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The Function of the *Saccharomyces Cerevisiae* Ribonucleotide Reductase Second β Subunit in DNA Repair

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The Function of Ribonucleotide Reductase Second β Gene in Yeast DNA Repair

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RÉSUMÉ

Les réductases de ribonucléotides (RNRs) catalysent la réduction des quatre ribonuclósides diphosphates en deoxyribonucleosides diphosphates et jouent également un rôle central dans le contrôle de la concentration intracellulaire des deoxynucleosides triphosphates (dNTPs). Ces deux étapes sont exigées pour le processus de réplication d'ADN à haute fidélité, ainsi que pour la réparation d'ADN. Dans les saccharomyces cerevisiae, les RNRs constituent des tétramères α2β2 représentés sous forme de deux grandes et de deux petites sous-unités. Parmi ces réductases, Rnr2 codent l'un des 2 petites sous-unités β. Un deuxième gène essentiel codant une petite sous-unité homologue a été identifié en 1997, il s'agit du *RNR4*. Dans la présente étude, nous sommes les premiers à analyser le rôle de Rnr4 dans la réparation d'ADN, en utilisant les contraintes du mutant rnr4Δ ainsi que les contraintes parentales (S. *cerevisiae* BY4741). Ceci était réalisé après un traitement avec différents types d'agents endommageant l'ADN. Voici quelques exemples d'agents de dommage d'ADN : Bleomycine (BLM, agent de stress oxydatif), 4-nitroquinoline-1-oxide (4-NQO, inducteur d'effets de dommage identiques à ceux des UV, UV-mimetic DNA damage agent) et Methanesulphonate méthylique (MMS). La contrainte du mutant rnr4Δ était sensible au BLM et au MMS en comparaison avec la contrainte parentale. L'analyse de la survie a révélé que la contrainte du mutant rnr4Δ avait un taux de survie significativement plus élevé que son parent après une heure de traitement avec le 4-NQO mais moins élevé après seulement 4 minutes de traitement avec les UV. La forte résistance à 4-NQO chez le mutant rnr4Δ après une heure vs son

parent est de 50% ± 1.9 vs. 6% ± 1.1= Δ vs. wt, 3μg/ml 4-NQO, $p<0.01$. L'analyse de la mutagenèse a démontré que le taux de mutation de rnr4Δ était significativement plus bas que celui du parent lors de l'administration de 4-NQO. Les résultats suggèrent alors que 4-NQO induit des dommages d'ADN identiques à ceux des UV en plus d'autres dommages. Nous avons démontré que les dommages d'ADN induits par 4-NQO ne soient ni "des dommages UV-MIMETIC d'ADN" ayant lieu principalement dans la voie de la réparation d'excision de nucléotide (REN), ni du stress oxydatif induit par BLM. Nous avons donc suggéré qu'un nouveau mécanisme de lésion d'ADN causée par 4-NQO existe. Cette lésion pourrait être réparée par la méthode 'error free DNA repair'. Ceci a lieu probablement par une voie de translésion (TLS) peu importe le pool de dNTP. Un bas pool de dNTP chez le mutant rnr4Δ mène à un plus haut taux de survie et un taux bas de mutation en prévenant la voie TLS mais sans aucun effet sur 'error free DNA repair'. De plus, une grande concentration de dNTP permet aux cellules parentales de réparer la lésion via la voie TLS causant ainsi beaucoup d'erreurs et conséquemment un bas taux de survie en augmentant le taux de mutation.

Nous avons finalement conclu que la faible concentration de dNTPs chez la contrainte mutant RNR4 ne joue pas le rôle dans la réparation de la lésion d'ADN provoquée par ce mécanisme.

Les mots clés :

Deuxième sous-unité β de la réductases de ribonucléotide (RNR4)

4-nitroquinoline-1-oxide(4-NQO)

Réparation d'ADN

ABSTRACT

Ribonucleotide reductases (RNRs) catalyze the reduction of all four ribonucleoside diphosphates into deoxyribonucleoside diphosphates and play a central role in controlling the pool size of cellular deoxynucleoside triphosphates (dNTPs), required for high fidelity DNA replication and DNA repair processes. In *Saccharomyces cerevisiae*. ribonucleotide reductase is an α2β2 tetramer consisting of two large and two small subunits. *RNR2* encodes one of the 2 small β subunits. A second essential gene *RNR4* encoding a homologous small β subunit was found in 1997. In the present study, we investigated for the first time the role of Rnr4 in DNA repair, using *rnr4*Δ mutant strains and parent strains (S. *cerevisiae* BY4741) after treatment with different type of DNA damage agents such as bleomycin (BLM, an oxidative stress agent), 4-nitroquinoline-1-oxide (4-NQO "UV-mimetic" DNA damage agent), methyl methanesulphonate (MMS). *rnr4*Δ mutant strain was sensitive to BLM and MMS as compared with parent strain. More interestingly, survival assay demonstrated that as compared with his parent, *rnr4*Δ mutant strains have significantly higher survival rate after 1 hour of treatment with 4-NQO (50% ± 1.9 vs. 6% ± 1.1= Δ vs. parent, 3μg/ml 4-NQO, $p<0.01$), but has significantly lower rate after 4 minutes of treatment with UVC. Mutagenesis assay showed the mutation rate of *rnr4*Δ mutant is significantly lowered as compared with the parent when 4-NQO is administrated. Taken together, our results suggested that besides "UV-mimetic" DNA damage, 4-NQO could lead to other DNA damage mechanism. This DNA damage is neither a "UV-mimetic DNA damage, which is processed largely by the nucleotide excision repair (NER) pathway nor oxidative stress like BLM induced, but a

different DNA lesion mechanism causing by 4-NQO. This DNA lesion could be repaired in both an error free DNA repair manner (no matter in lower dNTPs pool or in higher dNTPs pool) perhaps using a translesion (TLS) pathway. The lower dNTPs pool in *rnr4*Δ mutant strains prevent TLS pathway but doesn't affect an error free DNA repair manner and lead to higher survival rate but lower mutation rate. On the other hand, the higher level of dNTPs pool allowed parent cells to repair this lesion using the TLS pathway, which cause lots of errors and consequently lower survival rate by the higher mutation rate.

Key words:

Ribonucleotide reductase second β subunit (Rnr4)

4-nitroquinoline-1-oxide(4-NQO)

DNA repair

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LIST OF ABBREVIATIONS

4-NQO	4-nitroquinoline-1-oxide
A	adenosine
Å	angstrom
ATP	adenosine triphosphate
BLM	bleomycin
bp	base pair
BSA	bovine serum albumin
C	cytosine
cDNA	complementary DNA
°C	degree Celsius
dATP	2'-deoxyadenosine 5'-triphosphate
dNTP	deoxynucleotide triphosphate
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
EDTA	ethylenediaminetetra acetic acid
E. coli	Escherichia coli

Fig.	figure
G	guanosine
g	gram
GDP	guanosine diphosphate
HE buffer	10 mM Hepes-KOH, pH 7.0, 1 mM EDTA
[^{3}H]TTP	[3H]thymidine 5'-triphosphate
kb	kilobase (1000 bp)
L	litre
mM	millimolar (10^{-3} M)
M	molar (moles/litre)
ml	milliliter (10^{-3} L)
MMS	methyl methanesulfonate
mRNA	messenger RNA
NER	nucleotide excision repair
ng	nanogram (10^{-9} gram)
nt	nucleotide
o/n	over night (roughtly 12 hours)
^{32}P	radioactive isotope of phosphorus
PAGE	polyacrylamide gel electrophoresis

PBS	phosphate buffered saline
PCA	phenol:choroform:isoamyl alcohol (25:24:1)
PCR	polymerase chain reaction
pmol	picomole
RNA	ribonucleic acid
RNase A	ribonuclease A
RNR	Ribonucleotide reductase
Rnr1/R1/Y1	Ribonucleotide reductase α-subunit
Rnr3/R3/Y3	Ribonucleotide reductase α-subunit
Rnr2/R2/Y2	Ribonucleotide reductase β-subunit
Rnr4/R4/Y4	Ribonucleotide reductase β-subunit
RNR	Ribonucleotide reductase gene
RNR1	R1 gene
RNR2	R2 gene
RNR3	R3 gene
RNR4	R4 gene
RPA	Replication protein A
RR inhibitor	Ribonucleotide reductase inhibitor
SDS	sodium dodecyl sulfate

T	thymidine
TE	10 mM tris, 1 mM EDTA pH 8.0
Tris	tris(hydroxy methyl)aminoethane
TLS	Translesion repair
UVC	ultraviolet C (wavelength range 220-280 nm)
μg	microgram (10^{-6} gram)
μl	microlitre (10^{-6} L)
μM	micromolar (10^{-6} M)
YPD	1% yeast extract, 2% peptone, 2% dextrose
YPDa	YPD + 2% agar

This Thesis Is Dedicated to My Family for

Their Love, Support and Encouragement

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CHAPTER I: INTRODUCTION

SECTION 1.1: General Review

Ribonucleotide reductase (RNR) is a well known and probably the most investigated free radical enzyme (Thelander and Reichard 1979; Jordan and Reichard 1998; Eklund et al., 2001). Ribnucleotide reductases have been classified into three main classes according to the free radical generator (see Section 1.2). A wealth of study on structure and function of the class I and class III enzymes has been done, that provided detailed views on how these enzymes perform their tasks.

The RNR catalyses the reduction of ribonucleotides to deoxyribonucleotides, which is the rate-limiting step of DNA synthesis, and control of the optimal levels of dNTPs, which are required for DNA replication and DNA repair processes; a failure to control the size of dNTP pools and/or their relative amounts leads to cell death or genetic abnormalities. An enzyme of this type is also believed to be needed for the production of the DNA precursors at the time of the transition from the RNA to the DNA (Thelander and Reichard 1979). The common feature of the different classes of RNR is the initiation of the reaction by removal of the 3'-hydrogen of the ribose by a transient cysteinyl radical. The class I enzymes demonstrate a sophisticated pattern as to how the free radical is used in the reaction, in that it is only delivered to the active site at exactly the right moment (Eklund et al., 2001).

The RNR activity has been known to be transcriptionally regulated and is cell cycle dependent in higher organisms (Bjorklund et al, 1993). Normally, the overall enzymatic

activity is regulated by ATP (activation) and dATP (feedback inhibition at high concentrations). A unique additional allosteric regulation controls the substrate specificity such that a balanced supply of the different deoxyribonucleotides is present during DNA synthesis (Eklund et al., 2001).

