNR3-lacZ system was induced by more than 8-fold after 0.1% SDMH treatment (Fig. 3-2-2). Hence, RNR3-lacZ induction appears to be a useful and complementary system to existing genotoxic and mutagenic tests.

Antibiotics such as tetracycline and canavanine, which kill yeast cells without damaging DNA, were also tested. Under our experimental conditions, at doses that kill as much as four-fifths or one-quarter of the yeast cells, respectively, tetracycline and canavanine were unable to induce RNR3-lacZ expression (Fig. 3-2-3).

Table 3-1 summarizes various experimental results obtained from this study. It also attempts to compare the results of the RNR3-lacZ test with the Ames test and SOS Chromotests. Results of the Ames test and SOS Chromotests were obtained from the genotoxicity database: www.pasteur.fr/recherche/unites/pmtg/toxic/index.html.

The intercalating agent EtBr could not induce the RNR3-lacZ system (Fig. 3-3). Since it also produced uncertain results in both Ames (Mamber et al., 1986) and SOS
Chromotest assay (Mamber et al., 1986; Quillardet and Hofnung, 1993), its capacity to cause DNA damage and mutation remains unclear.

3.2.3. Effects of incubation time on induction efficiency

In order to optimize test conditions for the RNR3-lacZ system, setting MMS as a reference compound, several parameters were altered to identify their effects on RNR3-lacZ expression.

The effect of varying duration of MMS treatment on RNR3-lacZ expression was determined. As seen in Fig. 3-4, MMS-induced β-gal activity in RNR3-lacZ transformed cells increased with treatment time up to 4 hours and began to decrease after a 6-hour treatment. This result is consistent with the previous observation comparing a time course of MAG1-lacZ induction with MAG1 transcript by MMS (Xiao et al., 1993). In comparison, a recent report (Afanassiev et al., 2000) suggests that RNR2-GFP induction by MMS reached a maximum fluorescent signal after approximately 14-16 hours of incubation. Since the mRNA transcript level after MMS and other types of DNA damage treatment often reaches a peak after 30 minutes (Elledge and Davis, 1990), the delayed increase in β-gal activity after DNA damage treatment probably represents the time required to produce the fusion protein and accumulate cellular β-gal. The reduced β-gal activity after 6 hours of treatment is either due to toxic effects that kill wild type cells, or due to turnover of the fusion transcript and/or protein.

3.2.4. The effects of cell growth stage on induction efficiency

The effect of cell growth stage on RNR3-lacZ induction by DNA damaging agents
Fig. 3-2. The induction of $RNR3$-lacZ by HU (hydroxyurea); UV (ultraviolet light); EMS (ethyl methanesulfonate), γ-ray, MNNG (N-methyl-N'-nitro-N-nitrosoguanidine); SDMH (1,2-dimethylhydrazine); 4-NQO (4-nitroquinoline), phleomycin, 5-fluorouracil, $H_2O_2$, tetracycline and canavanine. The DBY747 cells carrying pZZ2 ($RNR3$-lacZ) were incubated in SD minimal medium, treated with agents as indicated for a given dose and the incubation was continued for another four hours before β-galactosidase activity assay. Each of the results was the average of three independent experiments. The fold of induction was calculated as a ratio of β-galactosidase expressed in cells with and without treatment in the same experiment.
**Fig. 3-3.** The response of *RNR3-lacZ* system to ethidium bromide. The DBY747 cells carrying pZZ2 (*RNR3-lacZ*) were incubated in SD-Ura medium, treated with ethidium bromide at doses and incubate for another four hours before assaying the β-galactosidase activity. The result was the average of three independent experiments. The fold of induction was calculated as a ratio of β-galactosidase of cells with and without treatment in the same experiment.
### Table 3-1: Summary of the \textit{RNR3-lacZ} test and comparison with other test results

<table>
<thead>
<tr>
<th>Agents</th>
<th>Basal level(^a) (Miller unit)</th>
<th>Highest Induction(^a)</th>
<th>Fold induction</th>
<th>Optimal dose(^b)</th>
<th>Survival (%)</th>
<th>SOS test(^c)</th>
<th>Ames test(^e)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMS</td>
<td>0.96±0.12</td>
<td>51.78±7.08</td>
<td>53.9</td>
<td>0.02%</td>
<td>19.6</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>(\gamma) ray</td>
<td>1.18±0.30</td>
<td>31.03±2.48</td>
<td>26.3</td>
<td>30 K rad</td>
<td>45.5</td>
<td>+/N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>EMS</td>
<td>0.59±0.06</td>
<td>13.16±1.56</td>
<td>22.3</td>
<td>0.5%</td>
<td>42.1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>UV light</td>
<td>1.09±0.07</td>
<td>16.80±2.51</td>
<td>15.4</td>
<td>50 J/m(^2)</td>
<td>84.5</td>
<td>+</td>
<td>N/A</td>
</tr>
<tr>
<td>HU</td>
<td>1.02±0.08</td>
<td>12.40±0.15</td>
<td>12.2</td>
<td>75 mM</td>
<td>28.8</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SDMH</td>
<td>0.69±0.08</td>
<td>5.72±0.37</td>
<td>8.3</td>
<td>0.1%</td>
<td>46.7</td>
<td>-</td>
<td>+/e</td>
</tr>
<tr>
<td>MNNG</td>
<td>1.12±0.09</td>
<td>7.85±0.40</td>
<td>7.0</td>
<td>5 (\mu)g/ml</td>
<td>80.1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4-NQO</td>
<td>0.93±0.05</td>
<td>7.76±0.32</td>
<td>8.3</td>
<td>0.8 (\mu)g/ml</td>
<td>27.1</td>
<td>+</td>
<td>+/f</td>
</tr>
<tr>
<td>Phleomycin</td>
<td>0.81±0.07</td>
<td>4.99±0.76</td>
<td>6.1</td>
<td>0.15 mg/ml</td>
<td>49.7</td>
<td>+</td>
<td>N/A</td>
</tr>
<tr>
<td>(H_2O_2)</td>
<td>0.96±0.12</td>
<td>4.48±0.75</td>
<td>4.7</td>
<td>0.6 mM</td>
<td>63.2</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>5-Fluorouracil</td>
<td>1.21±0.05</td>
<td>4.69±0.95</td>
<td>3.9</td>
<td>0.1 mg/ml</td>
<td>52.5</td>
<td>+/-</td>
<td>+/-f</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>1.03±0.16</td>
<td>1.34±0.04</td>
<td>1.3</td>
<td>100 (\mu)g/ml</td>
<td>21.2</td>
<td>-</td>
<td>-/f</td>
</tr>
<tr>
<td>Canavanine</td>
<td>0.77±0.18</td>
<td>&lt;1.0</td>
<td>100 (\mu)g/ml</td>
<td>54.9</td>
<td>N/A</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>EtBr</td>
<td>0.59±0.08</td>
<td>0.60±0.17</td>
<td>1.0</td>
<td>0.04 (\mu)g/ml</td>
<td>81.0%</td>
<td>+/-</td>
<td>+/-</td>
</tr>
</tbody>
</table>
Note: 4-NQO: 4-nitroquinoline; HU: hydroxyurea; MMS: methyl methanesulfonate; EMS: ethyl methanesulfonate; EtBr: ethidium bromide; SDMH: 1,2-dimethylhydrazine; MNNG: N-methyl-N'-nitro-N-nitrosoguanidine; UV: ultraviolet light; Tetracycline: hydrochloride tetracycline

