Molecular Regulatory Mechanisms Of DNA Damage-inducible Genes, *MAG1* And *DDI1*, From *Saccharomyces cerevisiae*

A Thesis Submitted to the College of

Graduate Studies and Research

in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy

in the Department of Microbiology

University of Saskatchewan

By

Yule Liu

January, 1997

UNIVERSITY OF SASKATCHEWAN

College of Graduate Studies and Research

SUMMARY OF DISSERTATION

Submitted in partial fulfillment of the requirement for the

DEGREE OF DOCTOR OF PHILOSOPHY

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January 1997

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DNA damage is an important and ubiquitous type of stress with which all cells have to contend. One of the cellular responses to DNA damage is the induction of specific genes that evolved to enhance the cellular capacity for DNA damage repair. The control and expression of the DNA damage-inducible genes remain to be fully understood. My research project involved dissecting *cis*-acting promoter elements and attempting to identify binding proteins that regulate the expression and mediate the DNA damage response of the yeast genes *MAG1* and *DDI1*.

MAG1 encodes a 3-methyladenine (3MeA) DNA glycosylase and protects cells against killing by MMS-induced DNA replication blocks (Chen et al., 1989 Proc. Natl. Acad. Sci. USA 86: 7961-7965). *DDI1* was recently identified as a gene upstream of *MAG1* and was inducible by DNA damaging agents (Xiao and Fontaine, unpublished). *MAG1* and *DDI1* are arranged in a head-to-head configuration and are transcribed divergently. These two genes are closely linked, with the first ATG's of the two open reading frames being separated by 282 base pairs. The transcription of *MAG1* is repressed by a URS (upstream repressing site) element and stimulated in response to DNA damage by a putative UAS (upstream activating site) (Xiao et al., 1993 Mol. Cell. Biol. 13: 7213-7221). The 46 bp sequence containing the putative UAS of *MAG1* (UAS_{MAG1}) is located within the coding region of *DDI1*. The arrangement of these genes appeared unique, and presented an opportunity to explore the molecular mechanism for the regulation and co-expression of these DNA damage-inducible genes.

The transcriptional and the translational starts of *MAG1* and *DDI1* were determined. My results showed that the two genes were indeed closely linked to each other. The UAS_{MAG1} was identified within the protein coding region of *DDI1*. This is the first demonstration in yeast that a transcriptional regulatory element for one gene can be located within the protein coding region of another gene.

Since *MAG1* and *DDI1* are co-induced by DNA damage in a similar manner, it was hypothesized that the two genes share one or more regulatory elements. A direct repeat sequence (DR) within the intergenic region between *MAG1* and *DDI1* was identified as a bi-directional transcriptional regulatory element for the expression of these two genes. Sequences similar to the direct repeat were also found in the promoters of several DNA repair, or DNA metabolism genes from *S. cerevisiae*. This is the first report of a situation where two DNA damage-inducible genes are co-ordinately regulated by physically sharing a regulatory element.

The yeast RPA (yRPA) protein binds to single-stranded DNA and is involved in DNA replication. The yRPA was recently reported to bind a double-stranded *MAG1* promoter sequence that includes half of the above noted direct repeat (Singh and Samson, 1995 Proc. Natl. Acad. Sci. USA 92: 4907-4911). Therefore, the potential involvement of yRPA protein in the function of the direct repeat was investigated. The results from an electrophoretic mobility shift assay showed that yRPA protein bound to the wild type and to mutated sequences of the direct repeat, suggesting that the formation of a yRPA-DR complex is not required for the expression of *MAG1* and *DDI1*.

MAG1 is one of the most extensively studied yeast DNA damage responsive genes. Previous studies (Xiao et al., 1993 Mol. Cell. Biol. 13: 7213-7221) have focused primarily on the mechanism of repression of *MAG1* expression. The UAS_{MAG1} element was not well defined and its role in the induction of *MAG1* following DNA damage was not established. This work defined the UAS_{MAG1} as a 24 bp sequence required for the expression of *MAG1*, but not *DDI1*. An UAS_{MAG1}-binding protein(s) was identified. The UAS_{MAG1}-binding protein(s) is probably a transcription activator that regulates the expression of *MAG1*.

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regulatory pathway. *MAG1* and *DDI1* are differentially expressed in the presence of the protein synthesis inhibitor cycloheximide.

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(2) Yule Liu and J. Mi. (1990) Plant regeneration from mesophyll protoplasts of axenic tomato shoots. Genetic Manipulation in Plants 6: 56-62.

(3) Yule Liu and J. Mi. (1990) Isolation and identification of nuclear DNA from corn and carrot. J. of Beijing Agricultural University. 16: 273-275.

(4) Yule Liu , J. Mi and J. King. (1988) Isolation and ultrastructural studies of nucleus from soybean protoplasts. Chinese J. of Genetics 15: 21-24.

(5) Saxena, PK., Yule Liu and J. King. (1987) Nuclear transplantation into protoplasts; optimal conditions for induction and determination of nuclear uptake. J. Plant Physiol. 128: 451-460.

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ABSTRACT

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In combination, my results suggest that *MAG1* and *DDI1* can be both co-ordinately and differentially expressed. It is proposed that the common UAS element (the direct repeat) regulates co-ordinate expression of *MAG1* and *DDI1*, while distinct URSs and UAS_{MAG1} elements influence their differential expression. It is proposed that the constitutive expression of *MAG1* and *DDI1* is regulated by the UAS and the URS elements through an antagonistic mechanism, whereas their damage-induced expression is achieved by de-repression at the URS site and by activation at the UAS site.

ACKNOWLEDGMENTS

First of all, I sincerely thank Dr. Wei Xiao for providing the research project and for the financial support. It is in his laboratory that I started learning and doing systematic research in Molecular Biology.

I wish to thank the members of my thesis advisory committee, Drs. S. Hemmingsen, P. Krone, K. Bonham, L. Loh, and S. Hayes for their time and valuable suggestions.

I extend my gratitude to external examiner, Dr. W. Siede, for his participation in the thesis defense.

I would like to thank the College of Medicine, University of Saskatchewn, for the Teaching Fellowship for the year 1995-1996.

I would like to thank Dr. Mahon, J. from Plant Biotechnology Institute, National Research Council of Canada for help using Densitometer.

I would like to thank all the members in Dr. Xiao's laboratory for their help and for providing a friendly and harmonious environment of work.

To my wife, Lirong, and my son, Tong, I extend sincere gratitude for their support and true love.

I dare not to forget to thank my beloved mother back in China for her understanding and support.

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LIST OF ABBREVIATIONS

Amp	ampicilin
AP site	apurinic or apyrimidinic site
ATP	adenosine 5'-triphosphate
β-gal	β-galactosidase
BER	base excision repair
bp	base pair
BSA	bovine serum albumin
CDKs	cyclin-dependent kinases
cDNA	copy DNA
CIP	calf intestine phosphatase
CS	Cockayne's syndrome
CsCl	cesium chloride
CTD	carboxy terminal domain
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
DDI	DNA damage inducible
DNA-PK	DNA-dependent protein kinase
dGTP	2'-deoxyguanosine 5'-triphosphate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	2'-deoxynucleotide 5'-triphosphate
DR	direct repeat
DSBs	double-stranded breaks
dsDNA	double-stranded DNA
DTT	dithiothreitol
dTTP	2'-deoxythymidine 5'-triphosphate
E. coli	*Escherichia coli*
ERCC	excision repair cross complementation
EDTA	ethylenediaminetetraacetic acid
EMS	ethylmethylsulfonate
EMSA	electrophoretic mobility shift assay
EtdBr	ethidium bromide
Fapy	formamidopyrimidine
GTF	general transcription factor

HEPES	N-2-hydroxylethylpiperazine-N'-2-ethanesulfonate
IPTG	isopropyl β-D-thiogalactopyranoside
kb	kilobase pair or kilobase (=10^3 base pair)
kDa	kilodalton
LB medium	Luria-Bertani medium
MCS	multi-cloning site
3MeA	3-methyladenine
MePTs	Methylphosphotriesters
mfd	mutation frequency decline
MMS	methylmethanesulfonate
MOPS	3-(N-morpholino) propanesulfonic acid
MNNG	*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine
mRNA	messenger RNA
NER	nucleotide excision repair
4NQO	4-nitroquinoline-1-oxide
OD	optical density
O^6MeG	O^6-methylguanine
O^6MeT	O^6-methylthymine
8-oxo-G	7, 8-Dihydro-8-oxo-guanine
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PEG	polyethylene glycol
PIPES	piperazine-N, N'-bis[2-ethanesulfonic acid]
PMSF	phenylmethylsulfonyl fluoride
PR	photoreactivation
RNA	ribonucleic acid
SAM	S-adenosylmethionine
S. cerevisaie	*Saccharomyces cerevisiae*
SD	synthetic dextrose (glucose) medium
SDS	sodium dodecyl sulphate
ssDNA	single-stranded DNA
TAFs	TBP-associated factors
TBP	TATA-box binding protein
TE buffer	Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, pH 8.0)

TRCF	transcription-repair coupling factor
TTD	trichothiodystrophy
UAS	upstream activating site
URS	upstream repressing site
UV	ultraviolet
Xgal	5-bromo-4-chloro-3-indoyl-β-D-thio-galactopyranoside
XP	xeroderma pigmentosum
YCp	yeast centromere plasmid
YEp	yeast episome plasmid
YIp	yeast integration plasmid

CHAPTER I: INTRODUCTION

DNA damage is unavoidable. The repair of DNA damage is a universal and ubiquitous process in all living cells. Threats to DNA can come from either endogenous source or from the environment. Cells have evolved mechanisms to combat these threats by repairing or tolerating DNA damage. Cells in multicelluar organisms respond by increasing the expression of specific genes and by delaying the progression of cell cycle; and when DNA damage is severe and beyond repair, they respond by committing suicide, a process called programmed cell death or apoptosis.

The discovery of a correlation between hereditary human cancers and deficiency in a DNA repair pathway, coupled with increased understanding of the regulation of DNA damage responses, repair of DNA damage and the regulation of cellular responses to DNA damage have occupied the scientific center stage in recent years (Cleaver, 1994; Marx, 1994). This review will endeavor to present the current state of understanding of the mechanisms of DNA damage repair and the regulation of cellular responses to DNA damage.

1.1 DNA Damage

DNA damage can be sub-classified into two main classes; spontaneous and environmental damage. The types of DNA damages were described (Friedberg et al., 1995). Lesions formed by some types of DNA damage are summarized in Fig. 1-1.

1.1.1 Spontaneous damage

DNA damage results from normal cellular metabolism, DNA replication, recombination and repair, and from the inherent instability of specific chemical bonds that constitute the normal chemistry of nucleotides under physiological conditions, e.g. temperature and pH.

1.1.1.1 Types of spontaneous damage

a). Mismatches: Mismatches are chief sources of DNA alterations arising during normal DNA metabolism such as DNA replication and recombination.

b). Tautometric shifts: Each of the common bases in DNA can undergo a spontaneous, transient rearrangement of bonding to form a structural isomer (tautomer) of the base, which alters its base-pairing properties and thus results in misincorporation.

c). Deamination of bases: Except for T, A C and G bases contain exocyclic amino groups. The loss of these groups (deamination) occurs spontaneously in pH- and temperature-dependent reactions of DNA, resulting in conversion of the affected bases to hypoxanthine, uracil, and xanthine, respectively.

d). Loss of bases: The loss of bases results in abasic sites, a kind of DNA damage induced by the spontaneous hydrolysis of bases and by repair of damaged bases caused by alkylation and oxidation.

e). Oxidative damage: Oxidative damage to DNA and nucleoproteins produces base and sugar damage, single-stranded breaks, abasic sites and DNA-protein cross-links (Demple, 1991; Demple and Harrison, 1994; Moradas-Ferreira et al., 1996).

1.1.2 Environmental damage to DNA

Environmental damage to DNA results from physical agents such as ionizing radiation and UV radiation and chemicals that are released into environments by cellular metabolism or decomposition of other living forms by the cell. Other environmental damaging agents, especially in recent decades, are man-made.

1.1.2.1 Physical agents that damage DNA

a). Ionizing radiation. Ionizing radiation causes base damage, sugar damage, and strand breaks.

b). Ultraviolet (UV) radiation. UV radiation causes the formation of cyclobutane pyrimidine dimers, pyrimidine-pyrimidone (6-4) photoproducts, DNA cross-links and strand breaks. UV also causes damage to DNA through an indirect process called

"photosensitization". In this way UV is absorbed by other molecules (sensitizer molecules) that then transfer the energy to DNA.

1.1.2.2 Chemical agents that damage DNA

a). Alkylating agents. Alkylating agents are classified as mono-functional and bi-functional, which, respectively, possess a single and double reactive groups. Numerous potential reaction sites for alkylation have been identified in all four bases; although, not all of them have equal reactivity. In general, the ring nitrogens of a base are more nucleophilic than the oxygens, with the N^7 position of guanine and N^3 position of adenine being the most reactive. Among the alkylated bases, O^6-methylguanine (O^6MeG) which is the major product of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), and 3-methyladenine (3MeA) which is the major product of methylmethanesulfonate (MMS), are the most biological relevant lesions. O^6MeG is mutagenic, producing A to T transitions (Saffhill et al., 1985), and 3MeA is lethal, producing DNA replication blocks (Singer and Granberger, 1983; Larson et al., 1985).

b). Cross-linking agents. The bi-functional alkylating agents can react with two different nucleophilic centers in DNA. Interstrand DNA cross-links result if the two sites are on opposite strands. The reaction product is referred to as an "intrastrand" cross-link if the sites are situated on the same chain of DNA duplex. Interstrand cross-links prevent strand separation and hence can block to DNA replication and transcription. For this reason, agents such as nitrous acid, mitomycin C, nitrogen mustard and sulfur mustard, various platinum derivatives (such as cis-platinum diaminodichloride), and certain photoactivated psoralens have been used extensively in cancer chemotherapy.

c). Some chemical agents can undergo a metabolic activation process in multicellular organisms, making them reactive to DNA. Cells in multicellular organisms have enzyme

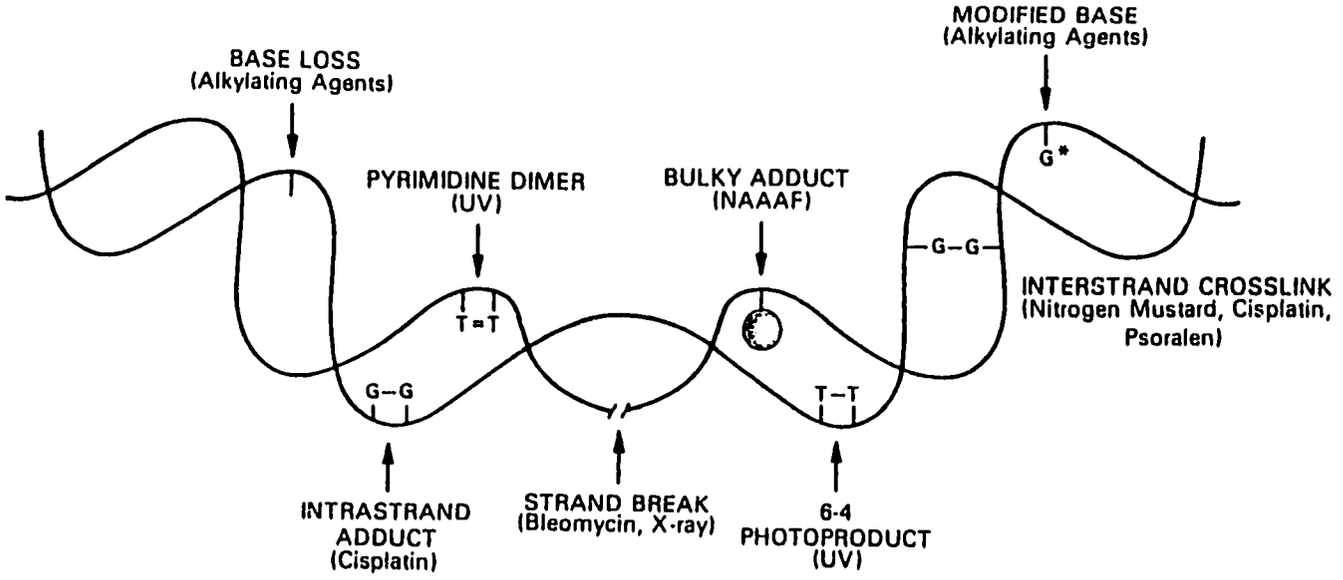


Figure 1-1 Lesions formed by some types of DNA damage

systems to protect them from cytotoxic effects by converting potentially toxic nonpolar chemicals to water-soluble forms. Some of the potentially toxic chemicals become activated to electrophilic forms that are particularly reactive with nucleophilic centers in organic macromolecules such as DNA. A liver cell extract is normally added into the assays used for testing potential mutagens and carcinogens in order to provide for metabolic conversion (Ames et al., 1975).

d). Base analogs of the four naturally occurring bases in DNA can be incorporated in place of the natural nucleotide triphosphate during DNA replication. The most extensively studied base analogs are the uracil derivatives, 5-bromouracil, 5-florouracil, and 5-iodouracil. All are thymine analogs that can produce mismatches when present in template DNA undergoing replication.

1.2 Repair of DNA damage

DNA repair refers to cellular events associated with the removal of damaged, inappropriate, or mispaired bases from the genome of living cells. DNA repair can be subdivided into direct reversal of DNA damage, excision repair which includes base excision repair (BER) and nucleotide excision repair (NER), and mismatch repair (Friedberg et al., 1991; Sancar, 1995).

1.2.1 DNA repair epistasis groups in *Saccharomyces cerevisiae*

Analysis of UV sensitivities of double-mutant combinations among the various *rad* mutants has revealed the existence of three epistasis groups for the repair of UV-induced DNA damage in *Saccharomyces cerevisiae* (*S. cerevisiae*), namely, *RAD3*, *RAD52*, and *RAD6* (Friedberg et al., 1991; Prakash et al., 1993). Each epistasis group is named for a prominent member of that group. Genes belonging to each of the three epistasis groups are shown in Table 1-1. For genes belonging to the same epistasis group (i.e. gene products involved in the same repair pathway) radiation sensitivity of the double mutant is equivalent to that of the more sensitive of the two single mutants,

whereas for genes belonging to different epistasis groups the double mutant is much more sensitive than either single mutant.

The *RAD3* group genes are required for NER of ultraviolet (UV)-damaged DNA. Mutations in *RAD1*, *RAD2*, *RAD3*, *RAD4*, *RAD10*, and *RAD14* confer extreme sensitivity to UV light, and a total defect in the incision step of excision repair. Because of the requirement of its human homolog *XPB* in this process, *RAD25*, another member of the *RAD3* epistasis group, is also likely to be indispensable in the incision step (Prakash et al., 1993).

The *RAD6* group genes are required for post-replication repair of UV-damaged DNA. Many of the genes in this group are also required for spontaneous and/or damage-induced mutagenesis.

Mutants in *RAD52* group are defective in genetic recombination and in the repair of strand breaks in DNA. These genes are therefore believed to be required for recombinational DNA repair.

The classification of DNA repair genes into only three epistasis groups has some limitations and should not be interpreted as a comprehensive definition of the multiple repair pathways in yeast. Other genes not included in the three major epistasis groups have been identified for specific cellular responses to DNA damage. These include genes for enzymatic photoreactivation (PR), for BER, and for mismatch repair.

1.2.2 Reversal of damage

Reversal of DNA damage involves the enzymatic action to restore DNA from the damaged state to its pristine state by a reverse process of DNA damage. The monomerization of pyrimidine dimers by DNA photolyase, a process called "photoreactivation" or PR, and demethylation of methylated bases by specific DNA methyltransferases are examples of this kind of damage repair.

Table 1-1 Epistasis groups for *Saccharomyces cerevisiae* DNA repair genes*

RAD3 group	*RAD52* group	*RAD6* group
RAD1	*RAD50*	*RAD5 (REV2)*
RAD2	*RAD51*	*RAD6*
RAD3	*RAD52*	*RAD18*
RAD4	*RAD53*	*REV1*
RAD7	*RAD54*	*REV3 (PSO1)*
RAD10	*RAD55*	*MMS3*
RAD14	*RAD56*	
RAD16	*RAD57*	
RAD23		
RAD25		
MMS19		

* Only genes for which firm genetic evidence for belonging to an epistasis group exists have been included (Adopted from Prakash et al., 1993).

1.2.2.1 Reversal of pyrimidine dimers

PR is a light-dependent process involving the enzyme-catalyzed monomerization of pyrimidine dimers. PR was the first recognized mode of DNA repair. However, it was not until the late 1950s that studies with extracts of *Escherichia coli* (*E. coli*) and yeast *S. cerevisiae* demonstrated that PR of pyrimidine dimers was in fact an enzyme-catalyzed phenomenon (for review see Friedberg, 1994; Sancar, 1994). The enzyme that catalyze the PR of pyrimidine dimers in DNA is referred to as DNA photolyase. A *S. cerevisiae* mutant defective in enzymatic photoreactivation defined the *PHR1* gene (Resnick, 1969; Resnick and Stetlow, 1972). The *PHR1* was cloned and shown to encode a monomeric protein of 60 kDa (Yasui and Chevallier, 1983; Schild et al., 1984). The protein was purified and shown to catalyze the monomerization of pyrimidine dimers in DNA (Sancar et al., 1987). *PHR1* was later shown to be inducible by DNA damage (Sebastian et al., 1990).

1.2.2.2 Repair of methylated bases by methyltransferases

When *E. coli* cells are exposed to low levels of MNNG and subsequently challenged with a much higher dose of the alkylating agent, there is a marked resistance to both the lethal and the mutagenic effects of the chemical in the "adapted" cell relative to the unadapted control. This resistance was found to be dependent on active protein synthesis by the cell prior to the challenge dose, suggesting that resistance involves the induction of one or more genes in response to low levels of alkylating agent. This led to the discovery of the adaptive response to alkylation damage in *E. coli* (Samson and Cairns, 1977). *E. coli* has two methylguanine transferases (MTase), MTaseI and MTaseII, encoded by *ada* and *ogt* genes respectively. The *ogt* MTase gene is constitutively expressed and protects cells against low levels of DNA alkylation damage (Rebeck et al., 1988; Rebeck and Samson, 1991). The MTaseI is induced several thousand fold when cells experience low levels of DNA alkylation (Samson and Cairns, 1977; Lindahl et al., 1988) and is also called Ada protein because of its central regulatory

role in the adaptive response (Lindahl and Sedgwick, 1988). Both MTases have one active cysteine that repairs O^6MeG or O^6-methylthymine (O^6MeT). Ada has a second active cysteine that repairs methylphosphotriesters (MePTs). The transfer of a methyl group from MePT DNA lesions to Cys-69 of Ada converts the protein into a strong transcription activator of several genes including *ada* itself (Teo et al., 1986).

Unlike its counterpart in *E. coli*, yeast *S. cerevisiae* has only one methylguanine transferase gene, *MGT1* (Xiao et al., 1991). *mgt1* deletion mutants lacks MTase activity and are sensitive to killing and mutagenesis following treatment with alkylating agents. Additionally, *mgt1* mutants have an increased rate of spontaneous mutagenesis, suggesting that an endogenous source of alkylation damage operates in *S. cerevisiae* (Xiao and Samson, 1993). Consistent with the absence of an adaptive response to alkylation damage in *S. cerevisiae, MGT1* is not inducible by alkylating agents (Xiao and Samson, 1992). Depending on the type of tissue and developmental stage, mammalian cells show considerable variations of O^6MeG DNA methyltransferase (MGMT) activity (Pegg, 1990).

1.2.3 Excision of DNA damage

The most general mode of DNA repair observed in nature is excision repair, by which damaged or inappropriate bases are excised from the genome and replaced by the normal nucleotide sequence. These include BER, NER, and mismatch repair.

1.2.3.1 Base excision repair

In BER, first described by Lindahl (Lindahl, 1976), damaged bases (e.g. alkylated) or inappropriate bases (e.g. uracil) are excised and released as free bases by specific classes of repair enzymes designated as DNA glycosylases. DNA glycosylases catalyze the hydrolysis of the N-glycosylic bonds linking bases to the deoxyribose-phosphate backbone. This initial enzymatic event during BER actually generates another type of DNA damage called apurinic or apyrimidinic (AP) sites. AP sites can also result from the depurination or depyrimidination of DNA owing to spontaneous hydrolysis of

N-glycosylic bonds. The removal of AP sites is initiated by AP endonucleases which specifically recognize these sites and hydrolyze the phosphodiester bond immediately 5' to AP sites. AP sites are also acted upon by AP lyases, which cleave 3' to the AP site. The remaining deoxyribose phosphate residue is excised by a phosphodiesterase (5'-phosphodiesterase for AP endonucleases and 3'-phosphodiesterase for AP lyases). The resulting gap is filled by a DNA polymerase (Pol I in *E. coli*, Pol δ in *S. cerevisiae*, and Pol β in mammalian cells), and the strand is sealed by DNA ligase. The BER pathway has evolved to protect cells from deleterious effects of endogenous DNA damage induced by hydrolysis, reactive oxygen species and other intracellular metabolites that modify DNA base structure. However, BER is also important to resist lesions produced by ionizing radiation and strong alkylating agents, which are similar to those induced by endogenous factors (Seeberg et al., 1995).

Exposure of cells to DNA methylating agents produces mutagenic and cell-killing lesions, such as O^6MeG and 3MeA. O^6MeG can mispair with deoxythymine (T) and cause transition mutations. 3MeA is cytotoxic and kills cells by blocking DNA replication. 3MeA is also produced by endogenous sources of methylation (reviewed in Marnett and Burcham, 1993). For example, the normal methyl group donor, S-adenosylmethionine (SAM) also reacts chemically with DNA and produces potential harmful methylations, such as 3MeA and 7-methylguanine (Rydberg and Lindahl, 1982). The major pathway for repairing O^6MeG is through the direct reversal of the damage by MTases as discussed above. The 3MeA is dealt with by N-glycosylase. *E. coli* has two N-glycosylases, Tag and AlkA. Tag is a highly specific constitutive enzyme which recognizes only 3MeA. AlkA comprises only about 10% of the glycosylase activity in cells growing under normal conditions. However, the *alkA* gene is part of the adaptive regulon and is induced tenfold when cells are exposed to a sublethal dose of alkylation. The AlkA enzyme appears to be a multifunctional protein with a much broader specificity than Tag, and excises 7-methylguanine in addition to several minor but important

products such as 3-methylguanine, O^2-methylcytosine and O^2-methylthymine (Bjøras et al., 1995). DNA glycosylase genes have been isolated from many eukaryotic cells (Chen et al., 1989; Berdal et al., 1990; O'Connor and Laval, 1990, 1991; Chakravarti et al., 1991; Samson et al., 1991; Engelward et al., 1993, 1996; Santerre and Britt, 1994). It appears that the mammalian and *Arabidopsis thaliana* enzymes are similar to each other and do not show significant homology to either Tag or AlkA (O'Connor and Laval, 1990; Santerre and Britt, 1994). The *S. cerevisiae* enzyme Mag1 is similar to AlkA (Berdal et al. 1990; Chen et al., 1990).

All aerobically growing cells, including yeast, have to face the toxic side-effects of molecular oxygens generated during normal cellular metabolism (e.g. by the mitochondria respiratory chain, and H_2O_2-generating reactions catalyzed by oxidases) or by exposure to ionizing radiation. The damage caused by oxidation includes single-stranded DNA (ssDNA) breaks with 3'-deoxyribose fragments, abasic sites (i.e. AP site) and DNA-protein cross-links (Demple and Harrison, 1994; Moradas-Ferreria et al., 1996). Two enzymes involved in the N-glycolytic removal of oxidative DNA damage have been extensively characterized in *E. coli*. Endonuclease III (also called Endo III or Nth) can excise a wide range of different oxidized pyrimidine derivatives, including ring-fragmented derivatives and 5-hydroxypyrimidine residues (Hatahet et al., 1994). The other N-glycosylase of *E. coli* for repair of oxidative damage is formamidopyrimidine (Fapy) DNA glycosylase (Fpg or MutM), which catalyses excision of imidazone-ring-opened purine residues, in addition to 7, 8-dihydro-8-oxo-guanine (8-oxo-G), another major spontaneous oxidative DNA product (Boiteux et al., 1992).

The most frequently observed endogenous DNA damage is probably the generation of AP sites. AP sites arise spontaneously by hydrolytic loss of purine bases at a frequency approaching 10,000 per human cell per day (Lindahl, 1993). AP sites in DNA are potentially lethal (by blocking DNA replication) and mutagenic (Sagher and Strauss, 1983; Loeb and Preston, 1986). The major cellular enzymes responsible for

initiating the repair of AP sites are class II AP endonucleases. These enzymes cleave immediately 5' to an abasic site to produce a normal 3'-OH nucleotide and a 5'-deoxyribose-5-phosphate (abasic) moiety. In addition, class II AP endonucleases possess repair diesterase activity for several 3'-damages in DNA, including 3'-phosphate (Demple and Harrison, 1994). In *E. coli*, the major proteins involved in the correction of AP sites and 3'-DNA fragments are Xth (exonuclease III) and Nfo (endonuclease IV) (Cunningham et al., 1986; Demple et al., 1986; Levin et al., 1988). Apn1 is the major (and perhaps sole) class II AP endonuclese/3'diesterase in *S. cerevisiae* and is present at about 7000 molecules per cell (Johnson and Demple, 1988). Apn1 is an endonuclease IV homolog. Unlike endonuclease IV, the level of Apn1 activity is not detectably regulated in response to oxidative stress or other toxic insults, nor is the level of the active enzyme or of the *APN1* transcript modulated by genotoxic challenges or during the cell cycle (Popoff et al., 1990; Ramotar et al., 1991). The major AP endonuclease of mammalian cells is Ape, an exonuclease III homolog (Demple et al., 1991; Robson and Hickson, 1991). Ape possesses only weak 3'-diesterase activity (Chen et al., 1991; Winters et al., 1994) but is a powerful hydrolytic AP endonuclease, displaying this activity at about 10-fold higher level than the bacterial or yeast enzymes (Demple and Harrison, 1994). Recently, Ape (also called Ref-1) was shown to have a dual function involved in base excision repair and in transcriptional regulation (Demple et al., 1991; Robson and Hickson, 1991; Seki et al., 1991; Xanthoudakis and Curran, 1992; Xanthoudakis et al., 1992). Ref-1 is essential for early embryonic development in mice (Xanthoudakis et al., 1996).

1.2.3.2 Nucleotide excision repair

NER is a process whereby oligonucleotide tracts containing distorted bulky base adducts are removed from the genome as nucleotides rather than free bases. NER was first discovered in *E. coli* in the mid 1960s by Setlow and Carrier (Setlow and Carrier, 1964). A few years later, the discovery by Cleaver (Cleaver, 1968) that the human

hereditary disease Xeroderma Pigmentosum (XP) was characterized by defective excision repair yielded the first evidence for a role of DNA repair in human health. The NER machinery is the sole repair system for bulky DNA adducts that result in large local distortions of the DNA structure. In addition, all other lesions that are repaired primarily by direct repair or BER are also excised by this repair system. There is no known covalent base modification that is not a substrate for the NER system (Huang et al., 1994). Therefore, NER is the most important repair system to maintain genetic integrity.

The basic strategy of excision repair is similar in prokaryotes and eukaryotes. In both systems, a multisubunit ATP-dependent nuclease (excision nuclease, excinuclease) makes dual incisions, one on either side of the lesion, and excises an oligonucleotide carrying the damage. In *E. coli*, three subunits are necessary and sufficient to carry out the dual incision. UvrA has DNA-independent ATPase and DNA binding activities, and is able to form a complex with UvrB protein. It has been suggested that the UvrA functions as a molecular matchmaker and brings the UvrB as an A2:B1 complex to the damaged site (Thomas et al., 1991). UvrA then dissociates, leaving behind a stable UvrB-DNA complex which is a specific target for UvrC. Upon binding to UvrC, UvrB makes the incision at the 5th phosphodiester bond 3' to the lesion, which causes a conformational change in the complex, enabling UvrC to make the 5' incision at the 8th phosphodiester. The oligonucleotide containing the damaged DNA is released from DNA duplex by UvrD encoded helicase II activity. The single-stranded gap left behind is filled by DNA polymerase I and sealed by DNA ligase. *uvrA, B* and *C* genes are under the control of the SOS response while *uvrD* gene is not. In *E. coli*, the repair of interstrand cross-links may occur in three basic steps; dual incision of one strand by ABC excinuclease, homologous pairing with a sister duplex mediated by RecA, and finally dual incision in the second strand by the ABC excinuclease.

The NER in eukaryotes is considerably more complex than that in *E. coli*. In *S. cerevisiae*, the genes involved in NER have been classified as *RAD3* epistasis group

(Table 1-1). The products of at least six genes (*RAD1, 2, 3, 4, 10* and *RAD14*), and probably *RAD25* appear to be absolutely required for early steps associated with the recognition and specific incision of damaged DNA (Prakash et al., 1993). At the damaged site, Rad1-Rad10 and Rad2 endonucleases hydrolyze the 5th phosphodiester bond 3' to the damaged site and the 24th phosphodiester bond 5' to the lesion respectively. Rad3 and Ssl2 (Rad25) have helicase activity and probably unwind the DNA duplex, releasing the oligonucleotide containing the damaged site. The *RAD3*, *TFB1*, *SSL1* and *SSL2* genes are essential probably because their presence in the transcriptional form of TFIIH. Extensive DNA damage leading to the induction of *RAD2* gene may result in assembly of the repairosome at the expense of Holo-TFIIH, resulting in diminished transcription initiation (Svejstrup et al., 1995).

In humans, 16 polypeptides, none of which has any homology to the *E.coli* excinuclease subunits, are required to perform the dual incisions (Mu et al., 1995). The complementation groups of human disease XP define seven polypeptides (XPA to XPG). The other genes were cloned by transfection of UV-sensitive rodent mutants with human DNA, and the genes are called *ERCC* (excision repair cross complementation). XPA binds to the damaged site and facilitates the entry of the replication protein HSSB (RPA); the XPA/HSSB complex recruits TFIIH and ERCC1/XPF complexes. The TFIIH unwinds DNA and permits the dual incisions. The ERCC1/XPF makes the 5' incision and XPG (recruited by HSSB/TFIIH) makes the 3' incision. In contrast to the bacterial excinuclease where the 3' incision precedes the 5' incision, in human excinuclease the incisions may form in random order (Matsunaga et al., 1996).

NER has clinical implications in humans, as three diseases have been associated with defects in this repair mechanism. XP patients are susceptible to sunlight-induced skin cancer, Cockayne's syndrome (CS) is characterized by growth defects, neurological deficiencies, and sunlight sensitivity, and trichothiodystrophy (TTD) patients suffer brittle hair, mental retardation, and some skeletal abnormalities (Sancar, 1995).