The role of RNR in DNA synthesis is so important that cell proliferation makes it highly interesting in the context of anti-bacterial, anti-viral and anti-cancer drug development. Just because of this, a great increase in interest on RNR as a target for cancer therapy has been observed recently, since a human ribonucleotide reductase regulated by p53 was identified (Lozano and Elledge 2000; Nakano and Vousden 2000; Tanaka and Nakamura 2000). The p53 protein actively suppresses tumor formation and when it is mutated, several kinds of cancer may develop. Moreover, more than 80% of human tumors have been found to contain mutations in p53 or the pathway that directly regulates it.

Mammals, *E. coli* and DNA viruses such as herpes viruses employ class I reductases, in which an iron-containing protein for the generation of the catalytically essential free radical is used. In these reductases, the quaternary organization of the holoenzyme is made up of the α dimer, called protein Rnr1, contains the active sites and binding sites for allosteric effectors, and the β dimer, called protein Rnr2, contains one dinuclear iron center and one stable tyrosyl radical per monomer, which are both essential for enzymatic activity. During the passed ten years, most of the structure function studies on class I RNRs have been performed on the *E. coli* enzyme (Fontecave and Eklund

1993) and mouse RNR. Except a normal gene for Rnr2, yeast also contains a homologous gene that codes for a Rnr4 protein lacking important iron ligands, which cannot form an active Rnr2. Although it is known that Rnr2 is unable to fold correctly by itself and is thus unable to form an iron-radical center, instead, Rnr4 has the crucial role of correctly folding and stabilizing an active Rnr2-Rnr4 complex (Chabes and Thelander 2000), but the real role of Rnr4 in DNA synthesis is not very clear yet.

SECTION 1.2: Three Different Classes of RNR

All of different RNR have radical generator; this has been the common feature of different RNR. The radical generator produces and stores a radical, which, as a first step of the reaction, is used to oxidize the substrate to a radical form. Surprisingly the radical generator is different for all RNRs, whereas the reductase component is more similar. Three main classes of ribonucleotide reductases have been described (Table 1), classified according to the radical generator (Reichard 1993). Class I enzymes produce a stable tyrosyl radical on one type of protein subunit through the action of a dinuclear iron center. Class II enzymes use a radical on the cofactor cobalamin. Class III enzymes form a stable glycyl radical with the help of an iron–sulfur protein and S-adenosyl methionine.

Table 1. Different Classes of RNR

Class	Occurrence	Subunit Structure	Cofactor	Active Radicals	Reduction System
Ia	Mammals Plants Yeast DNA. viruse *E.coli*	$\alpha_2\beta_2$	Fe^{3+}-O^{2-}-Fe^{3+}	Tyrosyl··cysteinyl·	Thioredoxi/glutaredoxin
Ib	Prokaryotes	$\alpha_2\beta_2$	Fe^{3+}-O^{2-}-Fe^{3+}	Tyrosyl··cysteinyl·	Redoxin
II	Prokaryotes	α and α_2	Adenosyl cobalamin	Adenylyl··cysteinyl·	
III	Prokaryotes (anaerobs)	$\alpha 2 + \beta 2$	[Fe-S] S-adenosin-methionine	Glycyl··cysteinyl·	Formate

PART 1: Class I

Class I enzymes are found practically in all eukaryotic organisms, from yeast and algae to plants and mammals, some prokaryotes and some viruses. They are all oxygen-dependent. Class I is further divided into two subclasses, Ia and Ib, according to their polypeptide sequence homologies and their overall allosteric regulation behaviour (Jordan and Reichard 1994). Class Ia exists in eukaryotes, prokaryotes, viruses, and bacteriophages, whereas Ib has only been found in prokaryotes. Class I RNRs are tetrameric enzymes and show αβαβ structure (Reichard 2002). The substrate binding active site is located in the large α- homodimer, which is called R1 in class Ia. The small β-homodimer contains a binding site for two ions in each polypeptide chain and is called R2 in class Ia. Because of the unusual β-subunit composition in the class Ia RNR from *Saccharomyces cerevisiae*, ααββ' (Huang and Elledge 1997), where only one of the R2 subunits (β) can harbour a diiron centre, the class Ia could also be divided into two subclasses, Ia1 and Ia2. However, the situation in *S. cerevisiae* seems to be quite exceptional, since even closely related yeast strains, such as *Schizosaccharomyces pombe* (Wood et al., 2002), won't be able to express this type of heterodimers.

For mammalian RNR, the mouse enzyme might be the best-studied model. There are a number of significant differences between the *E. coli* RNR and RNR in higher organisms. The sequence identity between the mouse R2 and the *E. coli* R2 is only 19%, whereas there is 50%, or higher, identity among the eukaryotic R2 proteins (Kauppi et al.,

1996; Demple et al., 1986) . For class Ib, the enzyme from *Salmonella typhimurium* is regarded as the prototype (Jordan et al., 1994).

PART 2: Class II

The classes II RNRs contain only one type of subunit, which are α or β proteins. The necessary radical is produced by adenosylcobalamin (Booker and Stubbe 1994). The radical is formed by cleaving the adenosyl-Co bond. This type of RNR is found in some microorganisms, while the best-studied example of this type RNR is from Lactobacillus leichmanni. Recently, several class II enzymes have been characterized, the sequences of these enzymes in catalytic domains are distantly related to the class I and III ribonucleotide reductases (Riera and Fontecave 1997).

PART 3: Class III

Class III anaerobic reductases use a glycine radical (Sun, and Sjoberg 1996), which is generated with the help of S-adenosyl methionine and an iron–sulfur cluster (Eliasson and Reichard 1990). The active form is a α2 dimer (Padovani et al., 2001); it contains the active site glycyl radical and binding sites for the allosteric effectors. It thus corresponds in most respects to the substrate binding R1 subunit of the aerobic reductases. The β2 subunits, a small iron–sulfur-containing protein, is essential for production of the glycyl radical. (Sun, and Reichard 1995) The overall reductant is formate (Mulliez, and Reichard 1995) rather than the enzymatic systems employed by class I.

SECTION 1.3: Structure of RNR

E. coli employ class I reductases; it use an iron-containing protein for the generation of the catalytically essential free radical. In this type of reductases, the quaternary organization of the holoenzyme is made up of the α dimer, called protein R1, contains the active sites and binding sites for allosteric effectors, and the β dimer, called protein R2, contains one dinuclear iron center and one stable tyrosyl radical per monomer; they are both essential for enzymatic activity.

High-resolution structures of several forms of *E. coli* R1 (Uhlin and Eklund 1994;Uhlin and Eklund 1996), R2 (Logan, et al., 1996; Aberg and Eklund 1993) and a plausible model for the R1:R2 holoenzyme (Padovani and Fontecave 2001) are now available. Unless stated otherwise the following discussion concerns the *E. coli* enzyme.

PART1: R1 Protein

The R1 protein is the large homodimer (2x85.5kDa) mediates both catalysis and allosteric interactions. Figure 1 shows the high-resolution structure of the R1 protein. Each monomer consists of three domains: one mainly helical domain comprising the 220 N-terminal residues; a barrel domain (480 residues), novel ten-stranded β/α barrel; and a small β/α domain comprising 70 residues. The barrel is composed of two halves connected in an antiparallel way, each containing five parallel strands and four connecting helices (Uhlin and Eklund 1994;Uhlin and Eklund 1996)

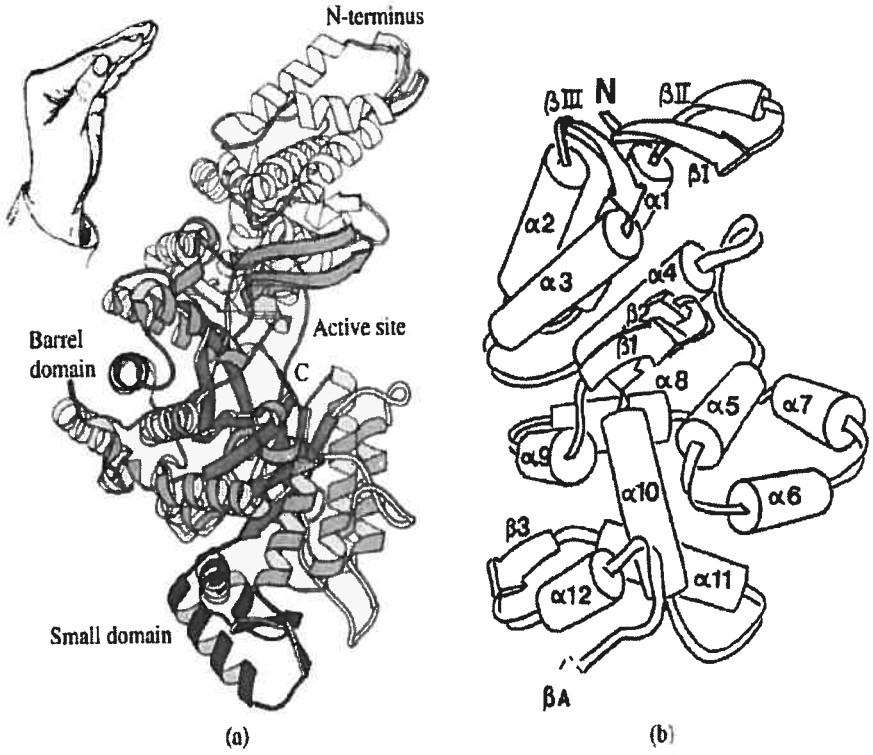


Figure 1. Structure of *E. coli* R1 Protein

PART 2: R2 Protein

Several excellent reviews (Fontecave and Reichard 1992; Graslund and Sahlin 1996) have discussed the R2 structure. Briefly, four carboxylates and two histidines organized the two μ-oxo linked irons of the oxidized center. On reduction the iron center is still coordinated by the same carboxylate-dominated ligand sphere, but several of the carboxylate-dominated ligands have been changed in conformation, with loss of the oxobridge and of two bound water molecules, and a decrease of the coordination number from six to four. The iron center has opened up and become accessible to dioxygen, a prerequisite for radical generation on the neighboring Y122.