“+” indicates positive results; “−” indicates negative results

a Average β-gal activity of untreated and treated samples at the highest induction level within experimental conditions, presented as Miller unit. All results are based on at least three independent experiments with standard deviation.

b Dose of treatment at which RNR3-lacZ reached highest induction, or the highest dose of treatment, within experimental limits.

c Results of SOS Chromostest assay and Ames test were obtained from database www.pasteur.fr/recherche/unites/pmtg/toxic/index.html

d Not available.

e Positive results were obtained only in DNA repair alkyltransferase deficient strains (Xiao et al., 1996).

f Unclear interpretation
was also explored. This study was focused on log phase cells, since it was anticipated that most DNA damaging agents attack replicating DNA during cell division and that RNR3 DNA damage induction requires cell cycle checkpoints (Zhou and Elledge, 1993; Navas et al., 1995) which function during cell division. The cell titer prior to the β-gal assay ranging from $1 \times 10^7$ to $3 \times 10^7$ cells/ml (OD$_{600} = 0.2-0.7$) affects β-gal specific activity up to 12-fold; however, the relative induction remained unaltered (Fig. 3-5). In contrast, the induction of RNR3-lacZ in stationary phase cells reached a maximum of 2.5 fold after treatment with 0.0025% MMS. Hence, the RNR3-lacZ test is relatively independent of cell titer as long as cells are in the log phase. Results obtained in this study were typically performed when asynchronized cells reached $1.5-1.8 \times 10^7$ cells/ml (OD$_{600}=0.4$).

3.2.5. **Creation and evaluation of a stable RNR3-lacZ testing system**

The RNR3-lacZ reporter gene used in the above studies was carried in a centromere-based YCp plasmid which is a single copy plasmid with a plasmid loss rate of $10^{-3}$ per cell per generation. The experiments were conducted in selective medium to ensure that only cells containing the plasmid were able to grow and divide. In order to increase the stability of the testing system and to make it more user-friendly, the RNR3-lacZ reporter was integrated into the host genome at the HO locus.

3.2.5.1. **Integration of RNR3-lacZ cassette into the yeast genome**

The HO gene encodes a site-specific endonuclease; however, HO in most laboratory strains, including the strains used in this study, is inactive (Baganz et al., 1997). The RNR3-lacZ cassette was delivered to the HO locus using a recently
Fig. 3-4. Effects of incubation time on the induction efficiency of RNR3-lacZ expression. The pZZ2 transformants were incubated in SD minimal medium, treated with MMS at the given final concentration, and incubated for 3 (○), 4 (●), 6 (○), 8 (▲) and 10 (△) hours before β-gal activity assay. Each of the results was the average of three independent experiments. The fold of induction was calculated as a ratio of β-galactosidase of cells with and without treatment in the same experiment.
Fig. 3-5: Effects of cell growth stage on induction efficiency. The pZZ2 transformants were grown in SD minimal medium to OD$_{600} = 0.06$ (○), 0.13 (●) and 0.21 (□), treated with MMS at the given final concentration, and incubated for 4 hours before β-gal activity assay. Cell titer at the time of β-gal assay for the above samples were approximately $1 \times 10^7$, $2 \times 10^7$ and $3 \times 10^7$ cells/ml, respectively. Each of the results was the average of three independent experiments.
developed integration system (Voth et al., 2001) such that, after initial integration and subsequent selectable pop-out through homologous recombination, only the $RNR3$-$\text{lacZ}$ cassette plus one copy of $\text{hisG}$ were integrated to replace the inactive host $HO$ sequence, resulting in a single-copy reporter gene in the haploid host chromosome. Hence, it is expected to be as stable as any other yeast nuclear gene. The genomic structure (Fig. 2-1) was confirmed by Southern hybridization (Fig. 3-6).

3.2.5.2: Evaluation of the integrated $RNR3$-$\text{lacZ}$ system

The resulting yeast strain was cultured in non-selective rich medium and the $\beta$-gal activity was compared to the same yeast strain carrying a plasmid-based reporter gene grown in the selective medium. As shown in Fig. 3-7, the induction profiles of three representative testing compounds (MMS, HU and 4-NQO) are indistinguishable between the integrant and plasmid-based systems. The results indicate that the $RNR3$-$\text{lacZ}$ integrant strain provides a suitable genotoxic testing system complementary to the existing microbial testing systems.
Fig. 3-6. The genomic structure of integrated RNR3-lacZ system was confirmed by Southern hybridization. Lane 1, 3: genomic DNA from DBY747 wild type strain; Lane 2, 4: genomic DNA from DBY747 integrated RNR3-lacZ cassette. The lacZ fragment was detected in the integrated cell (lane 4) but not in wild type DBY747 (lane 3). Because the integration of the RNR3-lacZ cassette into the HO locus, the fragment size increased from 3.2kb to 8kb when hybridized with HO probe (lanes 2 and 4).
Fig. 3-7: Comparison of RNR3-lacZ induction between the plasmid-borne and the integrated RNR3-lacZ reporter. DBY747 cells carrying pZZ2 (w) were incubated in SD minimal medium to an OD600 of 0.2-0.3, and those containing a RNR3-lacZ integrant at the HO locus (û) were incubated in YPD to an OD600 of 0.1. Cells were then treated with MMS (methyl methanesulfonate, A), HU (hydroxyurea, B) or 4-NQO (4-nitroquinoline, C) at the given concentration with further incubation of 4 hours before β-gal activity assay. All the results are an average of at least three independent experiments and are expressed as fold induction relative to untreated cells. The fold of induction was calculated as a ratio of β-galactosidase of the cells with and without treatment in the same experiment.
CHAPTER IV: ENHANCING THE SENSITIVITY OF RNR3-lacZ SYSTEM BY GENETIC MODIFICATION OF THE HOST STRAIN