Table 1-2 Genetic complexity of nucleotide excision repair in eukaryotes

S. cerevisiae genes	Human genes
RAD3	*XPD*
SSL2 (RAD25)	*XPB*
RAD2	*XPG*
RAD14	*XPA*
RAD4	*XPC*
RAD1	*ERCC4/XPF*
RAD26	*CSB*
RAD10	*ERCC1*
RAD23	*HHR23A*
	HHR23B
TFB1	*p62*
SSL1	*p44*
RAD7	
RAD16	

1.2.3.3 Mismatch repair

Contributions to the discovery and elucidation of mismatch repair were made by numerous investigators including Paul Modrich and his colleagues (Modrich, 1987). More recently this repair mode has occupied center stage in the unfolding drama of cancer pathogenesis, with the demonstration that defective mismatch repair contributes to a particular type of colon cancer (Service, 1994; Karran and Bignami, 1994), a second direct link between DNA repair, somatic cell mutagenesis, and cancer. In mismatch repair, mismatched bases generated during semiconservative DNA synthesis or during recombination are excised. The *E. coli* MutHLS mismatch repair pathway is the major mismatch repair system that recognizes and repairs all single base mispairs except C·C (Modrich, 1991, 1994; Modrich and Lahue, 1996). It also repairs small insertion/deletion mispairs, although it may not efficiently recognize more than 4 unpaired bases (Parker and Marinus, 1992). The repair process is initiated by binding of MutS to a mismatch. The subsequent binding of MutL is thought to increase the stability of the MutS-DNA-mismatch complex and is required to activate MutH, which then nicks the unmethylated strand at hemimethylated GATC sites. Nicking of the unmethylated strand is then followed by the excision and re-synthesis steps of mismatch repair. These interactions result in coupling of mismatch repair to DNA replication, so that mismatches formed during DNA replication are repaired using the methylated parental strand as template, resulting in a reduction of misincorporation errors. In *E. coli*, repair of mismatches caused by replication errors is initiated by specific enzymes which recognizes and corrects the mispaired base from the newly synthesized daughter strand. Normally, DNA in *E. coli* is methylated at GATC sites by the Dam methylase. This is a post-replication modification. The strand discrimination is provided by the hemimethylated state immediately after DNA replication. However, it is difficult for the enzymes to distinguish strands when both are methylated, which occurs shortly after replication. Therefore, the repair of mismatches caused by recombination tends to be bi-directional,

that is, either of the two mismatched bases can be corrected according to each other, resulting in "gene conversion" (Fogel et al., 1981). Other bacteria like *Streptococcus pneumoniae* have a mismatch repair system similar to MutHLS system but the *Streptococcus* system, referred to as Hex system, is not presently known to utilize a MutH homolog or DNA methylation as a mechanism for strand discrimination in the repair process (Modrich, 1991; Fishel and Kolodner, 1995; Kolodner, 1995; Modrich and Lahue, 1996). DNA methylation does not occur in *S. cerevisiae* (Proffitt et al., 1984), and no convincing evidence has implicated DNA methylation in strand discrimination during mismatch repair in other eukaryotes (Modrich, 1991; Fishel and Kolodner, 1995; Kolodner, 1995; Modrich and Lahue, 1996). Nevertheless, there is a mismatch repair system in eukaryotes related to the bacterial MutHLS system (for review, see Kolodner, 1996). Six yeast genes have been identified that bear homology to mutS and therefore called *MSH* for mutS homolog (Reenan and Kolodner, 1992a; New et al., 1993; Ross-MacDonald and Roeder, 1994; Hollingsworth et al., 1995; Johnson et al., 1996; Marsischky et al., 1996). Msh1 is responsible for mismatch repair in mitochondria (Reenan and Kolodner, 1992b) while Msh2 contains the predominant binding activity for single base mispairs in nucleus (Miret et al., 1993). The analysis of *S. cerevisiae MSH2, MSH3,* and *MSH6* has led to the proposal of a model in which there are two different pathways of *MSH2*-dependent mismatch repair: repair specific for single-base substitution mispairs that requires a Msh2-Msh6 complex, and repair specific for insertion/deletion mispairs that requires either a Msh2-Msh3 complex or Msh2-Msh6 complex (Johnson et al., 1996; Marsischky et al., 1996). Recently, an activity in nuclear extract of *S. cerevisiae* was identified that can recognize 4-9 insertion mispairs (Miret et al., 1996).

1.2.3.4 Transcription and repair

It has been known for some time that there are at least two sub-pathways of NER *in vivo*. One operates globally throughout an organism's genome. *RAD7* and *RAD16* ,

together with other genes, are essential for this mode of repair (Verhage et al., 1994; Verhage et al., 1996). A second class of NER acts specifically for the removal of DNA damage from the transcribed strands of active genes (Hoeijmakers, 1993; Hanawalt and Mellon, 1993). This specialized form of NER, called transcription-coupled repair, requires most of the same proteins required for genomic NER (Sweder, 1994). The presence of UV-induced DNA damage in the transcribed strand of an expressed gene blocks the progression of transcribing RNA polymerase complex (Selby and Sancar, 1990; Donahue et al., 1994). Such impeded polymerase complex may be perceived as substrates for the NER pathway and the result of recognition of stalled RNA polymerase complex may be the preferential repair of the transcribed strands of active genes over that of the nontranscribed regions of the genome. Mutations in the *mfd* (mutation frequency decline) gene in *E. coli* (Selby et al., 1991), the *RAD26* gene of *S. cerevisiae* (Van Gool et al. 1994), and the *ERCC6* (*CSB*) and *CSA* genes of humans (Venema et al., 1990; van Hoffen et al., 1993; Henning et al., 1995) results in repair of the transcribed strands of active genes that is no greater than that of the nontranscribed strands or the genome overall. Recently, mismatch repair was found to influence the process of transcription-coupled repair in *E. coli*; mutations in *mutS* and *mutL*, but not in *mutH*, abolished the transcription-coupled repair similar to the effect from mutations in *mfd* gene (Mellon and Champe, 1996). In contrast, mutations in mismatch repair genes in yeast have no discernible effect on either transcription-coupled repair or global NER of UV-induced DNA damage (Sweder et al., 1996).

The mechanistic aspects of transcription-coupled repair are reasonably well understood in *E. coli*. A transcription-repair coupling factor (TRCF) encoded by the *mfdl* gene was identified and its mechanism of action elucidated (Selby and Sancar, 1993). TRCF recognizes, binds and releases a stalled RNA polymerase while simultaneously recruiting the damage recognition subunit, UvrA, of the UvrABC

excinuclease. This results in a ten fold enhancement in the repair rate of lesions in the template strand.

Gene- and strand-specific repair in eukaryotes appear to be similar to that in *E. coli* in outline; however, the mechanistic details are more complex. A model for transcription-coupled repair in humans has been proposed (Sancar, 1995). According to the model, RNA PolII stalls at the lesion, and the complex is recognized by TFIIS and CSA/CSB proteins. TFIIS enables RNA PolII to back up while the CSA/CSB complex, with the aid of TFIIE, recruits XPA and TFIIH to the lesion site that is now accessible. The excinuclease then assembles at this site, and the dual incisions and repair synthesis restore a lesion-free duplex that can now serve as a template for PolII to elongate the truncated transcript. The main difference between this model and the prokaryotic model is that in *E. coli* the truncated transcript is discarded, whereas in humans it is reused (Sancar, 1995).

1.3 Tolerance of DNA damage

DNA damage tolerance can be defined as resistance to the cytotoxic effects of DNA lesions that does not involve their removal from DNA. Four mechanisms have been proposed: translesion DNA synthesis, template switching, recombinational strand transfer, and activation of alternative origins of replication which is unique for eukaryotic cells (Naegeli, 1994).

1.3.1 Translesion DNA synthesis

Most mutagenesis resulting from damage by UV radiation, ionizing radiation and various chemicals appears to be due to a process of translesion synthesis, in which a polymerase or replicative assembly encounters noncoding or miscoding lesions, inserts incorrect nucleotides opposite the lesion, and then continues elongation (Friedberg et al., 1995). In *E. coli*, mutagenesis by UV radiation and many chemicals requires the intervention of a specialized cellular system to process damaged DNA in such a way that

mutations result. This specialized processing system is induced by DNA damage as part of the SOS response and is therefore commonly referred to as SOS mutagenesis. Three genes, *recA*, *umuD* and *umuC*, are required for SOS mutagenesis to occur. *umuDC* genes form a single operon and are under the control of the SOS response (Elledge and Walker, 1983; Kitagawa et al., 1985; Perry et al., 1985). RecA plays three roles in SOS mutagenesis: first, a transcriptional derepression of the *umuDC* operon by mediating LexA cleavage; second, a post-translational activation of UmuD by mediating its cleavage to yield UmuD'; third, it has been hypothesized that RecA nucleoprotein filament interacts with Umu proteins and positions them correctly at the site of the lesion (for review, see Walker, 1995). Although the detailed molecular mechanism for the SOS mutagenesis is unknown, it is suggested that UmuD' and UmuC may alter the processivity of DNA polymerase III, suppress 3' to 5' proofreading activity, or alter some other aspects of polymerase behavior, such as the test for correct Watson-Crick geometry between the template nucleotide and the incoming nucleotide (Walker, 1995). Recently, a RecA-independent, inducible mutagenic phenomenon is described in *E. coli*. Pretreatment of *E. coli* cells with UV strongly enhances mutation fixation at a specific site, 3-N^4-ethenocytosine (εC) lesion, which is called UVM for ultraviolet modulation of mutagenesis (Palejwala et al., 1994). Further investigation shows that UVM is also induced by alkylating agents (Wang et al., 1995) and oxidative stress (Wang and Humayun, 1996).

In *S. cerevisiae* and other eukaryotes, DNA damage-induced mutagenesis is not well defined. Genes in the *RAD6* epistasis group are suggested to participate in error-prone modes of bypass during DNA replication. At least four genes, *RAD6*, *REV1*, *3*, and *7*, are required for DNA damage-induced mutagenesis in *S. cerevisiae*. The *REV1* gene (Larimer et al., 1989) encodes a protein that has weak homology with the *E. coli* umuC protein; the *REV3* gene encodes a protein with sequence motifs characteristic of a DNA polymerase and forms a complex (Nelson et al., 1996) with the *REV7* gene product

(Torpey et al., 1994). This polymerase replicates past a thymine-thymine dimer, a lesion that normally severely inhibits replication, with an efficiency of about 10%. In contrast, bypass replication efficiency with yeast DNA polymerase α is no more than 1%. The Rev3-Rev7 complex is the sixth eukaryotic DNA polymerase to be described, and is therefore called DNA polymerase-zeta (pol- ξ) (Nelson et al., 1996).

Error-prone strategy similar to the bacterial translesion replication may also exist in mammalian cells. For example, the processivity factor PCNA (Proliferating Cell Nuclear Antigen) considerably enhances the ability of DNA polymerase δ to elongate DNA primer past pyrimidine dimers in the template strand. Thus, polymerase δ may catalyze error-prone bypass of DNA damage in the presence of PCNA and other accessory factors specialized in mutagenesis (Naegeli, 1994)

1.3.2 Template switch

The main feature of Strauss and co-workers' model (Strauss, 1991) is to use a newly synthesized strand as template instead of the damaged parental strand. When replication of one DNA strand is blocked by DNA damage, replication of the other strand can continue. Template switching is then initiated by pairing interactions between the two daughter strands, allowing the nascent DNA initially blocked to bypass the lesion. Thus, the polymerase uses the complementary daughter strand instead of the damaged parental strand as a detour around the lesion, and then switches back to the parental strand after clearing the damaged site. It is viewed that template switching constitutes a major mechanism for the bypass of DNA lesions in mammalian cells (Naegeli, 1994).

1.3.3 Replicative bypass or Post-replicative repair

The replicative bypass appears to be the major route of replication recovery at sites of DNA damage in *E. coli*. This pathway is also called post-replicative repair and involves repriming of DNA synthesis some distance downstream from the damaged base, thus producing a daughter-strand gap opposite the site of damage. RecA protein presumably binds to this single-stranded segment and initiates repair of the gap by

homologous recombination, resulting in transfer of DNA from undamaged duplex to the homologous strand of the damaged duplex. Subsequently, the remaining damage on the parental strand is repaired by the excision-repair machinery. In *S. cerevisiae*, *RAD5, 6,* and *18* genes are required for the post-replication repair. A "channeling" function has been assumed for *Srs2*. When active, Srs2 protein channels the gap filling in favor of mutagenesis rather than by homologous recombination mediated by *RAD52* group of genes (Friedberg et al., 1995; Milne et al., 1995).

In addition to its importance in the repair of single-strand gaps, recombination is also important for the repair of double-strand breaks (DSBs) produced by DNA damage such as ionizing radiation (Dizdaroglu, 1992) and oxidative stress (Demple, 1994). It is known that rapidly growing bacteria with multiple replicons are more resistant to ionizing radiation, which induces DSBs, than those in stationary phase that have a lower copy number of replicons, and that haploid yeast cells in G1 phase are extremely sensitive to ionizing radiation, while the haploid cells in G2 are as resistant as diploid cells (Friedberg et al., 1995). These results suggested that two homologous DNA molecules are required for the repair of the damage. In *E. coli*, at least 20 genes are required for recombination, the most important ones are *RecBCD* and *RecA*. RecBCD complex has helicase and exonuclease activities and is involved in unwinding duplex DNA from DSB ends and degrading it from its 5' ends, leaving a 3'-OH ssDNA tail on the DNA. RecA forms a right-handed helical nucleoprotein filament on ssDNA and carries out the invasion of homologous duplex DNA. After pairing with the homologous DNAs, Holliday junctions are processed by RuvA, B, C, RecG and by a recently identified new endonuclease, Rus (Sharples et al., 1996). SOS-induced genes (*recN* and *uvrD*) and other gene products (RecF and J) are also necessary for the repair of DSBs (Shinohara and Ogawa, 1995).

In *S. cerevisiae*, the principal DSB repair mechanism requires a group of at least 11 genes comprising the *RAD52* epistasis group including *RAD50* to *RAD58, XRS2*, and *MRE11*. Mutations in any of these genes result in sensitivity to agents that cause DSBs,

such as ionizing radiation, with only a minor cross-sensitivity to agents that generate other types of DNA lesions. The *RAD52* group genes are divided into three classes (Shinohara and Ogawa, 1995). The first class consists of *RAD50, MRE11,* and *XRS2* genes. Yeast cells mutated in one of these genes are deficient in the repair of damaged DNA and meiotic recombination, but are proficient in mating-type switching and mitotic recombination. Mre11 can interact with Rad50 and Xrs2, but Rad50 and Xrs2 can not interact with each other. Rad50 is an ATP-dependent DNA binding protein and contains motifs common to a class of phosphoesterases (Sharples and Leach, 1995). The Rad50-Mre11-Xrs2 complex is required for generation and processing of DSBs. Mutants in the second class of genes, *RAD51, 52,* and *54* are defective in the repair of DSBs and in both mitotic and meiotic recombination (except for *RAD54*). These mutants accumulate unrepaired DSBs with long single-stranded tails, suggesting that these gene products function in a step subsequent to the processing of DSBs. Rad51 is a RecA homolog and can catalyze a strand-exchange reaction in the presence of the trimeric yeast ssDNA binding protein RPA. Rad52 is a ssDNA-binding protein that can anneal homologous ssDNAs to a duplex. Rad51 can bind to Rad52, 54, and 55. The third class of genes consists of *RAD55* and *57*. Rad55 and 57 interact with each other as well as with the gene products in the second class to form a big complex called "recombinosome" (Firmenich et al., 1995). However, the *in vivo* presence of the recombinosome has not yet been proved. Nonhomologous, often referred to as "illegitimate" recombination also play a role (minor role as compared to homologous recombination) in DSB repair. This nonhomologous end-joining pathway requires two Ku (the first two letters of a patient's name) homologs, Hdf1, a Ku70 homolog (Feldmann and Winnacker, 1993), and Ku80 (Milne et al., 1996) as well as Rad50. Rad50 may process DNA ends to form substrate for subsequent Ku- or Rad52-dependent recombination steps (Milne et al., 1996).

Higher eukaryotes appear to favor a nonhomologous DNA end-joining pathway for DSB repair. The well characterized mammalian genes involved in DSB repair are

DNA-PK (DNA-dependent protein kinase), *XR-1* (*XRCC4*) (yet to be cloned), and *XRCC1*. DNA-PK is a heterotrimer consisting of a p450 catalytic subunit and Ku regulatory subunits (Anderson, 1993), Ku80 (XRCC5) and Ku70 (XRCC10). Ku80 and Ku70 make a heterodimer with DNA end-binding activity. DNA-PK phosphorylates many proteins including p53 and the p34 subunit of RPA *in vitro*. The *Ku80*, *Ku70* and *XR-1* genes are involved in both mitotic recombination and DSB repair. Mutants of DNA-PK p450 subunit are not affected in mitotic recombination but are defective in DSB repair. The *XRCC1* gene corrects the repair defect in cells sensitive to alkylating agents and ionizing radiation.

1.3.4 Activation of alternative origins of replication

Prokaryotic chromosomes have only one origin of replication, from which DNA is replicated bi-directionally. In contrast, the chromosomes of eukaryotes contain many possible sites where replication can be initiated. Normally most of these sites are suppressed. However, when progression of an active replication fork is blocked by DNA damage, DNA replication may be completed by initiation of another adjacent site on the same chromosome. This compensatory replication fork would then approach the site of damage from the other side, complete DNA synthesis, and eventually leave a single-stranded gap in the daughter strand opposite the lesion.

1.4 Regulation Of Cellular Responses To DNA Damage

Cellular responses to DNA damage include enhanced protection, altered growth and programmed cell death (apoptosis), which have evolved to help cells or organisms survive the lethal effect of DNA damage. The cellular response to DNA damage is an actively regulated process involving a lot of gene products and their interactions.

Gene regulation directly influences many biological phenomena such as cell growth, response to environmental change, development of multicellular organisms, and

disease (Struhl, 1995). Therefore, it is important to understand the transcriptional regulation mechanisms before discussing the regulation of the DNA damage response.

1.4.1 Transcriptional regulation in yeast , a general consideration

The yeast *Saccharomyces cerevisiae* is a simple unicellular eukaryotic organism. The genome contains 16 linear chromosomes with a total DNA content of about 10^4 kb, of which about 80% is transcribed (compared to 7% in higher eukaryotic cells). Yeast genes are tightly packed with little spacer DNA and strikingly few pseudogenes. There is one gene for every 1.5-2 kb of DNA (Olson, 1992). This figure corresponds to an estimate of about 5800 genes for the whole genome (George et al., 1996; Dujon, 1996). *S. cerevisiae* has been extensively used as model system for the study of molecular biology in eukaryotes. Although *S. cerevisiae* has greater genetic complexity than bacteria, it still shares many of the technical advantages of prokaryotes and their viruses. These include:

a). Fast and simple growth.

b). Ease of mutant selection.

c). Development of DNA transformation has made yeast particularly accessible to gene cloning and genetic engineering techniques.

d). Availability of versatile cloning vectors (YEp, YIp and YCp).

e). Unlike many other organisms, strains of *Saccharomyces* are viable with a great many markers.

f). Because of efficient homologous recombination, targeting of DNA sequences to specific sites of yeast chromosomes is readily achievable in *S. cerevisiae*.

In addition, *S. cerevisiae* also possesses unique technical advantages of its own. It can exist in both haploid and diploid states which greatly facilitates the isolation and genetic analysis of recessive mutants. The completion of yeast genome sequencing project has already provided, and will continue to provide, valuable information on all aspects of yeast molecular genetics (Dujon, 1996).

Cells regulate the transcription of nearly all of their genes, and it is not surprising that a great number of cellular genes (some estimates range around 10%) is devoted to proteins involved in regulating gene expression, especially at the initiation of transcription. The initiation of transcription of all genes is controlled by the binding of proteins to DNA sequences called promoter elements.

1.4.1.1 Promoter elements

The term promoter is used to describe the DNA sequences found upstream of the coding region of genes. Three elements within the yeast promoters are necessary and sufficient for the efficient and accurate regulation of transcription initiation: the upstream regulatory sequences, the TATA element, and the initiator (I).

a). Upstream regulatory sequences: These include upstream activating sites (UASs), or enhancers, and upstream repressing sites (URSs).

UASs in yeast are similar to enhancer sequences in mammals. Like enhancers, UAS elements can function over far distances from the TATA box (up to at least 600 bp) in a bi-directional manner (Guarente and Hoar, 1984; Guarente, 1988). UASs are required for transcription, and they usually determine the particular regulatory properties of a given promoter (Guarente, 1984). The UASs were first recognized in the early 1980s by 5' deletion analyses of the *CYC1* and *HIS3* promoters (Guarente et al., 1982; Struhl, 1982). The key experiments in defining a role for a UAS in determining promoter specificity was carried out by Guarente et al (1982). Here, a hybrid promoter was constructed containing the *GAL* UAS linked to the TATA element and initiator element from the *CYC1* promoter. *CYC1* expression is now activated and properly regulated by *GAL* UAS. This construction of a hybrid promoter has become a standard test used to identify region(s) of a yeast promoter that contain transcriptional activating sequences. The *CYC1* promoter lacking the UASs and linked to the *E. coli* β-galactosidase coding region is commonly used.

Although upstream activation at the UAS by transcriptional activators is likely to be a basic mode of gene regulation in yeast and other eukaryotes (Guarente, 1988), other upstream elements can be identified which may modify the activity of UASs. One such element, URS, is involved in transcriptional repression. Repression can be achieved by competitive DNA binding of repressors with activators or by interference with the activity of activators. However, most eukaryotic repressors seem to act directly on the basal transcription machinery (Johnson, 1995). Some URSs can repress transcription when located upstream of UASs, but repression is generally much more efficient when the URS lies between the UAS and the TATA element. However, the mating-type silencer efficiently represses transcription when located 2 kb upstream or downstream from the initiation sites (for review, see Struhl, 1995).

b). The TATA elements and the initiator.

TATA elements (consensus sequence TATAAA) are necessary but not sufficient for transcriptional initiation of most yeast genes. The distance between the yeast TATA element and the mRNA start site ranges from 40 to 120 bp depending on the promoter (Struhl, 1987). In contrast, higher eukaryotic TATA sequences are almost always located 25 to 30 bp away from mRNA start site. *S. cerevisiae* promoters are extremely A/T-rich, making it difficult to distinguish which of the several potential sequences is a TATA box (Davison et al., 1983). TATA elements are presumed to have a general role in the transcription process such as the binding of general transcription factors (GTFs). However, approximately 20% of yeast promoters contain homopolymeric dA:dT tracts that function as an upstream regulatory element by virtue of their intrinsic DNA structure, not by interacting with a specific DNA-binding protein (Struhl, 1985; Iyer and Struhl, 1995). It is now known that a large number of genes, most of them being housekeeping genes, lack any recognizable TATA element. Although these promoters are not as strong as TATA-containing promoters, they can, to a somewhat lesser extent, modulate accurate

transcription initiation. Most TATA-less promoters still require all the GTFs including TFIID (Zawel and Reinberg, 1993).

In higher eukaryotes, the transcription start site is determined primarily by the distance from TATA box. In yeast, the precise position of initiation is encoded at the initiation site itself. A series of experiments performed by Smale and Baltimore (1989) identified a second core promoter element, the initiator. The initiator is a short , weakly conserved element that encompasses the transcription start site (for review see Weis and Reinberg, 1992). Consensus sequences of TCGA and PuPuPyPuPu account for the majority of initiation sites, and if inserted near a TATA box, they will function as initiators (Hahn et al., 1985). A protein that recognizes the initiator has not been identified.

1.4.1.2 General transcription factors and activators

In vitro, RNA Polymerase II cannot initiate promoter-specific transcription alone; it requires an additional set of proteins called the general transcription factors (GTFs) to assemble around the startpoint of transcription. Initiation requires transcription factors to act in a defined order to build a complex that is joined by RNA polymerase II (Lewin, 1994; Roeder, 1996). A single subunit of TFIID was identified as the TATA-box binding protein (TBP). The binding of TFIID to the TATA element is the essential first step in the stepwise assembly of the transcription complex (Fire et al., 1984; Cormack and Struhl, 1992). TFIID also contains a variety of small proteins called TBP-associated factors (TAFs). TFIIA contains several subunits (two in yeast, three in mammals) and joins the complex from farther upstream. Then, TFIIB joins the complex from downstream of the TATA box. TFIIF consists of two subunits. The large subunit has an ATP-dependent DNA helicase activity that could be involved in melting the DNA at initiation. The small subunit has some homology to regions of the bacterial σ factor that contact the core polymerase and binds tightly to RNA polymerase II. TFIIF may in fact bring RNA polymerase II to the assembling transcription complex. At this point, TFIIE

can bind to the complex, followed by TFIIH and TFIIJ. TFIIH has a kinase activity that can phosphorylate the CTD (carboxy terminal domain) tail of RNA polymerase II. It is possible that phosphorylation of the tail is needed to release RNA polymerase II from the complex of transcription factors so that it can leave the promoter and start elongation. In addition to its role in the initiation of transcription, recent studies have demonstrated a direct involvement of TFIIH in nucleotide excision repair (Svejstrup et al., 1995, 1996).

In vivo, efficient transcription also requires one or more activator proteins, which are usually bound to DNA upstream of the TATA box. Many site-specific transcription activators are modular in nature, consisting of a DNA-binding domain that directly contacts the DNA, a multimerization domain that allows the formation of homo- or heteromultimers, and a transcription activation domain. Examination of the DNA-binding domains of regulatory proteins from prokaryotes and eukaryotes has revealed at least three predominant motifs (Lewin, 1994). The first is the helix-turn-helix. The more C-terminal helix in this structure attaches to the major groove and makes the base-specific contacts with DNA. The second helix lies above the major groove and is connected to the C-terminal helix by a sharp β turn. Examples of this motif in yeast are found in the α2 and a1 mating-type regulators (Struhl, 1993). A second is the zinc finger motif. Pairs of cysteine and histidine residues are positioned to coordinate a zinc atom, and the residues in between loop out to form the finger. Yeast Adr1p and the Swi5p proteins (Struhl, 1993) contain this motif. A third motif found in mammalian AP1, Myc, Fos and C/EBP factors and in yeast Gcn4 (Struhl, 1993), is called the leucine zipper. This motif contains leucine residues positioned every seven amino acids, which would place them on the same face of an α-helical structure. Several different types of activation domains have been identified and classified as acidic, glutamine-rich, and proline-rich (Tjian and Maniatis, 1994). The transcription activation depends on the interaction between an activation domain and its target. Evidence suggests that TFIID is the major target for specific interaction with activators. This seems to be reasonable

since the binding of TFIID to the TATA box is the first, probably the rate-limiting step in the assembly of the preinitiation complex. TAFs in the TFIID multiprotein complex play a central role in RNA polymerase II transcriptional regulation. Some TAFs can function as co-activators and direct promoter selectivity by RNA polymerase II (Verrijzer and Tjian, 1996). TAFs are pin-pointed as the actual targets for some activators such as Sp1 (Gill et al., 1994). However, other components of the basal transcription complex such as TFIIA, B, E, F, and H also present potential targets for activators (Goodrich et al., 1996). Binding of transcription activators to DNA causes DNA conformational changes which are essential for the assembly of multiprotein complexes during transcription activation (Becker et al., 1995).

Based on the fact that transcriptional activation can still occur when the DNA binding domain and the activation domain of an activator are brought together from two different proteins, the two-hybrid system, a method used to identify protein-protein interactions, was developed (Fields and Song, 1989; Chien et al., 1991). Another similar method, designed for the identification of genes encoding proteins that recognize a specific DNA sequence, was developed and was named the one-hybrid system (Li and Herskowitz, 1993; Wang and Reed, 1993).

1.4.2 Induction of specific sets of genes

The induction of specific sets of genes is one of the hallmarks of the DNA damage response in living cells and is proposed to play an important role in cell survival and maintenance of the stability of the genetic material.

DNA damage-inducible genes are identified through different approaches. One approach involves differential hybridization using cDNA made from mRNA isolated from treated (with DNA damaging agents) and untreated cells (McClanahan and McEntee, 1984). The genes isolated are designated *DDR* for DNA damage responsive. The other method utilizes the power of transcriptional fusion (Ruby and Szostak, 1985), in which random yeast genomic DNA fragments were fused to the *E. coli lacZ* gene;

transformants were identified that displayed enhanced expression of β-galactosidase (β-gal) activity after treatment with the UV-mimetic chemical 4NQO (4-nitroquinoline-1-oxide). These genes are designated *DIN* for damage inducible. In yeast, there may be 80 or more DNA damage-inducible genes (Ruby and Szostak, 1985), which represent about 2% of the total number of genes. Inducible genes have been identified from all three *RAD* epistasis groups, from genes involved in repair of specific type of damage, such as BER (*MAG1*), cross-link repair (*SNM1*) and the repair of pyrimidine dimers by photoreactivation (*PHR1*), and also from genes involved in nucleic acid metabolism such as *RNR* (ribonucleotide reductase), *CDC8* (thymidylate kinase), *CDC9* (DNA ligase) and *CDC17* (DNA polymerase I), and other genes (e.g. *UBI4*) encoding proteins such as ubiquitin (see Table 13-1 in Friedberg et al., 1995). A large number of cDNA clones encoding UV-inducible transcripts were isolated by subtraction hybridization from mammalian cells and were named *DDI* (DNA damage inducible). This approach has the advantage that low abundant cDNA clones can be isolated (Fargnoli et al., 1990). In mammalian cells, DNA damage induces a large number of genes associated with many different cellular processes including signal transduction (e.g. transcription factors and certain oncogenes), intercellular signaling (e.g. cytokines), growth control (e.g. oncogenes and others), responses to tissue injury (e.g. collagenase), inflammation (e.g. IL-1 and TNF), DNA repair, response to oxidative stress, and other potentially protective responses (e.g. metallothionein) (see Table 1 in Fornace Jr., 1992).

These studies have generated several interesting general insights. First, the induction of most damage-inducible genes is not agent-specific, but occurs after treatment with a variety of different DNA damaging agents, including some that activate genes whose products are not required for repairing the damage generated by the agents. For example, the *PHR1* gene, which encodes the DNA photolyase that specifically removes pyrimidine dimers, can be activated by various chemical agents in addition to UV (Sebastian et al., 1990). Likewise, the alkylation damage-specific repair gene *MAG1* is

also inducible by radiation damage such as UV, even though *mag1* mutant does not exhibit enhanced sensitivity to killing by UV (Chen et al., 1990). Some DNA damage-inducible genes such as *UBI4* (Treger et al., 1988), *DDRA2*, and *DDR48* (Maga et al., 1986; Miralles and Serrano, 1996) are also induced by heat shock or by osmotic stress. Nevertheless, the spectrum of inducing agents varies considerably for different damage-inducible genes (McClanahan and McEntee, 1984; Ruby and Szostak, 1985). In contrast, the induction of *SNM1* gene, which repairs interstrand cross-links, is agent-specific. *SMN1* is induced solely by cross-linking chemicals such as nitrogen mustard, but not by monofunctional agents such as MMS (Wolter et al., 1996). Secondly, despite some overlap, heat-shock treatment induces a set of genes different from that induced by DNA-damaging agents (McClanahan and McEntee, 1986). None of the well-characterized DNA repair genes apparently responds to heat shock (Friedberg et al., 1995).

Unlike the isolation of damage-inducible genes with unknown functions, certain genes with known DNA repair functions were isolated by functional complementation of the corresponding mutants. The finding that some of the DNA repair genes from eukaryotes could functionally correct the repair deficiency of mutant bacterial cells (Chen et al., 1989; Berdal et al., 1990; Xiao et al., 1991) greatly facilitates the isolation of repair genes from eukaryotes.

1.4.3 Cell-cycle arrest and programmed cell death

The cell cycle is a continuous and highly ordered process consisting of various distinct chemical and physical events known as G1, S, G2 and M phases. In proliferating cells, DNA damage has great consequences in two major cell-cycle events: DNA replication and mitosis. Replication can render mutations irrepairable, while mitosis in the presence of unrepaired strand breaks can lead to gross chromosomal aberrations in the daughter nuclei. To counter these threats to genomic integrity, eukaryotic cells employ mechanisms that temporarily arrest cell-cycle progression. These controls have been termed as "checkpoints" (Hartwell and Weinert, 1989). Such cell-cycle delays provide

opportunities for DNA repair before or during replication and segregation of the affected chromosomes.

In *S. cerevisiae*, at least six genes have been identified so far that are involved in cell-cycle checkpoints following DNA damage. They are *RAD9, 17, 24, MEC1/ESR1, RAD53/MEC2/SAD1/SPK1* and *MEC3*. *RAD9, 24, 53* and *MEC1* gene products are required for the G1/S checkpoint, which is presumed to allow time for the repair of the damaged template prior to its replication (Siede et al., 1993; Siede et al., 1996). Recently, a S phase checkpoint was identified, and involves the *MEC1* and *RAD53* gene products (Paulovich and Hartwell, 1995). The delay in S phase is due to a decrease in either the "fireoff" of late replicons or in the elongation rate of pre-existing nascent strand or both. All of the six genes involved in cell-cycle checkpoints are required for G2 arrest, which prevents the onset of broken chromosomes from being segregated (Siede, 1995). Yeast cells also have a checkpoint that prevents the onset of mitosis when DNA replication is blocked with hydroxyurea. Two essential genes, *MEC1* (Weinert et al., 1994) and *RAD53* (Allen et al., 1994), transduce signals from DNA damage and replication block to downstream effectors (Elledge, 1996).

In mammalian cells, ATM and DNA-PK proteins probably serve as sensors to DNA damage (Meyn, 1995). DNA-PK consists of three subunits, Ku 70 and Ku 80 bind to free DNA ends. A large catalytic subunit of 450 kDa binds to DNA-bound Ku. Once bound to DNA, the protein is activated and can phosphorylate a variety of proteins. ATM encodes a protein with a C-terminal domain similar to the catalytic domain of a family of lipid kinases. In addition, ATM shows remarkable similarity to two yeast checkpoint genes, the *S. cerevisiae MEC1/ESR1* gene and the *S. pombe rad3* gene. ATM function is required for the rapid induction of p53 which in turn regulates p53-dependent checkpoints (Enoch and Norbury, 1995). The potential effector of cell-cycle arrest is $p21^{Cip1}$ (also known as WAF1, pic1, Sdi1 or p21CAP), an inhibitor of cyclin-dependent kinases (CDKs). After irradiation, *Cip1* gene expression is induced by p53, and $p21^{Cip1}$

activity has been detected in wild-type cells, but not in cells lacking p53 activity (Duilc et al., 1994). Activation of $p21^{Cip1}$ inhibits CDKs, preventing phosphorylation and inactivation of the retinoblastoma protein Rb. In turn, Rb is a negative regulator of the transcription factor E2F, which is required for expression of S-phase-specific genes (Chernova et al., 1995; Enoch and Norbury, 1995).