PART 3: The Holoenzyme

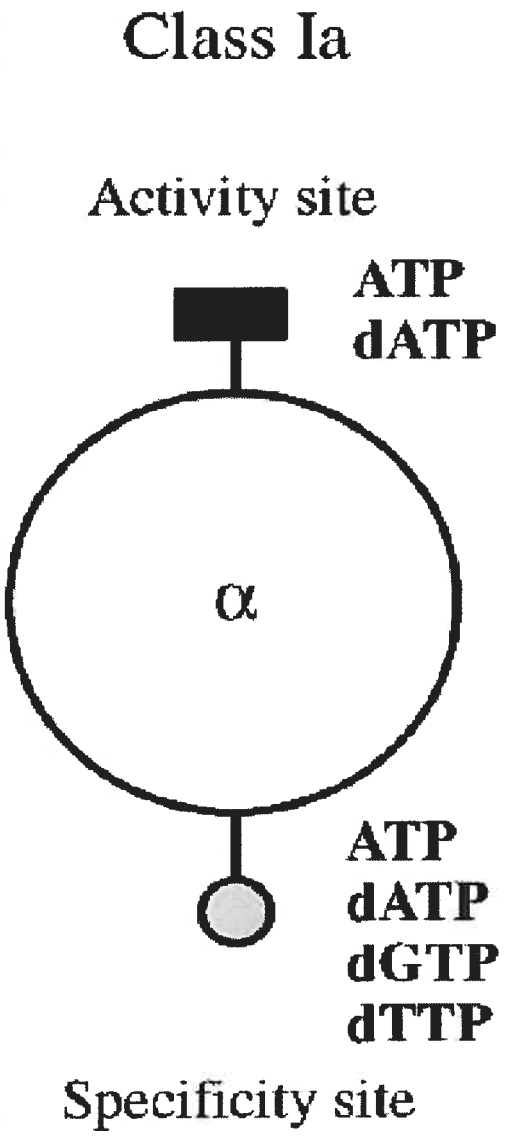
Crystals of the R1:R2 complex suitable for structure determination has not been obtained yet. However, a plausible model for the holoenzyme was created, mainly from considerations of symmetry, complementarity, and conserved amino acid residues. (Uhlin and Eklund 1994) The C terminus of R2 interacts with a region close to the C terminus of the R1 protein with some species-specificity. Just base on this model form, people designed peptidomimetic drugs that specifically inhibit the activity of the herpes reductase but not the mammalian enzyme. (Liuzzi et al., 1994)

PART 4: Allosteric Regulation

In general, allosteric regulation rapidly adapts an enzyme to changing requirements for its product by binding of effectors, which may efficiently increase or decrease its activity. Most class Ia reductases are regulated by ATP and dATP when binding either ATP (activating) or dATP (inhibitory) to the activity site of protein R1 (Brown and Reichard 1969). However, an additional and unique allosteric mechanism that regulates their substrate specificity and ensures that the enzyme produces equal amounts of each dNTP for DNA synthesis functions in the RNR regulation system. Disturbances in pool sizes may lead to genetic damage and in severe cases to cell death (Brown and Reichard 1969; Kunz et al., 1994). By binding of end products (dATP, dGTP, and dTTP) to the specificity site of the reductase this result may be prevented (Brown and Reichard 1969). The allosteric sites communicate with the substrate-binding site and they affect each other at the same time. A detailed model for class Ia enzymes involving various effectors and substrates was developed in 1979 (Thelander and Reichard 1979). It has been surviving well over the years. Figure 2 illustrates effector binding to the allosteric sites from class Ia reducates.

Allosteric regulation of the enzymes from different classes may shows similarities or differences. Here we talk about Class Ia only.

Figure 2. Models for the allosteric regulation of the ribonucleotide reductase classes



SECTION 1.4: Gene Organizations and Regulation of Enzyme Synthesis

Ribonucleotide reduction plays a central role in the regulation of the pool sizes of the four dNTPs required for DNA synthesis, even though deoxynucleoside kinases and nucleotidases are also important (Reichard 1988). The small dNTP pools suffice in some cells for only a few minutes of DNA replication and must therefore be renewed continuously during S-phase. The dNTPs are also required for DNA repair and mitochondrial DNA replication by cells that are not in S-phase. Cells also synthesize dNTPs de novo, albeit at a much slower rate (Bianchi and Reichard 1997). To satisfy the changing demands for dNTPs, the synthesis of ribonucleotide reductases is tightly adapted to the cell cycle throughout gene organization or regulation.

In S. *cerevisiae*, four genes involved in ribonucleotide reduction: *RNR1* and *RNR3* encode R1 proteins, *RNR*2 encodes an R2 protein (Elledge et al., 1992; Elledge et al.,1993), and *RNR4* encodes an R2-like protein (Wang et al., 1997). Rnr1 and Rnr2 are essential for normal growth. Rnr3 is not, but when present on a high-copy number plasmid, complements mutations in *RNR1*. *RNR4* is also required for viability but the coded protein by itself has no R2 activity. It may be required for the formation of a functional holoenzyme complex (Wang et al., 1997).

SECTION 1.5: Anti-tumor Activity of Ribonucleotide Reductase

As theoretically expected, some RNR inhibitors have been demonstrated to possess antitumor properties. They have been proved to be able to kill tumor cells preferentially with respect to normal cells (i.e., they have a sufficiently high therapeutic index). The high therapeutic index of antimetabolites is generally explained by the commitment of the neoplastic cells to replication and by their decreased adaptability and low responsiveness to regulatory signals making them more vulnerable than normal cells to drug-induced perturbations (Weber 1983). Some investigators recently used this reasoning specifically to the antitumoral activity of RR inhibitors.

It is known that before the progression of the cell to the next cycle stage, the control of factors, which acts as checkpoints, must ensure that the previous stage has been completed. Just because of this, one hypothesis was developed, that the effect of RR inhibitors (low concentration) on normal mammalian cells is just cytostatic because they are able to control cell cycle progression, while tumor cells have lower or no ability to control cell cycle progression and are more easily killed by RR inhibitors.

However, it is not known whether the drug-induced imbalanced growth is the main mechanism responsible for in vivo antitumoral activity of RR inhibitors or it is somehow related to the apoptotic death induced by these agents. Indeed, it is sufficient to note that, despite extensive research and the finding of a great number of powerful

compounds, hydroxyurea, a relatively weak RR inhibitor, is still the most used RR inhibitor in clinical settings.

SECTION 1.6: Yeast RNR (1-4)

The RNR gene in common bakers yeast S. cerevisiae is comprised of four genes: *RNR1, RNR2, RNR3* and *RNR4*. *RNR1* and *RNR3* encode polypeptides for the large subunit, called Rnr1 and Rnr3 (Elledge and Davis 1990), and *RNR2* and *RNR4* encode polypeptides for the small subunit, called Rnr2 and Rnr4 (Wang et al., 1997).

The different roles of Rnr1 and Rnr3 are not very clear now. Rnr1 and Rnr3 share 80% amino acid sequence identity, all the essential amino acid residues involved in catalysis and allosteric regulation are included in both Rnr1 and Rnr3.

It has been demonstrated that there are three dimeric combinations of these two polypeptides (i.e. R1R1, R1R3, and R3R3). All are catalytically active, although the specific activity of R3 dimer is significantly lower than that of the R1 dimer (Domkin et al., 2002). Expression of Rnr1 is essential for the cells to enter mitosis, and the cell cycle regulated the transcription of Rnr1, however, it seems that no expression of *RNR3* is explicitly demanded. The level of *RNR3* expression is very low during normal cell living; however, it is strongly inducible by DNA damage when the expression level can increase by a factor of up to 100 (Elledge and Davis 1990).

The differences between the two genes encoding small subunit, Rnr2 and Rnr4, are more striking in terms of the presence of functionally important amino acid residues. While R2 contains all the 16 critical residues conserved in almost all R2 proteins, six of

these residues are missing in R4, including three that would be expected to be involved in iron binding.

Moreover, alignments of the amino acid sequences of R2 and R4 reveal only 47% identity, and R4 is about 50 amino acid residues shorter at the N-terminus than normal R2 (Wang et al., 1997).

Deletion of R4 is lethal in some yeast strains (for example, S. cerevisiae) and impairs cell growth in others, indicating an important role of R4 in RNR function.

R2 and R4 can form both homodimeric and heterodimeric complexes, but only complexes involving R2 are catalytically active.

It has been proposed the important role of R4 in RNR is to deliver ions to R2 or/and to stabilize R2 in the proper conformation for ion cluster assembly and radical formation (Ge et al., 2001).

The crystal structure of the yeast R2R4 heterodimer has been solved (Voegtli et al., 2001) and the overall α-helical fold is very similar to other homodimeric class I enzymes, such as *E. coli* and mouse.

During the normal cell cycle, *RNR1* and *RNR3* are known to be predominantly localized to the cytoplasm and *RNR2* and *RNR4* are known to be predominantly present

in the nucleus. However under genotoxic stress (HU, MMS), *RNR*2 and *RNR*4 become redistributed to the cytoplasm in a checkpoint-dependent manner. Subcellular redistribution of *RNR2* and *RNR4* can occur in the absence of the transcriptional induction of the RNR genes but could be happened after DNA damage and likely represents a post translational event. These results strongly suggest that DNA damage checkpoint modulates RNR activity through the temporal and spetial regulation of its subunits. (Yao et al., 2003)

SECTION 1.7: Research Objective

*rnr4*Δ strain was initially isolated in genome wide screen for mutants that are sensitive to Bleomycine. Following cross examination of the mutant against a variety of the DNA damaging agents (MMS, 4-NQO, HU and H_2O_2). We observed that *rnr4*Δ strain was extremely resistant to 4-NQO.We therefore decide to characterize this observation. Herein we examined:

1. Whether there is any difference in sensitivity between the wild type cells and *rnr4*Δ mutant cells, while exposed to different kind DNA damage reagents, for example, 4-NQQ, BLM, MMS.
2. If so, what kind of DNA mechanisms lead to those difference in drug sensitive.

CHAPTER II: MATERIALS AND METHODS

SECTION 2.1: Strains and media

The wild-type strains used were *S. cerevisiae* BY4741. The collection of nonessential haploid *rnr4*Δ strains, derived from the S. *cerevisiae* BY4741, was obtained from EUROSCARF (Frankfurt, Germany).

Standard YPD (1% yeast extract, 2% peptone, 2% dextrose) growth media was used as patch colonies and cultured liquid.

SECTION 2.2: Chemicals and Equipment

Growth culture reagents, yeast extract, peptone and agar, were from Difco (Detroit, MI

4-NQO, MMS, and hydrogen peroxide were from Sigma (Saint Louis, MO).

Bleomycin A5 trihydrochloride was from Calbiochem (La Jolla, CA).