4.1. Introduction

The RNR3-lacZ genotoxicity testing system was developed as previously described (see Chapter III). It is a simple testing system with characteristics of high sensitivity, ease of assay, and time and cost saving. A variety of physical and chemical agents were tested in this system (Jia et al., 2002). The results correlate with the Ames test very well. Based on the comparison of minimum response dose with SOS Chromotest on several chemical agents, the sensitivity of the RNR3-lacZ system is comparable to or even higher than the SOS Chromotest (Jia et al., 2002).

There have been a variety of attempts to enhance the sensitivity of the Ames test. It has been shown that by adding S9 rat liver extract into the system, some carcinogens can be activated to mutagens and readily detected (Ames et al., 1973a). The rfa mutation in the strains leads to a defective lipopolysaccharide (LPS) layer that coats the bacterial surface and makes the cells more permeable to bulky chemicals (Ames et al., 1973b).

Modifying the DNA repair system is another choice to enhance the sensitivity of the Ames test. It is found that introducing an R factor (pKM101) into Ames strains can enhance chemical and UV-induced mutagenesis and make the test more sensitive (McCann et al., 1975; Walker and Dobson, 1979). Based on this theory, many R factor transformed strains such as TA100, TA98 (Ames et al., 1975), TA97 (Levin et al., 1982), TA102 and TA104 (Levin et al., 1982) were employed in the Ames test. Another attempt was to delete the uvrB gene, which is involved in nucleotide excision repair in E. coli. In the uvrB mutant strain, the incidence of mutagenesis was enhanced and therefore lowered.
the threshold of the Ames test (Moreau et al., 1976). Similar efforts were also reported with the SOS Chromotest. It was found that the introduction of a dam-3 mutation into a PQ37 derivative strain used in the SOS Chromotest enhanced the sensitivity of the system (Quillardet and Hofnung, 1987). The author provided evidence that the increase in SOS inducibility due to the dam-3 mutation is specific for compounds causing DNA mismatches. Previous research in our laboratory (Xiao et al., 1996) also demonstrated that SDMH could be detected as a bacterial mutagen if Ames test strains were defective in the DNA repair O6-methylguanine methyltransferase activity whereas the standard Ames test failed to detect it as bacterial mutagen (Lijinsky et al., 1985). The above results suggest that, as with the Ames test and SOS Chromotest, the sensitivity of eukaryotic genotoxicity testing systems may be further improved by inactivating certain DNA repair pathways. The fact that deletion of RAD16 in yeast cells enhances the sensitivity of a number of yeast DNA damage inducible genes to DNA damage treatment (Kiser and Weinert, 1996) supports this hypothesis. Most DNA repair pathways dealing with different types of DNA lesions have been well characterized in budding yeast and mutations in each of these pathways have been created in our laboratory. This hypothesis was systematically tested in the present study.

Unlike prokaryotic cells, the regulation of the DNA repair network seems much more complicated. Since the early 1970’s, an extensive collection of yeast mutants (rad) that are sensitive to UV radiation at 254 nm and/or to ionizing radiations has been established (Cox and Parry, 1968; Game and Mortimer, 1974). According to their responses to UV radiation and ionizing irradiations, these mutants were placed into three epistasis groups (Game and Cox, 1971). It became clear that these groups represent three distinct repair pathways, namely the RAD3 nucleotide excision repair pathway, the
RAD52 DNA recombinational repair pathway and the RAD6 post-replication repair and mutagenesis pathway (Friedberg et al., 1995). In addition to the above rad genes, another group of genes were found to be mainly involved in the repair of damaged bases and functioned in the base excision repair pathway.

In this study, mutant strains (Table 2-1) defective in DNA repair genes from different DNA repair pathways were employed as the host strain of the RNR3-lacZ system. The expression level of RNR3 was measured and compared with that of the wild type DBY747. Several mutant strains have been found to enhance the inducibility of the RNR3-lacZ reporter gene.

4.2. RESULTS

In this study, isogenic deletion mutants of DBY747 from different repair pathways were used as host strains of the RNR3-lacZ system. The peak induction of RNR3 as well as the dose level at which the induction was obtained were compared with the original RNR3-lacZ system in the wild-type strain DBY747.

4.2.1. The effects of base excision repair mutation on RNR3-lacZ induction

All BER reactions are initiated by the action of a specific class of DNA enzymes called DNA glycosylases. The glycosylase recognizes and binds to the damaged site, then mediates the cleavage of the damaged base from the sugar backbone. The resulting abasic site is incised exclusively by AP endonucleases (Wallace, 1994; Sancar, 1994; Seeberg, 1995). BER mediated by AP endonucleases is the primary defense against AP sites and 3’-blocked SSBs (Lindahl and Wood, 1999; Hoeijmakers, 2001)
*MAGI* encodes a 3-methyladenine (3MeA) DNA glycosylase (Chen et al., 1989), the first enzyme in a multistep BER pathway for the removal of lethal lesions such as 3MeA, and protects yeast cells from killing by DNA alkylating agents such as MMS (Sakumi and Sekiguchi, 1990).

Two endonucleases were found in *S. cerevisiae*, namely Apn1 and Apn2, encoded by *APN1* and *APN2*, respectively. Apn1 and Apn2 catalyze the hydrolytic cleavage of the phosphodiester backbone at the 5' side of an AP site, yielding an SSB with a 3'-OH group (Demple and Harrison, 1994; Unk et al., 2000). The *apn1* mutants were moderately sensitive to the killing action of alkylating agents such as MMS and exhibited enhanced spontaneous mutation rates (Ramotar et al., 1991). Deletion of *APN2* alone did not produce any noticeable phenotypic alterations; however, Apn2 provides a strong backup for the Apn1 AP endonuclease activity. Cells lacking both AP endonuclease are remarkably sensitive to the cytotoxic effects of alkylating agents, display an elevated spontaneous mutation rate and lose the ability to repair AP sites in genomic DNA (Johnson et al., 1998; Bennett, 1999). In this study, the *apn1 apn2* double mutant strain was used to test the effect of their deletion on the *RNR3* induction. Since it was also reported that the mutator phenotype of *apn1* is profoundly affected by 3MeA levels (Xiao and Samson, 1993; Glassner et al., 1998; Xiao et al., 2001), the *mag1 apn1 apn2* triple deletion mutant strain was also employed to determine whether *RNR3* induction can be increased in this genetic background.