Apoptosis or programmed cell death is a genetically controlled response for cells to commit suicide, which is evolved to protect the whole organism by eliminating heavily damaged cells. The process of apoptosis is controlled through the expression of a number of genes, with p53 as one of the most important regulators in the process. Through sequence-specific DNA binding, p53 can modulate the transcription of a variety of target genes involved in DNA damage repair, cell-cycle arrest, and apoptosis. Most of the p53 mutations sequenced from human tumors map within the sequence-specific DNA-binding domain and impair the ability of p53 to bind DNA (Lee et al., 1995). Therefore, sequence-specific DNA binding, and any functional consequences thereof, is essential for tumor suppression. Specific residues required for trans-activation within the activation domain have been identified by mutagenesis. Mutation of residue 22 and 23 dramatically impairs the activity of p53 as a transcription activator (Lin et al., 1994.). Provided with a death stimulus by E1A, p53 with mutantions in residues 22 and 23 failed to induce apoptosis in comparison to the wild-type (Sabbatini et al., 1995). p53 might activate the transcription of death genes (e.g. *BAX*) or/and repress the transcription of survival genes (e.g. *BCL-2*) (Miyashita and Reed, 1995). The *BAX* expression is depressed, and *BCL-2* expression is elevated in tissues from p53 null mice relative to p53-expressing wild-type controls (Miyashita et al., 1994). It is possible that the ratio of the expression of "death" genes (apoptosis promoters) to "survival" genes (apoptosis inhibitors) determines the fate of a cell. If this is true, then either up-regulation of Bax or down-regulation of Bcl-2 would be sufficient to induce apoptosis. Bax is up-regulated by wild-type p53 expression but not by expression of the transcriptionally defective p53 mutant. Furthermore, ectopic

Bax expression induces apoptosis where p53 is constitutively expressed in the mutant conformation, suggesting that Bax alone is sufficient to induce apoptosis and acts downstream of p53. Bcl-2 can bind to and neutralize Bax function and can thereby block p53-induced, Bax-dependent apoptosis (Oltvai et al., 1993). In addition to the induction of apoptosis, p53 can also induce cell-cycle arrest. It is suggested that under conditions of mild DNA damage, p53 is transiently activated and the growth arrest pathway is induced, the cell undergoes damage repair and then resumes cell cycle. However, if DNA damage is severe and beyond repair, p53 is permanently activated and the apoptosis pathway prevails. More recent results show that p53-induced apoptosis and growth arrest are functionally separable (Rowan et al., 1996), and are determined by at least two genetic factors in colorectal cancer cells: p21-mediated growth arrest that protects cells from apoptosis, and trans-acting factors that can overcome this protection, resulting in cell death (Polyak et al., 1996).

1.4.4 Regulation of DNA damage response in *E. coli* and eukaryotes

The regulation of DNA damage response has been most extensively characterized in bacterial cells. In *E. coli*, DNA damage-inducible genes belong to different regulons in response to specific types of damage. At least four such regulatory pathways have been identified, namely, adaptive response to alkylation damage (Lindahl et al., 1988), SoxR-mediated (Wu and Weiss, 1992) and OxyR-mediated (Kullik et al., 1995 a, b) responses to oxygen species (Demple, 1991; Farr and Kogoma, 1991), and the LexA/RecA-mediated SOS response (Walker, 1985). The SOS regulon is considered a global response to genotoxic stress because exposure of *E. coli* cells to UV leads to the temporally coordinated transcriptional induction of multiple genes involved in DNA repair, recombination, mutagenesis and cell cycle control (Walker, 1984). The coordinate

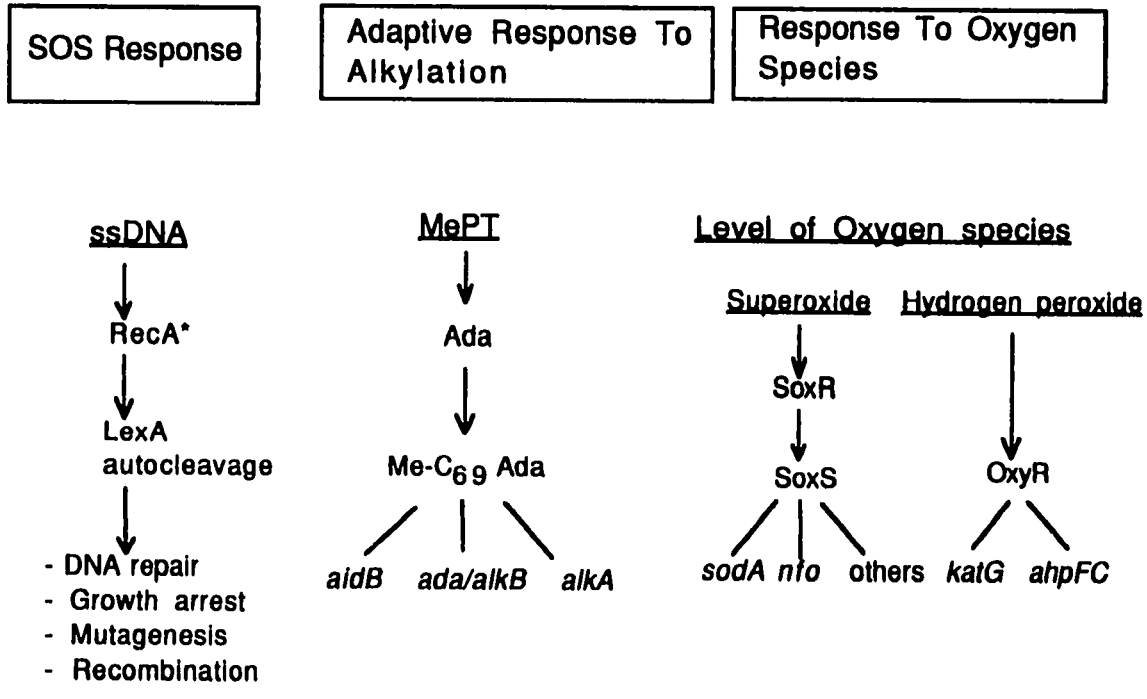


Figure 1-2 DNA Damage Responses In *E. Coli*

induction of about 20 genes is controlled by the products of *lexA* and *recA* during the SOS response (Walker, 1985). Thus in the absence of single-stranded DNA (ssDNA), the expression of genes in the SOS regulon is limited by the LexA repressor. The RecA protein is activated by ssDNA, which in turn mediates the LexA self-cleavage and the derepression of SOS genes (Walker, 1985). DNA damage responses in *E. coli* is shown in Fig. 1-2.

In eukaryotes, the molecular mechanism of damage response is less well understood and is obviously more complex. Mammalian cells possess multiple pathways that respond to DNA damage, and there is an extensive overlap between mammalian genes induced by DNA damage and those induced by other stimuli. For example, growth factor receptors (Sachsenmaier et al., 1994), transcription factors (Devary et al., 1991) and tyrosine kinases (Devary et al, 1992) are involved in mammalian UV response, and at least one of the pathways appears to be conserved in yeast (Engelberg et al., 1994). At post-translational level, p53 is responsive to induction by several DNA damaging agents (Kastan et al., 1992; Lu and Lane, 1993). However, the induction of some genes (e.g. *GADD45*) by MMS and other base-damaging agents is not mediated by p53 (Kastan et al., 1992), suggesting the existence of additional regulatory pathway(s). Most evidence suggests that p53 functions as a transcription factor. p53 contains three functional domains: an amino-terminal transcriptional activation domain, a central sequence-specific DNA binding domain, and a carboxyl-terminal oligomerization domain. It is proposed that the C-terminal domain recognizes the damage-induced lesions and this interaction could serve as a scaffold for the assembly of multiprotein complexes engaged in DNA repair and could enhance the ability of p53 to activate specific genes involved in cell cycle control, apoptosis, or DNA damage response (Jayaraman and Prives, 1995; Lee et al., 1995). Recently, Sturzbecher et al. (1996) showed that p53 seems to inhibit homologous recombination via direct interaction with Rad51/RecA protein. It is suggested that p53 may directly select the appropriate pathway for DNA repair and

control the extent and timing of the production of genetic variation via homologous recombination. p53 also exhibits 3' to 5' exonuclease activity (Mummenbrauer et al., 1996), which should substantially extend our view on the role of p53 as a "guardian of the genome" (Lane, 1992).

Yeast *S. cerevisiae* has been extensively used as a model system for the study of DNA repair and regulation of DNA damage response. There are two major approaches towards the understanding of DNA damage induction in yeast. The first approach is by dissecting the promoters of damage-inducible genes to identify *cis*-acting elements and their binding protein(s) that mediate the damage response. Only a few of these promoters have been carefully characterized, namely *RAD54* (Cole and Mortimer, 1989), *RNR2* (Elledge and Davis, 1989b; Hurd and Roberts, 1989), *RAD2* (Siede et al., 1989; Siede and Friedberg, 1992), *PHR1* (Sebastian and Sancar, 1991; Sancar et al., 1995), and *MAG1* (Xiao et al., 1993). Although some "consensus" sequences are indicated in a number of promoters (Sebastian et al., 1990; Yagle and Mcentee, 1990; Jones and Prakash, 1991; Siede and Friedberg, 1992; Xiao et al., 1993; Sancar et al., 1995), evidence for their co-regulation through these consensus sequences has not yet been documented. The second approach is by identifying transcription factors that control damage response. The damage uninducible mutants (*dun* mutants) were isolated based on the lack of the ability to induce the transcription of *RNR3-URA3* fusion gene upon DNA damage. The Dun2-Rad53(Sad1)-Dun1 pathway in yeast was identified by complementation of the corresponding mutant phenotype (Zhou and Elledge, 1993; Allen et al., 1994; Navas et al., 1995). More recent results showed that the ATM-like kinase *MEC1* controls the phosphorylation of *RAD53* (*SAD1*) which encodes a protein kinase that mediates DNA damage induction and cell cycle arrest (Sanchez et al., 1996). *DUN1* encodes a protein kinase that controls the DNA damage response of *RNR* genes but not other DNA damage inducible genes, such as the *RAD3* epistasis group genes (Sancar, 1995), *RAD51* (Torre-Ruiz and Fabre, unpublished result, see ref. Aboussekhra et al., 1996), *RAD54* (Wolter et

al., 1996), *DDR48* and *UBI4* (Zhou et al., 1993), suggesting the existence of multiple pathways in the regulation of DNA damage response in yeast.

Some evidence also suggests the involvment of other genes in the regulation of DNA damage induction. For example, induction of the *DDRA2* transcript by 4NQO or nitrosoguanidine is dependent on a functional *RAD3* gene (Maga et al., 1986) and *DDR48* is uninducible in *rad52* mutant strains following EMS (ethylmethylsulfonate) and heat shock treatments (Maga et al., 1986; Sheng and Schuster, 1993). RPA is a trimeric ssDNA binding protein and is known to play a role in DNA replication. Recently, it was shown that RPA bound to homologous sequences in the promoter of a number of DNA repair genes, suggesting that RPA may be involved in co-regulating the expression of these genes (Singh and Samson, 1995). Besides, RPA was identified as a component in a complex that binds the transcription start site of human metallothionein IIA gene and represses transcription (Tang et al., 1996). More recent results showed that the checkpoint gene, *RAD9*, was required for the induction of a variety of damage inducible genes, suggesting the existence of an analogous eukaryotic SOS network coordinating the cellular response to DNA damage (Aboussekhra et al., 1996).

Although a DNA damage induction system exists in eukaryotes and is suggested to have a protective role, the physiological significance of DNA damage inducibility is difficult to assess. Failure to induce *RAD54* does not affect DNA repair or recombination pathways (Cole and Mortimer, 1989). Deletion of the DRE promoter elements in *RAD2* (which eliminates the induction by UV) produces a moderate effect on survival, which is detectable only when cells are synchronized and UV irradiated in the G1 phase of the cell cycle (Siede and Friedberg, 1992). Nevertheless, the removal of pyrimidine dimers from DNA in both transcriptionally active and inactive genes (*MATa* and *HMLa*, respectively) exposed to UV radiation is enhanced after pretreatment of the cells with a lower dose (Waters et al., 1993).

MAG1 and *DDI1* are two clustered genes from *S. cerevisiae* and are subject to detailed analysis in this thesis. The data presented below show that these two genes are coordinately regulated by DNA damaging agents and are differentially expressed in other situations. Through dissection of the promoter region between *MAG1* and *DDI1*, some regulatory elements were identified. A common regulatory element (UAS_{DM}) in the promoter is required for the expression of both genes while others play a regulatory role in a gene-specific manner. Interestingly, one gene's regulatory element is found to be buried in the protein coding region of another gene, a phenomenon which has not been reported before.

CHAPTER II: MATERIALS AND METHODS

2.1 Yeast Genetics

2.1.1 Yeast strains and culture

The *S. cerevisiae* strains used in the thesis are listed in Table 2-1. DBY747 was obtained originally from D. Bostein (Stanford University, USA) and was used as plasmid recipient. Y203 and Y290 were obtained from S. J. Elledge (Baylor College of Medicine, Houston, Texas). Yeast cells can be stored in sealed agar plates for up to three months at 4°C. For longer term storage, vigorously growing cells were dispersed in 15% sterile glycerol and the vial was maintained at -70°C for an indefinite period of time.

Culture media used to support yeast cell growth were prepared based on the description of Sherman et al. (1983). Solid media were made by adding 2% Bacto-agar to the liquid media prior to autoclaving. Yeast cells were cultured in a 30°C growth chamber, and liquid cultures were shaken at 150 rpm (revolution per minute). A complete medium, YPD, which supports the growth of all auxotrophic strains, consists of 1% Bacto-yeast extract, 2% Bacto-peptone and 2% glucose. Minimal synthetic dextrose (SD) medium contains 2% glucose and 0.67% Bacto-yeast nitrogen base without amino acids. Individual nutrients required for auxotrophic mutants were supplied prior to autoclaving at the recommended "physiological" concentrations (Sherman et al., 1983). All the amino acids provided to culture media were in L-form.

2.1.2 Yeast cell transformation

Yeast cells were grown at 30°C in either complete YPD medium or SD medium supplemented with the appropriate nutrients. The transformation of intact yeast cells was performed essentially as described (Ito et al., 1983). In short, 0.5 milliliter (ml) to 1 ml overnight culture was centrifuged and the resulting pellet was resuspended in 80 µl H_2O

Table 2-1 *S. cerevisiae* strains

Strain	Genotype	Source/Reference
DBY747	*MATa his3-1, leu2-3,112 trp1-289 ura3-52*	Bostein, D.
WXY9216	DBY747 with *mag1::hisG*	Xiao et al., 1996
WXY9221	DBY747 with *rad50Δ::hisG-URA3-hisG*	Xiao et al., 1996
WXY9387	DBY747 with *rad52Δ::LEU2*	Xiao et al., 1996
WXY9327	DBY747 with *mag1::hisG-URA3-hisG* and *rad18Δ::TRP1*	Xiao et al., 1996
WXY9323	DBY747 with *mag1::hisG* and *rad50Δ::hisG-URA3-hisG*	Xiao et al., 1996
Y203	*MATa,ade2-1, his3, leu2-3, 112, lys2, trp1, ura3-Δ100rnr3:RNR3-URA3-TRP1*)	Zhou and Elledge, 1992
Y290	Y203 with *dun1-Δ100:HIS3*	Zhou and Elledge, 1993

plus 10 µl of 10x TE buffer (10 mM Tris, 1 mM Na_2EDTA (Sodium ethylenediaminetetraacetic acid), pH 8.0) and 10 µl of 10x lithium acetate (1 M LiOH pH 7.5 with glacial acetic acid). Cells were returned to 30°C water bath for at least 1 hr before adding 40 µg of carrier DNA (sonicated and boiled salmon sperm DNA) and 0.1 to 2 µg of transforming DNA (up to 10 µl of miniprep DNA). The tube was incubated at 30°C for 30 min with agitation. Then 0.7 ml of Lithium-PEG (polyethylene glycol) solution (40% PEG4,000 , 0.1 M lithium acetate in 1x TE) was added. After incubating at 30°C for 30 min, 50 µl of DMSO (dimethyl sulfoxide) was added and the tube was put in a 42°C water bath for 5 min. The cells were spun down and washed in H_2O before plating.

Two types of yeast plasmids (Parent et al., 1985), YEp (yeast episome plasmid) and YCp (yeast centromere plasmid) were used in this study. YEp and YCp plasmids generally have high transformation efficiency.

2.1.3 Total genomic DNA isolation from yeast

DNA isolation from yeast was adapted from Hoffman and Winston (1987). Yeast cells were grown overnight in YPD medium or selective medium and cells from 1 ml aliquots of the culture were collected by centrifugation in 1.5 ml microcentrifuge tubes. The cells were resuspended in 300 µl of extraction buffer (2% Triton X-100, 1% SDS (sodium dodecyl sulphate), 0.1 M NaCl, 1 mM EDTA and 10 mM Tris-HCl, pH 8.0). 300 µl of phenol and chloroform (a mixture of 24 parts of chloroform and one part of isopropyl alcohol) prepared in a 1:1 ratio volume and 0.3 g of acid-washed glass beads (D=0.4 to 0.5 mm) were added. The tube was vortexed at top speed for 2 to 2.5 min and spun for 5 min to separate the phases. The supernatant was then extracted with chloroform (24 parts of chloroform and one part of isopropyl alcohol), collected, and the DNA was precipitated by adding 15 µl of 5 M of NaCl and 2 volumes of 100% ethanol for 30 min at -20°C. DNA was obtained by centrifugation at the top speed (13,000 rpm) for 15 min. in a microcentrifuge.

2.1.4 Yeast RNA isolation

One ml of overnight culture was used to inoculate 4 ml fresh medium and cells were cultured for 2 h. For MMS induction or to inhibit protein synthesis, the chemicals were added at this point. MMS was added to a final concentration of 0.05% (or as indicated) and incubation continued for 30 min. (or as indicated) before RNA isolation. Cycloheximide was added to a final concentration of 100 μg/ml and cells were treated for 45 min. To determine MMS induction in the absence of *de novo* protein synthesis, MMS was added 15 min. after cycloheximide treatment. RNA was isolated by a glassbead method (Carlson and Botstein, 1982). Briefly, yeast cells were washed in DEPC (Diethylpyrocarbonate)-treated H_2O once and resuspended in 350 μl of lysis buffer (0.5M NaCl, 10 mM EDTA, 1% SDS and 0.2 M Tris-HCl at pH 7.6) in a microcentrifuge tube. 0.3 g of acid-washed glass beads (D=0.4-0.5 mm) and 350 μl of phenol/chloroform were added and the tube was vortexed at the top speed for 2 to 2.5 min. The tube was spun in a microcentrifuge for 30 seconds and the aqueous phase was extracted with chloroform. After centrifugation, the aqueous phase was transferred into a new tube. The RNA was precipitated by adding 1 ml of 100% ethanol, the content was mixed and centrifuged immediately for 1 min. The RNA pellet was washed in 70 % ethanol once and briefly dried by vacuum.

2.1.5 Preparation of yeast crude cell extracts

Crude yeast cell extracts were prepared as described previously (Xiao et al., 1993). Briefly, mid-log phase yeast cells were harvested and washed with buffer A (25 mM HEPES (N-2-hydroxylethylpiperazine-N'-2-ethanesulfonate) pH 7.5, 5 mM $MgCl_2$, 0.1 mM DTT (dithiothreitol), 10% glycerol, and 50 mM KCl). The cells were then resuspended in 1 ml of buffer A containing 0.3 M $(NH_4)_2SO_4$, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml of antipain, 1 μg/ml of leupeptin, and 1.4 μg/ml of pepstatin A. The cells were homogenized by a French Pressor for large scale

preparation or broken by either glassbeads or sonication for small scale preparations. After centrifugation at 8,000 rpm for 20 min at 4^0C, the supernatant was aliquoted and stored at -70°C.

2.1.6 β-galactosidase (β-gal) assay

Laboratory *S. cerevisiae* strains exhibit no β-gal activity. Yeast promoter sequences fused with the bacterial *lacZ* coding region generate a useful system for the study of yeast gene regulation. β-gal assay was performed as described previously (Guarente, 1983). Briefly, 0.5 ml overnight culture of yeast cells was used to inoculate 2.5 ml fresh medium and cultured for another 2 h. At this point, chemicals were added to the concentration indicated and cells were returned to incubation for 4 h. One ml of cells was used for determining cell concentration at OD_{600nm}. The remaining cells (2 ml) were collected by centrifugation and used for the β-gal assay. The cell pellet was resuspended in 1 ml of buffer Z (60 mM $Na_2HPO_4.7H_2O$, 40 mM $NaH_2PO_4.H_2O$, 10 mM KCl, 1 mM $MgSO_4.7H_2O$, 40 mM β-mecaptoethanol, pH 7.0). The cells were made permeable by adding 50 µl of 0.1% SDS , 40 ul of chloroform, and vortexed at top speed for 10 seconds. The reaction was started by adding 200 µl of 4 mg/ml ONPG (ortho-nitrophenyl-β-D-galactoside) and incubating at 28^0C for 20 min. After the incubation, the reaction was stopped by adding 500 µl of 1 M Na_2CO_3. The tube was centrifuged and the supernatant was used to determine the β-gal activity at OD_{420nm} using the following equation.

$$SA\beta\text{-gal} = 1000 \times \frac{OD_{420nm}}{\text{reaction time (min)} \times \text{culture volume (ml)} \times OD_{600nm}}$$

The β-gal activity was expressed as Miller unit (Guarente, 1983).

2.1.7 DNA damage and heat shock treatments

For UV treatment, cells in mid-log phase were exposed to a UV fluency rate of 2 J/m^2·s (using a UV cross-linker) for various doses as indicated. After irradiation, cells were grown in the dark for 4 h before the β-gal assay. For heat shock treatment, cells

were grown at 22^{0}C for 2 h and switched to 37^{0}C for 4 h. To reduce experimental variations, fresh yeast transformants were used and several independent cell colonies from a single transformation were assayed in the same experiment.

2.2 Molecular Biology Techniques

2.2.1 Bacterial culture and storage

Luria-Bertani (LB) medium containing 1% Bacto-tryptone, 0.5% Bacto-yeast extract and 0.5% NaCl was commonly used for all *E. coli* cell growth. LB plates contained 1.2% Bacto-agar. Selective medium for ampicilin (Amp) resistance was made by adding Amp (25 mg/ml) to LB at a final concentration of 50 μg/ml prior to pouring plates. Indicator plates for β-galactosidase expression were prepared as follows: A 25 μl of 2% IPTG (isopropyl β-D-thiogalactopyranoside) and 25 μl of 4% Xgal (5-bromo-4-chloro-3-indoyl-β-D-thio-galactopyranoside) dissolved in N' N-dimethylformamide were added onto the surface of a LB+Amp plate and spreaded quickly. The plate was put in 37^{0}C growth chamber to allow N' N-dimethylformamide to evaporate.

For short term storage, bacteria can be stored in parafilm-sealed plate at 4^{0}C for up to several weeks. For long term storage, DMSO (dimethylsulfoxide) was added into a microcentrifuge tube containing overnight culture to a final concentration of 10%. The content was mixed and the tube was stored at -70^{0}C. *E. coli* strain DH5α or NM522 (which grows faster than DH5α) was used for molecular cloning and plasmid amplification.

2.2.2 Small-scale preparation of plasmid DNA from bacteria (Miniprep)

The method used was essentially described in Maniatis et al. (1982). Briefly, single bacterial colony was cultured in 1 ml of LB+Amp liquid medium in a microcentrifuge tube and incubated at 37^{0}C incubator overnight. Cells were spun down at 13,000 rpm for 2 min. in a microcentrifuge. Cells were resuspended in 350 μl lysis buffer (0.8% sucrose, 0.5% TritonX-100, 50 mM EDTA and 10 mM Tris -HCl, pH8.0)

by vortexing at top speed. Then 20 µl of lysozyme solution (10 mg/ml) was added and the tube was put into boiling water for 50 seconds. After centrifugation (10 min, 13,000 rpm), the pellet was removed by a toothpick from the bottom of the tube. DNA was precipitated and pelleted as described before. Plasmid DNA was air-dried and resuspended into 30 µl of H_2O. For use in electroporation, the DNA pellet was washed with 70% ethanol (this step is especially important to prevent sparks during electroporation) and vacuum-dried.

2.2.3 Large scale plasmid DNA isolation (Maxiprep)

The CsCl (cesium chloride)-EtdBr (ethidium bromide) gradient method (Maniatis et al., 1982) was used to prepare pure, large amounts of plasmid DNA from *E. coli*. Overnight bacterial culture (5 ml) was used to inoculate 500 ml of LB+Amp medium and the culture was allowed to grow to OD_{600} 0.8-1.0. Chloramphenicol (1 ml from a stock of 100 mg/ml dissolved in ethanol) was added to the culture. This treatment stopped cell division but allowed relaxed plasmid to replicate. After overnight continuous incubation, the cells were harvested, resuspended in 12 ml ice-cold lysis buffer (10% sucrose, 50 mM Tris-HCl, pH 8.0.) to which 4 ml of 5 mg/ml of lysozyme and 6 ml of 0.2 mM EDTA were added. After standing on ice for 10 min, the cell suspension was treated with 1 ml of 2% Sarkosyl and was centrifuged at 13,000 rpm for 70 min. The aqueous phase was then extracted once with phenol/chloroform and once with chloroform, and the DNA was precipitated with two volumes of 100% ethanol and collected by centrifugation. The pellet was dissolved in CsCl solution (9.5 g CsCl in 10 ml TE buffer), and loaded into a Beckman Quick-Seal centrifuge tube with 0.35 ml of 10 mg/ml EtdBr. Gradient equilibrium was achieved by centrifugation at 55,000 rpm for 16 h. Under long wave UV light, the lower DNA band, which contained supercoiled circular plasmid DNA, was extracted. EtdBr was removed by repeated butanol-2 extraction and CsCl was diluted out by dialysis against large volume of TE buffer. DNA was precipitated and redissolved in

TE. The purity and the concentration of plasmid DNA were determined by spectrophotometry.

2.2.4 Preparation of bacterial competent cells

The method described by Chung et al. (1989) was used to prepare competent cells from *E. coli*. Briefly, *E. coli* DH5α cells were cultured in LB to OD_{600} 0.3-0.4. The culture was diluted 1:1 with 2x ice-cold transformation and storage solution, TSS (1x TSS consists of LB with 10% PEG8000, 5% DMSO, and 50 mM Mg^{+2} ($MgSO_4$ or MgCl), pH 6.5). The competent cells were ready for use or were quickly frozen in liquid nitrogen and stored in -70^{0}C.

2.2.5 Bacterial transformation

Bacterial transformation was performed using either heat shock or electroporation methods. In the heat shock method, 50 µl of the competent cells were mixed with up to 5 µl of the ligation product. The mixture was incubated in ice for 30 min. and heat-shocked at 42^{0}C for 2 min. About 500 µl of SOC medium (2% Bacto-tryptone, 0.5% Bacto-yeast extract, 20 mM glucose, 10 mM NaCl and 2.5 mM KCl, to which 1/100 volume of filter-sterilized 1 M $MgSO_4$ + 1 M MgCl was added and the medium was adjusted to pH 6.8-7.0) was added into the mixture and the cells were incubated at 37^{0}C for 1 h before plating. The electroporation method was performed as follows: Briefly, competent cells (50 µl) and miniprep DNA (5 µl) were mixed in an ice-cold microcentrifuge tube and the content was transferred to an ice-cold electroporation cuvette. Electroporation was performed at a voltage of 2.5 kv using *E. coli* Pulser (Bio-Rad). After electroporation, 500 µl of SOC medium was added into the cuvitte. The content was transferred into a microcentrifuge tube and the tube was incubated at 37^{0}C for 1 h before plating on LB+Amp plate.

2.2.6 DNA sequencing

Three bacterial plasmid minipreps were combined and treated with RNase for 10 min at 37^{0}C. The miniprep was then extracted with phenol/chloroform once, followed by

chloroform extraction. DNA was precipitated with 100% ethanol and washed in 70% ethanol. The DNA pellet was vacuum-dried and dissolved in H_2O. The DNA sequence was determined by the dideoxy-chain termination method (Sanger et al., 1977). The sequence reaction was performed with the T7 Sequencing Kit (Pharmacia) and followed the instructions provided by the company.

2.2.7 Enzymes and chemicals

Restriction and modifying enzymes were purchased from either New England Biolabs (NEB) or GIBCO/BRL and were used as recommended. Partial digestion was performed at room temperature with minimal amount of enzymes and with a limited time of incubation. MMS was from Aldrich, 4NQO, hydroxyurea and cycloheximide were from Sigma.

2.2.8 Agarose gel electrophoresis

As a general rule, lower concentrations of agarose (0.7%) were used for separations of larger DNA fragment while higher concentrations (up to 1.2%) were used for separating smaller fragments. The gel electrophoresis was performed in 1x TBE buffer (90 mM Tris-borate, 90 mM boric acid and 1 mM EDTA, pH 8.0). The gel was stained in 0.1% EtdBr solution before being photographed. *Hind*III digested phage λ DNA was included as molecular size markers. This digestion generates DNA fragments with molecular sizes (in kb): 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, 0.56 and 0.125 respectively.

2.2.9 Isolation of DNA fragment from the agarose gel

The method described by Wang and Rossman (1994) was applied for the recovery of DNA from gel following enzyme digestion. Briefly, enzyme digested DNA was run in 0.6% agarose gel, the band of interest was cut out from the gel and spliced into small pieces. To isolate the fragment from the gel, a Sephadex G-10 spin column was prepared. The column consisted of one 0.5 ml microcentrifuge tube within a 2 ml tube (without cap). A small hole (made by a hot needle) was made at the bottom of the 0.5 ml tube. The hole was blocked by sterilized and siliconlized glass wool and 150 μl of TE-saturated

Sephadex G-10 beads. The tube was spun for 2 min. before using. The sliced gel containing DNA was transferred into the spin column and centrifuged for 10 min at the top speed. The DNA was precipitated and collected as described above.

2.2.10 DNA ligation

To prevent vector self ligation, the 5' phosphate groups of vectors were removed by the activity of calf intestine phosphatase (CIP). The dephosphorylation was carried out at the end the enzyme digestion by adding 1 µl of CIP directly into 50 µl of the restriction digestion reaction. The reaction was incubated for 1 or 2 hrs at 37^0C. For vectors and the fragments that have compatible ends, they were directly mixed and incubated with T_4-DNA ligase (BRL) at 16^0C overnight. For incompatible ends, they were blunt-ended before ligation in the following manner. With 5' protrudings, the end was filled by Klenow fragment of *E. coli* DNA polymerase I in the presence of dNTPs (2'-deoxynucleotide 5'-triphosphates). Because Klenow fragment of *E. coli* DNA polymerase I is active in all of the NEB or BRL buffers, the blunt-end reaction was performed at the end of restriction digestion by directly adding the enzyme into the digestion mixture and treated for 30 min in the presence of dNTPs. The ends with 3' protrudings were blunt-ended by S1 nuclease treatment or by T_4-DNA polymerase in the absence of dNTPs.

After ligation, the DNA was precipitated with 100% ethanol and washed with 70% ethanol before transformation.

2.2.11 Labeling probes

a) Labeling of annealed double-stranded oligonucleotides using Klenow fragment of *E. coli* DNA polymerase I. The tube containing 1.5 µg to 2 µg of oligonucleotides were heated at 95^0C for 2 min. and slowly cooled to room temperature. The labeling reaction was performed in a total volume of 20 µl containing 2 µl of buffer #3 (NEB), 6 µl of dNTPs without dCTP (50 uM), 1 µl of α-^{32}P-dCTP and 1 µl (1U) of Klenow fragment of *E. coli* DNA polymerase I. The reaction was performed at room temperature

for 20 min., then 5 µl of dNTPs (50 µM) was added and the reaction was continued for another 10 min. At the end of the reaction, the volume was brought up to 100 µl with H_2O and was extracted with phenol/chloroform, chloroform. The labeled DNA was purified by repeated (usually twice) precipitation with ethanol and dried by vacuum.

b). Random primer labeling of double-stranded DNA fragment. The tube containing 25 ng of DNA fragment was treated in boiling water for 5 min. and cooled quickly on ice. A 15 µl of the random primer buffer mixture, 6 µl of dNTPs without dCTP (50 µM), 5 µl of α-^{32}P-dCTP and 1 µl (1U) of Klenow fragment of *E. coli* DNA polymerase I were added into the tube, the volume was adjusted to 50 µl with H_2O. The reaction was performed at room temperature for at least 1 h. After the incubation, the reaction was stopped by adding 5 µl of the stop buffer and the volume was brought up to 100 µl with H_2O. The DNA was precipitated as before and dried in air or under vacuum. The DNA was dissolved in 120 µl of H_2O, and 60 µl of the probe was normally used for each hybridization.

c). 5' terminal labeling. The reaction was performed in a 20 µl volume containing 200 ng of oligonucleotide, 1 µl of 10x T_4 kinase buffer, 10 µl of γ-^{32}P-ATP and 2 µl of T_4 polynucleotide kinase (10U/µl) at 37^{0}C for 10 to 30 min. The reaction was stopped by inactivating the enzyme at 90^{0}C for 2 min. The labelled DNA was extracted with phenol/chloroform and purified by repeated precipitation with ethanol.

2.2.12 Northern hybridization

RNA (8 µl) was mixed with 8 µl of formamide, 4.5 µl of formaldehyde, 1 µl of EtdBr solution, 1 µl of loading dye (containing sucrose or glycerol and bromophenol blue) and 2.5 µl of 10x MOPS (3-(*N*-morpholino) propanesulfonic acid) buffer. The RNA was separated in a 1% denaturing agarose gel containing 1 X MOPS buffer (20 mM MOPS, 5 mM Na acetate, 1 mM EDTA, pH7.0) and formaldehyde (2 ml in 40 ml gel). After electrophoresis, the separated RNA was blotted on a *GeneScreen* membrane (DuPont) against 20x SSC overnight. The membrane was treated in a UV crosslinker and

was hybridized with DNA probes made by the Random Primer Labeling Kit (BRL). The *DDI1*-specific probe was made from a 0.93-kb *Eco*RI fragment within the *DDI1* gene, whereas the *MAG1*-specific probe contains a 0.76 kb *Eco*RI-*Bgl*II fragment within the *MAG1* gene (Fig. 3-1). The *ACT1* probe was isolated as a 1.6 kb *Bam*HI-*Hind*III fragment from pAA93 (a gift from Dr. F. Sherman, Rochester University).

2.2.13 Southern and colony hybridizations

In Southern blot analysis, DNA samples were digested with appropriate restriction enzymes and separated by agarose gel electrophoresis. DNA in the gel was treated for 30 min. each in 0.25 M HCl solution for depurination, in 0.5 M NaOH + 1.5 M NaCl for denaturation, and in 0.5 M Tris-HCl (pH 8.0) + 1.5 M NaCl for neutralization. The DNA was then blotted onto a *GeneScreen Plus* membrane (DuPont) overnight in 10x SSC and crosslinked to the membrane by the UV crosslinker. For colony hybridization, a single colony of bacterium or yeast was streaked on plates and cultured overnight. The plate was put in the 4^0C cold room for 1 h and colonies were transferred onto a nylon or nitrocellulose membrane. The membrane was then autoclaved for 5 min. to lyse the cell. The cellular debris was then washed off the membrane in 2x SSC.

2.2.14 Partial purification of UAS_{MAG1} binding proteins (performed by Dr. Heping Dai)

The method used for the purification of the UAS binding proteins was essentially the same as described by Sweder *et al* (1988) with some modifications. Briefly, crude yeast cell extract (Fraction I) was precipitated by 80% saturation of $(NH_4)_2SO_4$ and the pellet was then dissolved in buffer A containing 2 M KCl and 60% polyethylene glycol (PEG_{8000}). The solution was gently stirred at 4°C for 45 min and centrifuged at 12,000 rpm for 25 min. The supernatant was collected and concentrated with solid sucrose at 4°C for at least 6 h. After centrifugation at 12,000 rpm for 10 min, the supernatant (Fraction II) was dialyzed against buffer A at 4°C overnight and loaded onto a DEAE (Diethylaminoethyl)-Sephacel column (2.5 x 40 cm) equilibrated with buffer A. The column was then eluted with 300 ml of 0.05 to 1 M of linear gradient in buffer A at a

flow rate of 0.5 ml/min and each fraction contained 8 ml. Electrophoretic mobility shift assay (EMSA) was performed after each purification step to monitor binding of yeast proteins to the UAS_{MAG1} probe made by annealing MAG1-23 and MAG1-24 (Table 2-1). Fractions #30 and #31 were combined (Fraction III) for further analyses.