Pfu DNA polymerase was from Stratagene (La Jolla, CA).

Primers (Table.2) were from Invitrogen (Carlsbad, CA)

Running buffer condition: 2.5 mM Tris pH7.0, 19.2 mM Glycine and 0.1% SDS.

Nitrocellulose membrane (Hybonc-C+, Amersham)

Transfer buffer (25 mM Tris pH7.0, 192 mM Glycine and 20% methanol)

Blocking buffer (5% non-fat dry milk in 10 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.1% Teween)

Primary antibody (Sigma)

Washing buffer (TBS containing 0.1% Tween 20)

Anti-rabbit IgG-HRP conjugate (Sigma)

ECL solution (Amersham)

Film (Kodak)

UVC lamp was from Fisher.

Shaker was from New Brunswick Scientific Co., Inc., Edison, N.J.

PCR machine: PTC-100^{TM} Programmable Thermal Controller MJ research INC

Table 2. PCR primers for *RNR4* gene amplification

Name	Sequence
pYEX-A4-F1 (forward)	5'-TTCGATGATGAAGATACCCCACCAAACCCAAAAAAAGAGATCGAAATGGAAGCACATAACCAATTTTTGAAG-3'
PYEX-A4-R1 (reverse)	5'-TTCAGTATCTACGATTCATAGATCTCTGCAGGTCGACGGATCCCTTAGAAGTCATCATCAAAGTTAATTTCCTTGG-3'

SECTION 2.3: Survival Curves

Overnight cultures grown to saturation at 30°C in YPD medium were diluted into fresh YPD medium to an optical density at 600 nm of 0.4 (2 x10^6 cells per ml) and incubated to an optical density at 600 nm of 0.8 to 1.0. Aliquots of the cultures were then treated with BLM (with different time course 0, 10, 20, 30, 40 minutes) at 20 μg/ml or MMS (ranging from 0 to 0.2%) for 1 hour, or 4-NQO (ranging from 0 to 3 μg/ml) for 1 hour, at 30 °C with shaking (250 rpm) in an incubator shaker. Diluted the samples in sterilized water to 10^{-4} and plated 100μl on YPD agar plate. Colonies were counted after 2 to 3 days of growth at 30 °C.

Overnight cultures grown to saturation at 30°C in YPD medium were diluted into fresh YPD medium to an optical density at 600 nm of 0.4 (2 x 10^6 cells per ml) and incubated to an optical density at 600 nm of 0.8 to 1.0. Diluted the samples in sterilized water to 10^{-4} and plated 100μl on YPD agar plates, then treated with UVC (0.4 J per second and per square meters with different time courses in 1, 3, 5, 8, and 10 minutes) at RT. Colonies were counted after 2 to 3 days of growth at 30 °C.

SECTION 2.4: Mutagenesis Assays

Yeast cultures were started from single colonies and grown to stationary phase in liquid YPD at 30 °C, then diluted into fresh YPD medium to an optical density at 600 nm of 0.4 ($2x10^6$ cells per ml) and incubated to an optical density at 600 nm of 0.8 to 1.0. Freshly dissolved 4-NQO was then added to a final concentration of 1.0 μg/ml. Cells were treated with aeration for 1 h at 30°C, washed, and resuspended in water at a density of 10^7 cells per ml. Both treated and untreated cells were plated after appropriate dilutions onto complete medium containing L-canavanine (40 mg/ml) but lacking arginine for Can^r mutant count and complete medium but lacking arginine for viable cell count. Plates were incubated for 3 to 5 days at 30°C before counting. The frequencies Can^r mutants in each culture were calculated by dividing the Can^r mutant count by the viable cell count.

SECTION 2.5: Western Blot

Yeast cell were harvested from liquid culture, washed twice with sterilized water, and centrifuged at 4000 rpm with ss-24 rotor in 4 ^{0}C. The pellet was resuspended in yeast extract buffer (50 mM Tris pH 7.0, 30 mM KCl, 10% glycerol, 1 μg/ml leupeptin, 1 μg /ml aprotinin, 1 mM PMSF, 1 mM DTT). The proteins were homogenized by using a MINI beadbeater (BioSpec Products, Bartlesville, OK) at 5000 rpm for 20 seconds to six times. Cellular debris was partially removed by centrifugation at 9000 g for 10 minutes in SS-24 rotor for 10 minutes. Proteins were quantified according to the method of Bradford.

50 microgram of whole cell lysate per lane was loaded in an SDS-PAGE mini gel. Run gel at 120V/20 mA per gel untill the dye front was close to the bottom. Running buffer condition: 2.5 mM Tris pH7.0, 19.2 mM Glycine and 0.1% SDS.

The proteins was transferred to a nitrocellulose membrane (Hybonc-C+, Amersham) at 100V/250 mA in transfer buffer (25 mM Tris pH7.0, 192 mM Glycine and 20% methanol) for 1.5 h. the blot was incubated with blocking buffer (5% non-fat dry milk in 10 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.1% Teween) for 2h at room temperature.

The blot was incubated with primary antibody (rabbit anti-Rpa32, diluted to 1:500 in blocking buffer) for 1h in blocking buffer at RT. The blot was washed 3 x 10 min in washing buffer (TBS containing 0.1% Tween 20) with shaking.

SECTION 2.6: Primer Extension Assay

Exponentially growing cells in 5 ml of YPD were either treated or untreated with 4μg/ml BLM for 1 h at 30°C. Cells were harvested, washed twice with M9 buffer, and the cell pellet was stored at -80°C for 1 h. Extraction of the chromosomal DNA was performed as usual. To measure the incorporation of [methyl-3H] dTMP, 150 μM of chromosomal DNA in 25 μl of HE buffer (10 mM Hepes-KOH, pH 7.0; 1 mM EDTA) was added to 225 μl of an ice-cold reaction mixture. This mixture was consist of 25 mM Hepes-KOH pH 7.6; 25 mM KCl; 10 mM MgCl2; 50 μg/ml bovine serum albumin; 100 μM dATP; 100 μM dCTP; 100 μM dGTP; 30 μM dTTP, and 3 units of Escherichia coli DNA polymerase per ml. Labeled [methyl-3H] dTTP (NET221X from PerkinElmer Life Sciences; 37.0 MBq) was next added to a specific activity of 1260 cpm/pmol. The reaction was started when the samples were immersed into a 30 °C water bath. At the indicated times, 40 μl samples were withdrawn and added to tubes containing 200 μl of 0.1 M sodium pyrophosphate and 1 mg/ml of bovine serum albumin, followed by the addition of 200 μl of 0.8 M trichloroacetic acid. Samples were vortexed and placed on ice for 10 min. The samples were processed on a 12-hole filtration apparatus (Millipore, Bedford, MA) using GF/C circle filters (Whatman). The trapped DNA was washed three times with 3 ml of 0.1 M sodium pyrophosphate, briefly rinsed with ethanol, air-dried, and counted with 5 ml of scintillation fluid (BCS, Amersham Biosciences).

Incubated blot with anti-rabbit IgG-HRP conjugate (Sigma) (diluted 1:10,000 in blocking buffer) for 1 h in blocking buffer at RT.

Washed 3 x 10 min in washing buffer with shaking. Drained washing buffer, added ECL solution (Amersham) and developed for 1 min. Exposed to X-ray Kodak film for 1 to 30 min.

SECTION 2.7: Preparation of Yeast Genomic DNA

Each single colony of S. *cerevisiae* BY 4741 strains was cultured into 5 ml YPD liquid at 30^0C. In second day, centrifuged at 3600 rpm for 5 minutes, resuspended the cell into 0.5 ml TE in eppendorf tubes. Centrifuged again, resuspended the pellet into 0.5 ml of spheroplast buffer (1 M sorbital, 10 mM NaHPO4 pH7.0, 10 mM EDTA), and added 5 µl 20 µg/ml of lyticase (Sigma) and 3 µl beta-mercapthoethonol (Fisher), incubated 37 ^{0}C for 20 minutes. Centrifuged 15 second in 14000 rpm. Resuspended into 0.5 ml buffer (50 mM EDTA, 0.3% SDS 5 µl protease K 20 mg/ml) incubated 60^0C for 30 minutes. Added 0.2 ml 5 M Potassium acetic acid on ice for 20 minutes, and centrifuged 10 minutes in 12,000-rpm 4^0C. Supernatant were extracted with twice phenol, twice chloroform, precipitated with 2.5 fold of volume 95% ethanol. Centrifuged 10 minutes 12000 rpm 4^0C, added 20 µl TE into pellet.

SECTION 2.8: PCR Program

PCR machine: PTC-100^{TM} Programmable Thermal Controller MJ research INC

Master Mixture: DNA or genomic DNA 10-50 ng, 10x taq buffer 5µl, oligo upstream 0.5µl (1µg/µl), oligo downstream 0.5µl (1µg/µl), 10 mM dNTP 3 µl, 25 mM $MgCl_2$ 3 µl, Tap DNA polymerase 1 µl, add H_2O up to 50 µl.

PCR designed program:

1. 95 ^{0}C 5 minutes
2. 80 ^{0}C 10 seconds, during this period, add pfu DNA polymerase 1 unit.
3. 94 ^{0}C 50 seconds
4. 50 ^{0}C 50 seconds
5. 72 ^{0}C 3 minutes
6. 10 times repeat go to step 3. Each cycle decrease by 1 ^{0}C in step 4.
7. 94 ^{0}C 50 seconds
8. 50 ^{0}C 50 seconds
9. 72 ^{0}C 3 minutes
10. Repeat 20 cycles go to step 7.
11. 72 ^{0}C 10 minutes
12. 4 ^{0}C o/n

SECTION 2.9: Clone of plasmid Gst-rnr4

The entire *RNR*4 coding sequence was amplified by PCR with two primers which contains upstream pYEX-A4-F1 and downstream pYEX-A4-R1 (Table 2) in vector pTW340. Co-transformation with fragment and pTW340 vector into yeast strain YW607 and selected with URA mark. The positives clones were confirmed by Western blot and enzyme digestions (Fig. 3).