With the *mag1* null mutant strain, the expression of *RNR3* after MMS treatment was increased (Fig. 4-1). In particular, the maximum induction of *RNR3-lacZ* increased from 48-fold in wild type cells to 59-fold in the *mag1* null mutant. More importantly, the minimum and maximum induction doses decreased dramatically in the *mag1* mutant.
Hence, with MMS doses up to 0.01%, the \textit{magI} mutant strain displays a 3-4 fold enhancement of induction (Fig. 4-1). This phenomenon appears to be true for other DNA alkylating agents, since the \textit{magI} mutant shows an enhanced sensitivity to another alkylating agent, EMS. In this case, although the maximum fold induction is similar between wild type and mutant cells, the \textit{magI} mutant reaches the maximum at 0.2% EMS, instead of 0.5% in wild type cells. A 4-5 fold enhancement of \textit{RNR3-lacZ} induction at low doses of EMS by \textit{MAGI} deletion was observed and the response is in the linear range (Fig. 4-2). In contrast, deletion of \textit{MAGI} does not affect the \textit{RNR3-lacZ} induction profile by UV treatment (Fig. 4-3), and actually decreases the \textit{RNR3-lacZ} response to \textit{\gamma-ray} treatment at high doses (Fig. 4-4). These results indicate that the enhancement of \textit{RNR3-lacZ} sensitivity by \textit{MAGI} deletion is probably specific to DNA alkylating agents and limited to lesions repaired by the Mag1 DNA glycosylase.

The enhanced sensitivity of the \textit{RNR3-lacZ} genotoxic testing system to DNA alkylating agents in a \textit{MAGI} deletion strain seems to suggest that it is due to the increased sensitivity of \textit{magI} cells to killing by MMS. Deletion of both AP endonuclease genes in yeast results in extreme sensitivity to killing by MMS (Johnson et al., 1998; Bennett, 1999) and other base-damaging agents (Bennett, 1999). \textit{RNR3-lacZ} induction was tested by MMS in the \textit{apnl apn2} double mutant and surprisingly found that it was actually decreased (Fig. 4-1). Deletion of the \textit{MAGI} gene in the AP endonuclease deficient cells partially rescues the severe sensitivity to killing by MMS (Xiao et al., 2001); however, the \textit{magI apnl apn2} triple mutant almost completely lost the \textit{RNR3} induction (Fig. 4-1).
Fig. 4-1. Comparison of MMS-induced RNR-lacZ expression in the magl null mutant (●), the apn apn2 mutant (▲) and maglapn1apn2 mutant (△) with that in the wild type DBY747 strain (○). Yeast cells carrying pZZ2 (RNR3-lacZ) were incubated in SD-Ura medium, treated with MMS as indicated doses and incubated for an additional four hours before assaying β-galactosidase activity. The result was the average of three independent experiments. The fold of induction was calculated as a ratio of β-galactosidase expressed in cells with and without treatment in the same experiment.
Fig. 4-2. Comparison of RNR3-lacZ induction in the mag1 null mutant (○) and in the wild type DBY747 (●) after EMS treatment. RNR3-lacZ induction can be detected at much lower EMS dose in mag1 mutant than in DBY747. Yeast cells carrying pZZ2 (RNR3-lacZ) were incubated in SD-Ura medium, treated with EMS as indicated dose and incubated for an additional four hours before assaying the β-galactosidase activity. The result was the average of three independent experiments. The fold of induction was calculated as a ratio of β-galactosidase expressed in cells with and without treatment in the same experiment.
Fig. 4-3. UV-induced RNR3-lacZ expression in wild type DBY747 (•) and the magl null mutant (○). The two strains show no significant difference in the response. The yeast cells carrying pZZ2 (RNR3-lacZ) were incubated in SD-Ura medium, treated with UV as indicated dose and incubated for additional four hours before assaying β-galactosidase activity. The result was an average of three independent experiments. The fold of induction was calculated as a ratio of β-galactosidase expressed in cells with and without treatment in the same experiment.
Fig. 4-4. Comparison of RNR3-lacZ induction in the magl deletion mutant (•), the rad2 deletion mutant (▲) and the wild type DBY747 (▼) after γ-ray treatment. Yeast cells carrying pZZ2 (RNR3-lacZ) were incubated in SD-Ura medium, treated with agents as indicated dose and the incubated for additional four hours before assaying β-galactosidase activity. The result was an average of three independent experiments. The fold of induction was calculated as a ratio of β-galactosidase expressed in cells with and without treatment in the same experiment.
4.2.2. Deletion of nucleotide excision repair gene *RAD2* enhances the induction of RNR3-lacZ to UV radiation

NER consists of the process of damage recognition, dual incision, release of an oligonucleotide fragment carrying the damage, re-synthesis of the gap, and finally ligation of the free ends. The incision step of NER is mainly completed by the *RAD3* epistasis group. This group was further divided into two classes: Class 1 consists of *RAD1, RAD2, RAD3, RAD4, RAD10, RAD14* and *RAD25*; Class 2 contains *RAD7, RAD16, RAD23* and *MMS19*. It is suggested that Class 1 genes are essential for all the steps leading to the incision of damaged DNA, while Class 2 genes only affect the proficiency of the reaction. Indeed, mutations in Class 1 genes confer a much higher degree of sensitivity to UV light and other DNA damaging agents and cause a high level of defect in the incision of UV damaged DNA and crosslinked DNA (Prakash and Prakash, 2000).

Based on the essential roles of Class 1 genes in the NER pathway, one of them, *RAD2*, was selected in this study to determine how its deletion mutation affects the RNR3-lacZ induction, in order to assess the potential application to enhance the sensitivity of the RNR3-lacZ system. Rad2 protein has a Mg$^{2+}$ dependent endonuclease activity that is specific for single-stranded DNA (Habraken et al., 1993) and cleaves at the 3'-side of the lesion (Habraken et al., 1995). Furthermore, *RAD2* expression is UV inducible at the transcriptional level (Siede and Fridberg, 1992).