2.2.15 Electrophoretic mobility shift assay (EMSA)

Probes used for the assay were made by annealing a pair of oligonucleotides and then end-labeled with [α-^{32}P]dCTP and Klenow fragment of *E. coli* DNA polymerase I (see 2.2.11). Protein-DNA binding reaction was performed in a reaction containing 13 µl of buffer A, 1 µl (1µg) of poly (dI-dC), 1 µl (2 µg) of BSA (bovine serum albumin), 5 µl of 50% glycerol, 0.5 µl of probe (about 20 ng) and different amounts of proteins. After incubation at room temperature for 15 min., the reaction mixture was separated in a 6% polyacrylamide gel containing 2.5% glycerol. The gel was dried and exposed to X-ray film.

2.2.16. Southwestern (DNA-protein) hybridization

Yeast cell extract proteins were separated by 12% SDS-PAGE and transferred to a nitrocellulose membrane. The filter was treated with a blocking renaturation solution [50 mM Tris-HCl, (pH 8.0), 50 mM NaCl, 2 mM EDTA, 2 mM β-mercaptoethanol, 0.1% NP-40, 5% nonfat dry milk] for 1 h at room temperature with gentle shaking. The membrane was then incubated at room temperature for 20 min with the binding solution used for the gel shift assay. The radioactive DNA probe was added at about 10^6 cpm/ml and the incubation was continued for 1 h. The filter was washed three times with buffer A for 20 min each before being exposed to X-ray film.

2.2.17 Screening yeast cDNA library

The yeast λYES cDNA library and the bacterial strains required for screening the library and the conversion of phage λ into plasmid clone (Elledge et al., 1991) were obtained from S. Elledge (Baylor College of Medicine, TX). The library was screened with the *MAG1*-specific probe (Fig. 3-1). The phage clones containing the *MAG1* cDNA

were then converted into plasmids as described (Elledge et al., 1991). The 5' ends of the *MAG1* cDNA clones were determined by sequencing using MAG1-138 (Table 2-2) as primer.

2.2.18 RNA primer extension analysis and the S1 nuclease mapping

For primer extension experiment, primer DDI1-2 (Table 2-2) covering a region from +253 to +270 in *DDI1* coding region was end-labeled as described (see 2.2.11). Freshly prepared total RNA was mixed with the labeled primer and co-precipitated. The RNA and the primer was resuspended in 19 µl of H_2O by vortexing at top speed. The tube was heated at 75°C for 5 min. The extension reaction was performed at 42°C for 1 h in the reaction mixture containing 1.6 µl of M-MLV (Moloney-Murine Leukemia Virus) reverse transcriptase (NEB), 2.5 ul of MLV buffer (10 X) and 2.5 ul of dNTPs (2.5 mM). The reaction was stopped by adding EDTA to a final concentration of 20 mM and the RNA was removed by RNase A (1 hr. at 37°C). The reaction mixture was then treated with phenol/chloroform and DNA was precipitated by ethanol. The DNA pellet was resuspended in 4 µl of water and 4 µl of the stop/loading solution (from the T_7 sequencing kit). Half of the sample was loaded on a 4% sequencing gel and run side by side with the standard M13 sequencing reactions (with the M13-UP primer, Table 2-2). Part of the M13 DNA sequence is shown in Fig. 2-1.

For S1 nuclease mapping, a PCR (polymerase chain reaction) fragment was amplified from plasmid DDI1-E8A (*EcoR*I fragment from *DDI1* cloned in pUC18) using DDI1-2 and the M13-UP primer pairs (Table 2-2). The amplified fragment covered the region from +270 to -156 (relative to the *DDI1* ORF) and was end-labeled as described (see 2.2.11). Freshly prepared RNA and the labeled fragment were resuspended in 30 µl of hybridization buffer (40 mM PIPES (piperazine-N, N'-bis[2-ethanesulfonic acid]), pH 6.4, 1 mM EDTA, 0.4 M NaCl and 80% of formamide) by pipetting (for at least 20 times) and vortexing vigorously. The tube containing the RNA and the labeled fragment was heated at 75°C for 5 min. The hybridization was performed at 30°C overnight. After

Table 2-2. Oligonucleotide sequences

Name	Sequence[a]	Position	Source
DDI1-2	GTCGGACAAAGTAGCAGC	+253 to +270	*DDI1*
DRm	GGAATTCCCGTCGATATCACGCT CTGTAAATTCATAGTCACCGAAAAG	-133 to -179	*MAG1*
DRSS1	P- GGTGGCGATGAATTTACAGGGCG GGGTGGCGACATG	-173 to -140	*MAG1*
DRSS2	P- TCGCCACCCCGCCCTGTAAATTCA TCGCCACC	-142 to -173	*MAG1*
DRm1	TCGACGGTGACTATGAATTTACA GAGCGTGATATCGAC	-174 to -140	*MAG1*
DRm2	TCGAGTCGATATCACGCTCTGTA AATTCATAGTCACCG	-140 to -174	*MAG1*
DR1	TCGACGGTGGCGATGAATTTACA GGGCGGGGTGGCGAC	-174 to -140	*MAG1*
DR2	TCGAGTCGCCACCCCGCCCTGTA AATTCATCGCCCACCG	-140 to -174	*MAG1*
MAG1-1	GGCAGTGGCCAATTCTC	-199 to -185	*DDI1*
MAG1-5	GTATTACCGCCTTTGAG		pUC18
MAG1-6	TCGAGTATACTTTCTTATTCG ACCTACTTTATATAT	-228 to -197	*MAG1*
MAG1-7	TCGAGATATATAAAGTAGGT CGAATAAGAAAGTATA	-196 to -227	*MAG1*
MAG1-17	ATTTTGGCACTCCATGGGCC	+560 to +579	*MAG1*
MAG1-23	GAGATATACGGCCAATTGAAGT	+34 to +56	*DDI1*
MAG1-24	GACTTCAATTGGGCCGTATATCT	+57 to +35	*DDI1*
MAG1-25	GAGATATATGGAACAATTGAAGT	+34 to +56	*DDI1*
MAG1-26	GACTTCAATTGGTCCATATATCT	+57 to +35	*DDI1*
MAG1-138	CTTTCTCTAAAATGTGTTCGCAAG	+138 to +163	*MAG1*
MSC1	GAATTCGAGCTCGGTACC		
MSC2	GGTACCGAGCTCGAATTC		
M13-UP[b]	GTAAAACGACGGCCAGT		

[a] Oligonucleotide sequences are arranged from 5' to 3'. Oligonucleotides with a phsphate group at the 5' end were indicated. The mutated bases are underlined.

[b] M13 Universal primer.

Figure 2-1. Partial sequence of M13 used as size reference for primer extension experiment

5851	GGATTTTGCC	GATTTCGGAA	CCACCATCAA	ACAGGATTTT
5891	CGCCTGCTGG	GGCAAACCAG	CGTGGACCGC	TTGCTGCAAC
5931	TCTCTCAGGG	CCAGGCGGTG	AAGGGCAATC	AGCTGTTGCC
5971	CGTCTCGCTG	GTGAAAAGAA	AAACCACCCT	GGCGCCCAAT
6011	ACGCAAACCG	CCTCTCCCCG	CGCGTTGGCC	GATTCATTAA
6051	TGCAGCTGGC	ACGACAGGTT	TCCCGACTGG	AAAGCGGGCA
6091	GTGAGCGCAA	CGCAATTAAT	GTGAGTTAGC	TCACTCATTA
6131	GGCACCCCAG	GCTTTACACT	TTATGCTTCC	GGCTCGTATG
6171	TTGTGTGGAA	TTGTGAGCGG	ATAACAATTT	CACACAGGAA
6211	ACAGCTATGA	CCATGATTAC	GAATTCGAGC	TCGGTACCCG
6251	GGGATCCTCT	AGAGTCGACC	TGCAGGCATG	CAAGCTTGGC
6291	ACTGGCCGTC	GTTTTACAAC	GTCGTGACTG	GGAAAACCCT

The underlined DNA sequence represents the M13-UP primer binding site.

hybridization, 300 ul of S1 nuclease buffer containing 300 U of S1 nuclease (BRL) was added and the tube was incubated at 30^0C for 1 h. The reaction was then stopped by adding 5 µl of 0.5 M EDTA and the reaction mixture was extracted with phenol/chloroform. DNA was precipitated by ethanol and resuspended in 4 µl of water plus 4 µl of the stop/loading solution (from the T_7 sequencing kit).

2.3 Plasmid Construction

The maps of plasmids used for making various constructs were shown in Fig. 2-2.

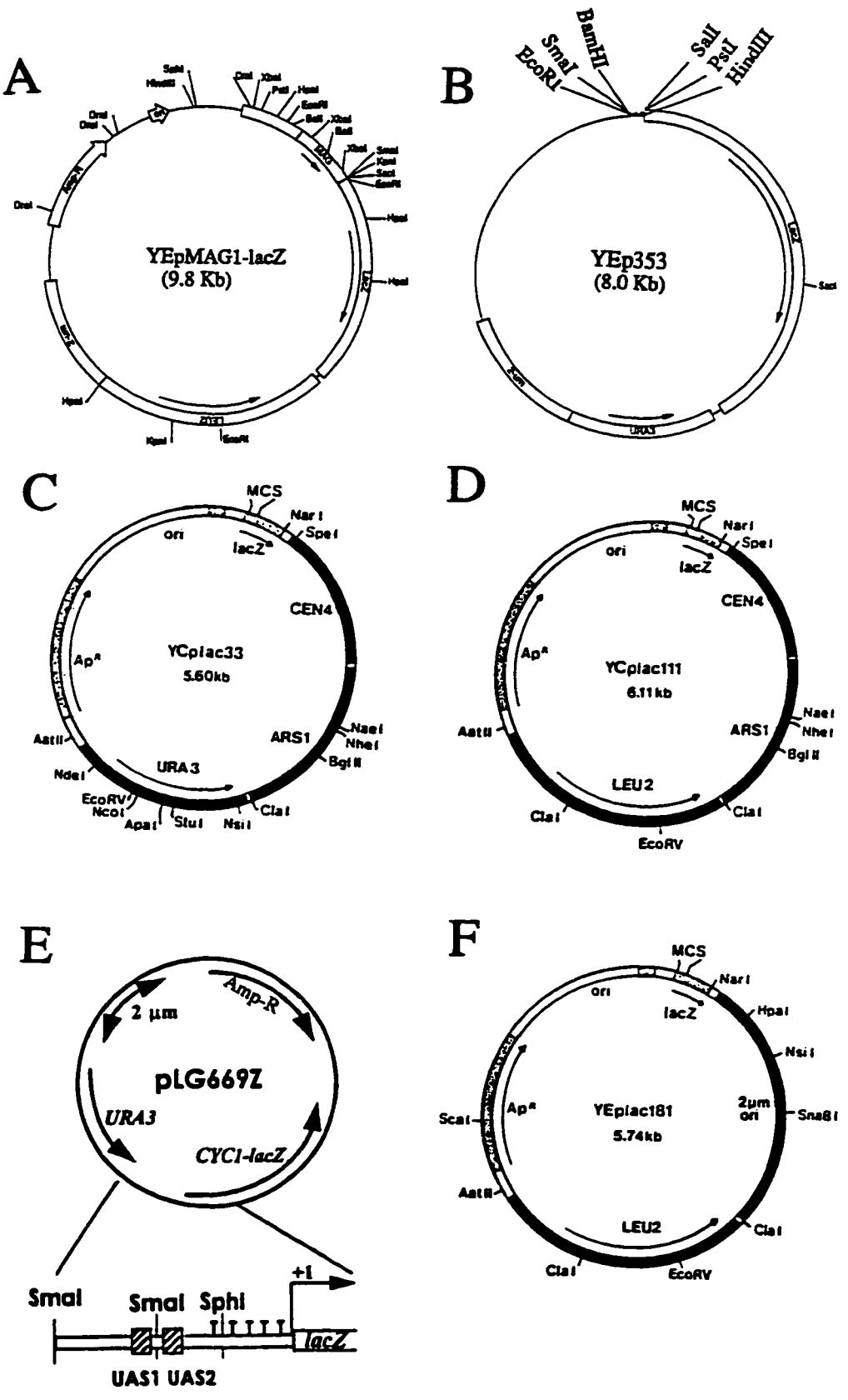
2.3.1 Construction of plasmid YEpDDI1-lacZ

The construction of plasmid YEpMAG1-lacZ (Fig. 2-2A) was described previously (Xiao et al., 1993). Plasmid pWX1807 was made by cloning a 5.0 kb *Xho*I fragment containing both *MAG1* and *DDI1* genes into YCplac33 (Fig. 2-2C, Gietz and Sugino, 1988). YEpDDI1-lacZ (Fig. 3-3A) was constructed by cloning a 1.08 kb *Dra*I-*Bgl*II fragment from pWX1807 into the *Hind*III (blunt ended prior to ligation) and *BamH*I sites of YEp353 (Fig. 2-2B, Myers et al., 1986) so that the *DDI1* open reading frame was in-frame with *E. coli lacZ* gene.

2.3.2 5' deletions of *DDI1-lacZ*

5' upstream deletions to -191 (relative to the translation initiation) in the *DDI1* promoter was obtained by *Sma*I digestion followed by *Msc*I digestion of YEpDDI1-lacZ. The deletion to -149 was made by *Eco*RI digestion. To make stepwise deletions, plasmid YEpDDI1-lacZ was cleaved at the unique *Afl*III (-468) or *Msc*I (-191) sites relative to the *DDI1* ORF and then digested with *Bal*31 (NEB) under standard conditions for 2, 5 and 10 min. The reaction was stopped by phenol extraction. The plasmid DNA was purified, digested with *Sma*I, self-ligated and then used to transform bacterial cells. Clones containing potential deletions were isolated and the sequence at the deletion and the rejoining point was determined by DNA sequencing using MAG1-5 (Table 2-2) as primer. Representative plasmids were used to transform DBY747.

Figure 2-2. Maps of plasmids used for making various constructs. The construction of YEpMAG1-lacZ was described previously (Xiao et al., 1993) and used frequently in the current study. The region containing *MAG1* sequence is indicated, which consists of 608 bp upstream sequence and 627 bp of the coding sequence (indicated as MAG in the Figure). YEp353 was constructed as described (Myers et al., 1986) and was used to construct YEpDDI1-lacZ (Fig. 3-1). The plasmid YCplac33, YCplac111 and YEplac181 were from Gietz and Sugino (1988) and used to express *MAG1* in a single copy and in multiple copies respectively. The multicloning site (MCS) is from pUC19 and the restriction sites labeled are unique sites outside the MCS. The plasmid pLG669Z was constructed as described (Guarente, 1983) and the map is from Sancar et al. (1995). The hatched boxes indicate the *CYC1* UASs. +1 is the first A in the translational start site of the fusion protein. 'T' indicates the positions of TATA boxes in the *CYC1* promoter.


A
YEpMAG1-lacZ
(9.8 Kb)
B
BamHI
SmaI
EcoRI
SalI
PstI
HindIII
YEp353
(8.0 Kb)
C
MCS
NarI
SpeI
ori
lacZ
CEN4
YCplac33
5.60kb
ARS1
URA3
D
YCplac111
6.11kb
LEU2
E
2 µm
Amp-R
pLG669Z
URA3
CYC1-lacZ
SmaI
SmaI SphI
+1
lacZ
UAS1 UAS2
F
YEplac181
5.74kb
2µm ori

2.3.3 Internal deletions, insertion and mutagenesis within the UAS $_{MAG1}$

The internal deletions within the region of UAS$_{MAG1}$ were made by cleaving at the unique *PstI* site of plasmid YEpMAG1-lacZ or at the unique *Mfe*I site of the plasmid YEpDDI1-lacZ. The overhangs were dephosphorylated and the plasmid was digested with *Bal*31 under standard conditions for a very short time (0.5 and 1 min). The reaction was stopped by phenol extraction. The treated plasmid was purified, self-ligated and transformed into bacterial cells. Colonies containing potential deletions were isolated and sequenced using primer MAG1-1 (Table 2-2). The deletion of +30 to +69 was introduced into plasmid YEpMAG1-lacZ by cloning the *Pst*I-*Nco*I fragment from the plasmid YEpDDI1-lacZ into YEpMAG1-lacZ. To make an insertion, the plasmid YEpDDI1-lacZ was cleaved at *Mfe*I site and the 5' overhangs were blunt-ended with the Klenow fragment of DNA polymerase I of *E. coli*. To introduce the insertion into YEpMAG1-lacZ, the *Pst*I-*Nco*I fragment from the YEpDDI1-lacZ plasmid carrying the insertion was cloned into YEpMAG1-lacZ. To introduce mutations at positions +42 and +45, a PCR fragment was amplified by MAG1-26 and MAG1-17 (Table 2-2) at conditions of 94^{0}C, 55 sec., 57^{0}C, 55 sec. and 72^{0}C, 80 sec. for 30 cycles. The PCR fragment was digested with *Mfe*I and *Afl*II and cloned into YEpDDI1-lacZ. The plasmids carrying the mutations were distinguished from the wild type plasmid by digesting with *Eco47*I. The mutations were introduced into YEpMAG1-lacZ by cloning the *Pst*I-*Nco*I fragment from the *DDI1-lacZ* (carrying the mutations) into the *MAG1-lacZ* The mutations were confirmed by sequencing using DDI1-2 as primer (Table 2-2). Note, the sequence of the entire promoter region was checked to make sure that no other mutations were introduced during the PCR amplification.

2.3.4 Deletions of DNA sequences containing the direct repeat (DR)

Internal deletions from -149 to -58 and from -149 to -113 were obtained by replacing the *Eco*RI fragment of the -58 and -113 upstream deletion constructs with the *Eco*RI fragment from YEpDDI1-lacZ. The internal deletion from -159 to -137 was made

by two steps. a). The *EcoR*I site at the junction between the yeast and the bacterial sequences was eliminated by *Bal*31 at the *Sma*I site. b). The resulting plasmid was opened up at the second *EcoR*I site located in the intergenic region of *MAG1/DDI1* and treated with *Bal*31 as describe above. The deletion was determined by using MAG1-1 as primer (Table 2-2).

2.3.5 Introduction of mutations in DR

a). Vector modification. There are two *EcoR*I sites in the plasmid YEpDDI1-lacZ. One is in the intergenic region between *MAG1* and *DDI1*, and another in the multiple cloning site next to *Sma*I. The *EcoR*I site in the multiple cloning site was eliminated by *Sma*I digestion followed by *Bal*31 treatment.

b). Introduction of point mutations in DR sequence. To introduce point mutations in DR sequence, a PCR fragment, obtained by using Drm and DDI1-2 primers (Table 2-2), was digested with *EcoR*I and *Pst*I and cloned into the" modified vector". The plasmid carrying mutations were distinguished from the wild type plasmid by the presence of an additional *EcoR*V created in Drm. *EcoR*V digestion produced 3 fragments from the wild type sequence while four fragments were obtained from the mutated sequence. The mutations were confirmed by sequencing analysis using DDI1-2 (Table 2-2) as primer. Note, the sequence of the entire PCR amplified fragemnt was checked to make sure that no other mutations were introduced during the PCR amplification. The same mutations were introduced into *MAG1-lacZ* by cloning the mutated *Pst*I-*Nco*I fragment from YEpDDI1-lacZ into YEpMAG1-lacZ.

2.3.6 Insertion of DR sequence into the *CYC1* promoter

Plasmid pLG669Z (Fig. 2-2E) is a yeast-*E coli* shuttle vector containing the 5' regulatory region of the yeast *CYC1* gene and the translation start site fused in-frame to *lacZ* (Guarente and Ptashne, 1981; Guarente, 1983) and was used to test the function of DR sequence in a heterologous promoter. pLG669Z was digested with *Sma*I and *Sph*I to remove all the *CYC1* sequence containing the two *CYC1* UASs but left four of the five

TATA boxes (Guarente et al., 1984; Li and Sherman, 1991). Oligonucleotides DRSS1 and DRSS2 (Table 2-2) were annealed and inserted into the *Sma*I-*Sph*I sites of pLG669Z so that the DR sequence replaced UAS_{CYC1} in the *MAG1* orientation. As a ΔUAS_{CYC1} control, the *Sma*I-*Sph*I digested plasmid pLG669Z was blunt-ended by S1 nuclease treatment and self ligated. Cells containing plasmid pLG669Z with the insert were selected by colony hybridization using the direct repeat sequence (labeled DR1/DR2) as a probe and verified by sequencing using DRSS1 as primer.

2.4 Determination Of Biological Function Of DR

2.4.1 Plasmids used for the functional assay

A single copy plasmid carrying the functional *MAG1* gene with the deletion of DR sequence from the promoter was constructed as follows: First, the *Pst*I-*Sma*I fragment from YEpDDI1-lacZ carrying the deletion between nucleotides -149 to -113 (which deleted theDR sequence) was cloned into the corresponding sites of a centromere plasmid YCplac111 (Fig. 2-2D, Gietz and Sugino, 1988); the *Eco*RI fragment from the resulting plasmid was then replaced by the 2.2-kb *Eco*RI fragment from pWX1807. The insertion of the *Eco*RI fragment produced two different orientations: One of the orientation reconstituted *MAG1* promoter but with a deletion of the DR sequence, this plasmid was called YCpMAG1ΔDR. The other orientation is the reverse of the former, and was named YCpMAG1ΔRm. YCpMAG1ΔRm contained the promoter with a 5' deletion of URS_{MAG1} and therefore overexpressed *MAG1*. *MAG1* was also overexpressed in a YEp multi-copy plasmid by cloning a 4.2 kb *Sph*I-*Kpn*I fragment (containing *MAG1* and *DDI1* sequences) from pWX1807 into YEplac181 (Fig. 2-2F, Gietz and Sugino, 1988). The resulting plasmid was named YEpMAG1. To serve as the wild type promoter control, *MAG1* sequence within the *Pst*I-*Kpn*I fragment derived from pWX1807 was cloned into YCplac33, the resulting plasmid was named YCpMAG1.

The plasmids were transformed into *mag1* disruption strain WXY9216 (Table 2-1) and tested for the MMS sensitivity.

2.4.2 MMS liquid killing and gradient plate assay

Two types of quantitative killing experiments were performed. For a liquid killing experiment, overnight yeast cultures were used to inoculate fresh YPD at 1/10-fold dilution. Cells were allowed to grow up to about 2 x 10^7 cells/ml. MMS was added to the culture at a final concentration as specified (0.3%) and aliquots were taken at given intervals. Cells from each sample were collected by centrifugation, washed, diluted, and plated on YPD. The colonies were counted after a 3-day incubation. Untreated cells were also plated and scored as 100% survival. The experiment was repeated once and the average numbers were used. For a gradient plate assay, a MMS gradient was formed by pouring a bottom layer of YPD +MMS medium in a tilted square Petri dish. After agar solidification, the Petri dish was returned flat and a top layer of YPD medium was added. Cell cultures were printed onto each plate across the gradient using a microscope slide and the plates were incubated for 2-3 days as specified.

2.5 Isolation of mutants that affect the expression of *MAG1/DDI1*

2.5.1 Xgal plate

Cells were allowed to grow to individual colonies on SD selective plate and covered by a layer of Xgal medium consisting of 5 ml of KPO_4 buffer (1 M K_2PO_4+1 M $KHPO_4$, pH 7.0), 5 ml of SD agar medium (autoclaved and cooled to 50-60^{0}C), 50 µl of 2% SDS and 50 µl of Xgal (40 mg/ml).

2.5.2 Mutagenesis

Cells in log phase were treated with 0.25% EMS (ethylmethanesulfonate) for 4 h. After the treatment, cells were washed with culture medium twice and cultured in fresh medium overnight to fix the mutations. The cells were diluted and plated onto SD

selective medium. Colonies from a 3-day culture were covered with the Xgal medium for the selection of desired colonies.

2.5.3 Identification of putative mutants

Cell colonies with the desired color were selected and streaked onto SD selection plate. The selected cells were tested by the β-gal assay to see if they continue to show the desired phenotype. To test whether or not the mutant phenotype is plasmid-borne, the plasmid that present inside the cell during the mutagenesis was eliminated by repeated culturing in non-selective medium. The original plasmid was then reintroduced into the cell by transformation. If cells continued to show the mutant phenotype, it suggested that the phenotype is not associated with the plasmid. To determine if the mutant has a specific effect on *MAG1* and *DDI1* or it has a general effect on the expression of other genes, plasmid pLG669Z which contains the *CYC1-lacZ* in a YEp plasmid was used as a control. The procedure for the selection of mutants are shown in Fig. 2-3.

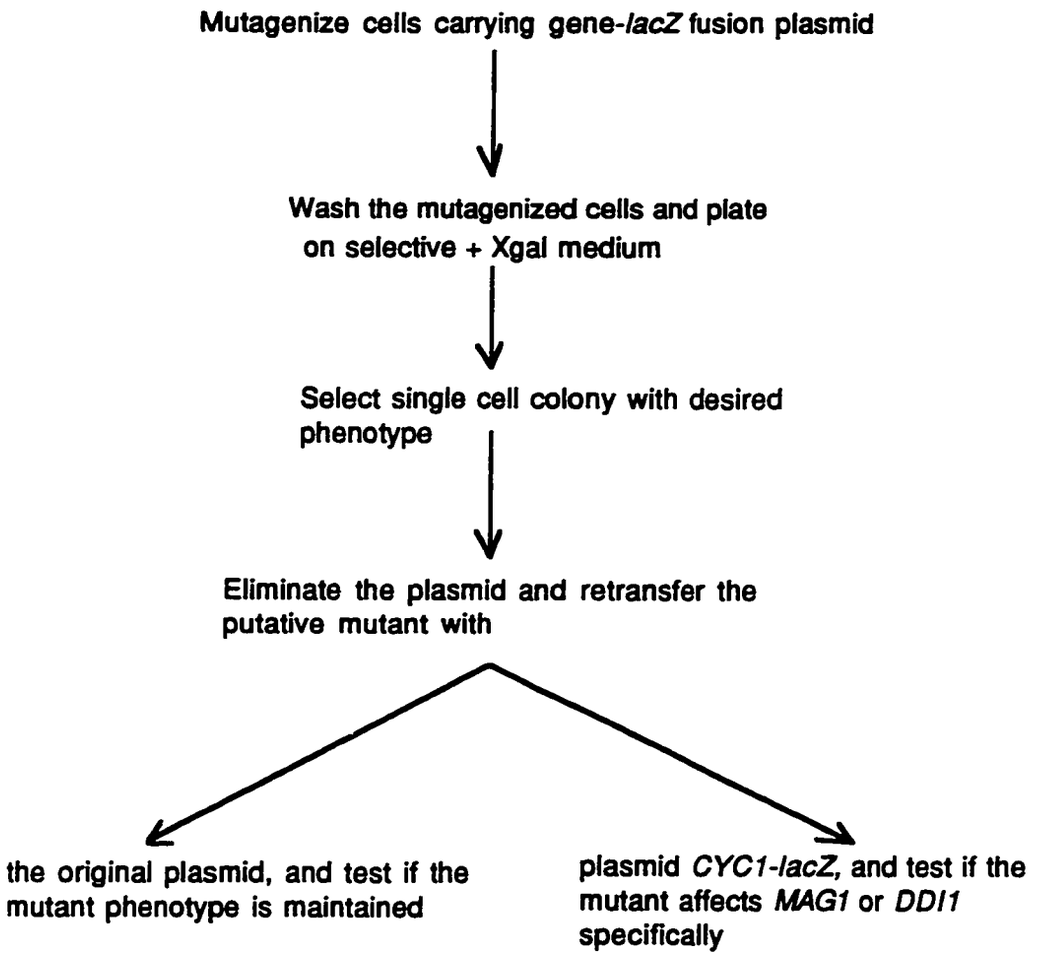


Figure 2-3 Procedure for selection of mutants affecting the expression of *MAG1* and *DDI1*

CHAPTER III: RESULTS

3.1. Features Of The Intergenic Region Between *MAG1* And *DDI1*

By sequencing the upstream region of *MAG1*, a new open reading frame (ORF), named *DDI1* (for DNA damage inducible) was revealed. *DDI1* is a novel gene, it encodes a putative protein of 428 amino acid residues with molecular weight of 47.3 kDa (Fig. 3-1), and has no apparent homology to any proteins in the public database GenBank. The biological and biochemical functions of *DDI1* remain unknown. Disruption of the *DDI1* gene did not result in noticeable phenotypic alterations, including cell growth rates and resistance to high temperature (39°C) and DNA damaging agents such as UV and MMS (W. Xiao and T. Fontaine, unpublished). However, a partial human cDNA clone that bears 51% identity and 75% similarity to the 102 amino acid sequence of the Ddi1 polypeptide has been identified from the Human Genome Sciences, Inc. database (Y. Wei and W. Xiao, unpublished), suggesting that this gene is probably conserved in eukaryotes. The sequence of the entire yeast genome, recently made public by GenBank, confirmed the *DDI1* sequence.

Concerning the structure in the regulatory region between *MAG1* and *DDI1*, several interesting features stand out: First, *MAG1* and *DDI1* are arranged in a head-to-head configuration and are divergently transcribed from each other. Secondly, the two genes are closely linked, with the first ATGs of the two ORFs being separated by only 282 bp, as compared to the average of 731 bp for divergently transcribed genes in *S. cerevisiae* chromosome 8 (Johnston et al., 1994). Finally, the sequence required for the damage induction of *MAG1* (UAS_{MAG1}) indicated by a previous study (Xiao et al., 1993) is located within the coding region of *DDI1* (Fig. 3-1).

Figure 3-1. Divergent arrangement of *MAG1* and *DDI1*. (A). The nucleotide and deduced amino acid sequences of *DDI1* (GenBank accession number U14002). The *DDI1* ORF of 1,287 bp is translated into a putative 428 amino acid protein with calculated molecular weight of 47.3 kDa. The translation starts of *DDI1* and *MAG1* open reading frames are at +1 and -283 respectively. The transcription starts of *DDI1* and *MAG1* are shown in arrows. Several restriction sites described in the text are indicated. The putative TATA box for TFIID binding is marked as [TATA]. The putative polyadenylation signal sequence AATAAA downstream of the *DDI1* ORF is underlined. The URS_{MAG1} is marked by a broken line and the region required for *MAG1* induction is shown in bold. The 8-bp direct repeat is marked as DR. (B). A diagram showing the divergent arrangement of *MAG1* and *DDI1*. Restriction enzyme sites: A, *Afl*III; B, *Bgl*II; D, *Dra*I; E, *Eco*RI; M, *Msc*I; Mf, *Mfe*I; N, *Nco*I; P, *Pst*I; and X, *Xba*I. Rm, URS_{MAG1} and Am, UAS_{MAG1} (as defined in Xiao et al., 1993). *MAG1* and *DDI1* specific probes are shown on the top.