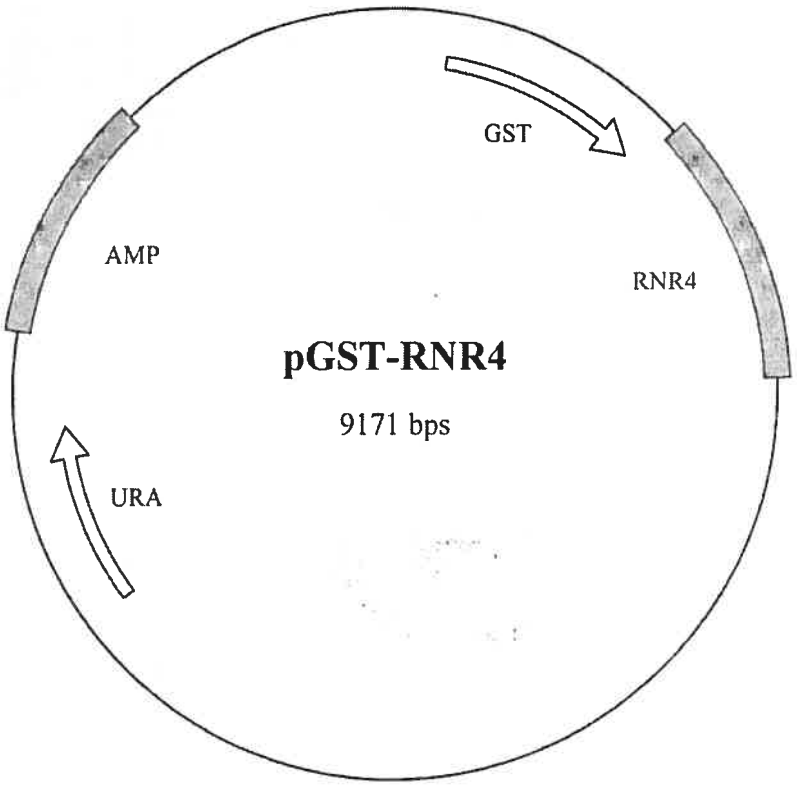


Figure. 3. Clone of plasmid Gst-Rnr4. The entire *RNR*4 coding sequence is amplied by PCR with two primers which contains upstream and downstream in vector pTW340. Co-transformation with fragment and pTW340 vector into yeast strain YW607 and selected with URA mark. The clones are positives confirmed by western blot and enzymes digestion.

CHAPTER III: RESULTS

SECTION 3.1: Strains *rnr4*Δ Mutant Display Extreme Resistance to 4-NQO as Compared With the Parent

In order to determine the contribution of the Rnr4 to the repair of damage sites, we first chose 3 different type of DNA damage agent, 4-NQO, BLM and MMS, and examining whether *rnr4*Δ strains conferred the same level of sensitivity to these agents as the parent strains.

In this experiment, exponentially growing cultures were treated with 4-NQO, MMS and BLM then scored for fractional survivors as described in the material and methods. Interestingly, strains *rnr4*Δ mutant displayed extreme resistance to 4-NQO as compared with the parent strains (50% ± 1.9 vs. 6% ± 1.1= Δ vs. wt, 3μg/ml 4-NQO, 1 hour, $p<0.01$). (Figure .4) On the contrary, *rnr4*Δ mutant strains were sensitive to BLM and MMS as compared with the parent strain. (Figure .5.6)

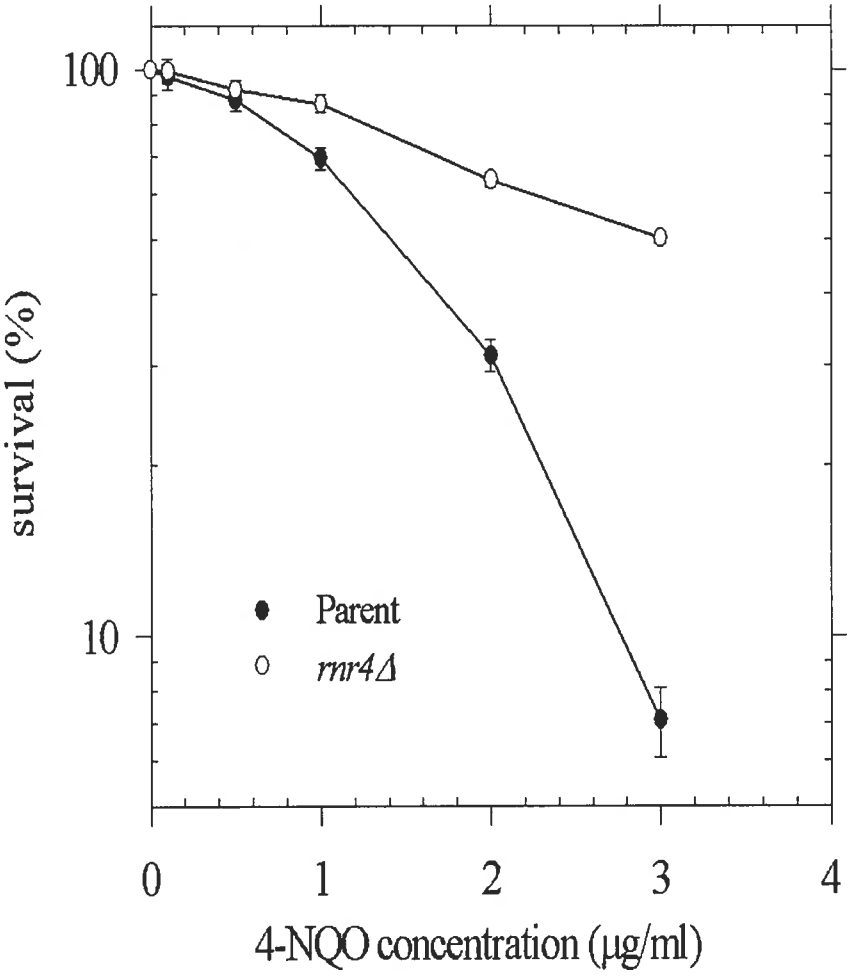


Figure 4. Survival curve assay after treatment of 4-NQO

The Log phase strains were treated with different concentration of 4-NQO 1 hour at 30°C. *rnr4*Δ strains (o) were resistant to 4-NQO as compared with parent strains (•). Results were expressed as the mean ±SD from three separate experiments

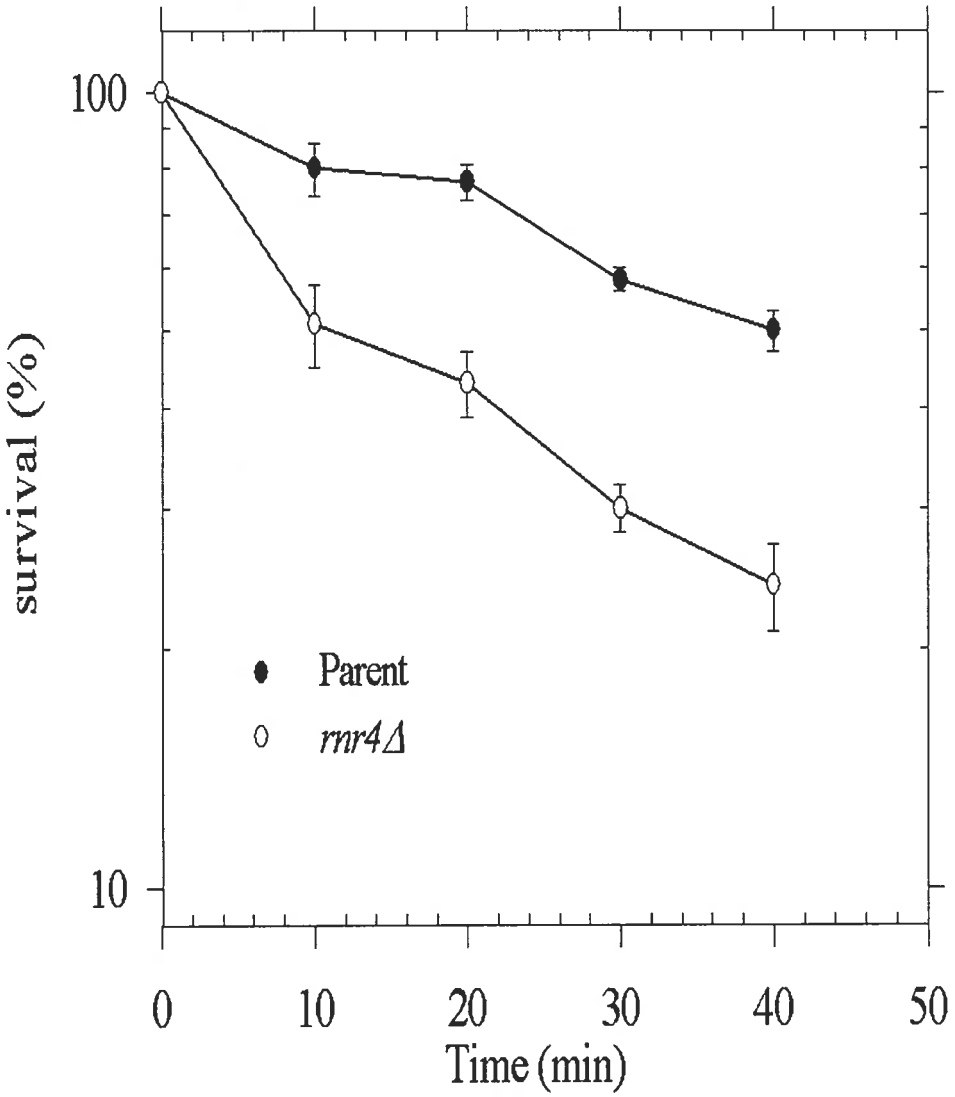


Figure 5. Survival curve assay after treatment of BLM

The Log phase cells were treated with 20 μg/ml BLM for different times at 30°C. *rnr4*Δ strains (o) were sensitive to BLM as compared with parent strains (•). Results were expressed as the mean ±SD from three separate experiments.