The induction of RNR3-lacZ after UV treatment was less than 20-fold in wild type
cells (Jia et al., 2002; Fig. 4-5). It was surprising to find that the peak level reached more than 40-fold in the rad2 null mutant (Fig. 4-5). More importantly, the maximum induction was reached at 10 J/m² in the rad2 mutant compared to 50 J/m² in the wild type cells (Fig. 4-5). A UV mimetic agent 4-NQO was tested to see if inactivation of NER is able to help detection of genotoxic chemicals. Indeed, deletion of RAD2 enhances the induction of RNR3-lacZ by about 3 fold in the dose range examined (Fig. 4-6). It was also reported that NER participates in the repair of DNA methylation damage (Xiao et al., 1996; 1998). Hence, the effect of deleting RAD2 on the MMS-induced RNR3-lacZ expression was examined. It was interesting to notice that at low MMS doses, the rad2 mutant displayed an enhanced sensitivity. However, at doses higher than 0.1% MMS, RNR3-lacZ induction was compromised in the rad2 mutant (Fig. 4-7). Deletion of RAD2 also compromised RNR3-lacZ induction by γ-ray treatment at high doses, although no significant differences between the rad2 mutant and wild type cells was observed at low doses (Fig. 4-4).

4.2.3. Effects of recombination repair mutations on the induction of RNR3-lacZ

Recombination repair appears to be much more complicated than other DNA repair pathways. The present understanding about the mechanism of this pathway especially regarding the homologous recombination has lagged behind that of other DNA metabolic processes. DSBs are induced by a variety of DNA-damaging agents, including ionizing radiations like γ-rays and X-rays, as well as radiomimetic chemicals such as MMS.

The DSBs may also arise “spontaneously” from replication past SSBs, processing
Fig. 4-5. Comparison of RNR3-lacZ induction level in wild type DBY747 (α) and in rad2 mutants (●) after UV treatment. The peak level in the rad2 deletion mutant reached more than 40 fold at 10 J/m², while the peak level in DBY747 was less than 20-fold, which was reached at a much higher dose level of 45 J/m². The yeast cells carrying pZZ2 (RNR3-lacZ) were incubated in SD-Ura medium, treated with UV as indicated dose and incubated for additional four hours before assaying β-galactosidase activity. The result was an average of three independent experiments. The fold of induction was calculated as a ratio of β-galactosidase expressed in cells with and without treatment in the same experiment.
Fig. 4-6. Comparison of RNR3-lacZ induction in wild type DBY747 (○) and its rad2 null mutant (●) after 4-NQO treatments. The fold induction in the rad2 mutant is higher than that in DBY747 and reached >20 fold, whereas that in DBY747 was less than 10-fold. The yeast cells carrying pZZ2 (RNR3-lacZ) were incubated in SD-Ura medium, treated with 4-NQO as indicated dose and incubated for additional four hours before assaying β-galactosidase activity. The result was an average of three independent experiments. The fold of induction was calculated as a ratio of β-galactosidase expressed in cells with and without treatment in the same experiment.
Fig. 4-7. Comparison of RNR3 induction level in DBY747 (o) and rad2 null mutant strain (•) after MMS treatment. At lower doses, the rad2 mutant displayed an enhanced sensitivity; at doses higher than 0.1% MMS, the RNR3-lacZ induction was compromised in the rad2 mutant. The yeast cells carrying pZZ2 (RNR3-lacZ) were incubated in SD-Ura medium, treated with MMS as indicated dose and incubated for additional four hours before assaying β-galactosidase activity. The result was an average of three independent experiments. The fold of induction was calculated as a ratio of β-galactosidase expressed in cells with and without treatment in the same experiment.
of endogenous damage and endonuclease activities (Friedberg et al., 1995).

RAD52 epistasis group are involved in the repair of DSBs in S. cerevisiae.

Although these genes share the property of conferring resistance to ionizing radiation, their mutants display considerable variation in assays for DSB repair and recombination. RAD50, XRS2, and MRE11 form a subgroup with similar properties. Physical interactions among their gene products have been detected by the two-hybrid system (Johzuka and Ogawa, 1995; Chamankhah and Xiao, 1999). It has been suggested that the complex is nucleolytic and plays a role in the initiation of homologous recombination as well as nonhomologous end rejoining (Johzuka and Ogawa, 1995; Moore and Haber, 1996).

Rad52 is known to bind to the ends of DNA molecules (Dyck et al., 1999) to promote DNA strand annealing between single-stranded oligonucleotides (Mortensen et al., 1996; Sugiyama et al., 1998), and to potentiate the strand-exchange activity of Rad51 (Shinohara and Ogawa, 1995; Sung, 1997; New et al., 1998;). Its mutants produce the most severe defects in DSB repair and recombination (Friedberg et al., 1995).

In this study, both rad50 and rad52 null mutant strains were employed. The induction of RNR3-lacZ after treatment of \( \gamma \)-ray was dramatically reduced in both rad50 and rad52 cells compared with the wild type cells (Fig. 4-8). It is noticed that the basal levels of \( \beta \)-gal activity in rad50 and rad52 mutants are approximately 4.0 and 7.0, respectively, while it is less than 1.0 in wild type cells, which may partially explain the compromised fold induction in the recombination repair deficient cells. Similarly, MMS-induced RNR3-lacZ expression was also compromised in the rad52 mutant (Fig. 4-9) to a level comparable to the \( \gamma \)-ray treatment (Fig. 4-8).
Fig. 4-8. Comparison of RNR3 induction level in DBY747 (□), rad50 deletion mutant strain (●) and rad52 deletion mutant (○) after γ-ray treatments. Both of the two mutant strains reduced the induction of RNR3 dramatically comparing to DBY747. The yeast cells carrying pZZ2 (RNR3-lacZ) were incubated in SD-Ura medium, treated with γ ray as indicated dose and incubated for additional four hours before assaying β-galactosidase activity. The result was an average of three independent experiments. The fold of induction was calculated as a ratio of β-galactosidase expressed in cells with and without treatment in the same experiment.
Fig. 4-9. Comparison of RNR3-lacZ induction in the wild type DBY747 (○) and rad52 mutant strain (●) after treatment with MMS. Yeast cells carrying pZZ2 (RNR3-lacZ) were incubated in SD-Ura medium, treated with MMS as indicated and the incubated for additional four hours before assaying β-galactosidase activity. The result was an average of three independent experiments. The fold of induction was calculated as a ratio of β-galactosidase expressed in cells with and without treatment in the same experiment.
4.2.4. *RNR3-lacZ* induction in post-replication repair deficient mutant strains