A

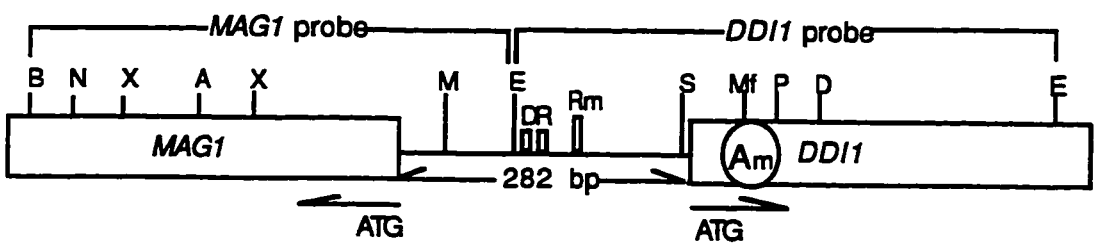
```
                                                                                                  BglII
                                                                                                  AGATCTTCAGG  -901
                                                                  NcoI
AGCAAATACATCCATTCTTTTCAATCCGGAGATCAAGAACATTTTGGCACTCCATGGGCCTATACCTTTTACATTCGTAACTAAACTTTC  -811
                                                                                                  XbaI
AATCACTTCCTCATCATTATCTTTTTGACCGAAGAGCTTTTCGATATCCTTATATTTTTCAGTAAAATAGACAGCAAGAGACTCTAGATA  -721

TATCATTTTCCTTTTACTCAATCCACATTTTGCGATTTCTGCACATTTTGCTGGGTCTTTGAAGTCTTCGAAAAGGATTTTGTAATCAGG  -631

AAATGCACCGCCATAAAGACTGACAACCCTTGCCTTGATGCTTTCAGCTGCTTGGCCACTGATCTGTTGAGACAAAATTGTGCTTGCAAG  -541

                                                                  AflII
CCTAATAAAATAATCTTCGAGTGTATTAGGGACTTGAGTCTCCTTCAAGTACAACGTAAATTCATTATTCTTAAGTATGGGAAAAAGTGA  -451
                                                                                                  XbaI
TGGATCTTTCTCTAAAATGTGTTCGCAAGCCATATTGAACTTTTCTTCATGTCTAGCAATATATTTCTCAGGAAGAGCAACCTCTAGAGG  -361
                                                                                                  +1MAG1
TCGAGACCCTAATTCTTTTGCTATTTCCTTAACAGCGTCTGCTTTTATTAACTCATCATACTCCCTTTTTAGTTTCATTGAGACCAATCC  -271
                                                                  <                  MscI
CTCTCTAGCTTCTCTTGATTCTCAGTAAAAAATTAAAAAGGAAAAGTACCACTTCCATCGTCATATCAGAGGGCAGTGGCCAATTCTCAT  -181
                                  EcoRI          DR                        DR
TGCTTCATAGATAACTTAAACTTTATGTAAGAATTCCCGTCGCCACCCCGCCCTGTAAATTCATCGCCACCGAAAAGATAATAATACCAA  -91
                [TATA] ----------  >                                                 SnaBI
AATGATATATATAAAGTAGGTCGAATAAGAAAGTATACTTAACGTAGTTAACAAAGTACATACCAAACATAACAGCAAAAATATACGTAAAG  -1
+1DDI1                                  MfeI
ATGGATTTAACAATTTCAAACGAACTTACTGGCGAGATATACGGCCCAATTGAAGTCAGTGAAGATATGGCTTTAACTGATCTAATAGCA  +90
 M  D  L  T  I  S  N  E  L  T  G  E  I  Y  G  P  I  E  V  S  E  D  M  A  L  T  D  L  I  A   30
    PstI                                                                                 DraI
TTACTGCAGGCCGATTGCGGGTTTGACAAAACAAAACACGACTTATATTACAATATGGACATTCTAGACTCAAACAGAACTCAGTCTTTTA  +180
 L  L  Q  A  D  C  G  F  D  K  T  K  H  D  L  Y  Y  N  M  D  I  L  D  S  N  R  T  Q  S  L   60
AAAGAACTAGGGCTCAAAACCGATGACTTATTATTGATTAGGGGTAAGATTTCCAATTCCATTCAAACAGATGCTGCTACTTTGTCCGAC  +270
 K  E  L  G  L  K  T  D  D  L  L  L  I  R  G  K  I  S  N  S  I  Q  T  D  A  A  T  L  S  D   90
GAAGCGTTCATAGAGCAATTTAGACAAGAGCTACTTAATAATCAAATGCTTAGATCACAACTCATTTTGCAAATCCCTGGATTGAATGAT  +360
 E  A  F  I  E  Q  F  R  Q  E  L  L  N  N  Q  M  L  R  S  Q  L  I  L  Q  I  P  G  L  N  D  120
TTAGTAAATGACCCACTGTTGTTTAGGGAAAGATTGGGCCCGCTCATATTACAAAGACGTTATGGTGGCTATAACACCGCCATGAATCCT  +450
 L  V  N  D  P  L  L  F  R  E  R  L  G  P  L  I  L  Q  R  R  Y  G  G  Y  N  T  A  M  N  P  150
TTTGGCATCCCCCAAGATGAATATACTAGACTAATGGCCAACCCCGATGATCCTGACAACAAGAAGAGGATTGCAGAGCTTTTAGACCAA  +540
 F  G  I  P  Q  D  E  Y  T  R  L  M  A  N  P  D  D  P  D  N  K  K  R  I  A  E  L  L  D  Q  180
CAAGCTATCGATGAACAGTTGCGCAATGCTATCGAATATACACCTGAAATGTTTACCCAGGTCCCTATGCTTTACATTAATATCGAAATA  +630
 Q  A  I  D  E  Q  L  R  N  A  I  E  Y  T  P  E  M  F  T  Q  V  P  M  L  Y  I  N  I  E  I  210
AATAACTACCCCGTAAAGGCATTTGTAGATACAGGGGCTCAAACAACGATTATGTCCACCAGGTTAGCGAAGAAAACTGGCCTGTCGAGA  +720
 N  N  Y  P  V  K  A  F  V  D  T  G  A  Q  T  T  I  M  S  T  R  L  A  K  K  T  G  L  S  R  240
                                                                  EcoRI
ATGATTGACAAGAGATTTATTGGAGAAGCTCGTGGCGTAGGAACCGGCAAAATTATTGGGAGAATTCACCAAGCCCAAGTCAAAATAGAA  +810
 M  I  D  K  R  F  I  G  E  A  R  G  V  G  T  G  K  I  I  G  R  I  H  Q  A  Q  V  K  I  E  270
ACACAATATATTCCATGCAGTTTTACCGTCTTAGATACTGACATCGATGTTTTGATAGGACTAGACATGCTGAAAAGGCATTTGGCTTGT  +900
 T  Q  Y  I  P  C  S  F  T  V  L  D  T  D  I  D  V  L  I  G  L  D  M  L  K  R  H  L  A  C  300
GTGGACTTAAAGGAAAACGTTCTTAGGATAGCAGAAGTGGAGACAAGCTTTTTGAGTGAAGCGGAAATCCCAAAAAGTTTTCAAGAAGGA  +990
 V  D  L  K  E  N  V  L  R  I  A  E  V  E  T  S  F  L  S  E  A  E  I  P  K  S  F  Q  E  G  330
CTTCCAGCACCAACGTCAGTTACAACTTCGTCTGATAAGCCCCTAACACCCACCAAGACTAGTTCAACTCTACCACCACAACCGGGGGCT  +1080
 L  P  A  P  T  S  V  T  T  S  S  D  K  P  L  T  P  T  K  T  S  S  T  L  P  P  Q  P  G  A  360
GTTCCTGCGCTTGCTCCAAGGACTGGTATGGGGCCAACACCTACGGGAAGAAGTACAGCCGGTGCTACAACGGCAACCGGGAGAACGTTT  +1170
 V  P  A  L  A  P  R  T  G  M  G  P  T  P  T  G  R  S  T  A  G  A  T  T  A  T  G  R  T  F  390
CCCGAACAAACGATTAAACAGCTTATGGATTAGGTTTCCCTAGGGATGCTGTGGTAAAGGCTCTTAAACAGACTAACGGAAATGCAGAAT  +1260
 P  E  Q  T  I  K  Q  L  M  D  L  G  F  P  R  D  A  V  V  K  A  L  K  Q  T  N  G  N  A  E  420
TTGCTGCATCCCTCCTTTTCCAATGAGCAACCACTGATATTGTGATCGGCCTCTACGTATGTAGCCCATAACACAAATAGATAAGTATA  +1350
 F  A  A  S  L  L  F  Q  *                                                               428
AGGATGTACATACTTTCATTGTTTCGTCAATTGTTGTTTGTATTGTATATGATTGTACTTCTGTTAAGTATATAATATGCAGATTGTATT  +1440
TGTGCCTCAATGTGCGCCATTCATGTGTTGCAAACCGGTAATTTGATTCAAAAAATTCTTTCGTAAAATAAAACATATGCACTGGGTGTG  +1530
ATAAATTCAGTACTAAACGTTATAGGACGATAAAGCGAGTCATCGAAAAAATACCAACCTTTTTTTGGTCCCTTATAAACGTATGAAGTA  +1620
TAGTGTCCCCCATACAAACTCCCCGAATGACATGCAACCCCATACAGTCTGTATCTAAATGGTGGTACTTGGCCCCTGGTAGGAATGTCC  +1710
                                                                  BglII
TCATTAACAATAGCTTCATGATTAAAATCTCTCGCCCAATATGGTGTAAGATCT                                          +1763
```

B



3.2 The *MAG1* UAS Is Located In The Protein Coding Region Of *DDI1*

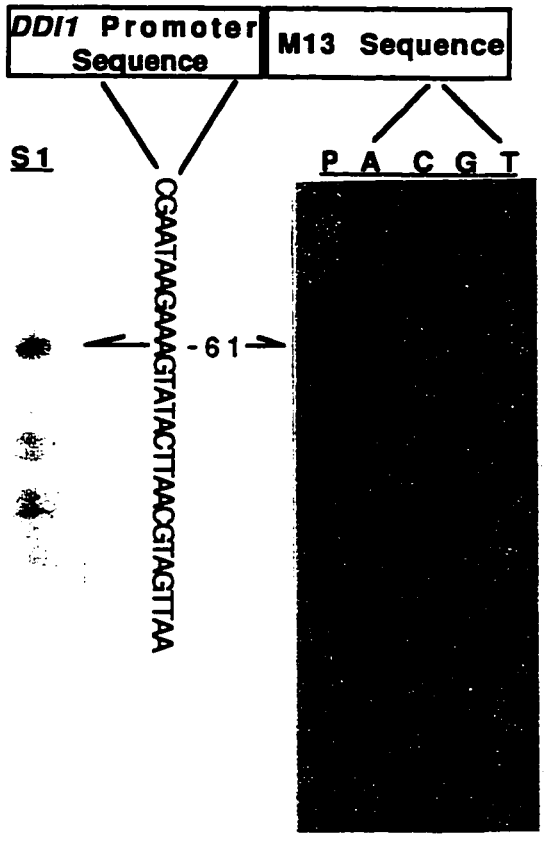
As noted above, the UAS element of *MAG1* is located within the *DDI1* ORF and the intergenic sequence between *MAG1* and *DDI1* is extremely short (Fig. 3-1). To confirm this curious genome organization, the transcriptional and translational starts for both genes were determined.

The transcriptional start of *DDI1* was determined by primer extension analysis. As shown in Fig. 3-2, one major *DDI1* primer extension fragment and several minor ones were revealed. By comparison to the size of the corresponding band from M13 sequencing reactions, the major primer extension fragment was mapped to -61 bp relative to the translation start of *DDI1* ORF. The result from S1 nuclease mapping also showed multiple bands comparable to that from the primer extension, suggesting that *DDI1* transcription involves multiple initiations.

Probably due to the low abundance of the *MAG1* transcript, the transcriptional start site could not be mapped by the primer extension method. Instead, the approximate 5' end of the *MAG1* transcript was estimated by sequencing the cDNA clones selected from a λYES yeast cDNA library (a gift from Dr. Stephen J. Elledge, Baylor College of Medicine, Texas). From about 10^5 pfu (plaque forming unit), 4 *MAG1* cDNAs were obtained. The longest cDNA had the 5' end at -68 relative to the first ATG of *MAG1*. Other three cDNA clones had 5' ends at +5, +59 and +61, probably resulting from an incomplete synthesis of *MAG1* cDNA. This result differs from the previously reported result (Berdal et al., 1990) which claimed that the *MAG1* transcriptional start was mapped to -31 by primer extension.

To determine whether or not the first AUG in the *DDI1* mRNA serves as the translation initiation codon, a 4 bp "AATT" was inserted in the *DDI1-lacZ* fusion construct at the unique *Mfe*I site located between the first and the second in-frame ATGs (Fig. 3-1). It is expected that if the first ATG is indeed the translation start, the 4-bp

Figure 3-2. Mapping the *DDI1* transcription initiation sites. The transcription initiation sites were determined by primer extension analysis and S1 nuclease mapping using RNA isolated from untreated (i.e. no MMS treatment) DBY747 cells. Lanes A, C, G, T are M13 sequence reactions which were used as molecular size reference; lane P contains the primer extension reaction and lane S1 is the result from S1 nuclease mapping. Part of the sequence from *DDI1* promoter was shown. Arrows indicate the major primer extension or S1 protected fragment. The molecular size of the major extension fragment was determined by comparing with the M13 sequence and the transcription initiation site was mapped in the *DDI1* promoter region at nucleotide -61 relative to the translation start.


DDI1 Promoter Sequence
M13 Sequence
S1
P A C G T
CGAATAAGAAAGTATACTTAACGTAGTTAA
-61

insertion would introduce a +1 frameshift and create a stop codon immediately 3' to the *Mfe*I site (Fig. 3-1A). As a result of the insertion, the β-gal activity was totally abolished (Fig. 3-14C), indicating that the first AUG is the translation initiation codon of *DDI1*.

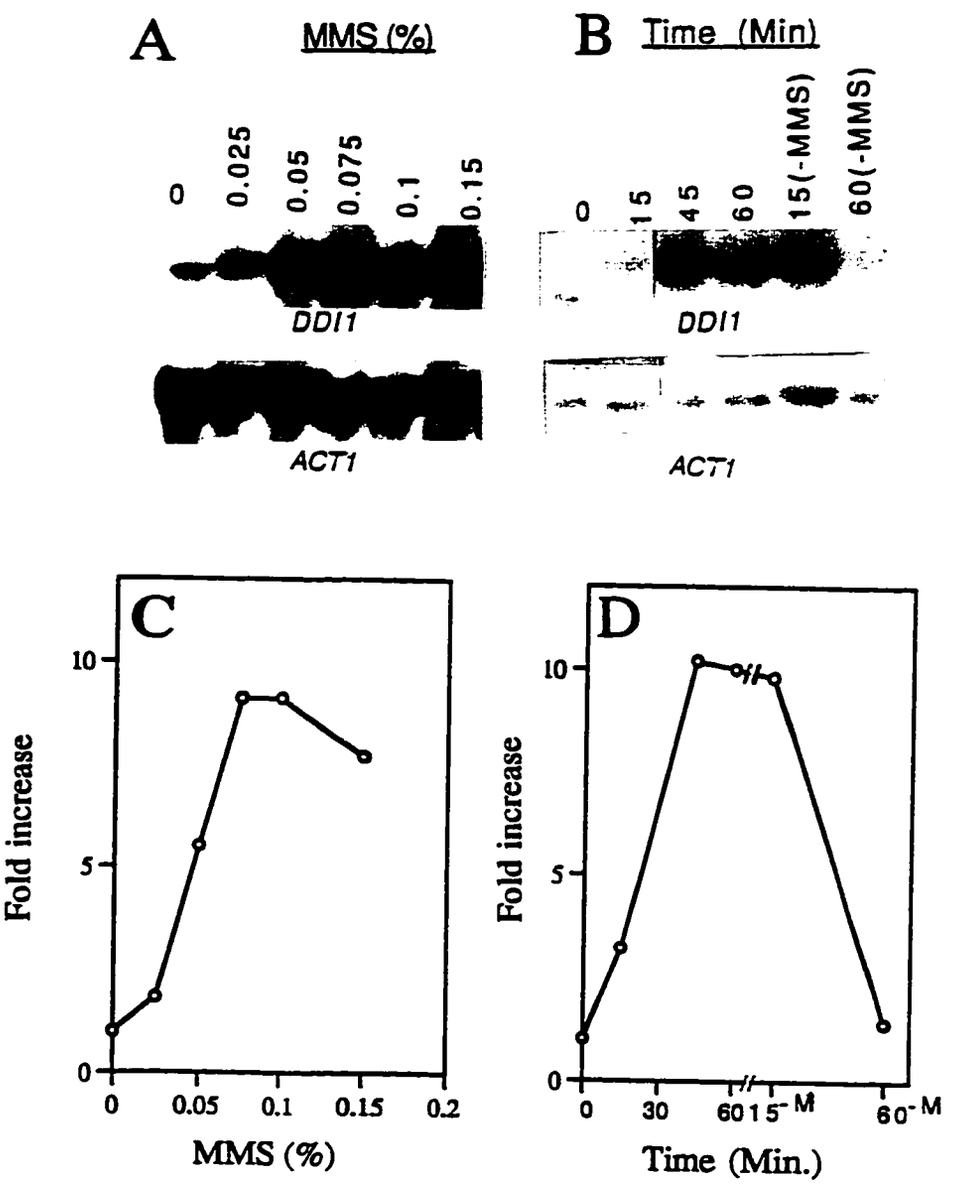
YEpMAG1-lacZ contains several in-frame ATGs from the *MAG1* coding region. To determine if the first ATG is required for *MAG1* mRNA translation, all but the first ATG were removed by *Xba*I-*Sma*I deletion from the *MAG1* coding region in YEpMAG1-lacZΔ-133 (Xiao et al., 1993) which lacks an upstream *Xba*I site. This deletion maintained the reading frame with *lacZ* and did not alter the level of β-gal activity compared with YEpMAG1-lacZΔ-133. The experiment suggests that the first AUG is used as the *MAG1* mRNA translational start, which agrees with the molecular size of the native Mag1 protein (Berdal et al., 1990).

3.3. Co-regulation Of *MAG1* And *DDI1* By DNA Damage

3.3.1. The transcription of *DDI1* is also inducible by MMS

Since *DDI1* shares a very short upstream sequence with *MAG1* known to be inducible by DNA damage, it was asked if the new gene was also inducible by DNA damage. The expression of *DDI1* gene in response to MMS was studied quantitatively by Northern blot analysis using a *DDI1*-specific probe (0.9 Kb *Eco*RI fragment, see Fig. 3-1). The result showed that *DDI1* was inducible by MMS treatment in a dose- and time-dependent manner with a maximum induction of about 10-fold (Fig. 3-3). The fold of induction increased linearly within the concentration of 0.075% MMS (Fig. 3-3, A and C), comparable to the induction of *MAG1* gene (Chen and Samson, 1991; Chen et al., 1990). The optimal concentrations under the experimental conditions were found to be 0.075 and 0.1% of MMS. Increasing the concentration above 0.1% resulted in a decrease in the fold of induction, similar to the pattern of induction of *MAG1* gene (Chen and Samson, 1991; Chen et al., 1990). With 0.05% MMS treatment, *DDI1* transcripts

Figure 3-3. Dose response of *DDI1* mRNA accumulation after MMS treatment. Total RNA (approximately 20 μg per lane) was isolated from yeast strain DBY747 that had been exposed to MMS . The same blots were hybridized with the *DDI1* and *ACT1* probes respectively. (A). Northern blot analysis showing that *DDI1* transcript increases after MMS treatment in a concentration-dependent manner. Total RNA was isolated from DBY747 cells exposed to the indicated concentrations of MMS for 30 min. (B). Northern blot analysis showing changes of *DDI1* transcripts after MMS treatment in a time-dependent manner. Total RNA was isolated from DBY747 cells exposed to MMS (0.05%) for the indicated period of time. -MMS represents the post-MMS treatment. After exposure to MMS for 1 h, the treated cells were washed in YPD medium twice and cultured in YPD medium for the indicated period of time (post-MMS treatment). (C) and (D). Graphic representation of the Northern results. The Northern hybridization bands from the X-ray negatives were measured by a Computing Densitometer Model 300A (Molecular Dynamics). The MMS treatment and the post-MMS treatment is divided by "//" sign in (D).


A
MMS (%)
0
0.025
0.05
0.075
0.1
0.15
DDI1
ACT1
B
Time (Min)
0
15
45
60
15(-MMS)
60(-MMS)
DDI1
ACT1
C
Fold increase
10
5
0
0
0.05
0.1
0.15
0.2
MMS (%)
D
Fold increase
10
5
0
0
30
60
15'-M
60'-M
Time (Min.)

increased linearly with the time of exposure and reached the highest level (about 10 fold) by 45 min. The level of *DDI1* transcript dropped to the uninduced level within 1 hour after release from MMS treatment (B and D, Fig. 3-3).

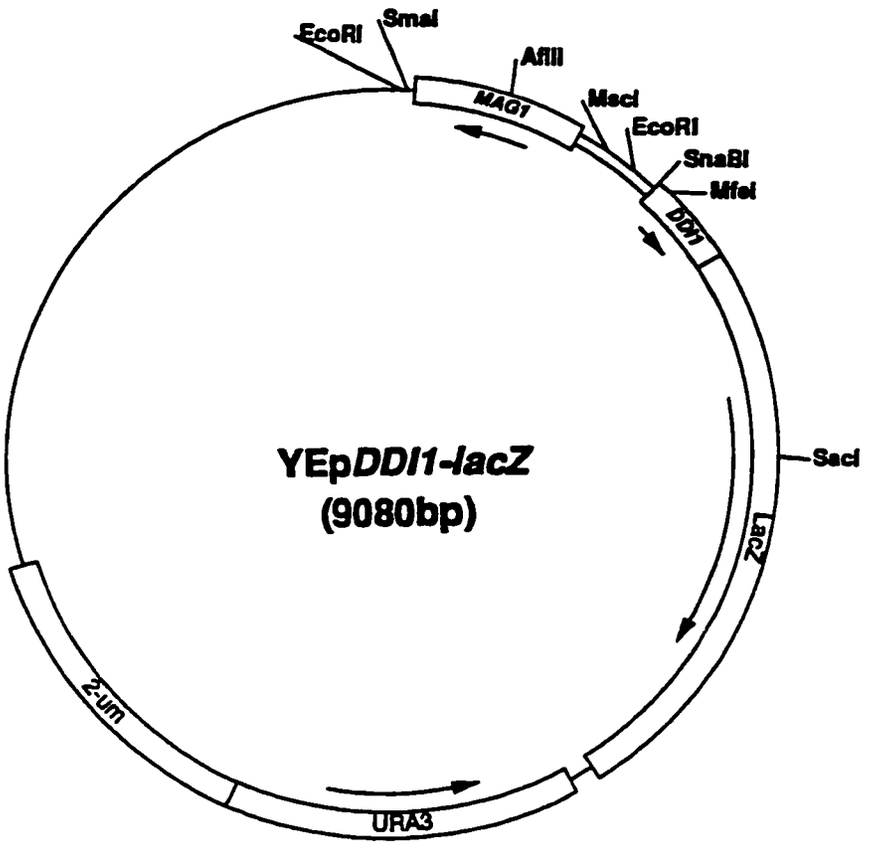
3.3.2. *MAG1-lacZ* and *DDI1-lacZ* are inducible by a variety of DNA damaging agents

To define the *cis*-acting elements in the intergenic region between the two divergently transcribed genes, *MAG1* and *DDI1*, a *DDI1-lacZ* gene fusion construct was made in a YEp vector (Fig. 3-4A). Comparison of β-gal activities of *DDI1-lacZ* and *MAG1-lacZ* (Fig. 2-2) under basal and MMS-induced conditions showed that both of the fusion genes were inducible by MMS. However, the basal and the induced levels of *DDI1-lacZ* were about 3 fold higher than that of *MAG1-lacZ* (Fig. 3-4B).

It is known that the *MAG1* gene is induced not only by DNA alkylating agents, but also by other DNA damaging agents (Chen and Samson, 1991). Therefore, it is of interest to know if *DDI1* is also inducible by multiple DNA damaging agents. Indeed, both *DDI1* and *MAG1* responded to the same sets of DNA damaging agents, including MMS, UV, 4NQO and hydroxyurea, in a dose-dependent manner within a certain range of treatments (Table 3-1). Like *MAG1-lacZ*, *DDI1-lacZ* showed little induction by heat shock treatment, suggesting that the induction of both genes is DNA damage specific, and that general stresses that do not produce DNA damage such as heat shock do not induce *MAG1/DDI1*. With respect to DNA damage induction, *MAG1-lacZ* and *DDI1-lacZ* showed a number of similarities: First, both genes were inducible by the same set of damaging agents, namely, MMS, MNNG, UV, 4NQO and hydroxyurea, but not by heat shock. Secondly, both required similar dosages of the DNA damaging agents for the maximum induction. Finally, the fold of induction ratio was also similar (Table 3-1).

Figure 3-4. Induction of *MAG1-lacZ* and *DDI1-lacZ* by MMS treatment. (A). Structure of plasmid YEpDDI1-lacZ. The plasmid contains an 1.09 kb *Bgl*II-*Dra*I fragment including 628 bp *MAG1* coding region, 179 bp *DDI1* coding region and 282 bp intergenic region. The *DDI1* coding region was fused in frame to *lacZ* at the *Dra*I site by cloning the *Bgl*II-*Dra*I fragment into the *Bam*HI-*Hind*III sites of YEp353 (Myers et al., 1986). The direction of transcription of the fusion gene is shown by an arrow and the relevant restriction sites are indicated. (B). Basal and MMS-induced levels of β-galactosidase activity from *DDI1-lacZ* and *MAG1-lacZ* transformants. *MAG1-lacZ* was from YEpMAG1-lacZ transformants (Xiao et al., 1993) and *DDI1-lacZ* was from YEpDDI1-lacZ transformants. +MMS and -MMS, DBY747 transformants were treated with MMS (0.05%, 4 hours) and without MMS treatment respectively. The results are the average of three independent experiments with standard deviations.

A


EcoRI
SmaI
AflII
MscI
EcoRI
SnaBI
MfeI
MAG1
DDI1
YEpDDI1-lacZ
(9080bp)
SacI
LacZ
2-um
URA3

B

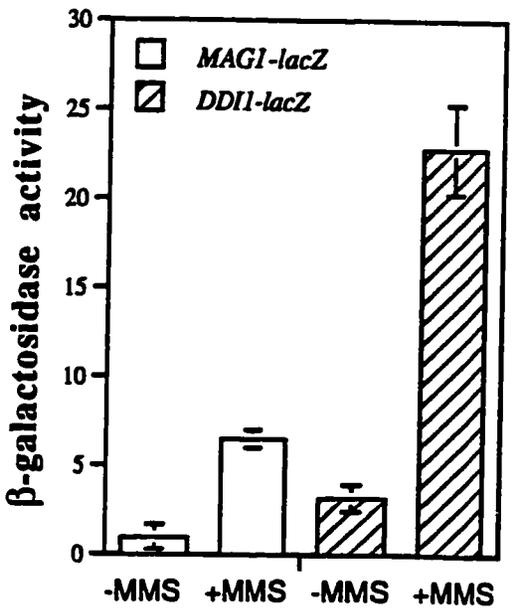

30
25
20
15
10
5
0
β-galactosidase activity
MAG1-lacZ
DDI1-lacZ
-MMS
+MMS
-MMS
+MMS

Table 3-1. Induction of *MAG1-lacZ* and *DDI1-lacZ* by DNA damaging agents

Agent	Dose (range)[a]	Fold Increase	
		DDI1-lacZ	*MAG1-lacZ*
MMS	0.05% (0.015-0.12%)	7.1[b]	6.6
UV	20 J/m^2 (10-60 J/m^2)	4.5	4.2
4NQO	0.8 μg/ml (0.2-1.6 μg/ml)	4.0	4.5
HU	80 mM (20-120 mM)	3.8	4.1
Heat Shock	3 hours (1-4 hours)	1.6	1.2

[a] The dose of treatment listed gave the optimal induction within experimental range of treatments shown in brackets. Chemical treatments were all for 4 hours. UV treatment was followed by 4-hour incubation in the dark. Heat shock was from 22 to 37°C for the length of incubation as indicated.

[b] All data presented are the average of at least two experiments.

3.4 *DDI1-lacZ* Upstream Deletions Identify A Repression Element

To determine the *cis*-acting elements that regulate *DDI1* expression, 5' deletions were introduced into plasmid YEp*DDI1-lacZ* such that all constructs had the same sequence 5' to the *DDI1* upstream region. Therefore, any changes in the level of β-gal activity will likely reflect manipulations in the *DDI1* promoter sequence. β-gal activities of yeast transformants harboring a series of promoter deletions were determined in the absence and presence of MMS.

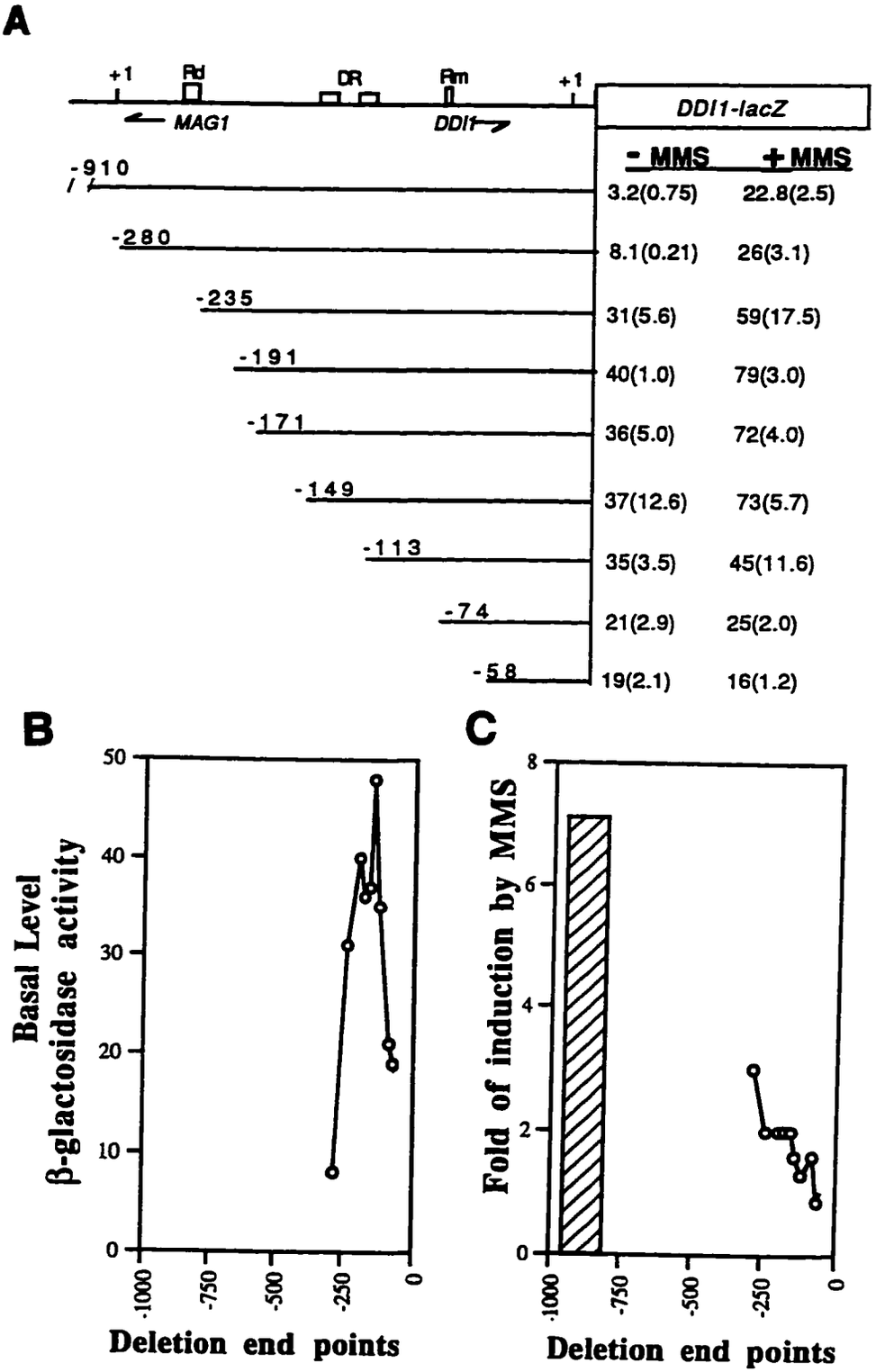
The 5' deletion from -910 to -280 relative to the *DDI1* ORF resulted in a 2.5-fold increase in basal -level expression. Deletion between -280 and -235 resulted in a additional 4-fold increase in the basal level β-gal activity (Fig. 3-5A), indicating the presence of a URS element between -280 and -235. As the basal level increased by the 5' removal of the URS (Fig. 3-5B), the fold of induction decreased accordingly (Fig. 3-5C), suggesting that the mechanism of *DDI1* induction may involve a release of repression at the URS site. However, the promoter remained partially inducible (with a 2-fold induction) after the URS was deleted. However, the deletion of DNA sequences between -149 and -113 abolished DNA damage induction, suggesting that sequences between -149 to -113 might contain another regulatory element.

Between -76 and -67 (relative to the first ATG of *DDI1*) lies the URS of *MAG1* (Xiao et al., 1993). When the URS_{MAG1} was removed, however, no further increase in the basal level of *DDI1-lacZ* was observed (Fig. 3-5A), suggesting that the URS_{MAG1} does not function as a URS of *DDI1*.

3.5 Bi-directional Regulation of *MAG1* and *DDI1* Through UAS_{DM}

Based on the facts that *MAG1* and *DDI1* share a short intergenic region (Fig. 3-1) and both are inducible by DNA damage in a similar manner, it was hypothesized that the short intergenic region between *MAG1* and *DDI1* might contain regulatory element(s) that co-regulates the expression of both genes.

Figure 3-5. 5' end deletions of the *DDI1-lacZ* promoter. (A). Deletion end points and β-gal activity with and without MMS treatment. The arrows indicate the initiation site and the direction of the *DDI1* and *MAG1* transcription. +1 marks initiation of *MAG1* and *DDI1* translation. Rm, the URS_{MAG1} (Xiao et al., 1993); Rd, putative URS_{DDI1}; DR, the 8 bp direct repeat. The solid lines represent the sequence remaining after deletion, with the deletion end points indicated. Basal level expression was determined in cells grown without (-) MMS and induced expression was determined in cells treated with (+)MMS (0.05% for 4 h). The experiment was repeated three times and the average values are presented with standard deviation in brackets. (B). Graphic representation of the basal levels of β-gal activity. (C). Graphic representation of the fold induction after MMS treatment. Data are based on the results from Fig. 3-5A.

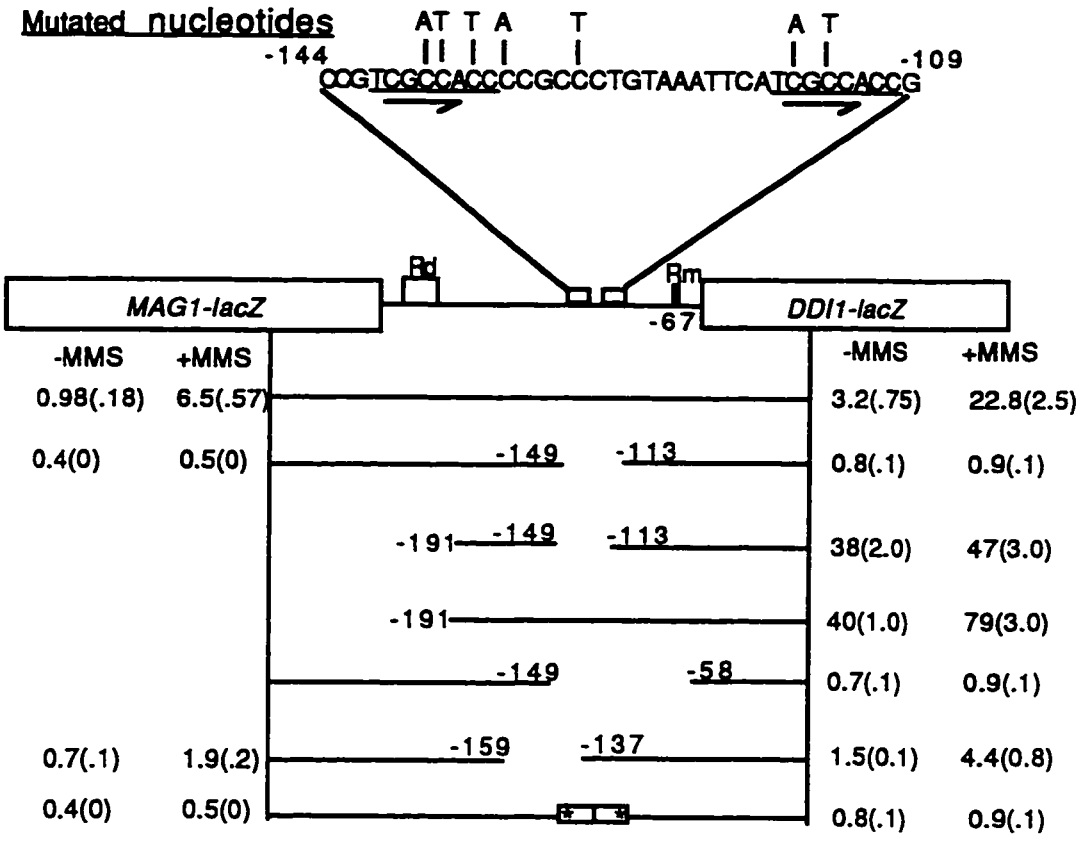

A
+1
Rd
DR
Rm
+1
MAG1
DDI1
DDI1-lacZ
- MMS
+ MMS
-910
3.2(0.75)
22.8(2.5)
-280
8.1(0.21)
26(3.1)
-235
31(5.6)
59(17.5)
-191
40(1.0)
79(3.0)
-171
36(5.0)
72(4.0)
-149
37(12.6)
73(5.7)
-113
35(3.5)
45(11.6)
-74
21(2.9)
25(2.0)
-58
19(2.1)
16(1.2)
B
Basal Level
β-glactosidase activity
50
40
30
20
10
0
-1000
-750
-500
-250
0
Deletion end points
C
Fold of induction by MMS
8
6
4
2
0
-1000
-750
-500
-250
0
Deletion end points

The 5' deletion of YEpDDI1-lacZ identified a putative URS_{DDI1} and suggested the presence of another possible regulatory element between -149 to -113 (Fig. 3-5A). It is noticed that the region between nucleotide -149 and -113 contained a G/C-rich direct repeat sequence (DR), an 8 bp sequence "GGTGGCGA" or "TCGCCACC" is repeated once and is located around the center between the coding regions of *MAG1* and *DDI1* (Fig. 3-1). To address the regulatory role of the direct repeat, a series of internal deletions in YEpDDI-lacZ and YEpMAG1-lacZ were made and the β-gal activity of yeast transformants carrying these deletion constructs was measured. The results are summarized in Fig. 3-6. When the entire DR sequence (between -149 and -113 relative to the translation start of *DDI1*) was deleted (ΔDR), the basal β-gal activity of *DDI1-lacZ* transformants was reduced by 4 fold and DNA damage induction was totally abolished. Interestingly, the effect of ΔDR on *DDI1* expression was alleviated by removing URS_{DDI1} (ΔDR plus 5' deletion to -191) but not by URS_{MAG1} deletion (internal deletion of -149 to -58).

To test if the DR sequence has a bi-directional function, this sequence was also deleted from YEpMAG1-lacZ. Again, ΔDR reduced basal level of *MAG1-lacZ* expression by at least 2.5 fold (the β-gal activity dropped to a level too low to be measured with accuracy) and completely abolished its DNA damage induction.

To distinguish if the results from the above deletions were due to loss of a regulatory element (DR), or due to shortening of the promoter, point mutations were created in both halves of the repeat by site-directed mutagenesis and the resulting plasmids were named YEpDDI1-lacZDRm and YEpMAG1-lacZDRm. The mutant oligonucleotide was designed to abolish the tandem repeats, to reduce GC content within each repeat, and to create a selectable restriction site (*EcoR*V). DRm sequence did not support wild type basal level expression, nor did it confer DNA damage response, for either *MAG1-lacZ* or *DDI1-lacZ*. As a matter of fact, it behaved exactly as ΔDR.