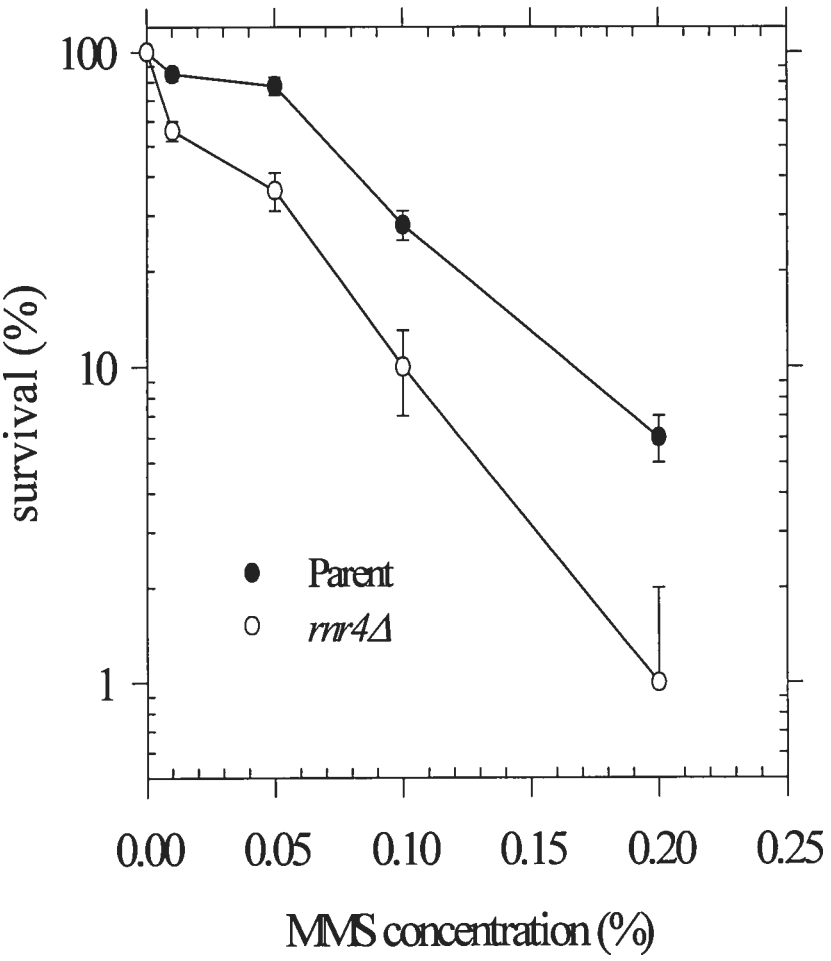


Figure 6. Survival curve assay after treatment of MMS

The Log phase strains were treated with different concentration of MMS for 1 hour at 30°C *rnr4*Δ mutant strains (o) were sensitive to MMS as compared with parent strains (•). Results were expressed as the mean ±SD from three separate experiments.

SECTION 3.2: The *rnr4*Δ Strain Is Sensitive to UVC

Since the apparent strong similarity in modes of cellular processing (nucleotide excision repair) for UVC light-induced and 4-NQO-induced DNA damage in both prokaryotic and eukaryotic systems, The 4-NQO has been catalogued in "UV-mimetic" (Felkner and Kadlubar 1968). However, this classification is not accurate enough, because several recent investigations have clearly demonstrated that 4-NQO, unlike 254-nm UV light, can generate a substantial degree of intracellular oxidative stress (Ramotar et al., 1998). Strains *rnr4*Δ mutant display extreme resistance to 4-NQO as compared with the parent, in order to know further if strains *rnr4*Δ mutant is resistant to "UV-mimetic" DNA damage or the other DNA damage mechanism, the UVC ray was applied for exposure on petri dishes containing 100 µl of 10^{-4} diluted wild type or *rnr4*Δ strains as described in the methods. The *rnr4*Δ mutant showed sensitive to UVC as compared with parent cells. This result suggests that *rnr4*Δ strains are specifically resistant to the cell killing effect, which is not "UV-mimetic". (Figure.7)

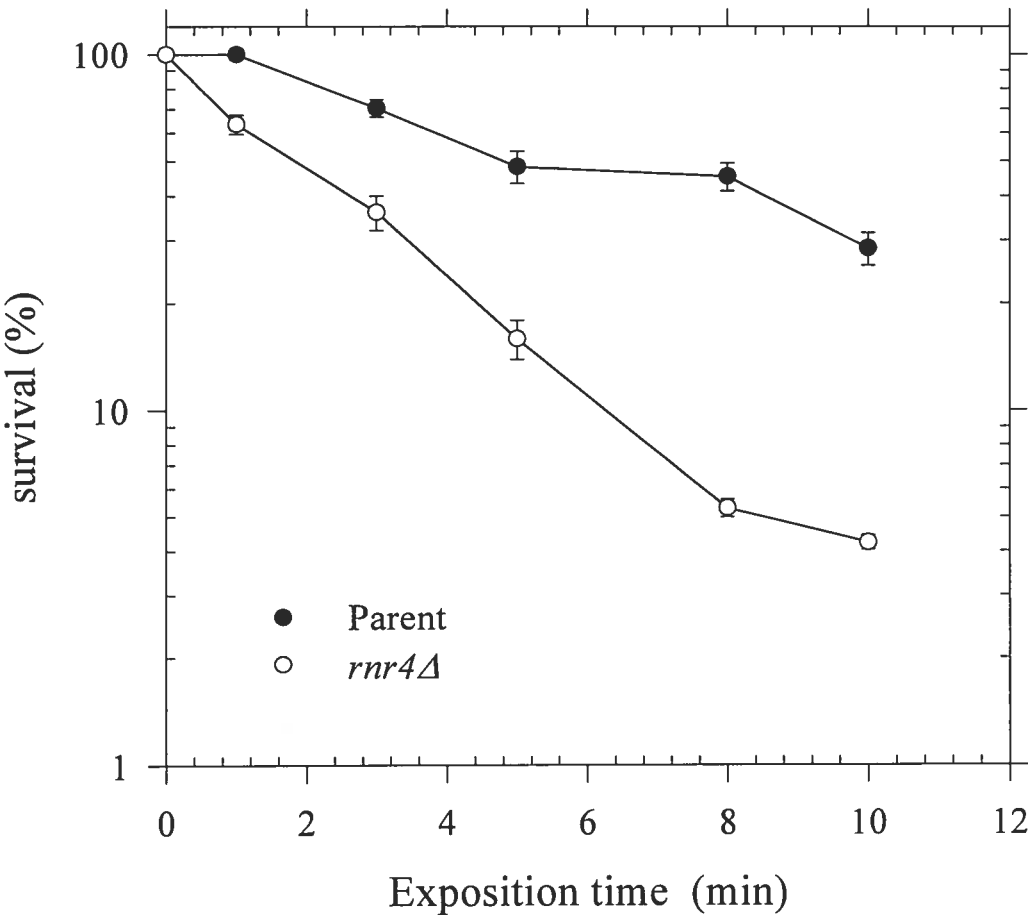


Figure 7. Survival curve assay Analysis after Treatment UVC Exposure

The parent (wild type BY4741) and strains *rnr4*Δ mutant grow in YPD liquid 30°C over night to be saturated. 100 ml of each saturated cells are made 10^4 dilutions and plated on the YPD agar plate. Triple plates of each strain are exposed on UVC on 0.4 J per second and per square meters with different time courses in 0, 1, 3, 5, 8, and 10 minutes. Counting the all colonies numbers in each plate after 48 hours incubation in 30°C. *rnr4*Δ mutant strains (o) were sensitive to BLM compare with parent strains (•). Results are expressed as the mean ±SD from three separate experiments

SECTION 3.3: The Frequency of 4-NQO-induced can^r Mutations Rise Sharply in the Wild Type Strain

Our data showed that strains *rnr4*Δ mutant displayed extreme resistance to 4-NQO as compared with the parent strains. If Rnr4 plays a role in the repair of 4-NQO-induced DNA lesions, then parent cells might be expected to show hypermutable phenotype in 4-NQO induced DNA lesions. We therefore measured the reversion mutation rate on -arg plate containing 25 μg /ml canavanine in -arg plate for a wild type and *rnr4*Δ mutant strains as described in material and methods.

Under normal growth conditions, parent cells showed no significant difference mutation rate as compared with *rnr4*Δ mutant strains (Table.3). The mutation rate was increased as much as 27-fold when the wild type stains was treated with 4-NQO compare with no treatment. In contrast, the mutation rate increase only 2-fold when the *rnr4*Δ strain were treated with 4-NQO. This result suggested that Rnr4 played an important role in allowing 4-NQO-induced mutagenic effects.

Table 3. Spontaneous rates and spectrum of can^r mutation in *rnr4*Δ deficient mutant

Strains	Expts	Mutation rate (can^r/cell, 10-8)	Fold increase
BY 4741	1	158.70 ± 0.43	1
	2	114.49 ± 1.46	1
BY4741 + 1 μg/ml 4-NQO 1h	1	4200.35 ± 0.22	26.47
	2	3412.13 ± 1.42	29.80
*rnr4*Δ	1	286.64 ± 0.59	1.81
	2	107.12 ± 0.14	0.94
*rnr4*Δ +1 μg/ml 4-NQO 1h	1	305.75 ± 0.36	2.21
	2	283.68 ± 0.41	2.48

SECTION 3.4: Western Blot

Replication protein A (RPA) is essential for multiple processes in DNA metabolism including DNA replication; recombination and DNA repair pathways (including nucleotide excision, base excision and double-strand break repair). It is a heterotrimeric single-stranded DNA-binding protein composed of subunits of 70-, 32- and 14-kDa, which is highly conserved in eukaryotes. RPA binds ssDNA with high affinity and interacts specifically with multiple proteins. Cellular DNA damage causes the N-terminus of the 32-kDa subunit of human RPA (RPA32) to become hyper-phosphorylated. Current data indicates that hyper-phosphorylation of human RPA32 causes a change in RPA conformation that down-regulates activity in DNA replication but does not affect DNA repair processes. This suggests that the role of RPA32 phosphorylation in the cellular response to DNA damage is to help regulate DNA metabolism and promote DNA repair. RPA32 is phosphorylated during the normal cell cycle and after cellular DNA damage in a number of different organisms including humans, *Drosophila melanogaster* and *Saccharomyces cerevisiae*. By now it has been accepted that changes happen in RPA32 in response to DNA damage induced by various agents and RPA modification may be associated with a loss of replication competence. For review, see ref.(Iftode et al., 1999)

In order to see changes in RPA32 in response to DNA damage induced by 4-NQO, parent BY4741 and *rnr4*Δ mutant strains were treated with 0, 1, 2 μg/ml of 4-NQO for one hour at 30 degree. The total protein extract was loaded onto 10% PAGE gel, and

probed by western blot using 1: 5000 dilution of antibody Rpa 32 from rabbit was used. We did not observe any difference between treated and untreated cells in both strains (Figure 8)

Since 4-NQO has been proved to be a DNA damage agent which easily enters the cells and that the phosphorylation of Rpa32 was the same in both the parent and mutant, we excluded the possibility that resistance of the mutant to 4-NQO is a the level of drug entry.