Post-replication repair is required by the cell to survive an S-phase with unrepaired lethal DNA lesions in the genome. After the replication machinery bypasses the damage, the PRR processes fill in the resulting gaps. In *S. cerevisiae*, members of the *RAD6* pathway are responsible for PRR. Many lines of evidence suggested that the error-prone mechanism of PRR is not the preferred form of tolerance in eukaryotes (Broomfield et al., 2001). It is now clear that there are at least two independent error-free PRR pathways mediated by the product of *RAD5* and *POL30*, and both of them are under the control of *RAD6/RAD18* (Xiao et al., 2000).

Rad6 is one of 13 ubiquitin-conjugating enzymes (Ubcs) in *S. cerevisiae* and is involved in diverse cellular functions. A *rad6* null mutant is defective in PRR, resulting in an extreme sensitivity to a wide variety of DNA damaging agents and a defect in induced mutagenesis (Prakash et al., 1993). *RAD6* is also necessary for sporulation (Morrison et al., 1988), telomere silencing (Huang et al., 1997), and protein degradation based on the amino-end rule (Dohmen et al., 1991).

Like *rad6* null mutants, *rad18* null mutants are sensitive to UV, X-rays, γ-rays, 4-NQO, bleomycin and alkylating agents (Friedberg, 1991). However, it seems that *RAD18* is only involved in PRR. Physical interactions between Rad6 and Rad18 have been identified (Bailly et al., 1994). The Rad6-Rad18 heterodimer possesses a Ubc activity, a ssDNA binding activity and an ATPase activity (Bailly et al., 1997a). It has been postulated that the function of this heterodimer is similar to that of RecA in *E. coli.*
Rad18 binds ssDNA formed at a stalled replication fork and targets Rad6 to the site of damage (Bailly et al., 1997a; b).

Since PRR is centrally controlled by RAD6 and RAD18, in this study, UV radiation and the radiomimetic agent MMS were chosen to test RNR3-lacZ induction in rad6 and rad18 deletion mutant strains. Compared with wild type strains, the induction of RNR3-lacZ decreased dramatically after MMS treatment in both rad6 and rad18 null mutant strains (Fig. 4-10). Since the RNR3-lacZ induction in rad6 mutants was compromised more severely than in rad18 cells, only rad18 cells were tested for its response to UV radiation in this study (Fig. 4-11).
Fig. 4-10. Comparison of the RNR3-lacZ induction in the wild type DBY747 (∇) with that in the rad6 deletion mutant (●) and the rad18 deletion mutant (●) after MMS treatment. The yeast cells carrying pZZ2 (RNR3-lacZ) were incubated in SD-Ura medium, treated with MMS as indicated and incubated for additional four hours before assaying β-galactosidase activity. The result was an average of three independent experiments. The fold of induction was calculated as a ratio of β-galactosidase expressed in cells with and without treatment in the same experiment.
Fig. 4-11. Comparison of \textit{RNR3-lacZ} induction in wild type DBY747 strains (\ast) with that in \textit{rad18} strain (\sigma) after UV radiation treatment. The yeast cells carrying pZZ2 (\textit{RNR3-lacZ}) were incubated in SD-Ura medium, treated with UV as indicated and incubated for additional four hours before assaying \(\beta\)-galactosidase activity. The result was an average of three independent experiments. The fold of induction was calculated as a ratio of \(\beta\)-galactosidase expressed in cells with and without treatment in the same experiment.
CHAPTER V. DISCUSSION

5.1. The yeast system is suitable for genotoxicity testing

Microorganism based short-term genotoxicity testing is important in the assessment of environmental toxins and carcinogens. The Ames test is used to determine whether an agent is a mutagen and the results from the Ames test correlate highly to its action as a rodent carcinogen (Ames et al., 1972; 1975; McCann et al., 1975; 1983). Since its first development, it has been extensively employed for several decades. In general, this method is sensitive and convenient to perform, but is less effective in detecting DNA damaging agents that are mainly toxic but not mutagenic. A second method is based on the potential cytotoxicity of an agent. By performing a quantitative killing experiment, the toxicity of the test agent can be determined. The SOS Chromotest is based on cellular responses to DNA damage, especially based on the noticeable induction of gene expression. This method appears to be highly sensitive; however, a major concern is that the *E. coli* SOS system only responds to a specific type of DNA damage that results in single-stranded DNA recognized by RecA. Most importantly, both the Ames test and the SOS Chromotest employed prokaryotic cells. The differences between eukaryotes and prokaryotes and the fact that the well-known potent carcinogen SDMH cannot be detected in bacterial systems have been discussed in Chapter I. However, in the *RNR3-lacZ* system, SDMH showed high genotoxic potency and induced the *RNR3* expression by more than 8-fold. This perhaps indicates that at least in some cases, yeast as a lower eukaryote is a more faithful model than bacteria to represent mammalian cells in mutagenic and geotoxicity tests. This is likely due to similar DNA
repair, tolerance and response pathways within eukaryotic cells which are different from prokaryotic cells (Friedberg et al., 1995).

In this study, we examined the feasibility of employing the budding yeast DNA damage response to develop a microbial genotoxic testing system. The results allow us to draw several conclusions. Firstly, our RNR3-lacZ testing system responded to treatment with a broad range of DNA damaging agents and a DNA synthesis inhibitor, regardless of whether they are mutagenic or genotoxic, whereas, all the tested non-genotoxic compounds did not induce RNR3-lacZ expression. Secondly, the sensitivity of the RNR3-lacZ system is comparable to that of SOS Chromotests. Thirdly, the reporter cassette can be integrated into the host genome and achieve a stable inheritance, thus facilitating applications beyond laboratory use. Fourthly, as a unicellular organism, S. cerevisiae cells can be manipulated in a manner similar to bacterial cells. Finally, S. cerevisiae, known as a baker’s yeast, is biologically safe and environmentally friendly. In combination with using lacZ as a reporter gene, it allows visual examination through a color reaction and can be potentially developed into a field test.