Figure 3-6. Deletion analyses of the direct repeat (DR) sequence. The sequence from nucleotide -144 to -109 containing the entire direct repeat is shown on the top panel. Rd and Rm represent URS_{DDI1} and URS_{MAG1}, respectively. The solid lines represent the sequence that remained after deletion with numbers indicating the regions deleted. Asterisks at the bottom construct represent point mutations in both halves of the direct repeat with nucleotide sequences as indicated on top of the figure. The values were the average of at least three experiments with standard deviation in brackets.

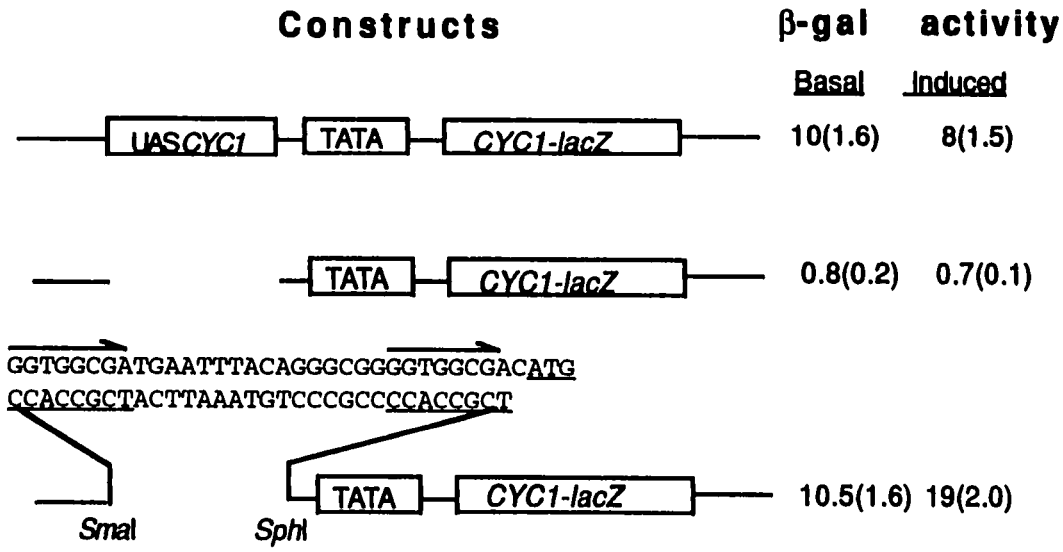

Mutated nucleotides
AT T A T A T
-144
CCGTCGCCACCCCGCCCTGTAAATTCATCGCCACCG
-109
Bd
Bm
MAG1-lacZ
DDI1-lacZ
-67
-MMS
+MMS
-MMS
+MMS
0.98(.18)
6.5(.57)
3.2(.75)
22.8(2.5)
0.4(0)
0.5(0)
-149
-113
0.8(.1)
0.9(.1)
-191
-149
-113
38(2.0)
47(3.0)
-191
40(1.0)
79(3.0)
-149
-58
0.7(.1)
0.9(.1)
0.7(.1)
1.9(.2)
-159
-137
1.5(0.1)
4.4(0.8)
0.4(0)
0.5(0)
0.8(.1)
0.9(.1)

To see if half of the repeat could be still functional, a DNA sequence from -159 to -137 was deleted from both *DDI1-lacZ* and *MAG1-lacZ* promoters. The results (Fig. 3-6) indicated that half of the repeat was partially functional for both genes as compared to the entire repeat. Based on this result, the sequence "GGTGGCGA" or "TCGCCACC" was proposed as a bi-directional UAS and the duplication of the sequence enhances the UAS function. In view of its bi-directional role in the intergenic region between *DDI1* and *MAG1*, the regulatory sequence is named UAS_{DM}.

3.6 The Direct Repeat Activates Expression And Confers Some Damage Responsiveness To A Heterologous Promoter

To determine if the direct repeat is capable of function as UAS within the context of a heterologous promoter, a transcriptional fusion was made by replacing the *Sma*I-*Sph*I fragment of plasmid pLG669Z with the direct repeat sequence (DRSS1/DRSS2, Table 2-1). pLG669Z is a multi-copy plasmid containing a *CYC1-lacZ* fusion (Guarente and Ptashne, 1981; Guarente, 1983). *Sma*I-*Sph*I deletion of pLG669Z removed all the *CYC1* sequence containing the two *CYC1* UASs but left four of the five TATA boxes (Guarente et al., 1984; Li and Sherman, 1991). DBY747 (Table 2-1) transformants carrying plasmid pLG669Z expressed about 10 U of β-gal activity, which was slightly reduced following MMS treatment. *CYC1* TATA minimal promoter (ΔUAS_{CYC1}) decreased the β-gal level to about 0.8 U, whereas insertion of the *MAG1-DDI1* DR sequence in place of UAS_{CYC1} brought the β-gal activity up to a level similar to that from UAS_{CYC1}. In contrast to UAS_{CYC1}-*lacZ*, where β-gal activity was slightly reduced following MMS treatment, MMS treatment of the DR-*CYC1-lacZ* transformants increased β-gal activity by about 2-fold (Fig. 3-7).

Figure 3-7. Direct repeat functions in the heterologous *CYC1* promoter. The diagram shows promoter constructs used in the study. The broken solid line represents the deletion of UAS_{CYC1} sequence. Double-stranded oligonucleotides containing the direct repeat (shown in arrows) were inserted in the upstream of the *CYC1* minimal promoter, the underlined ATG on top strand was synthesized for cloning and is not part of the promoter sequence from *MAG1/DDI1*. Right panel shows β-gal activity of corresponding DBY747 (Table 2-1) transformants under basal and MMS-induced conditions. The values were the averages from three experiments with standard deviation in brackets.


Constructs
β-gal activity
Basal
Induced
UASCYC1
TATA
CYC1-lacZ
10(1.6)
8(1.5)
TATA
CYC1-lacZ
0.8(0.2)
0.7(0.1)
GGTGGCGATGAATTTACAGGGCGGGGTGGCGACATG
CCACCGCTACTTAAATGTCCCGCCCCACCGCT
SmaI
SphI
TATA
CYC1-lacZ
10.5(1.6)
19(2.0)

3.7 Deletion Of The Direct Repeat Increases The Sensitivity Of Cells to MMS

The *MAG1* gene encodes a 3MeA DNA glycosylase and protects cells from killing by MMS-induced DNA replication block (Chen et al., 1989). To determine the physiological significance of the direct repeat and to correlate the level of *MAG1* transcription with cellular sensitivity to MMS, a *mag1* disruption strain WXY9216 (Table 2-1) was transformed with a single-copy YCp plasmid carrying a functional *MAG1* gene with wild type or altered promoter sequence. To achieve a high level *MAG1* expression, WXY9216 (*mag1::hisG*) was transformed with a multi-copy plasmid YEpMAG1 and a single-copy plasmid YCpMAG1ΔRm carrying a promoter deletion of URS of *MAG1*. Fig. 3-8A showed the *MAG1* transcript level of these transformants with or without MMS treatment. Compared to the level of *MAG1* transcript from the wild type promoter (wt), the basal (-) level of the transcript was reduced and MMS induced (+) expression was abolished with DR deletion (ΔDR). Like endogenous *MAG1*, the *MAG1* gene borne in a multi-copy plasmid (YEpMAG1) was still inducible by MMS; however, both the basal and the induced levels were much higher than that of the endogenous *MAG1*.

The cellular MMS sensitivity was determined by both the liquid killing experiment and the gradient plate assay (Materials and Methods). In the presence of 0.3% MMS, WXY9216 (DBY747 with *mag1* disruption, Table 2-1) cells were extremely sensitive to MMS-induced killing, while WXY9216 transformants carrying a functional *MAG1* gene with a ΔDR promoter provided only partial complementation compared to that with a wild type promoter (Fig. 3-8B). However, over-expression of *MAG1* with a multi-copy plasmid (YEpMAG1) or with the promoter deletion of URS_{MAG1} (YCpMAG1ΔRm) did not provide additional MMS resistance (Fig. 3-9B).

Figure 3-8. The level of *MAG1* expression correlates with cellular sensitivity to MMS-induced killing. (A). Northern analyses of the *MAG1* transcript. YEpMAG1 is a multi-copy plasmid carrying the *MAG1* gene. wt represents transformants carrying the *MAG1* gene with a wild-type promoter and ΔDR represents the promoter with DR deletion (-170 to -137). + and - represent RNAs from MMS-treated and untreated cells, respectively. The Northern blot was first hybridized with a *MAG1* probe. The same membrane was stripped and then hybridized with an *ACT1* probe. (B). 0.3% MMS-induced killing of various transformants. The experiment was repeated twice and the average numbers were presented. ◎WXY9216/YCpMAG1; ▲ WXY9216/YCpMAG1ΔDR; ● WXY9216.

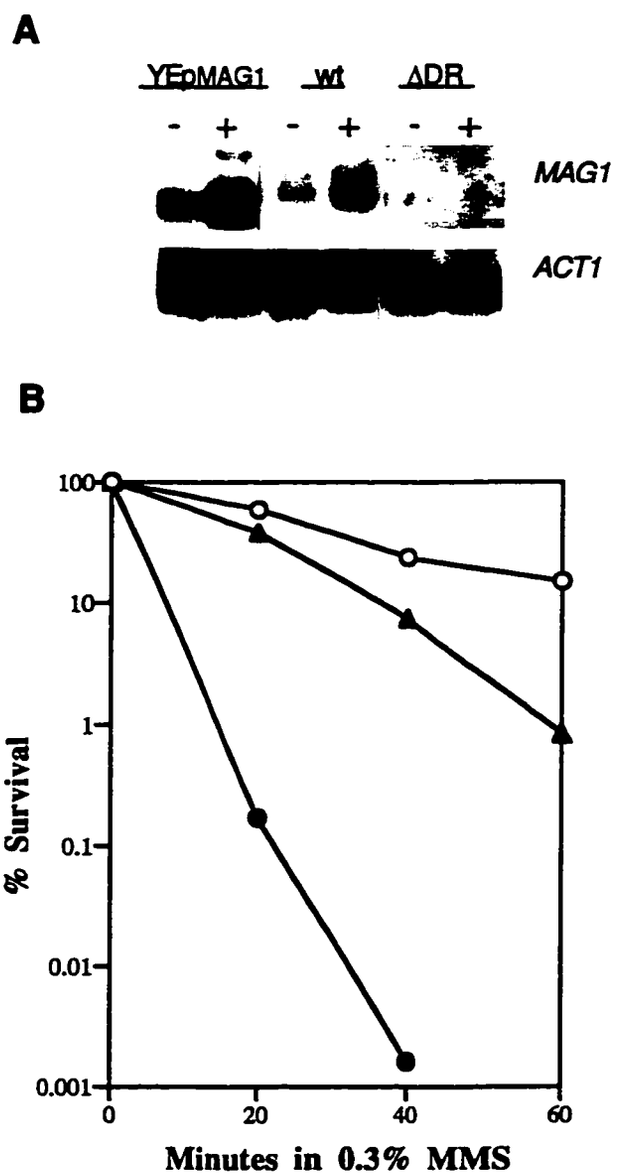
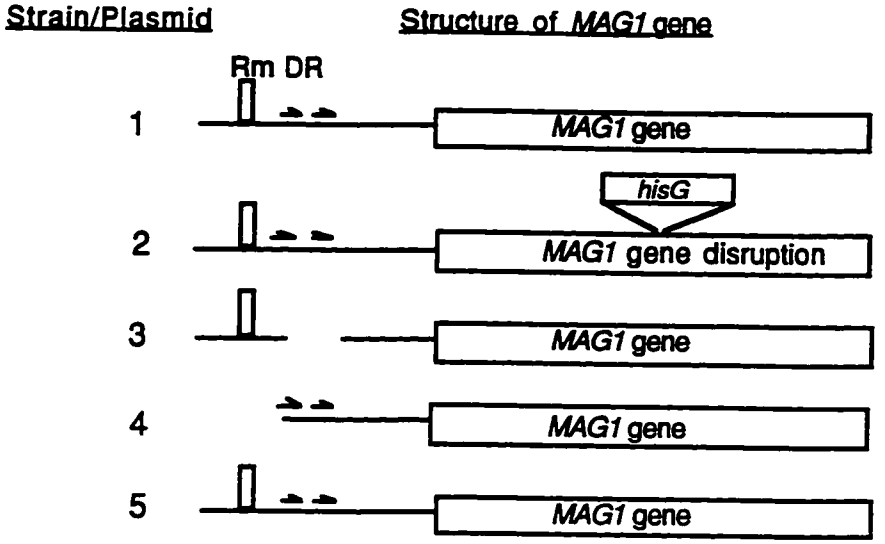
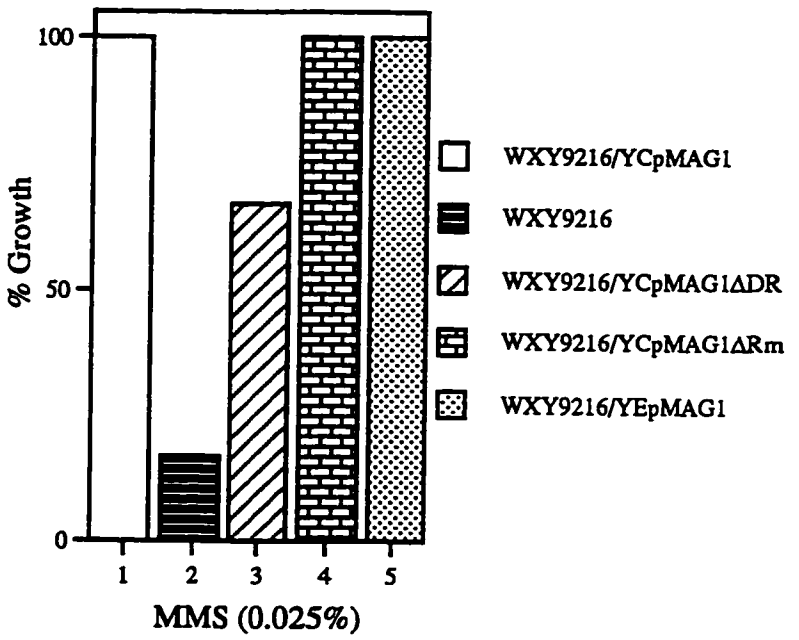

A
YEpMAG1
wt
ΔDR
- + - + - +
MAG1
ACT1
B
100
10
1
0.1
0.01
0.001
% Survival
0
20
40
60
Minutes in 0.3% MMS

Fig. 3-9 MMS gradient plate assay of yeast strains carrying *MAG1* promoter alterations. (A). Graphic representation of yeast strains carrying *MAG1* gene with different promoter alterations. Rm, URS_{MAG1}; DR, direct repeat shown in arrows. (1). WXY9216 with a single-copy plasmid YCpMAG1. (2). WXY9216, a DBY747 strain with *mag1* disruption. (3). WXY9216 with a single-copy plasmid YCpMAG1ΔDR. (4). WXY9216 with a single-copy plasmid YCpMAG1ΔRm. (5). WXY9216 with a multi-copy plasmid YEpMAG1. (B). Gradient plate assay. The plate contains a linear gradient of MMS with concentrations ranging from 0 to 0.025%. The length of region of cell growth on the plate was measured after a 3-day incubation and the % growth was calculated as specified in Materials and Methods.

A


Strain/Plasmid
Structure of MAG1 gene
Rm DR
1
MAG1 gene
hisG
2
MAG1 gene disruption
3
MAG1 gene
4
MAG1 gene
5
MAG1 gene

B


100
50
0
% Growth
1
2
3
4
5
MMS (0.025%)
WXY9216/YCpMAG1
WXY9216
WXY9216/YCpMAG1ΔDR
WXY9216/YCpMAG1ΔRm
WXY9216/YEpMAG1

3.8 The Formation Of DR-protein Complexes *In Vitro*

3.8.1 Formation of yRPA-DNA complexes

Yeast RPA (yRPA) is a ssDNA-binding protein consisting of three subunits with molecular weights of 70 (RPA1), 30 (RPA2) and 14 (RPA3) kDa (Heyer et al., 1990; Brill and Stillman, 1991). It was recently reported that yRPA interacted with dsDNA probes from promoters of several yeast DNA repair and DNA metabolism genes, including a DNA sequence, called $URS2_{MAG}$, from the promoter region of *MAG1*. Besides, $URS2_{MAG}$ was able to repress the function of UAS_{CYC1} (Singh and Samson, 1995). $URS2_{MAG}$ is located between nucleotide -180 and -161 (relative to the translation start of *MAG1*) and contains half of the DR sequence. Therefore, this report raised a possibility that the *in vivo* DR function could be related to its interaction with yRPA. To test the possibility, it was first asked if purified yRPA could form complexes with the DR probe. The electrophoretic mobility shift assay (EMSA) was performed and the result (Fig. 3-10) showed that purified yRPA formed protein-DNA complexes with the DR probe. EMSA revealed a single band resulting from the interaction of yRPA with the DR probe. The specificity of yRPA-DR interaction was evidenced by the competition with unlabeled DR and the lack of competition with pUC18 or double-stranded DNA consisting of oligonucleotides MSC1/MSC2 (Table 2-2). On the other hand, yRPA also formed protein-DNA complexes with labeled probes consisting of oligonucleotides MAG1-23/-24 and MAG1-25/-26 (Table 2-2). Besides, formation of the yRPA-DR complex was efficiently competed by the single-stranded oligonucleotide MSC1 (Table 2-2).

3.8.2 Interactions of proteins from cell extract with DR probe

The interaction of cell extract with the DR probe formed two retarded bands (Fig. 3-11B), one of which (Band II) co-migrated with yRPA-DR complex. Band I did not seem to contain yRPA as evidenced by faster migration than yRPA-DR (lane 9). The specificity of Band I and II was evidenced by the competition with unlabeled DR and the

Figure 3-10. Binding of yRPA to DNA probes of different sequences. Each reaction contains 20 ng ^{32}P-labeled probe and 100 ng of RPA. The competitor consists of 2μg of dsDNA or 1 μg ssDNA. DR1/DR2 (2 μg) is used as double-stranded sequence-specific competitor of the DR probe. pUC18 (2 μg) and MSC1/MSC2 (2 μg) are used as double-stranded sequence-nonspecific competitors. MSC1 (1 μg) is used as single-stranded sequence-nonspecific competitor. Band I, RPA-DNA complex; FP, free probe.

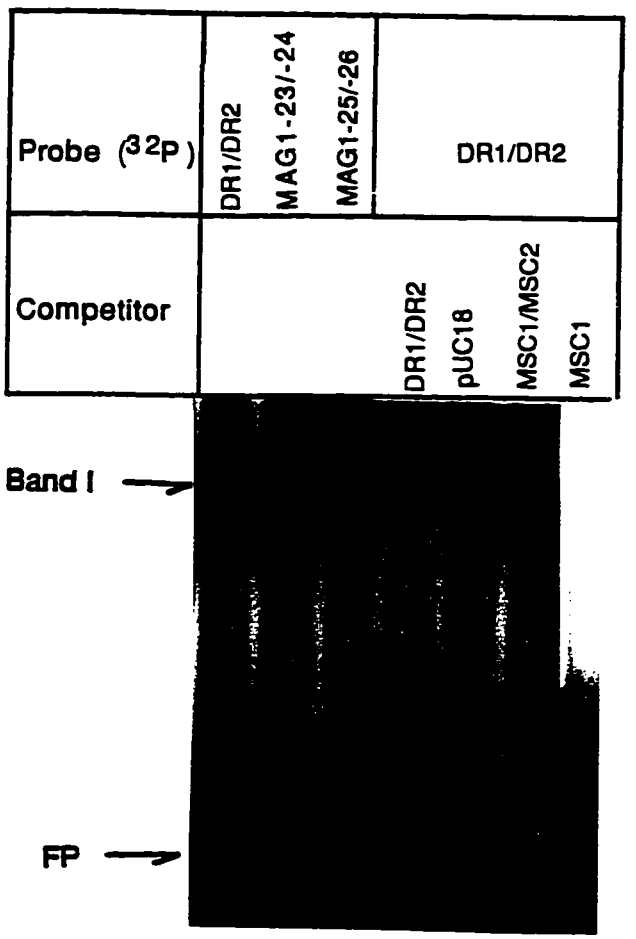
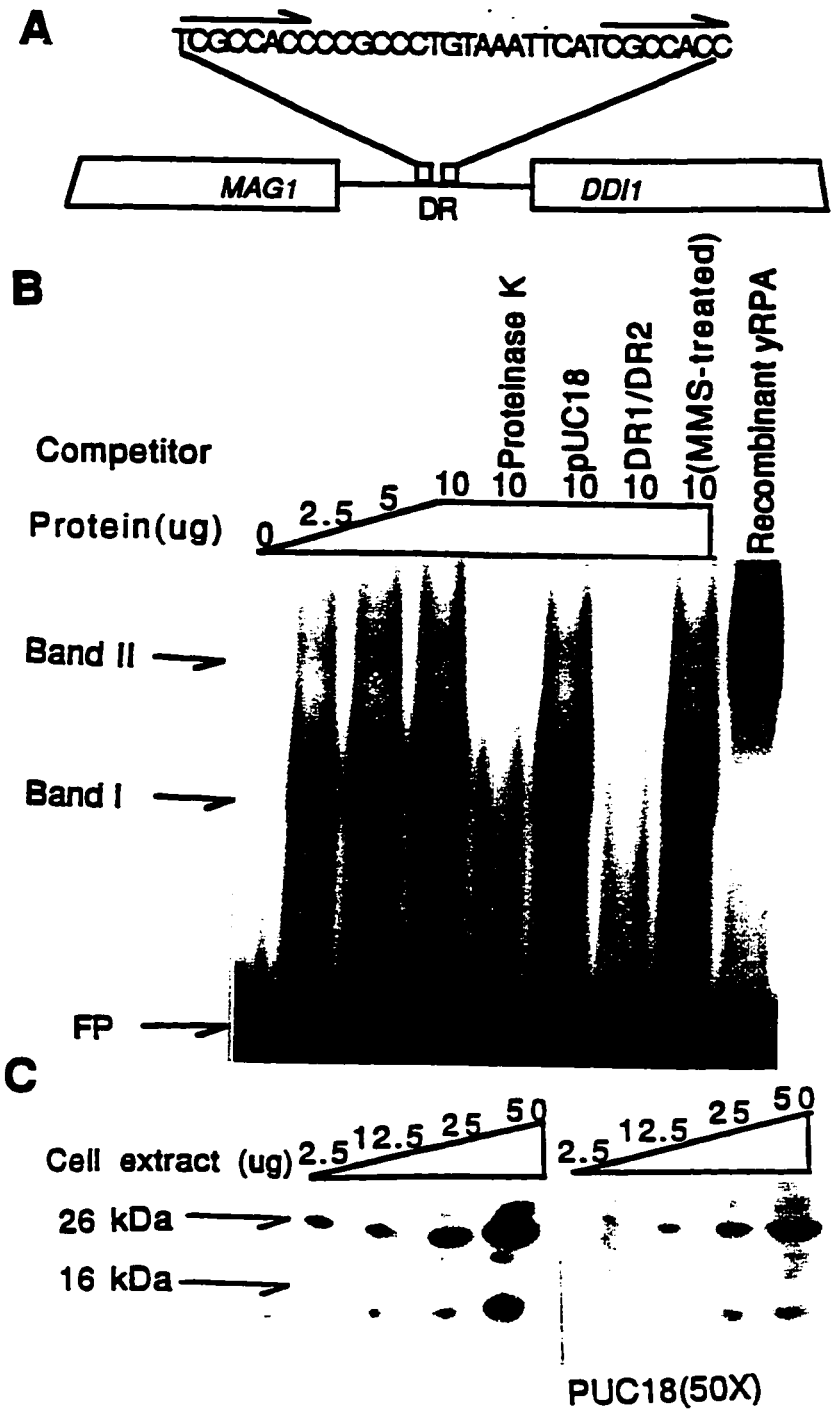

Probe (^{32}P)
DR1/DR2
MAG1-23/-24
MAG1-25/-26
DR1/DR2
Competitor
DR1/DR2
pUC18
MSC1/MSC2
MSC1
Band I
FP

Figure 3-11. Interactions of proteins from cell extract with DR probe. (A). A diagram of the *DDI1/MAG1* promoter region showing the sequence and location of the DNA probe. (B). Gel mobility shift assay using the direct repeat (DR) probe. Each reaction contains 20 ng ^{32}P-labeled probe. The amounts of proteins and competitors used in each reaction are either indicated or as follows: proteinase K (10 μg), annealed DR1/DR2 (2 μg), pUC18 (2 μg) and yRPA (50 ng). Band I and Band II indicate protein-DNA complexes. FP, free probe. (C). Southwestern analysis using the direct repeat probe. Different amounts of yeast cell extract from DBY747 cells were separated by 12% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane-bound proteins were renatured and hybridized with DR probe (1μg/ml) in buffer A containing 50 μg/ml nonspecific DNA competitor poly(dI-dC) with (50 μg/ml) or without pUC18 DNA. The estimated molecular sizes of proteins interacting with the DR probe are indicated.


A
TCGCCACCCCCGCCCTGTAAATTCATCGCCACC
MAG1
DR
DDI1
B
Competitor
Protein(ug)
0
2.5
5
10
10 Proteinase K
10 pUC18
10 DR1/DR2
10 (MMS-treated)
1 Recombinant yRPA
Band II
Band I
FP
C
Cell extract (ug)
2.5
12.5
25
50
2.5
12.5
25
50
26 kDa
16 kDa
PUC18(50X)

lack of competition with pUC18. It is important to note that cell extracts from MMS treated (Lane 8) and un-treated cells formed similar retarded bands.

Southwestern analysis was applied to investigate the identity of the protein(s) bound to the DR probe revealed by the gel retardation assay. In the absence of pUC18 as a nonspecific competitor, two proteins of 26 and 16 kDa were detected, while in the presence of excess (50 fold by weight) pUC18, only the 26 kDa band was prominent (Fig. 3-11C).

3.8.3 Both yRPA and cell extract formed protein-DNA complexes with the mutated DR

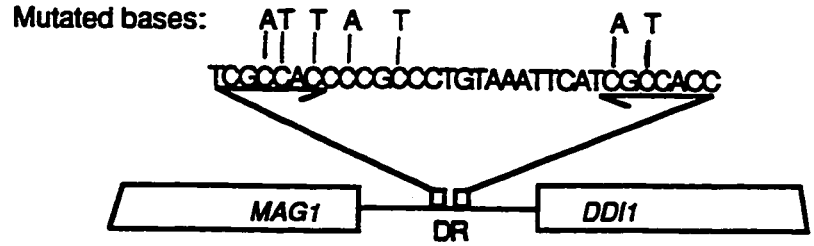
It was shown that introduction of point mutations within the DR sequence greatly reduced the expression and abolished the damage inducibility of both *MAG1* and *DDI1* (Fig. 3-6), and that both yRPA and crude yeast cell extract formed protein-DNA complexes with the DR probe (Fig. 3-10 and Fig. 3-11). To determine if there was a correlation between the UAS_{DM} function and the formation of protein-DNA complex, EMSA was used to see if the DR point mutations affect the formation of protein-DNA complex *in vitro*. Surprisingly, the mutated DR probe was able to form the protein-DNA complex with both the purified yRPA and the crude yeast cell extract. In addition, the formation of protein-DR complex was competed equally well when unlabeled wild type and the mutated DR oligonucleotides were used as competitors (Fig. 3-12).

3.9 Further Localization of UAS_{MAG1}

Previous studies on the regulation of *MAG1* were focused on the identification of an URS element that represses *MAG1* expression (Xiao et al., 1993). The UAS element that regulates damage-induced expression of *MAG1* was not well defined. The present investigation was focused on further defining the DNA sequence important for the UAS function, identifying protein(s) that interacts with the UAS, and determining if the UAS plays a role in DNA damage induction of the *DDI1* gene.

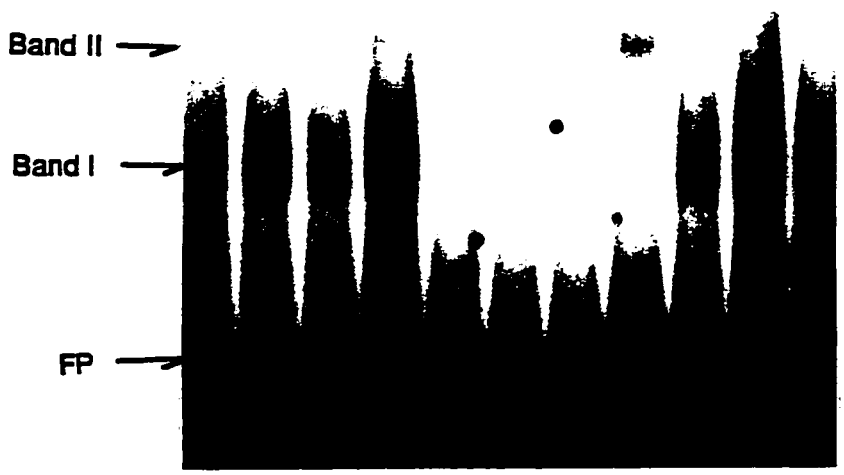
Figure 3.12 Both yRPA and yeast cell extract formed protein-DNA complexes with the mutated DR probe. (A). A diagram of the *DDI1/MAG1* promoter region showing the sequence (wild type DR sequence with the mutated bases on the top) of the DNA probes. (B). Interaction of RPA and cell extract with wild type or mutated DR probe. Each reaction contains 20 ng ^{32}P-labeled DR (consisting of oligonucleotides DR1 and DR2) or Drm (the mutated DR sequence consisting of oligonucleotides DRm1 and DRm2) probe. The competitors consist of double-stranded DR (0.5 μg) or the mutated DR (0.5 μg). Band I and Band II indicate protein-DNA complexes. FP, free probe. Note, the difference in the intensity of Band II formed by the DR and the Drm probes was particular to this experiment rather than a consistent observation.

A



B

Probe	DR	Drm	DR	Drm	DR	
Competitor (ug)					DR(0.5)	Drm(0.5)
Protein (ug)	Cell extract 1	5	1	5	vRPA .01	.05 .01 .05	Cell extract 5	5	5



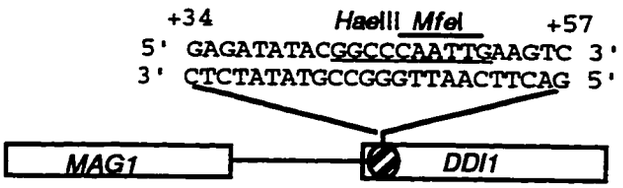
3.9.1 Determination of DNA sequences within the UAS$_{MAG1}$ important for the formation of protein-DNA complex *in vitro*

If the sequence designated UAS$_{MAG1}$ is an activator binding site, it would be expected to interact with one or more proteins. A 83-bp PCR fragment (nucleotide -382 to -300 relative to *MAG1* ORF, or nucleotide +17 to +99 relative to *DDI1* ORF) covering the UAS region was initially used as a probe for a gel retardation assay. A single band was identified as a result of sequence-specific DNA-protein complex formation. In the middle of the 83 bp fragment, there is a *Hae*III restriction site. Cleavage of the 83 bp probe by *Hae*III completely abolished the DNA-protein interaction, whereas C-5 methylation at the *Hae*III site by the *Hae*III methylase interfered with the complex formation (Heiping Dai and Wei Xiao, unpublished), suggesting that the sequence around the *Hae*III site is probably important for DNA-protein complex formation. Subsequently, a 24 bp oligonucleotide probe (MAG1-23 and MAG1-24, Table 2-2) covering sequences between nucleotide +34 to +57 (relative to the *DDI1* ORF) and centered at the *Hae*III site was used and was found to support the DNA-protein complex formation comparable to the 83-bp probe. Fig. 3-13B showed a typical gel mobility shift assay result using partially purified yeast cell extract (Fraction III, see Materials and Methods) and the 24-bp UAS probe. In the absence of cell extract, labeled DNA migrated as a free probe (FP) to its expected position at the bottom of the gel. A single retarded band was revealed in the presence of cell extract (Band I). The nature of the DNA-protein complex in Band I was confirmed since proteinase K treatment abolished band formation. Furthermore, the addition of excess unlabled competitor DNA abolished the formation of the protein-DNA complex, whereas addition of excess non-specific DNA (pUC19) did not compete out the complex formation.

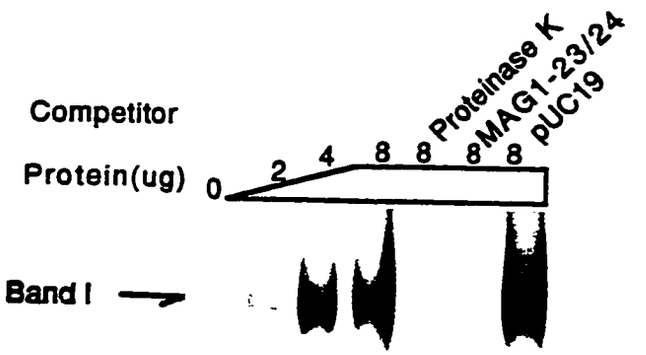
To take one step further, point mutations were introduced within the 24 bp sequence to test the effect of the mutations on the formation of the protein-DNA complex. Compared to the wild type sequence, a 24 bp probe with mutations at position

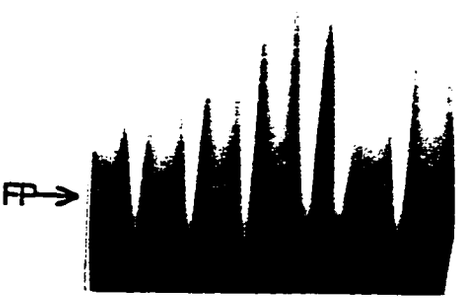
Figure 3-13. Protein-DNA interactions with a 24 bp probe within *MAG1* UAS region . (A). A diagram of the *DDI1/MAG1* promoter region showing the sequence and location of the 24-bp DNA probe. The open boxes represent the protein coding region, the solid line represents the intergenic region and the UAS_{MAG1} region is in the oval shape. (B). Gel mobility shift assay using the 24-bp probe (MAG1-23/-24, Table 2-2). Each reaction contains 20 ng ^{32}P-labeled probe and 1 μg poly(dI-dC). The amounts of proteins and competitors used in each reaction are either indicated or as follows: proteinase K (10 μg), annealed MAG1-23/-24 (1.7 μg), pUC19 (0.8 μg). FP, free probe. Band I indicates the protein-DNA complex. (C). Gel mobility shift assay using either the 24 bp probe or the mutated 24 bp (MAG1-25/-26, Table 2-2) probe. The amount of proteins (μg) and competitors (μg) is indicated on the top panel.