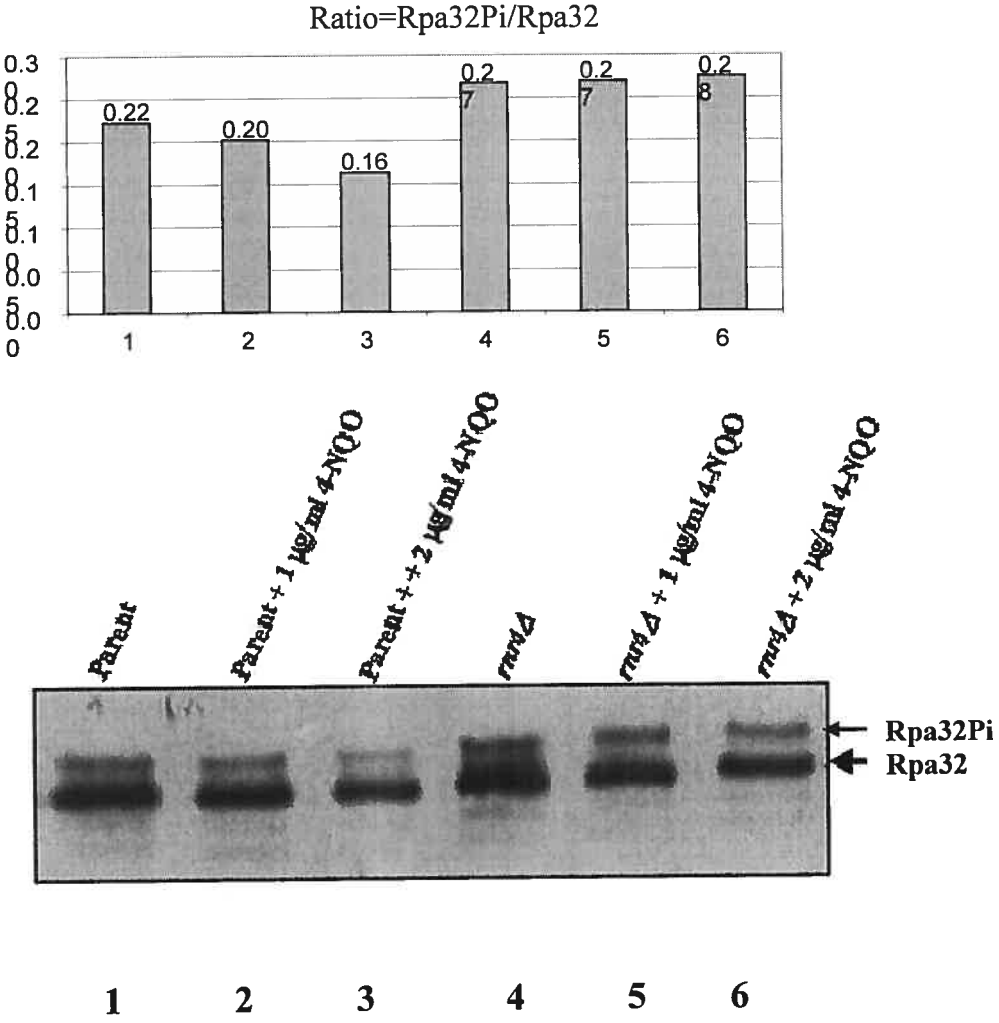


Figure 8. Western blot detection of Rpa32 phosphalation

The parent BY 4741 and *rnr4*Δ strains were treated with zero concentration, 1 μg/ml, and 2 μg/ml of 4-NQO for one hour in 30°C. The 50 μg of total proteins extract were loaded on the 10% PAGE gel. RPA32 phosphorylated was not significantly increased in *rnr4*Δ mutant cells (**5.6**) as compared with the untreated cells (**4**); the percentage of RPA phosphorylated was not significantly increased in parent cells (**2,3**) compared with the untreated cells (**1**).

SECTION 3.5: Primer Extension Assay

By now we had already known that compared with the parent strain, *rnr4*Δ strain was more sensitive to BLM, the inability of the *rnr4*Δ deletion mutant to repair BLM-damaged DNA could be probably due to a defect in processing DNA strand breaks containing blocked 3′ termini. To test this possibility, we examined whether chromosomal DNA isolated from cells treated with BLM at 40 μg/ml for 1 h could allow *in vitro* DNA repair synthesis by *E. coli* DNA polymerase I (Demple et al., 1986;Henner et al., 1983;Levin and Demple 1988;Masson and Ramotar 1996;Ramotar and Demple 1991;Seki et al., 1991).

In this experiment, chromosomal DNA was isolated from the both strains with or without 40 μg/ml BLM concentration in 1-hour 30^0C incubation. The 200 ng BLM treated or without treated genomic DNA and 0.05 U of DNA polymerase I (New English BioBar) were added into each reaction. 200 μl 0.5M TCA stopped each reaction in 0, 3, 6, and 9 minutes. As shown on the graph: first DNA isolated from untreated cells (both parent and mutant trains) showed lower [*methyl*-3H] dTMP incorporation compared with the treated cells in both parent strain and *rnr4*Δ mutant strains; secondly, DNA isolated from treated parent cells showed great higher [*methyl*-3H] dTMP incorporation compared with the untreated parent cell; DNA isolated from treated *rnr4*Δ cells showed only slight higher [*methyl*-3H] dTMP incorporation compared with the untreated *rnr4*Δ cells (Figure. 9). These results suggested:

1. BLM-mediated cell killing was a direct result of damage to DNA.

2. The defect of the *rnr4*Δ deletion mutant to repair BLM-damaged DNA was due to a defect in processing DNA strand breaks containing blocked 3` termini.

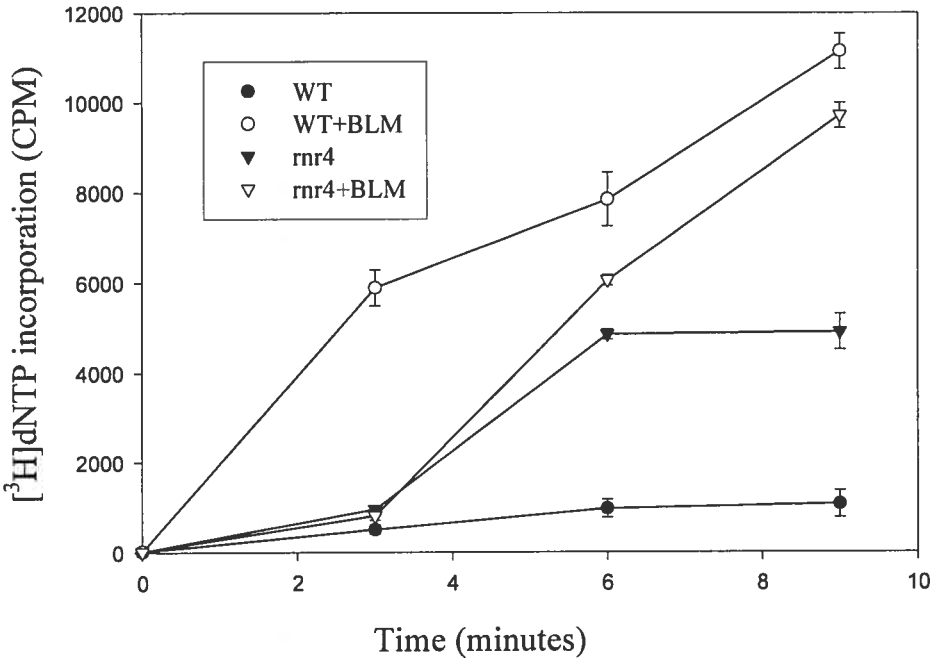


Figure 9. *In vitro* incorporation of [methyl-^{3}H] dTTP

To detect accumulation of bleomycin-induced DNA lesions in parent (BY4741) and *rnr4*Δ strains. The substrat of genomic DNA were prepared from the both strains with or without 40 μg/ml BLM concentration in 1 hour 30°C incubation. The 200 ng BLM treated or without treated genomic DNA and 0.05 U of DNA polymerase I (New English BioBar) were added into each reaction. 200 μl 0.5M TCA stops each reaction in 0, 3, 6, and 9 minutes. Results were expressed as the mean ± SD from three separate experiments.

SECTION 3.6: The Survival Curve Assay for Gst-Rnr4 Containing Strain

*rnr4*Δ strain displayed extreme resistance to 4-NQO as compared with the parent strain was the most important result in our study. In order to verify this result, Gst-Rnr4 in *rnr4*Δ strain was created in our lab, as described in material and methods.

Over night grown cells of *rnr4*Δ/ BY 4741 /Gst-Rnr4 in *rnr4*Δ strain were used for survivalcurve assay. In *rnr4*Δ strain, the resistance to 4-NQO always showed. The *rnr4*Δ mutant containing plasmid Gst-Rnr4 lost the resistance to 4-NQO (Fig.10)

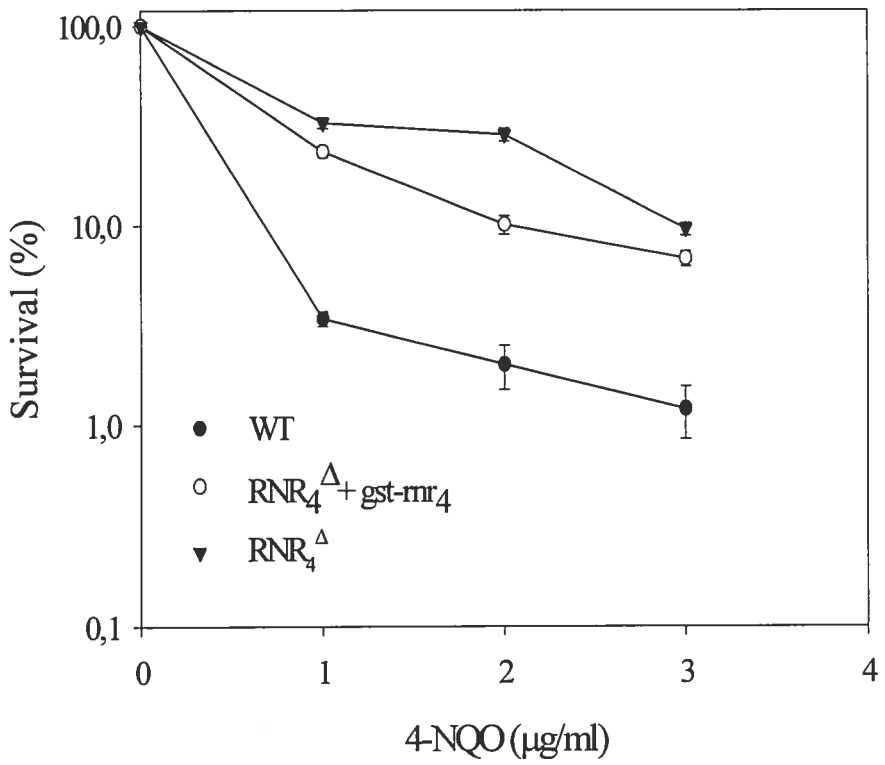


Figure. 10 The survial curve assay for Gst-Rnr4 containing strain.