5.2. **RNR3 gene is the best choice among yeast DNA damage-inducible genes**

Previous studies in our lab on the molecular mechanisms of cellular transcriptional response to DNA-damaging agents (Xiao et al., 1993; Liu and Xiao, 1997; Liu et al., 1997; Zhu and Xiao, 1998; 2001) led to this study to investigate the possibility of using a yeast DNA damage-inducible gene as a reporter to detect genotoxic agents. Results obtained from previous studies from our lab (Liu and Xiao, 1997) as well as other
laboratories (Elledge et al., 1993; Friedberg et al., 1995) indicate that, unlike the case in *E. coli*, *S. cerevisiae* DNA damage-inducible genes appear to respond to different classes of DNA-damaging agents, making yeast more suitable for the development of a genotoxic testing system. Furthermore, it has been observed that different yeast genes not only have different levels of induction after DNA damage, but the kinetics may also be different (Zhu and Xiao, 1998). It was shown in this study that the *RNR3* gene is probably the best choice among all yeast DNA damage-inducible genes studied to date (see section 3-2-1). The *RNR3* gene not only has one of the highest rates of induction after DNA damage, it is also induced at a much lower dose of DNA damage than most other genes. It is interesting to note that in a genome-wide investigation of DNA damage induction in budding yeast, some genes can be induced by MMS up to 250-fold (Jelinsky and Samson, 1999). However, under the same experimental conditions (0.1% MMS for 60 minutes), *RNR3* was only induced by up to 7-fold (Jelinsky and Samson, 1999). It was found that *RNR3* induction reached a peak after 0.02% MMS treatment, and was severely inhibited by higher dose of MMS treatment. Nevertheless, the present results do not rule out the possibility that other yeast genes may be more sensitive to DNA damage and more suitable for the genotoxic test than *RNR3*.

5.3. Quantitative assessment of the *RNR3-lacZ* testing system

5.3.1. Using the “2-fold rule” to evaluate the results

In the Ames test, to determine whether or not the result is positive is a complex question. This problem also exists in SOS chromotest and the present system. A number
of statistical methods and programs have been developed to analyze Ames test data (Margolin et al., 1981; Bernstein et al., 1982; Simpson and Margolin, 1990; Edler, 1992). An approach that has been widely used is referred to as "2-fold rule". The 2-fold method considers a compound significantly mutagenic if its mean number of revertants per plate at any dose is equal to or greater than twice the mean number of revertants per plate in the concurrent control (Cariello and Pieorsch, 1996). A non-statistical procedure has also been widely used to evaluate the results of Ames test (Zeiger et al., 1992). In this procedure, if a substance can produce a reproducible, dose-related increase than over the control group and the dose-related increase is less than two fold of the background, the chemical is considered as a weak mutagen. Most of the data evaluation methods are first used with Ames test. However, they are also applied to SOS system and other testing systems.

In this study, the 2-fold method was used to evaluate the results. There are at least two reasons for applying this method. Firstly, statistical methods tend to complicate the evaluation procedure and are obviously inappropriate for a system such as ours that is only designed for preliminary screening of genotoxic agents. Secondly, although it has been noticed that some aspects of the 2-fold method of analysis do not have a sound scientific foundation (Margolin, 1985; Cariello and Pieorsch, 1996), more than 40% of the recent publications and most of the publications from 1970's to 1990's on the Ames assay and SOS Chromotest still used this method for analysis (Kim and Margolin, 1999). Hence, employing the same evaluation method makes the comparison with previous reports feasible.
In order to make the results more reliable, the variance of the experimental data in
this study was maintained at a very low level (See Chapter III, Table 3-1).

5.3.2. Comparison of the RNR3-lacZ testing results with other genotoxicity testing
systems

Among the tested 14 agents, the two known non-genotoxic agents showed negative
results in the RNR3-lacZ system. With regard to the other 12 agents, by the 2-fold rule,
most of the results correlated with the Ames test and the SOS Chromotest very well
(Table 3-1). According to the results from MMS, EMS, HU and H2O2, the lowest
response dose of this system is comparable to or even better than the SOS Chromotest
(See section 3-2-2). One agent (SDMH) showed positive result that was different from
the standard Ames test which was discussed in Chapter III to demonstrate that it was one
of the advantages of using the yeast system. The result from EtBr basically agreed with
the SOS Chromotest and Ames test. It showed uncertain results in both Ames test
(Mamber et al., 1986) and SOS Chromotest (Mamber et al., 1986; Quillardet and
Hofnung, 1993), and the RNR3-lacZ system evaluated it as a non-genotoxic agent.

Ethidium bromide is an intercalating agent and commonly used as a non-radioactive
marker to identify and visualize nucleic acid bands in electrophoresis and in other
methods of gel-based nucleic acid separation. Intercalating agents often have a flat,
multi-ring aromatic group of similar size as a nucleotide base pair. These compounds can
insert or intercalate between adjacent base pairs, thus pushing the nucleotides apart.
When a single-stranded DNA containing an interacting molecule is used as template
during DNA replication, an extra nucleotide is often added to the newly synthesized chain, resulting in a +1 frameshift mutation. If this process occurs during transcription, the DNA reading frame for RNA synthesis is changed and produces an altered protein. However, it should be noted that not all the intercalating agents cause frameshift mutations. That is probably the reason why EtBr does not induce RNR3-lacZ expression. Result obtained from RNR2-GFP and RAD54-GFP systems (Afanassiev et al., 2000) agree with the negative evaluation of EtBr. Another possible explanation for the negative results is the employment of the sensing genes. The corresponding sensing genes used in these systems may not respond to the specific DNA damage caused by EtBr. It remains possible that the use of appropriate sensing genes will lead to different results.