A



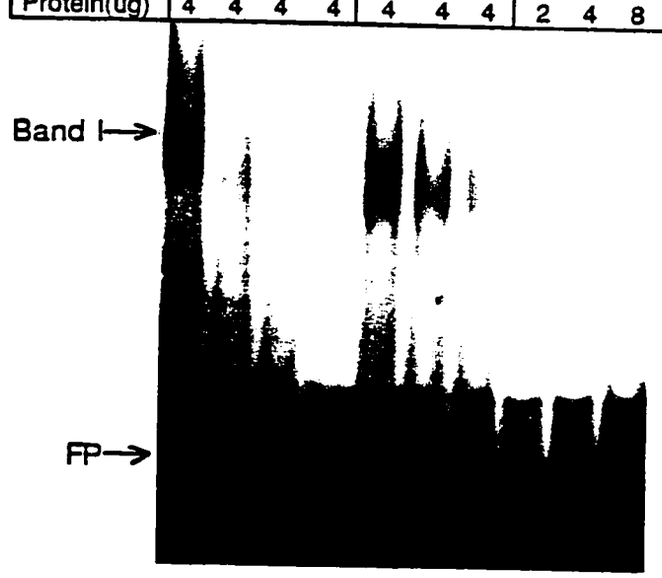
B





C

Probe	MAG1-23/24							MAG1-25/26		
Competitor (ug)	MAG1-23/24				MAG1-25/26					
	0	1	2	4	1	2	4			
Protein(ug)	4	4	4	4	4	4	4	2	4	8



+42 (C to T)and +45 (C to A) greatly reduced the formation of the protein-DNA complex (Lanes 8-10, Fig. 3-13C). Indeed, the mutated double-stranded oligonucleotide was relatively inefficient (compared to the wild type sequence) in competing with the UAS probe for the formation of protein-DNA complex (Lanes 5-7, Fig. 3-13C).

3.9.2 Determination of DNA sequences important for the function of UAS_{MAG1}

To determine the functional importance of the 24 bp sequence identified by *in vitro* EMSA, internal deletions, insertion, and mutagenesis were introduced within the 24 bp sequence and β-gal assay was performed to determine the effects of these alterations on the expression of *MAG1-lacZ* and *DDI1-lacZ*. These results are summarized in Fig. 3-14. Compared to the *MAG1-lacZ* with a full length promoter (wt), deletion between +30 and +69 (Δ30-69) relative to the first ATG of *DDI1* ORF (or -312 to -351 relative to the *MAG1* ORF) reduced the basal level of *MAG1-lacZ* by 50% and decreased the induced expression by 70%. Moreover, the point mutations (C to T at position +42 and C to A at position +45) and a 4 bp insertion at the *Mfe*I site lowered both the basal and the induced expression of *MAG1-lacZ*. These results were consistent with the *in vitro* protein-DNA binding assay (Fig. 3-13) and suggested that the sequence important for the UAS function is likely to be around the *Mfe*I site.

To see if the UAS_{MAG1} also regulates *DDI1* expression, the deletion Δ30-69, which maintained *DDI1* ORF, was tested in the *DDI1-lacZ* fusion construct. Interestingly, a decrease in the expression of *DDI1-lacZ* was also observed. The *Mfe*I site is located between the first and the second ATGs of *DDI1* ORF. Insertion of 4 bp sequence "AATT" at the *Mfe*I site introduced a +1 frameshift and totally abolished β-gal activity, suggesting that the first ATG is the translation start.

From the internal deletion (Δ30-69), it appears that *MAG1* and *DDI1* share the UAS. However, the deletion result could be either due to a UAS being present in the deleted sequence, or due to the effect of deletion on the stability of *DDI1* transcript or/and the polypeptide. It is argued that if the two genes indeed share a UAS element, carefully

designed base substitutions within the UAS_{MAG1} should alter the expression of both genes. Mutations at position +42 (C to T) and +45 (C to A) were introduced by PCR-mediated mutagenesis (Materials and Methods). Two important factors that might affect *DDI1* gene expression were considered: First, the nucleotide changes at the position +42 and +45 would not change the amino acids by base substitution at the wobble sites. Secondly, new codons would have a similar codon usage (TAT=16.27, GGA=8.55) to the previous ones (TAC=16.59, GGC=8.92). The codon usage of *S. cerevisiae* is obtained from 435 genes found in GeneBank release V63.0 compiled by J. Michael Cherry (Stanford). While these mutations reduced the expression of *MAG1-lacZ* by more than 50%, little change in *DDI1-lacZ* expression was observed (Fig. 3-14). It is thus suggested that the N-terminus of the Ddi1 polypeptide may contain sequence important for the stability of the fusion protein, and that this sequence incidentally overlaps with the UAS_{MAG1}. Alternatively, the internal deletions may affect the stability of the fusion transcript.

3.10. Determination Of Plasmid Copy Number

YEp is a 2μm-based plasmid and, therefore exists in multiple copies in yeast cells. The problem concerning the use of YEp plasmid is the possible variation of plasmid copy number. To confirm that the results from the promoter deletions were not associated with the variations in plasmid copy number, selected yeast strains carrying different constructs (having a wide range of β-gal activities) were subject to Southern analysis using *URA3* as a probe (a selection marker carried in YEpDDI1-lacZ). Fig. 3-15 showed a similar intensity of the hybridization signals among these transformants, indicating that there was little variation in the plasmid copy number among cells carrying different constructs. Therefore, the difference in β-gal activities among cells carrying different plasmids are due to the deletions of promoter sequences rather than the variation in plasmid copy number.

Figure 3-14. Effect of sequence alterations at the UAS_{MAG1} region on the expression of *MAG1-lacZ* and *DDI1-lacZ*. (A). A diagram of nucleotide alterations. Internal deletion, site-specific mutagenesis and the 4-bp insertion were made as described in Materials and Methods. (B). Effects of sequence alterations at the UAS_{MAG1} on the *MAG1-lacZ* expression. (C). Effects of sequence alterations at the UAS_{MAG1} on *DDI1-lacZ* expression. The β-gal activity is an average of three experiments with standard deviation as shown. No β-gal activity was observed with transformants carrying the 4-bp insertion of YEpDDI1-lacZ due to a frameshift in the coding region.

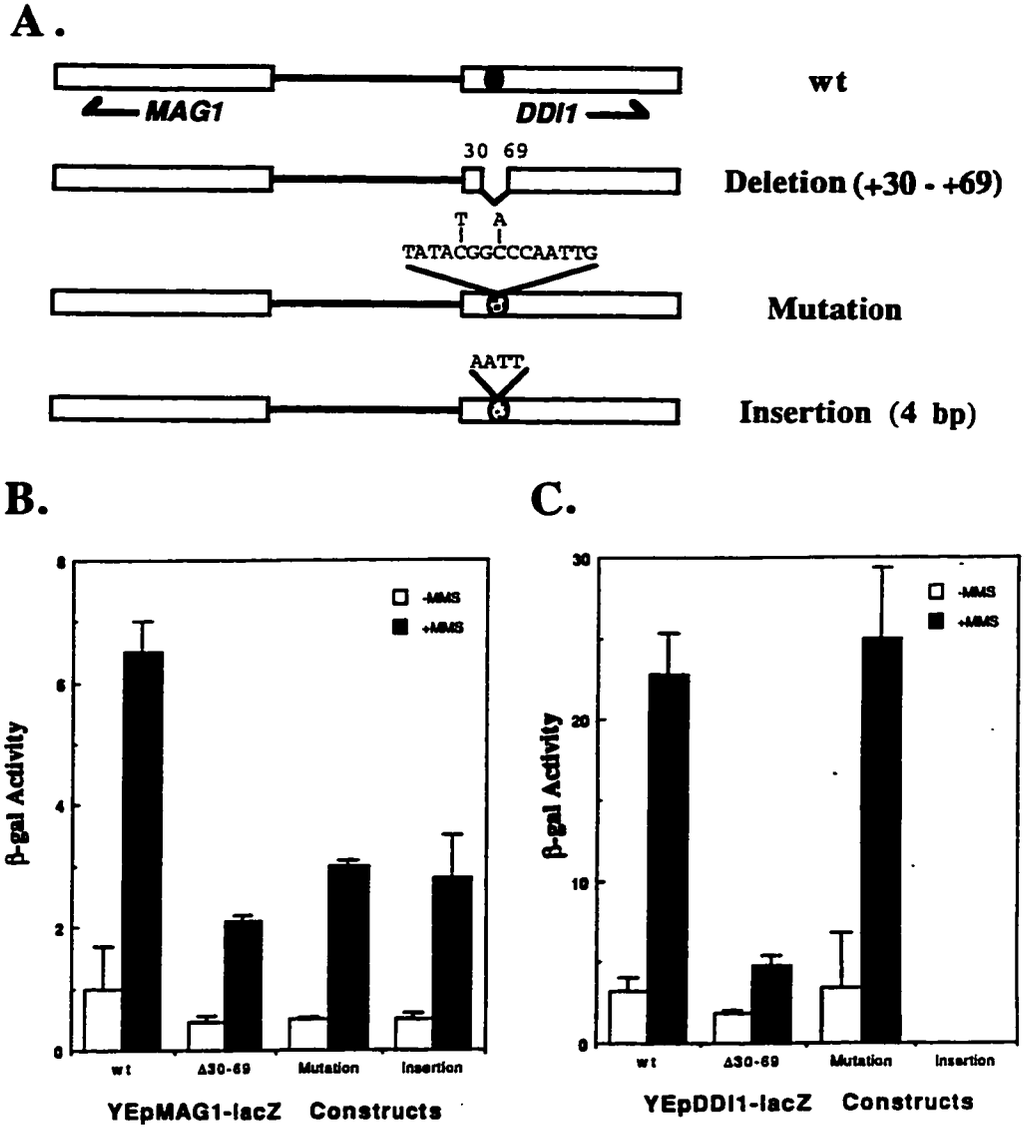
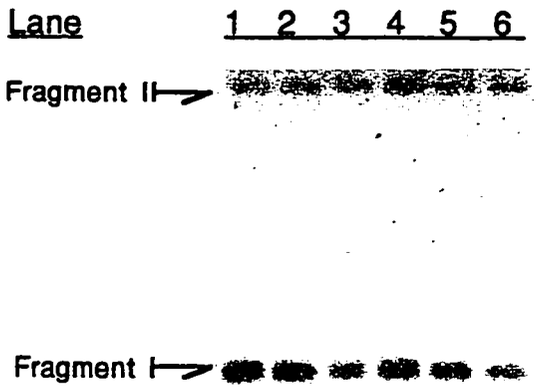

A.
MAG1
DDI1
wt
30 69
Deletion (+30 - +69)
T A
TATACGGCCCAATTG
Mutation
AATT
Insertion (4 bp)
B.
C.
β-gal Activity
-MMS
+MMS
wt
Δ30-69
Mutation
Insertion
YEpMAG1-lacZ Constructs
YEpDDI1-lacZ Constructs

Figure 3-15. Southern analysis of plasmid copy number. Total DNA was extracted from cells carrying different plasmid constructs and the DNA was digested with *Nde*I to release a fragment from *URA3* gene carried in YEp plasmid (1181 bp). The digested DNA was separated by agarose gel electrophoresis. The Southern blot was hybridized with *URA3* probe (1.2 Kb *Hind*III fragment from YEp24) radioactively labeled by random primer labeling method (Materials and Methods). Fragment I represents the *URA3* fragment from the YEp plasmid, while Fragment II comes from the chromosomal *URA3* which can be used as an internal control for the amount of DNA loaded in each lane. Lane 1, DBY747/YEpDDI1-lacZ; lane 2, DBY747/YEpDDI1-lacZΔDR, containing the internal deletion from -149 to -113 (Fig. 3-6); lane 3, DBY747/YEpDDI1-lacZΔDR-Rd containing ΔDR plus the 5' deletion to -191 (Fig. 3-6); lane 4, DBY747/YEpDDI1-lacZDRm, containing the mutated DR sequence (Fig. 3-6); lane 5, DBY747/YEpDDI1-lacZ(UAS_{MAG1})Mut, containing the mutated within the UAS_{MAG1} region (Fig. 3-14C); lane 6, DBY747/YEpDDI1-lacZ(Δ30-69), containing the internal deletion from +30 to +69 (Fig. 3-14C).


Lane 1 2 3 4 5 6
Fragment II
Fragment I

3.11 Differential Regulation Of *MAG1* And *DDI1*

3.11.1 Differential expression of *DDI1* and *MAG1* in the absence of protein synthesis

In a previous study (Chen and Samson, 1991), the effect of cycloheximide (CH) was examined at the transcriptional level and was found to have two effects on *MAG1* transcription. First, it reduced the basal level of *MAG1* mRNA, and secondly it totally abolished MMS inducibility. In the present study, the effect of protein synthesis inhibition (by cycloheximide) on the transcription of two divergently transcribed, DNA damage inducible genes (*DDI1* and *MAG1*) was examined. The previous results were reproduced in the case of *MAG1* (Fig. 3-16, Lanes 1-4). However, under the same conditions, the basal level of *DDI1* transcript actually increased with cycloheximide treatment (Lanes 6 and 8). Nevertheless, the MMS induction of *DDI1* was completely abolished by prior treatment with cycloheximide since *DDI1* mRNA level in the cells subsequently treated with MMS remained the same as that with cycloheximide treatment alone (Lanes 7 and 8).

3.11.2 The expression and inducibility of *MAG1* and *DDI1* in several mutation backgrounds

DUN1 encodes a protein kinase that controls the DNA damage response of *RNR* (Ribonucleotide reductase) (Zhou and Elledge, 1993) and *SNM1* (Wolter et al., 1996) genes. To see if *MAG1* and *DDI1* belong to the Dun1 regulatory pathway, the expression and MMS inducibility (fold of induction) of *MAG1* and *DDI1* were studied in the background of *dun1* mutation by Northern analysis. Compared to *MAG1* induction in *DUN1* wild type strain (Y203), the MMS inducibility of *MAG1* was abolished in the *dun1* mutant (Y290). In contrast, *DDI1* clearly remained inducible in *dun1* (Fig. 3-17A). Consistent with the Northern result, β-gal assay (Fig. 3-18A) showed that the MMS inducibility of *MAG1-lacZ* in *dun1* mutant was abolished. In addition, the basal level expression of *MAG1-lacZ* was reduced by about 3 fold.

Figure 3-16. The effects of cycloheximide and MMS treatments on the expression of *MAG1* and *DDI1* genes. Total RNA was isolated from either non-treated (-) or cycloheximide (CH) and/or MMS treated (+) cells. Each lane contains about 30 μg RNA. Same RNA samples were loaded in Lanes 1&5, 2&6, 3&7, 4&8. Northern hybridization: *MAG1* (Lanes 1-4) and *DDI1* (Lane 5-8) probes. *ACT1*, actin probe.

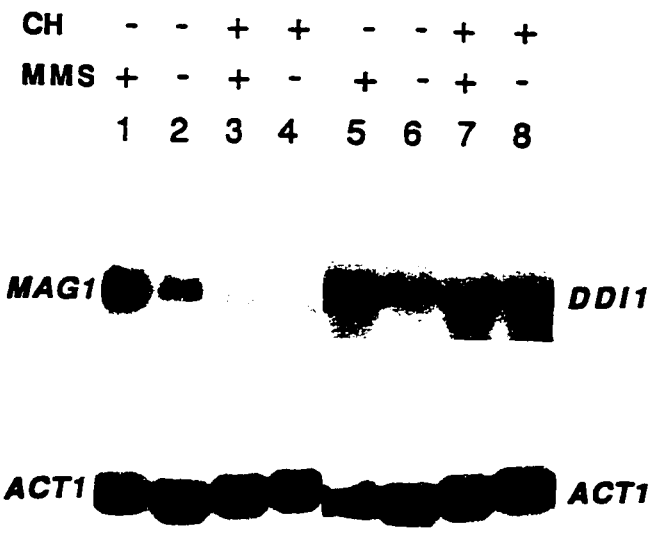
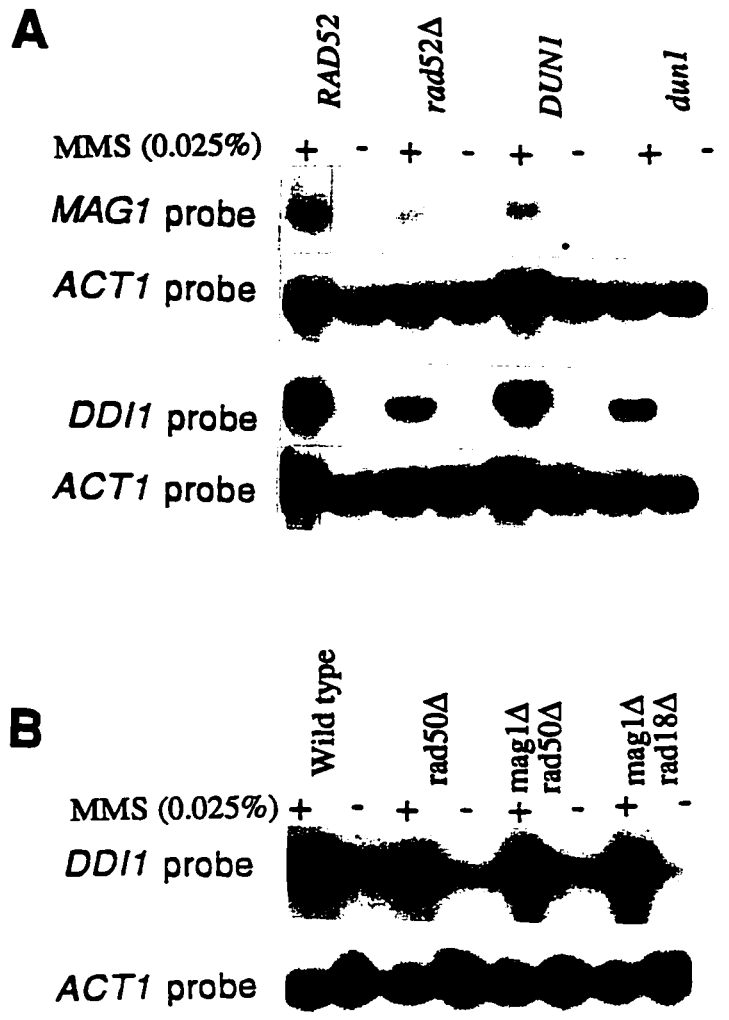

CH - - + + - - + +
MMS + - + - + - + -
1 2 3 4 5 6 7 8
MAG1
DDI1
ACT1
ACT1

Figure 3-17. The expression of *MAG1* and *DDI1* in several different mutants. (A). Effect of *dun1* and *rad52* on the transcription of *MAG1* and *DDI1*. Each lane contains about 20 μg of total RNA isolated from, DBY747 (*RAD52*), WXY9387 (*rad52 Δ*), Y203 (*DUN1*), and Y290 (*dun1*). +, MMS treated; -, non-treated. The same Northern blot was hybridized with *MAG1*, *DDI1* and *ACT1* probes subsequently. (B). Induction of *DDI1* in various isogenic mutant strains. Each lane contains about 15 μg of total RNA isolated from, DBY747 (wild type), WXY9221 (*rad50 Δ*), WXY9323 (*mag1Δ* and *rad50Δ* double mutants), and WXY9327 (*mag1Δ* and *rad18 Δ* double mutants). The blot was hybridized with *DDI1* and *ACT1* probes respectively.


A
RAD52
rad52Δ
DUN1
dun1
MMS (0.025%)
+ - + - + - + -
MAG1 probe
ACT1 probe
DDI1 probe
ACT1 probe
B
Wild type
rad50Δ
mag1Δ rad50Δ
mag1Δ rad18Δ
MMS (0.025%)
+ - + - + - + -
DDI1 probe
ACT1 probe

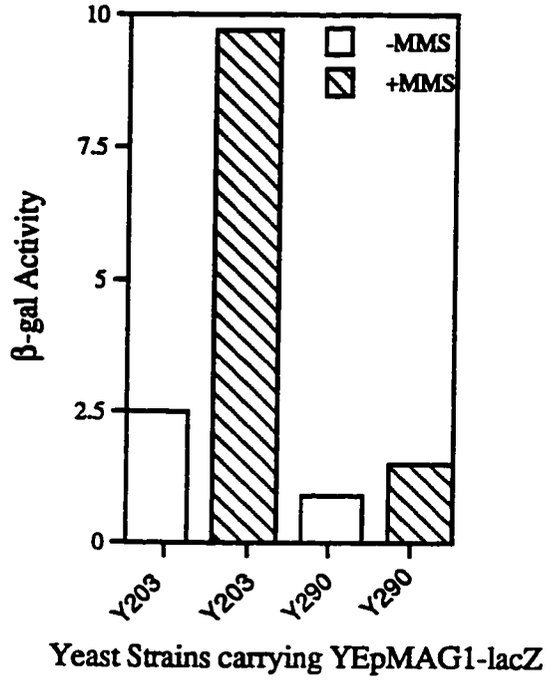
The *dun1* mutant was known to be sensitive to both UV and MMS treatments (Zhou and Elledge, 1993), and since Mag1 is the first enzyme in a multi-step pathway to repair 3MeA and protect cells from killing by MMS-induced replication block, it was asked if the reduction in the basal level of *MAG1* transcription observed in *dun1* could be correlated with the MMS sensitivity of the mutant. Strain Y290 (*dun1* mutant) was transformed with plasmid YCpMAG1ΔRm. YCpMAG1ΔRm contained the *MAG1* gene with a promoter deletion of URS_{MAG1} (ΔRm) and expressed at a constitutively high level (Xiao et al., 1993). Compared to strain Y203 with a wild type *DUN1* gene , strain Y290 was moderately sensitive to MMS (column 1 and 2, Fig. 3-18B). Over-expression of *MAG1* in Y290 (Y290/YCpMAG1ΔRm) did not enhance the MMS resistance (column 3, Fig. 3-18B), suggesting that the MMS sensitivity of *dun1* is not soly due to its effect on the expression of *MAG1*. The alterations in the expression of genes in other pathways involved in repairing MMS-induced lesions (Xiao et al., 1996) might be responsible for the MMS sensitivity of *dun1*.

Since some data have suggested a direct or indirect role of *RAD52* in the regulation of DNA damage inducible genes (Maga et al., 1986; Sheng and Schuster, 1993), the expression of *MAG1* and *DDI1* was studied in a *rad52* mutant background. Compared to its isogenic wild type (DBY747), *rad52* reduced the MMS inducibility of *MAG1* and *DDI1*. But the basal level of *DDI1* transcript remained unchanged (Fig. 3-17A). It is known that mutants in the *RAD52* epistasis group are very sensitive to treatment with DNA damaging agents especially those that produce double-stranded breaks. Therefore, the reduced expression of *MAG1* and *DDI1* in *rad52* mutant could be due to the compromised cellular capacity to repair MMS-induced DNA lesions. However, the inducibility of *DDI1* in the *rad50* mutant (which has a similar sensitivity to MMS as *rad52* mutant) appeared normal (Fig. 3-17B).

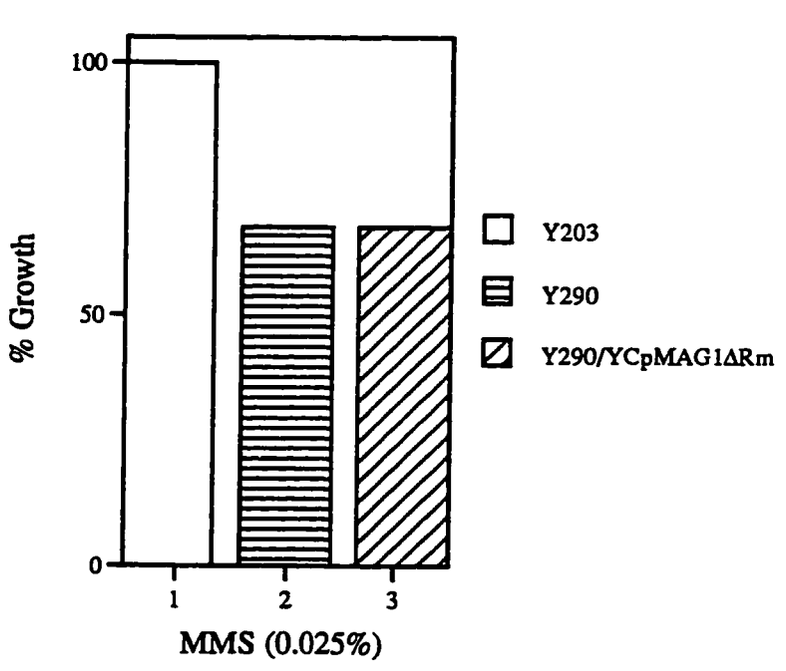
Treatment of cells with methylating agents such as MMS mainly produces N-methyl lesions, including the replication-blocking 3-MeA (Beranek, 1990). MMS is also

Fig. 3-18 MMS sensitivity of *dun1* is not due to its effect on the expression of *MAG1*. (A). Expression of *MAG1-lacZ* in *DUN1* wild type and *dun1* mutant. Yeast strains Y203 (*DUN1*) and Y290 (*dun1*) were transformed with plasmid YEpMAG1-lacZ. The β-gal activity was measured with (+MMS) and without (-MMS) MMS treatment, and was expressed in Miller's unit. Note, the β-gal activity of Y203 transformants is higher than that of the corresponding DBY747 transformants. This discrepancy is probably due to the difference in the genetic background of different strains. (B). Gradient plate assay. Yeast cells, Y203, Y290, and Y290 carrying plasmid YCpMAG1ΔRm were grown on MMS gradient plate which contained a linear gradient of MMS with concentrations ranging from 0 to 0.025%. The length of region of cell growth on the plate was measured after a 3-day incubation and the % growth was calculated as specified in Materials and Methods.

A


10
7.5
5
2.5
0
β-gal Activity
-MMS
+MMS
Y203
Y203
Y290
Y290
Yeast Strains carrying YEpMAG1-lacZ

B


100
50
0
% Growth
1
2
3
MMS (0.025%)
Y203
Y290
Y290/YCpMAG1ΔRm

regarded as an X-ray mimetic agent and it is thought that processing of the methyl lesions gives rise to DNA strand breaks (Schwartz, 1989). To see whether *DDI1* induction depends on specific types of DNA damage, *DDI1* mRNA from several mutant strains (including *rad50Δ* mutant, *mag1Δ* and *rad50Δ*, and *mag1Δ* and *rad18Δ* double mutants) was analyzed. The result (Fig. 3-17B) indicated that the induction of *DDI1* by MMS was unaffected in all the mutants tested, suggesting that the *DDI1* induction does not depend on a specific type of lesion or the processing of methyl lesions, consistent with observation that *DDI1* was inducible by multiple DNA damaging agents.

3.12 Isolation of Mutants Affecting The Expression of *MAG1/DDI1*

3.12.1 Phenotypes of yeast cells carrying different fusion constructs on Xgal plate

The phenotype of DBY747 carrying different fusion constructs was determined on the Xgal plate and was summarized in Table 3-2. Cells carrying YEpMAG1-lacZ on the Xgal plate appeared white while those carrying YEpDDI1-lacZ showed blue color. Deletion of DR from YEpDDI1-lacZ (YEpDDI1-lacZΔDR) resulted in colonies with white color on the Xgal plate, while cells carrying plasmid with deletion of both the DR and the URS_{DDI1} (YEpDDI1-lacZΔDR-Rd) formed blue colonies. These observations were in good agreement with the previous β-gal assay of the deletion constructs (Fig. 3-6). The phenotype on the Xgal plate could be utilized to isolate mutants that affect the expression of *MAG1/DDI1*.

3.12.2 Isolation of mutants affecting the activation of *MAG1/DDI1* at the UAS_{DM} site

Deletion analysis has identified the UAS_{DM} as the most prominent *cis*-acting regulatory element for the expression of both *MAG1* and *DDI1*. Deletion of UAS_{DM} from YEpDDI1-lacZ (YEpDDI1-lacZΔDR) resulted in a phenotypic change from blue to white on the Xgal plate (Table 3-2). It is expected that the mutations in the gene encoding a transcription activator that binds to the UAS_{DM} should also give the same phenotype (white) as the deletion construct does. Cells carrying plasmid YEpDDI1-lacZ

Table 3-2 Phenotype of DBY747 carrying different fusion constructs on Xgal plate

Fusion construct	Color on the Xgal plate
DBY747/YEpMAG1-lacZ	White
DBY747/YEpDDI1-lacZ	Blue
DBY747/YEpDDI1-lacZΔDR[a]	White
DBY747/YEpDDI1-lacZΔRd[b]	Dark Blue
DBY747/YEpDDI1-lacZΔDR-Rd[c]	Dark Blue

[a] DBY747/YEpDDI1-lacZΔDR contains the internal deletion from -149 to -113 (Fig. 3-6).

[b] DBY747/YEpDDI1-lacZΔRd contains the 5' deletion to -191 (Fig. 3-5).

[c] DBY747/YEpDDI1-lacZΔDR-Rd contains ΔDR plus the 5' deletion to -191 (Fig. 3-6).

were mutagenized with EMS and selected on the Xgal plate for white colonies from among the blue ones. It was not difficult to isolate many white colonies in a single experiment. However, further analysis of these putative mutants showed that majority of them were plasmid-borne mutations since the mutant phenotype disappeared upon introduction of the original plasmid into these cells. One mutant, named "W6" continued to show the mutant phenotype even after the reintroduction of the original plasmid. However, W6 reduced the expression of *CYC1-lacZ* (plasmid pLG669Z) as well as *DDI1-lacZ*, suggesting that W6 may have a general effect on gene expression, either at transcriptional or at translational levels.

3.12.3 Isolation of mutants resulting in constitutive high expression of *DDI1-lacZ*

Constitutive high-level expression can be achieved if the negative regulatory pathway is turned "off" or the positive regulatory pathway is permanently turned "on". The deletion of URS_{MAG1} (Xiao et al., 1993) or removal of the region containing putative URS_{DDI1} (Fig. 3-5) resulted in a constitutive high level of expression of *MAG1-lacZ* or *DDI1-lacZ*. It is expected that mutations in the gene that encodes the repressor should have the same effect on gene expression as the deletion of the URS does. Cells carrying YEpDDI1-lacZΔDR (white color on the Xgal plate) were mutagenized and blue colonies were selected as putative repressor mutants. This experiment was not successful, no blue colonies were isolated. It is possible that the mutation frequency of the gene of interest may be very low and either a large number of colonies needs to be screened or a powerful selection method is required for mutant detection.

CHAPTER IV: DISCUSSION

4.1 What Is The Possible Function(s) Of *DDI1* ?

Unlike most other yeast genes whose cloning are performed after their biochemical or genetic studies became available, *DDI1* was identified by sequencing the upstream region of the *MAG1* gene. Presently, the function of *DDI1* remains unknown and no detectable phenotype is associated with its disruption. Nevertheless, studies on its regulation and the genome organization between *DDI1* and *MAG1* have provided some suggestive information about the function of *DDI1*. First of all, *DDI1* is an expressed gene as evidenced by the presence of *DDI1*-specific transcripts and the expression of *DDI1-lacZ* fusion gene. Secondly, *DDI1* is clustered with *MAG1* and co-induced by a variety of DNA damaging agents. Gene clustering and divergently transcribed genes are a common feature in yeast genome organization (Beck and Warren, 1988). In a few cases, such as *HIS3-PET56* (Struhl, 1986), the clustered genes are related by neither function nor regulation, but in many other instances the two genes either function in the same metabolic pathway or the functions of their gene products are related (Johnston and Davis, 1984; Yao et al., 1994). Therefore, it is reasonable to suggest that *DDI1* might be involved in stress conditions such as DNA damage, which points to the direction of future research on *DDI1* function. Putative *DDI1* homologs have been found in *S. pombe* (Z69728, $P=1.9\times10^{-57}$) and *Caenorhabditis elegans* (U50068, $P=4.2\times10^{-15}$). Besides, *DDI1* also shares sequence homology with a cDNA from humans. All these suggest that the *DDI1* sequence is well conserved among eukaryotes. *C. elegans* is a genetically well defined multi-celluar organism and has served as a model organism for studies of development. Therefore, it is now possible that the function of *DDI1* and its homolog can be studied, not only at the cellular level, but also in a whole organism.

4.2 Structural Organizations Between *MAG1* And *DDI1*

The genomes of a variety of organisms contain genes that are divergently transcribed, ranging from simple organisms like bacteria (Guha et al., 1971) to humans (Shinya and Shimada, 1994). This form of gene organization is also common in yeast. The best known example is the yeast *GAL1-GAL10* gene cluster. *GAL1* and *GAl10* transcripts are separated by 606 bp and are co-induced in the presence of galactose (Johnston and Davis, 1984; Yocum et al., 1984). The co-ordinated expression are regulated by several UAS elements (Johnston, 1987) and possibly other *cis*-acting sequences (West et al., 1990). Other examples include the mating type loci such as *MATa1 /MATa2* (Astell et al., 1981) and *MATα1 /MATα2* genes (Siliciano and Tatchell, 1984), and histone genes *H2A/H2B* (Osley et al., 1986). By dissecting the regulatory region between two divergently transcribed DNA damage inducible genes, *MAG1* and *DDI1*, some interesting features about genome organization were revealed.

4.2.1 A bi-directional regulatory element

The intergenic region between the translation starts of the two ORFs is only 282 bp, much shorter than the average of 731 bp derived from divergently transcribed genes in *S. cerevisiae* chromosome 8 (Johnston et al., 1994). The 5' termini of the major transcripts of *MAG1* and *DDI1* are separated by 155 bp. This curious structural arrangement, like that found in the divergent promoter of *DHFR* (dihydrofolate reductase) and *Rep-3* (a *mutS* homolog) genes from mammalian cells (Smith et al., 1990; Wells et al., 1996), suggests that the two genes might share common regulatory elements.

Clustering of genes with divergent promoters is common in yeast (Beck and Warren, 1988). In a number of cases, the clustered genes are so tightly linked that a bi-directional promoter co-ordinates their expression (Geever et al., 1989; Punt et al., 1995; West et al., 1984). In the promoter of *MAG1/DDI1*, a UAS element (UAS_{DM}) was found to drive the expression of *MAG1* and *DDI1* in a bi-directional manner. To the best of my knowledge, this is the first example of two DNA damage-inducible genes that are co-

ordinately expressed by physically sharing a regulatory element. Obviously, it is a simple means to co-ordinate the expression of two divergently transcribed genes by sharing a common regulatory element. Furthermore, yeast genes are compact in their relatively small genome and about 80% of the genome is transcribed (Olson, 1992). This structural arrangement is economical on genetic information, which might be important for simple eukaryotes with small genomes such as *S. cerevisiae*.

4.2.2 One gene's regulatory element is buried in the coding region of another gene

The divergent promoters have been classified into three possible forms (Beck and Warren, 1988): a), back-to-back promoters with intervening sequence in between; b), overlapping promoters where two genes share a single bi-directional promoter; and c), face-to-face promoters where one gene's promoter is buried within the coding region of another gene. To the best of my knowledge, the last form of divergent promoters have not been reported in eukaryotes. Therefore, the localization of UAS_{MAG1} in the protein coding region of *DDI1* provides the first example. It is not known at the present time the functional significance of this unique organization of regulatory element. It is tempting to propose that, being located within a protein coding region, the activator-UAS_{MAG1} complex will be difficult to maintain when *DDI1* is being actively transcribed in response to DNA damage or under the inhibition of protein synthesis. Therefore, *MAG1* transcription will be diminished unless a stimulus (e.g. DNA damage) persists. This ensures that the expression of *MAG1* is up-regulated only in the presence of DNA damage and returns automatically to the uninduced level after the induction signal is removed. Secondly, with functions both in protein coding for Ddi1 and in gene regulation of *MAG1*, the UAS_{MAG1} will less likely to sustain mutations that could disrupt both of these functions.