In *rnr4*Δ strain,the resistance of 4-NQO always showed. While the *rnr4*Δ strain

Containing plasmid Gst-Rnr4 brought its phenotype with more sensitive with drug 4-NQO. Results were expressed as the mean ±SD from three separate experiments.

CHAPTER IV: DISCUSSION

RNR catalyze the reduction of all four ribonucleoside diphosphates into deoxyribonucleoside diphosphates and play a central role in controlling the levels of cellular deoxynucleoside triphosphates (dNTPs), which are essential for high-fidelity DNA replication and DNA repair processes. In S. *cerevisiae,* RNR is a α2β2 tetramer consisting of two large (Rnr1/Rnr3) and two small subunits (Rnr2/Rnr4). Rnr4 and Rnr2 appear to have nonoverlapping functions and cannot substitute for each other even when overproduced. To investigate the role of Rnr4 in DNA repair processes, except the survival curve assay which show that *rnr4*Δ mutant strain is resistant to 4-NQO, different kind of techniques (western blot/primer extension assay/mutation assay) were applied step by step. As a result a new DNA damage mechanism induced by 4-NQO was disclosed and an error free DNA repair manner may play an important role when *rnr4*Δ mutant strain is treated with 4-NQO.

First survival curve assay for strains *rnr4*Δ mutant and parent (S. *cerevisiae* BY 4741) after treatment with different type of DNA damage agents (BLM, MMS, and 4-NQO) was applied in our study.

Bleomycin is used extensively to treat a variety of cancers, including those of the lungs, testicles, head, and neck. The antitumor effect of BLM is exerted through oxidative lesions in chromosomal DNA, formed via a free radical-reactive complex that is produced when BLM binds to iron and oxygen in vivo. The activated Fe-BLM complex takes a hydrogen atom from the 4'-carbon of deoxyribose, resulting in two types of

lesions: (i) oxidized apurinic/apyrimidinic (AP) sites and (ii) DNA single-strand breaks that terminate with 3'-phosphoglycolate. Noncoding AP sites lead to the incorporation of incorrect nucleotides by DNA polymerase. While 3'-phosphoglycolate is known to block DNA synthesis, the mutagenic effect of this adduct is unknown. Activated Fe-BLM complex can also produce double-strand breaks at certain DNA sequences, such as CGCC. In any case, the lesions produced by BLM are toxic and are considered to be mutagenic.

People now use the agent 4-nitroquinoline 1-oxide (4-NQO) in mammalian systems as a paradigm for DNA damage-induced carcinogenesis. To damage DNA, 4-NQO must first undergo metabolic activation to the proximate carcinogen 4-hydroxyaminoquinoline 1-oxide, which, following acylation, reacts with DNA to form stable quinoline-purine monoadducts, i.e. at the exocyclic N-2 and N-6 positions of guanine and adenine, respectively. In bacteria, yeast, and mammalian cells, these genotoxic "bulky" DNA lesions are repaired largely by the nucleotide excision repair (NER) pathway in a manner analogous to classical dipyrimidine photoproducts. Since the apparent strong similarity in modes of cellular processing (nucleotide excision repair) for UVC light- and 4-NQO-induced DNA damage in both prokaryotic and eukaryotic systems. The 4-NQO is catalogued as "UV-mimetic".

In our present study, *rnr4*Δ mutant strain was sensitive to BLM and MMS as compared with parent. This is not strange, because even without DNA damage agent, *RNR4* deletion is lethal in *Saccharomyces cerevisiae*, and the *rnr4*Δ mutant strains show

slow growth phenotype as compared with parent strains in normal condition (Huang M and Elledge SJ 1997). Moreover, like the other RNR genes, *RNR*4 is inducible by DNA-damaging agents through MEC1, RAD53 and DUN1 transduction pathway. So the possible mechanism for the lethality of deletion of *RNR*4 is that the mutant cell has a lower level of functional RNR complex, resulting in dNTP levels below the threshold critical for mitoic viability. In fact, the level of dNTPs in yeast increase dramatically after DNA damage and the survival of DNA damage in yeast directly depends on increased dNTP levels (Chabes A et al., 2003).

Interestingly, strains *rnr4*Δ mutant display extreme resistance to 4-NQO as compared with the parent, this suggests us that *RNR*4 deletion mutations show strong ability in repair for DNA damage induced by 4-NQO.

Since 4-NQO is a "UV-mimetic" DNA damage agent, whose genotoxic "bulky" DNA lesions are processed largely by the nucleotide excision repair (NER) pathway. Does it mean that an *RNR*4 deletion mutant has a strong ability in NER pathway? We next applied survival curve assay for strains *rnr4*Δ mutant and parent (BY 4741) after treatment with UVC. More interestingly, the strain *rnr4*Δ mutant showed sensitivity to UVC as compared with parent.

This suggests that the resistant to 4-NQO of *rnr4*Δ mutant is not result from a strong ability in NER pathway as compared with parent.

During S phase, when DNA damage is encountered, TLS (translesion DNA repair) plays a major role in bypassing the lesions. In spite of increased mutagenicity, such a process may be inherently more advantageous than the other repair mechanisms where interruptions in the newly synthesized strands persist for long periods. An increase in dNTP concentration above normal S phase levels are essential for translesion DNA repair (Prakash S and Prakash L., 2002). If *rnr*4 mutant enhance translesion DNA repair, then the mutation rate must be raised. We therefore measured the reversion mutation rate on -arg plate for a wild type and *rnr4*Δ mutant strains.

Under normal growth conditions, parent cells showed no significant difference mutation rate as compared with *rnr4*Δ mutant strains (Table.3). The mutation rate was increased by as much as 27-fold when the wild type stains was treated with 4-NQO as compared with no treatment and 13-fold as compared with *rnr4*Δ mutant strains 4-NQO treated. We believe that the lower level of dNTP may prevent the translesion bypass pathway from function by not allowing the bypass polymerase from being fully active.

We do not think that the 4-NQO cell killing effect is the result of defect in drug entry or DNA damage, which *rnr4*Δ plays an important role. In order to answer this, Western-Blot assay was applied. Although, we couldn't see any difference in Rpa32 between treated and untreated cells in both strains (Figure 8), suggesting that 4-NQO must enter the cell at the same rate in both strains to cause induce phosphorylation of Rpa32.

Both BLM and 4-NQO induce DNA damage to yeast cells, but strains *rnr4*Δ

mutant display only resistance to 4-NQO as compared with the parent. Does the cell killing effect of BLM is a direct result of DNA damage induced by BLM? Our primer extension assay verifies this result and shows that cell-killing effect of BLM is a direct result of DNA damage. The defect of the *RNR*4 deletion mutant to repair BLM-damaged DNA could be due to a defect in processing DNA strand breaks containing blocked 3` termini, which is likely regulated by the availability of dNTPs. The cell might sense that the level of dNTP is low and therefore prevent the action of DNA repair proteins and the subsequent action of DNA polymerase.

Taken together, *rnr4*Δ mutant strains display extreme resistance to 4-NQO induced DNA damage as compared with the parent. In addition, the mutant showed very little increase in 4-NQO-induced mutants when compared to the parent. We propose that this DNA damage is neither a "UV-mimetic" DNA damage, which is processed largely by the nucleotide excision repair (NER) pathway nor oxidative stress like BLM induced, but a different DNA lesion caused by 4-NQO. This DNA lesion could be repaired in both an error free DNA repair manner (Prakash S, et al., 2005.) (Huang M and Elledge SJ 1997), even when the dNTPs level are low. The lower dNTPs pool in *rnr4*Δ mutant strains prevent may prevent TLS pathway but doesn't affect the error free DNA repair manner and lead to higher survival rate but lower mutation rate; on the other hand, the higher level of dNTPs pool allowed WT cells to repair this lesion in TLS pathway (Chabes and Thelander 2000), but since a lot of error remained this lead to lower survival rate and higher mutation rate.

At the end the most important result that *rnr4*Δ mutant strains display extreme resistance to 4-NQO induced cell killing as compared with the parent was verified by survival curve assay (Figure 10).

CHAPTER V: CONCLUSION

In conclusion, strains *rnr4*Δ mutant display extreme resistance to 4-NQO induced DNA damage compared with the parent; this DNA damage is neither a "UV-mimetic DNA damage which is processed largely by the nucleotide excision repair (NER) pathway nor oxidative stress like BLM induced, but a different DNA lesion mechanism causing by 4-NQO. The lower dNTP pool in *rnr4*Δ mutant doesn't affect the repair of DNA lesion causing by this DNA lesion mechanism, which could be an error free DNA repair manner and lead to higher survival rate; on the other hand, the higher level of dNTP pool in WT allow cells to repair this lesion in TLS, which has a lot of errors and lead to lower survival rate. Base on this result we believe that *RNR*4 gene play an important role not only in controlling the levels of cellular dNTPs, but also in DNA repair including TLS and an error free DNA repair manner. We propose that like some RNR inhibitors, *RNR*4 gene may play a role in cell antitumor properties.

CHAPTER VI: PROSPECT

In my project, the strongly 4-NQO resistance phenomenon of *rnr4*Δ knockout may come from a third DNA damage role of this drug which is neither a "UV-mimetic DNA damage which is processed largely by the nucleotide excision repair (NER) pathway nor oxidative stress like BLM induced. For identifying the 4-NQO involved DNA repair pathway in mutant *rnr4*Δ strain and further characterization. The following studies are suggested as future work:

1.Create different gene knockout from parent strain BY4741 and BY4741 *RNR*4Δ. For example, knockout NER pathway gene rad1, mismatch repair gene msh6, recombination rad51.

2. Microarray assay for find related genes. Immunoprecipitation assay to detect *RNR*4 interacting proteins.

CHAPTER VII: REFERENCE

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