5.3.3. The threshold of genotoxicity tests

It has been commonly accepted that risk assessments of genotoxic chemicals are based on linear extrapolation methods and therefore the risk at low doses is considered to be directly proportional to that at high doses. However, there is substantial evidence that some chemicals may be genotoxic only at high doses by mechanisms that do not occur at low doses. In the present study, it was found that for some substances, the induction does not increase linearly (Fig. 3-2-1; Fig. 3-2-2). This may to some extent reflect the threshold dose for cells and the threshold dose may reflect the cellular repair capacity that undermines low dose DNA damage. In this study, a wide range of doses were tested to avoid the situation that all experimental doses were below the possible threshold dose. In general, the highest dose set for each agent was around LD50.
Several mechanisms may be responsible for the existence of threshold dose. In this study, the cell wall of yeast may reduce the availability of some types of chemicals inside cells and therefore delay the cellular response. Another possible mechanism is the organism's tolerance to DNA damage. During evolution, organisms, from prokaryotic organisms to both lower and higher eukaryotic organisms, developed different mechanisms to tolerate DNA damage at the cost of decreased genomic stability. In this system, the capability of tolerance may result in the non-responsiveness of cells to low dose treatment.

5.4. Improvement of the RNR3-lacZ system

5.4.1. Alteration of host strains to enhance the sensitivity of RNR3-lacZ system

In order to further enhance the sensitivity of the RNR3-lacZ system, several deletion mutants defective in different DNA repair pathways were employed as host strains and compared with wild type cells. All the mutant strains tested were created by a one-step targeted gene deletion of the same parental strain DBY747 (Chen and Samson, 1991; Xiao et al., 1996; 2001). Thus, the difference in the response to treatment can be solely attributed to the gene(s) under investigation. The results indicated that the deletion of DNA repair genes variably affected RNR3-lacZ induction by genotoxic agents. Nevertheless, in some cases, the deletion of a DNA repair gene would enhance the response of the RNR3-lacZ system to agents that primarily repaired by the corresponding pathway, and this enhancement of sensitivity is potentially applicable to the improvement of RNR3-lacZ testing system.
Comparing with wild type cells, the magl null mutation enhanced the sensitivity of the RNR3-lacZ system to MMS and EMS. Since MAGl encodes the DNA glycosylase that is specifically involved in the repair of alkylated lesions (Chen et al., 1989) and plays a critical role in the BER pathway, it is not surprising to observe increased RNR3-lacZ induction after treatment with alkylating agents. It indicates that inefficient repair of alkylated damage amplifies the messages sent to the regulation system and enhances the transcription of RNR3. In contrast, RNR3-lacZ induction in the magl mutant strain remains unaltered after UV treatment and dramatically decreased after γ-ray treatment. These results collectively suggest that the magl mutant strain can be used in the RNR3-lacZ test to detect DNA alkylating agents.

The Rad2 protein is critical for NER (Habraken et al., 1993). The mutation of its human homologue, XP-G protein, results in hereditary cancer. In this study, the RNR3-lacZ induction dramatically increased in the rad2 null mutant strain after UV treatment. The enhanced induction was also seen after treatment with a UV mimetic agent 4-NQO. Eukaryotic NER is primarily responsible for the repair of DNA damage caused by UV, bulky DNA adducts and DNA cross-links (Friedberg et al., 1995). The effects of the rad2 mutation on RNR3-lacZ expression appear to agree with the hypothesis that inactivation of NER is able to enhance the detection of a broad range of genotoxic agents that cause distortion of double-helix DNA structure. Our observations also agree with the enhancement of Ames test by inactivation of uvrB (Moreau et al., 1976). With regard to MMS treatment, although maximum RNR3-lacZ induction in the rad2 mutant is lower than in wild type cells, deletion of RAD2 does enhance RNR3-lacZ induction by MMS at
low doses. This result probably reflects the complicated involvement of NER in the repair of DNA methylation damage in yeast cells (Xiao et al., 1996; 1998). Further investigation is needed for other DNA alkylating agents to assess the usefulness of NER inactivation in the detection of this group of agents by the \textit{RNR3-lacZ} system.

In this study, no mutant strain was found to enhance \textit{RNR3-lacZ} induction after treatment with ionizing irradiation. In both of the \textit{rad50} and \textit{rad52} null mutant strains, \textit{RNR3-lacZ} induction decreased dramatically after γ-ray treatment. It should be noted that although ionizing radiation has been extensively used to induce DSBs, DSBs are only a minor component of the radiation damage. The complex mixture of lesions makes it difficult to determine if a specific biological effect is caused by a given lesion.

It was found that after the deletion of certain DNA repair genes, the basal-level \textit{RNR3-lacZ} expression was increased. For example, in \textit{rad52} cells, the basal-level increased up to 7 fold after γ-ray treatment. The enhanced basal level probably indicates the higher level of spontaneous DNA damage that leads to the de-repression of \textit{RNR3} gene expression. Indeed, \textit{RNR3-lacZ} has been employed as a reporter of endogenous DNA damage in yeast cells (Hryciw et al., 2002). For a genotoxicity testing system, not only is a high-level of gene expression (in this study referred to the specific β-gal activity) desired, but the relative induction potential is also important.

5.4.2. **Considerations to develop a more user-friendly \textit{RNR3-lacZ} system**

While our research was in progress, a testing system based on a \textit{GFP} fusion to yeast DNA damage inducible promoters was reported (Afanassiev et al., 2000). The
authors found that the \textit{RNR2} fusion is more sensitive than the \textit{RAD54} fusion to DNA damage treatment. However, as hinted in previous studies (Elledge and Davis, 1989; 1990) and directly demonstrated in this study, the fold induction of \textit{RNR2} is much less than that of \textit{RNR3}. Furthermore, the \textit{GFP} fusion protocol described in that study requires overnight (14-16 hours) incubation of cells with the test agents even though \textit{RNR2-GFP} was carried in a high-copy plasmid. Nevertheless, the idea of using a 96-well plate and automation is attractive, and may be complementary to our approach of employing a more sensitive \textit{RNR3-lacZ} reporter and stable gene integration.

In fact, the SOS-Chromotest kit (EBPI, Ontario, Canada) has been in use since 1982. It is licensed from Institute Pasteur in France. The reporter used in this kit is the same as that of SOS-Chromotest, however, the reaction is carried out on a 96-well plate. In addition to the reaction reagent, only a plate reader is needed. This is much faster and less costly than the \textit{RNR2-GFP} system.

The present \textit{RNR3-lacZ} test is carried out in test tubes. The advantage of this system is that it is time and cost saving. To make the system more user-friendly and suitable for field tests, during the present study, solid \(\beta\)-gal assay was considered initially to replace the liquid assay. It was found that the replacement made the system even more complicated in treating the cells and quantifying the response. The SOS Chromotest kit inspired us to consider the possibility of developing the \textit{RNR3-lacZ} system for field application.
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