4.3 Potential Uses For Bi-directional Promoters

By definition, bi-directional promoters are those that are capable of co-regulating the expression of two divergently transcribed genes, which may present potential usefulness. Most genetic traits are controlled by multiple genes and it has been technically cumbersome to introduce and co-express more than one gene in a given host. Bi-directional promoters offer an attractive approach. A pair of genes can be cloned separately on either side of such a control region and could be introduced as well as co-expressed as a single unit. For example, to express a functional protein consisting of two different subunits, two genes encoding each subunit can be cloned on either side of a bi-directional promoter, thereby the two genes are co-transfered and co-expressed as a single unit. Furthermore, a bi-directional promoter may find itself very useful in the genetic modification of species (such as plant) for multiple resistance to pesticides or to viruses. In this case, two resistant genes can be brought together into a cell and co-expressed through a bi-directional promoter to produce a novel cell with dual resistance. In cases where genes of interest are not selectable, bi-directional promoters may also find their use. A selectable gene (such as a drug resistant gene) can be cloned on one side of the regulatory region while the non-selectable gene of interest can be cloned on the other side. Because the selectable gene is closely linked to the gene of interest, disruption of the gene linkage will be less likely to occur.

4.4 Further Localization Of UAS_{MAG1}

Through deletion analysis and electrophoretic mobility shift assay, the promoter region containing a UAS_{MAG1} was further defined to a 24 bp region between nucleotide -339 and -316 relative to the translation start of *MAG1* ORF, or +34 and +57 relative to the translation start of *DDI1* ORF. Several lines of evidence suggest that the sequence "CGGCCC" and possibly flanking AT-rich sequence are important for the UAS_{MAG1} function. First, deletion encompassing the 24 bp sequence centered at the "CGGCCC"

reduced *MAG1* expression. Secondly, the 24 bp sequence was found to support the formation of DNA-protein complex in a sequence-specific manner. The cleavage, modification (by methylation) and mutation within the sequence reduced the formation of DNA-protein complex and reduces the UAS function. Importantly, the correlation between the formation of UAS-protein complex *in vitro* and the function of the UAS *in vivo* has been established, which suggests that the UAS_{MAG1}-binding protein(s) is probably the transcription activator that regulates *MAG1* expression. Based on the observation that the UAS_{MAG1}-binding activity is detectable in both the MMS-treated and un-treated cell extracts, it is proposed that post-translational modification rather than *de novo* synthesis of activator(s) is likely involved in DNA damage induction of *MAG1*.

4.5 Identification of A Bi-directional Regulatory Element

Cells respond to DNA damage by enhancing the expression of a large number of genes that participate either directly or indirectly in DNA repair/synthesis. One of the most often asked questions is how all the inducible genes are co-regulated. Analyses of the SOS response, adaptive response, OxyRS, and SoxRS pathways (Walker, 1985; Lindahl et al., 1988; Demple, 1991) provide detailed mechanism of how *E. coli* cells adapt to various genotoxic environments. However, little is known about the molecular mechanisms of co-ordinated expression of eukaryotic genes in response to DNA damage. In particular, it has not been shown that the proposed "common" promoter sequences mediate co-regulation *in vivo* (Friedberg et al., 1995). Through the analysis of two head-to-head arranged DNA damage inducible genes from *S. cerevisiae*, one of the mechanisms that a cell could utilize for co-regulating two genes was revealed, i.e., two damage-inducible genes physically share a bi-directional UAS element. In the intergenic region between *MAG1* and *DDI1*, an 8 bp sequence "GGTGGCGA" is repeated. Several lines of evidence presented in this study strongly suggest that the DR sequence functions as a UAS. First, deletions or point mutations of the direct repeat reduced the expression

of both *MAG1-lacZ* and *DDI1-lacZ*, indicating that both *MAG1* and *DDI1* are positively regulated by the DR sequence. Secondly, deletion of the repeat from the *MAG1* promoter resulted in a decreased basal level and non-inducible *MAG1* expression, which correlated with an increased MMS sensitivity. Finally, the direct repeat alone in place of UAS_{CYC1} is capable of activating the expression and confers partial DNA damage responsiveness in a heterologous *CYC1* promoter. The observation that half of the direct repeat was still functional (albeit not as efficient as the entire repeat) indicates that the regulatory effect of the direct repeat is additive and that the sequence "GGTGGCGA" itself is a bi-directional UAS element. For the convenience of description, the "GGTGGCGA" sequence was referred to as UAS_{DM} in lieu of its bi-directional feature.

In a previous study (Xiao *et al.*, 1993), the disruption of half of the repeated sequence by 5' deletion of *MAG1* promoter resulted in an increase in the basal level expression of *MAG1-lacZ*. Removing the entire repeat, however, reduced the β-gal activity. In another study (Singh and Samson, 1995), the insertion of $URS2_{MAG}$ (containing half of DR sequence) between *CYC1* UAS and *CYC1* TATA box reduced the expression of *CYC1-lacZ*. First of all, it is noticed that the deletion between -186 to -171 (Xiao *et al.*, 1993) only removed two bp from the 8 bp repeat sequence. Therefore, it is probably the deletion of sequences other than the direct repeat that resulted in the observed increase (2-fold). Secondly, the $URS2_{MAG}$ probe and our DR probe formed different mobility shift patterns with yeast cell extract; the band I in our assay was the major form of complex and had an apparent mobility different from the complex I (Singh and Samson, 1995), suggesting that different proteins may be involved in the binding to the $URS2_{MAG}$ and to the DR sequence. Finally, the only functional test of $URS2_{MAG}$ was the insertion of the oligonucleotide between *CYC1* UAS and *CYC1* TATA box (Singh and Samson, 1995), which is different from the construct used in this study.

4.6 Sequences Similar To The UAS_{DM} Are Found In The Promoters of Other Yeast Repair and DNA Metabolism Genes

A number of reported "consensus" sequences (Sebastian et al, 1990; Jones and Prakash, 1991; Xiao et al, 1993; Sancar et al, 1995) was found to contain either an identical "GGTGGCGA" sequence (e.g. *RAD23*, Madura and Prakash, 1990) or share significant sequence homology to UAS_{DM} (UAS_{DM}-like element). A 7 bp sequence, "GG(T/A)GGCA" was derived based on UAS_{DM} with each sequence differing no more than one base from the consensus sequence. Among genes listed in Table 4-1, UAS_{DM} and UAS_{DM}-like elements have been found in both DNA damage-inducible and non-inducible genes. Thus UAS_{DM} does not appear to be intrinsically damage-responsive, a conclusion consistent with the fact that damage induction of *MAG1/DDI1* requires the presence of URS elements.

Promoter regions containing the derived consensus sequence have been defined as UAS (e.g. *RAD23*, Jones and Prakash, 1991; *RAD2*, Siede et al., 1989; *MAG1/DDI1*, this work) or proposed as UAS (DREII of *RAD51*, Shinohara et al., 1992). Two of the consensus sequences, however, have been indicated as URS (Elledge and Davis, 1989b; Xiao and Samson, 1992). That the same protein binding site acts as either UAS or URS in different promoters is not unprecedented; several transcriptional regulatory proteins, such as Rap1 (Brand et al., 1987; Shore and Nasmyth, 1987), Mcm1 (Keleher et al., 1988; Passmore et al., 1989) and Abf1 (Buchman and Kornberg, 1990), function as either an activator or a repressor, depending on the sequence context.

4.7. DR-binding proteins and their role in regulation of *MAG1/DDI1* expression

The finding that purified yRPA binds to the DR probe is not surprising since the DR sequence contains the consensus ($URS2_{MAG}$-like element) previously shown to bind to yRPA (Singh and Samson, 1995). However, it is surprising that yRPA also binds to

Table 4-1. Nucleotide sequences found in the promoters of some yeast DNA repair and metabolism genes that are similar to UAS_{DM}

Gene	Location[a]	Function	Sequence[b]	References[c]
MAG1/DDI1[d]	-165/-120	UAS	G G T G G C g	this work
MAG1/DDI1[d]	-141/-144	UAS	G G T G G C g	this work
RAD23[d]	-295	UAS	G G T G G C g	
RAD2[d]	-166	UAS	G G A G G C A	
RAD51[d]	-154	DREII*	G G T G G g A	Shinohara et al., 1992
MGT1[e]	-207	URS	G G A G G C c	
RNR2[d]	-371	URS	G G T c G C A	Elledge and Davis, 1989
RNR3[d]	-458	?	G G T a G C A	
RAD1[e]	-448	?	G G A G G g A	
RAD1[e]	-198	?	G G T G G a A	
RAD4[e]	-487	?	c G A G G C A	
RAD7[d]	-121	?	G G A a G C A	
RAD51[d]	-451'	?	G G T G G C A	
Consensus			G G T/A G G C A	
Occurrence			12 13 13 10 13 10 9	
			13 13 13 13 13 13 13	

[a] Location relative to the first A in the initiating ATG. ' indicates transcribed strand.

[b] Nucleotide sequences different from the consensus are in lower case.

[c] Sequences without references are cited from Sancar et al., 1995.

[d] Genes are damage-inducible.

[e] Genes are not damage-inducible.

* DREII is a homologous sequence upstream of the *PHR1* gene (positions -103 to -94 in the sequence of Sebastian et al., 1990).

DNA sequences (such as MAG1-23/-24 as well as its mutated sequence MAG1-25/-26) which bear no apparent homology to the consensus sequence. Besides, yRPA-DR complex can be efficiently competed by single-stranded DNA, indicating that the affinity of RPA to single-stranded DNA is stronger than to the DR sequence which is in contrast to the yRPA-URS2$_{MAG}$ complex (Singh and Samson, 1995). Nevertheless, the lack of competition by pUC18 or another nonspecific double-stranded oligonucleotide (MSC1/MSC2) indicates that the binding of yRPA to DNA is not completely random.

Two protein-DR complexes were identified in EMSA using crude cell extract, one of which (Band II) co-migrated with purified yRPA. It is noticed that extracts from yeast cells with or without MMS treatment gave the same mobility shift pattern, indicating that the DR-binding protein is synthesized in both non-induced and induced conditions.

The ability of the mutated DR to form protein-DNA complexes *in vitro* but without transcriptional activation *in vivo* raises two questions. First, are the DR-binding proteins (including RPA) identified by EMSA transcriptional regulators? The lack of correlation between the *in vitro* binding to the DR and the *in vivo* DR function seems to suggest that the identified proteins that bind to the DR may not be the transcriptional regulators, which probably escaped detection by the EMSA. However, in the absence of specific inhibitors or mutants, the *in vitro* binding studies are only suggestive. Therefore, other possibilities need to be explored. Although the binding of transcription regulators to a *cis*-acting element is the first event occurring during transcriptional activation, subsequent events such as DNA bending (Becker *et al.*, 1995) and protein-protein contacts between transcription regulators and components of the general transcriptional machinery (Goodrich *et al.*, 1996) are also required. It is possible that DR-binding proteins could recognize and bind to the mutated DR sequence, but transcriptional activation would not occur due to alterations in steps following the initial binding.

Secondly, the binding of proteins from cell extract and purified yRPA to the mutated DR (which has a different sequence from the wild type DR) questions the

binding specificity. Although DR-binding proteins can form complexes with the mutated DR probe, the binding is not a random protein-DNA interaction, as evidenced by the lack of competition with nonspecific pUC18 DNA or non-homologous sequence with a length similar to DR. The sequence-independent binding of the proteins characterized in this study is not an isolated case. For example, the yeast DNA-binding protein RAPI or SBF-E binds to sequences with very little or no sequence homology (Shore and Nasmyth, 1987) and the yeast HAP1 activator binds to two upstream activation sites of different sequences (Pfeifer *et al.*, 1987). Similarly, a human mitochondrial transcription factor binds to two promoters in a sequence-independent manner (Fisher and Clayton, 1988) and the Hela cell protein TEF-1 binds specifically and coorperatively to two SV40 enhancer motifs of unrelated sequence (Davidson *et al.*, 1988). Finally, the PolI trans-acting factor UBF1 (Bell et al., 1989) and E1BF (Zhang and Jacob, 1990) bind several DNA regions in a sequence-independent manner.

4.8 The Constitutive Expression of *MAG1* And *DDI1* Is Controlled Through An Antagonistic Mechanism By The UAS_{DM} And URS

5' deletions of *DDI1-lacZ* indicated the presence of a putative URS located between nucleotide -280 and -235 relative to the translation start of *DDI1* ORF. Deletion of this sequence, plus the region from -910 to -280 (that resulted in a 2.5-fold increase in the basal level β-gal activity) increased the basal level expression of *DDI1-lacZ* by 10-fold. It is not clear from the present data whether URS_{DDI1} consists of two separate elements located between -910 to -280 and between -280 to -235, or whose function is dependent on the surrounding sequences. To test the possibilities and to further define the URS_{DDI1}, a fine-tuned dissection of the region from -910 to -235 is required. Nevertheless, like *MAG1*, *DDI1* expression is also negatively regulated by a URS (URS_{DDI1}).

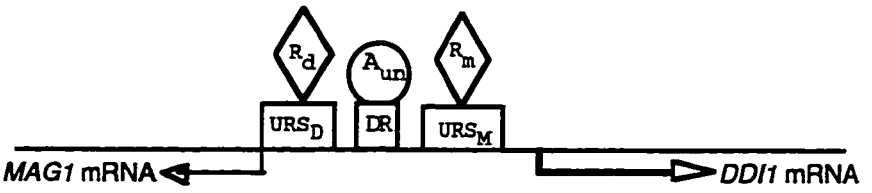
The presence of a bi-directional UAS (UAS_{DM}) indicates that the two genes, *MAG1* and *DDI1*, are positively regulated. The experimental results seem to suggest that the constitutive expression of *MAG1* and *DDI1* is controlled by two functionally opposite regulatory elements, UAS and URS, probably through an antagonistic mechanism. Thus deletion of the URSs results in a constitutive high-level expression regardless of the presence of UAS_{DM}, whereas removal of UAS_{DM} leads to a URS-dependent constitutive low-level expression. The observation that the UAS_{DM} alone can activate *CYC1*-TATA minimal promoter and confer partial damage induction suggests that the UAS_{DM} acts directly on the basal transcriptional machinery. Transcriptional repression can be achieved by a variety of distinct mechanisms (Herschbach and Johnson, 1993). It seems unlikely that the repression of *MAG1* and *DDI1* expression involves a simple steric hindrance because (i) the URSs of both genes are located upstream of UAS_{DM}, and (ii) the repression function is independent of UAS_{DM}. Like most of the eukaryotic repressors (Johnson, 1995), the formation of repressor-URS of *MAG1/DDI1* may affect the general transcription machinery directly. The antagonistic mechanism might be achieved through the activator-UAS_{DM} and the repressor-URS that act on the basal transcriptional machinery; the activator-UAS_{DM} promotes transcription while the repressor-URS inhibits transcription. A model for the regulation of constitutive expression is summarized in Fig. 4-1A. According to gel retardation assays with UAS_{DM} in this study and with the URS_{MAG1} in the previous study (Xiao et al., 1993), it is suggested that under uninduced conditions transcription regulatory proteins bind to their cognate *cis*-acting sites and both *MAG1* and *DDI1* are under repression.

4.9 Mechanisms of DNA Damage Induction of *MAG1/DDI1* Gene Cluster

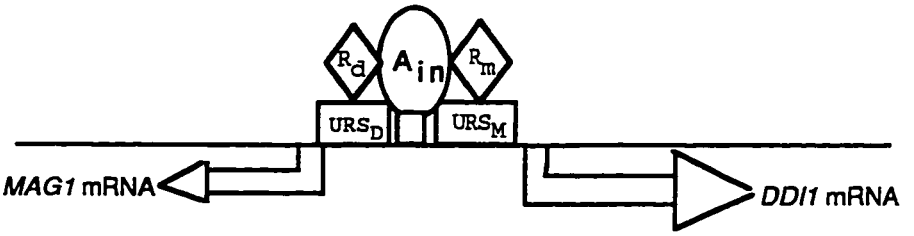
It appears that DNA damage-induced expression of *DDI1* is regulated through two distinct mechanisms. The induction by a derepression mechanism is suggested by

Figure 4-1. A proposed model describing the common and distinct regulation of *DDI1* and *MAG1* expression. URS_D, tentative URS_{DDI1}; DR, direct repeat (also known as UAS_{DM}) ; URS_M, URS_{MAG1}; R_d, proposed repressor binding to the URS_{DDI1}; R_m, proposed repressor binding to URS_{MAG1} (Xiao et al., 1993); A_{un}, an activator in an uninduced form binding to the DR, and A_i, the induced form of DR-binding protein in response to DNA damage, which might interact with repressors and derepress *DDI1* and *MAG1* gene expression. Sizes of arrows depict relative level of *DDI1* and *MAG1* expression.

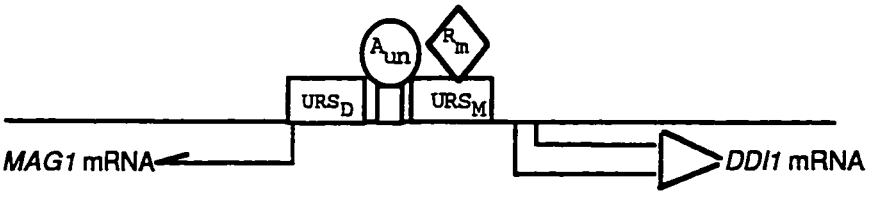
A. Basal level



B. Coinduced by DNA damage



C. Under the inhibition of protein synthesis



the observation that increase in the basal level expression through URS_{DDI1} deletion was accompanied by a decrease in the fold of induction from 7 to 2, accounting for >70% of the induced expression. The derepression mechanism was also found in DNA damage induction of *RNR2* (Hurd and Roberts, 1989). The second mechanism of induction, which is responsible for the remaining 30% of induced expression, probably involves the activation at the DR site. This mechanism of regulation is suggested by the observation that DR alone conferred a 2-fold induction in a heterologous *CYC1-lacZ* promoter, which is also observed in the native promoter containing UAS_{DM} alone (i.e., when URS_{DDI1} is removed). In addition to UAS_{DM}, DNA damage induction of *MAG1* also requires UAS_{MAG1}, deletion or point mutations of the UASs either abolished (UAS_{DM}) or significantly decreased (UAS_{MAG1}) DNA damage induction. A model for DNA damage induction is shown in Fig. 4-1B. It is proposed that, in the case of DNA damage, the activator-UAS_{DM} is modified into an induced form (A_{in} in Fig. 4-1B) which relieves the repression (derepression mechanism) and enhances the transcription of *MAG1* and *DDI1* (activation mechanism).

4.10 DNA Damage-responsive Element of *MAG1/DDI1*

Although all the DNA damage inducible genes respond to a common signal, that is, DNA damage, the damage responsive elements identified in the promoter regions of damage-inducible genes are different. Full induction of *RAD2* is mediated by several UAS elements (Siede and Friedberg, 1992) while a negative regulatory element (URS_{PHR1}) is responsible for *PHR1* damage induction (Sancar et al., 1995). Up to now, two UAS elements , UAS_{MAG1} (Xiao et al., 1993; this study) and UAS_{DM} (this study), and two URS elements, URS1 (Xiao et al., 1993) and URS2 (Singh and Samson, 1995) have been identified in the promoter region of *MAG1*. One URS and a UAS_{DM} have been identified in the promoter of *DDI1*. Previous studies showed that the two URS elements of *MAG1* could repress the expression of but could not confer damage induction

to a heterologous promoter (Xiao et al., 1993; Singh and Samson, 1995). Indeed, *MAG1* induction requires both UAS_{MAG1} and UAS_{DM}. It appears that the function of UAS_{MAG1} depends on the presence of UAS_{DM} as evidenced by the lack of induction in the absence of UAS_{DM}. It is not known if *DDI1* induction also requires another UAS element besides UAS_{DM}. In any events, UAS_{DM} will be the prominent element for regulating the expression of *MAG1* and *DDI1* as well. Although UAS_{DM} is absolutely required for *MAG1/DDI1* induction, it appears that UAS_{DM} alone can not confer a full level induction as it is demonstrated for the UAS elements of *RAD2* (Siede and Friedberg, 1992). Actually, the 10-fold induction (demonstrated by Northern analysis) of *DDI1* expression is only observed in the presence of URS_{DDI1}. It is possible that the UAS-URS interaction would also present another potential target for regulation of DNA damage response of *MAG1* and *DDI1*, a phenomenon observed for regulation of damage response of *PHR1* gene (Sancar et al., 1995).

4.11 Differential Regulatory Mechanisms of *MAG1* And *DDI1*

Although *MAG1* and *DDI1* are expressed co-ordinately in the presence of various damaging agents, their expression is distinguished in the presence of cycloheximide, an agent considered to inhibit protein synthesis. Cycloheximide treatment decreases the basal level of *MAG1* transcript while increases the steady-state level of *DDI1* transcript. The latter phenomenon is similar to that seen for *RAD2* (Siede et al., 1989). Thus, there are probably two induction pathways for the activation of *DDI1*. The first pathway for *DDI1* activation comes from the induction by DNA damaging agents, most likely through the UAS_{DM}-activator interaction, which is shared by the *MAG1* gene. The second pathway of *DDI1* induction results from the inhibition of protein synthesis, probably through de-repression at the URS_{DDI1} site since the deletion of URS_{DDI1} resulted in a phenotype similar to that of cycloheximide treatment. It is proposed that the function of the repressor-URS_{DDI1}, not the repressor-URS_{MAG1}, probably requires a continuous

protein synthesis. The absence of *de novo* protein synthesis, therefore, could result in a constitutively high-level expression of *DDI1*. The differential expression of *MAG1* and *DDI1* under the inhibition of protein synthesis is shown in Fig. 4-1C.

Among DNA damage-inducible genes studied so far, *RNR* (Zhou et al., 1993), *SNM1*(Wolter et al., 1996) genes have been assigned to the Dun1 regulatory pathway while damage induction of *RAD3* epistasis group genes (Sancar, 1995), *RAD51* (unpublished result of Torre-Ruiz and Fabre, source from Aboussekhra et al., 1996), *RAD54* (Wolter et al., 1996), *DDR48* and *UBI4* (Zhou et al., 1993) are independent of Dun1. Although damage induction of *MAG1* in *dun1* mutant requires further detailed analysis (such as varying MMS concentration as well as incubation time), the preliminary study on *MAG1* induction indicates that *MAG1* is probably another member of the Dun1 regulatory pathway. Interestingly, the divergently transcribed *DDI1* appears to have another induction pathway(s) distinct from Dun2-Rad53(Sad1)-Dun1 pathway (Navas et al., 1995). It is suggested that the co-ordinate regulation of *DDI1-MAG1* probably relies on their physical sharing of a common UAS element (i.e. UAS_{DM}) while the unique UAS_{MAG1} as well as the distinct URS elements of *MAG1/DDI1* may determine their differential regulation in the absence of *de novo* protein synthesis or in the genetic background of *dun1*. It is important to note that among genes whose induction depends on Dun1, *RNR* and *MAG1* are inducible by several DNA damaging agents including MMS, while *SNM1* is soley inducible by cross-linking agents and UV but not by MMS. Besides, *RNR* and *MAG1* differ during protein synthesis inhibition where the MMS induction of *MAG1* is abolished, while 4-NQO induced expression of *RNR2* is not affected (Elledge and Davis, 1989a), suggesting that the detailed mechanism of DNA damage induction may differ among genes in the Dun1 pathway.

4.12 The Level of *MAG1* Expression And Cellular Resistance to MMS

MAG1 encodes a 3MeA DNA glycosylase, the first enzyme in a multi-step base excision repair (BER) pathway for the removal of lethal lesions such as 3MeA (Friedberg et al, 1995). In the present study, the level of *MAG1* transcription and the cellular MMS sensitivity were studied. It was found that while severely compromised *MAG1* transcription (by deletion of DR) resulted in a decreased level of MMS resistance, increase in *MAG1* transcription above the wild type level did not provide further protection. While the result indicates that the transcription status of *MAG1* is an important determinant of cellular resistance to MMS, the wild type level of *MAG1* transcription does not seem to be a limiting factor in the base excision repair pathway. Cellular repair capacity to 3MeA may be limited by subsequent enzymes, such as Apn1 AP endonuclease, which is neither induced by DNA damage nor regulated during the cell cycle (Popoff et al., 1990; Ramotar et al., 1991), deoxyribophosphodiesterase or DNA polymerase and DNA ligase (Friedberg et al., 1995). Alternatively, the entire cellular capacity to repair MMS-induced lesions may be limited by other repair pathways such as homologous recombination (Petes et al., 1991) or Ku-dependent DNA end-joining (Feldmann and Winnacker, 1993; Milne et al., 1996; Feldmann et al., 1996). Actually, it has been demonstrated that *S. cerevisiae* has multiple pathways for the repair of MMS-induced lesions (Xiao et al., 1996). In addition to the removal of 3MeA, Mag1 also has a 7-methylguanine glycosylase activity (Berdal et al., 1990). Therefore, high levels of Mag1 glycosylase may result in the removal of this otherwise benign lesion and increase the number of mutagenic abasic sites. Indeed, in the absence of AP endonuclease, *MAG1* over-expression resulted in a 12-fold increase in spontaneous mutation rate compared with wild type cells (Xiao and Samson, 1993).

4.13 Selection of Mutants Affecting The Expression of *MAG1/DDI1*

Gene expression is regulated by *cis*-acting elements and sequence-specific transcription regulatory proteins. In response to intra- or extra-cellular signals, transcription regulators are activated through a cascade of cellular events. This sequential relay from signals to the target genes is termed signal transduction pathways. It is expected that the expression of a target gene can be affected through alterations in any of the components in its signal transduction pathway.

Identification of components that constitute the signal transduction pathway of *MAG1/DDI1* expression was based on the color selection of mutants with changes in the expression of *MAG1-lacZ* or *DDI1-lacZ*. The advantage of this method is that the putative mutants can be visually selected. However, due to the lack of mutant-enriching means, large numbers of cells have to be screened. Yeast cells carrying a *MAG1-lacZ* or *DDI1-lacZ* fusion construct were mutagenized and colonies with a desired phenotype (i.e. color) were selected. Since the mutagenesis treatment causes mutations not only in chromosomes, but also in the plasmids, the plasmid-borne mutations must be distinguished. This was done by repeatedly sub-culturing putative mutant cells in non-selective medium to eliminate the plasmids and by re-transforming with the original plasmid (that is, the plasmid that has not been subject to the mutagenesis treatment). If the mutant phenotype is maintained after the reintroduction of the original plasmid, then the possibility that the mutant phenotype is associated with plasmid can be eliminated. Plasmid pLG669Z carrying *CYC1-lacZ* fusion was used to test if a mutant has a specific effect on *MAG1* and *DDI1* or it has a general effect on other genes as well.

Plasmid-borne mutation appears to be the major problem in isolating white colonies from the original blue ones, probably due to the fact that mutations in many places of the plasmid could reduce expression of the *lacZ* gene (e.g. mutations in the promoter region and/or in the coding region, etc.). This problem could be solved by introducing plasmids (without mutagenesis treatment) into mutagenized cells. In

contrast, plasmid-borne mutations that result in blue colonies from the original white ones are expected to be low in frequency. Cells carrying YEpDDI1-lacZΔDR could produce blue colonies only when mutations occurred within the URS_{DDI1} or in the gene that encodes the repressor. Probably due to the same reason, selection of the blue colonies was unsuccessful. To increase the chance of success in the future experiment, a positive selection method is advised; the *lacZ* coding region in the plasmid YEpDDI1-lacZΔDR could be replaced by the coding region of *HIS3*. If the gene encoding the repressor is mutated, the derepression of *HIS3* will occur. The mutant cell will then express high level of histidine and can be selected on medium containing 3-amino-1',2',4' triazole (3AT) which eliminates cells that express no or low level of histidine. Since large number of cells can be plated on the 3AT selection plate, the chance to recover the mutants of interest will be greatly improved.

With respect to the identification of genes encoding transcriptional regulators, two recent methods are worth mentioning. A genetic selection based on a UAS2-dependent *ADH2* reporter was devised to isolate genes capable of activating UAS2-dependent transcription (Donoviel and Young, 1996). The second method was based on the sequence-specific binding between a transcription factor and its cognate *cis*-acting sequence, a gene that encodes Msn2p was isolated by direct screening of the yeast genomic library in λgt11 with the stress response promoter element (Schmitt and McEntee, 1996).

REFERENCES

Aboussekhra, A., Vialard, J., Morrison, D. E., Torre-Ruiz, M. A. de la., Cernakova, L., Fabre, F., and Lowndes, N. F. 1996. A novel role for the budding yeast *RAD9* checkpoint gene in DNA damage-dependent transcription. EMBO J. 15: 3912-3922.

Allen, J.B., Zhou, Z, Siede,W, Friedberg, E.C, and Elledge, S.J. 1994. The SAD1/RAD53 protein kinase controls multiple checkpoints and DNA damage-induced transcription in yeast. Genes & Dev. 8: 2401-2415.

Ames, B. N., Mccabh, J., and Yamasaki, E. 1975. Methods for detecting carcinogens and mutagens with the Salmonella/mammalian microsome mutagenicity test. Mutat. Res. 31: 347-363.

Anderson, C. W. 1993. DNA damage and the DNA-activated protein kinase. Trends Biochem. Sci. 18: 433-437.

Astell, C. R., Ahlstrom-Jonasson, L., Smith, M., Nasmyth, K. A., and Hall, B. D. 1981. The sequence of the DNAs coding for the matting type loci of *Saccharomyces cerevisiae*. Cell 27: 15-23.

Beck, C.F., and Warren, R.A.J. 1988. Divergent promoters, a common form of gene organization. Microbiolo. Rev. 52: 318-326.

Becker, J. C., Nikroo, A., Brabletz, T., and Reisfeld, R. 1995. DNA loops induced by cooperative binding of transcriptional activator proteins and transcription complexes. Proc. Natl. Acad. Sci. USA 92: 9727-9731.

Bell, S. P., Pikaard, C. S., Reeder, R. H., and Tjian, R. 1989. Molecular mechanisms governing species-specific transcription of ribosomal RNA. Cell 59: 489-497.

Beranek, D. T. 1990. Distribution of methyl and ethyl adducts following alkylation with monofunctional alkylating agents. Mutation Res. 231: 11-30.

Berdal, K.G., Bjøras, M., Bjelland, S., and Seeberg, E. 1990. Cloning and expression in *Escherichia coli* of a gene for an alkylbase DNA glycosylase from *Saccharomyces cerevisiae*; a homologue to the bacterial *alkA* gene. EMBO J. 9: 4563-4568.

Bjøras. M., Klungland, A., Johansen, R. F., and Seeberg, E. 1995. Purification and properties of the alkylation repair DNA glycosylase encoded by the *MAG* gene from *Saccharomyces cerevisiae*. Biochemistry 34: 4577-4583.

Boiteux, S., Gajewski, E., Laval, J., and Dizdaroglu, M. 1992. Substrate specificity of the *Escherichia coli* Fpg protein (Formamidopyrimidine-DNA glycosylase): Excision of purine lesions in DNA produced by ionizing radiation or photosensitization. Biochemistry 31: 106-110.

Brand, A.H., Micklem, G., and Nasmyth, K. 1987. A yeast silencer contains sequences that can promote autonomous plasmid replication and transcriptional activation. Cell 51: 709-719.

Brill. S.J., and Stillman, B. 1991. Replication factor-A from *Saccharomyces cerevisiae* is encoded by three essential genes coordinately expressed at S phase. Genes & Dev. 5: 1589-1600.

Buchman, A.R., and Kornberg, R.D. 1990. A yeast ARS-binding protein activates transcription synergistically in combination with other weak activating factors. Mol. Cell. Biol. 10: 887-897.

Carlson, M., and Botstein, D. 1982. Two differentially regulated mRNAs with different 5' ends encode secreted and intracellular forms of yeast invertase. Cell 28:145-154.

Chakravarti, D., Ibeanu, G. C., Tano, K., and Mitra, S. 1991. Cloning and expression in *Escherichia coli* of a human cDNA encoding the DNMA repair protein N-methylpurine-DNA glycosylase. J. Biol. Chem. 266: 15710-15715.

Chen, J., Derfler, B., Maskati, A., and Samson, L. 1989. Cloning a eukaryotic DNA glycosylase repair gene by the suppression of a DNA repair defect in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 86: 7961-7965.

Chen, J., Derfler, B., and Samson, L. 1990. *Saccharomyces cerevisiae* 3-methyladenine DNA glycosylase has homology to the AlkA glycosylase of *E. coli* and is induced in response to DNA alkylation damage. EMBO J. 9: 4569-4575.

Chen, D. S., Herman, T., and Demple, B. 1991. Two distinct human DNA diesterases that hydrolyze 3'-blocking deoxyribose fragments from oxidized DNA. Nucleic Acids Res. 19: 5907-5914.

Chen, J., and Samson, L. 1991. Induction of *Saccharomyces cerevisiae MAG* 3-methyl adenine glycosylase transcript levels in response to DNA damage. Nucleic Acids Res. 19: 6427-6432.

Chernova, O. B., Chernov, M. V., Agarwal, M. L., Taylor, W. R., and Stark, G. R. 1995. The role of p53 in regulating genomic stability when DNA and RNA synthesis are inhibited. TiBS 20: 431-434.

Chien, C.T., Bartel, P.L., Sternglanz, R., and Fields, S. 1991. The two-hybrid system : A method to identify and clone genes for proteins that interact with a protein of interest. Proc. Natl. Acad. Sci. USA 88: 9578-9582

Chung, C. T., Niemela, S. L., and Miller, R. H. 1989. One-step preparation of competent *Escherichia coli*: Transformation and storage of bacterial cells in the same solution. Proc. Natl. Acad. USA 86: 2172-2175.

Cleaver, J. 1968. Defective repair replication of DNA in Xeroderma pigmentosum. Nature 218: 652-656

Cleaver J. E. 1994. It was a very good year for DNA repair. Cell 76: 1-4.

Cole, G.M., and Mortimer, R.K. 1989. Failure to induce a DNA repair gene, *RAD54*, in *Saccharomyces cerevisiae* does not affect DNA repair or recombination phenotypes. Mol. Cell. Biol. 9: 3314-3322.

Cormack, B. P., and Struhl, K. 1992. The TATA-binding protein is required for transcription by all three nuclear RNA polymerases in yeast cells. Cell 69: 685-696.

Cunningham, R. P., Saporito, S. M., Spitzer, S. G., and Weiss, B. 1986. Endonuclease IV *(nfo)* mutant of *Escherichia coli.* J. Bacteriol. 168:1120-1127.

Davidson, I., Xiao, J. H., Rosales, R., Staub, A., and Chambon, P. 1988. The Hela cell protein TEF-1 binds specifically and coorperatively to two SV40 enhancer motifs of unrelated sequence. Cell 54: 931-942.

Davison , B. L., Egly, J-M., Mulvihill, E. R., and Chambon, P. 1983. Formation of stable preinitiation complexes between eukaryotic class B transcription factors and promoter sequences. Nature 301: 680-686.

Demple, B. 1991. Regulation of bacterial oxidative stress genes. Annu. Rev. Genet. 25: 315-337.

Demple, B., and Harrison, L 1994. Repair of oxidative damage to DNA: Enzymology and Biology. Annu. Rev. Biochem. 63: 915-948.

Demple, B., Herman, T., and Chen, D. S. 1991. Cloning and expression of APE, the cDNA encoding the major human apurinic endonuclease: Definition of a family of DNA repair enzymes. Proc. Natl. Acad. Sci. USA 88: 11450-11454.

Demple, B., Johnson, A., and Fung, D. 1986. Exonuclease III and endonuclease IV remove 3' blocks from DNA synthesis promers in H_2O_2 damaged *Escherichia coli.* Proc. Natl. Acad. Sci. USA 83:7731-7735.

Devary, Y., Gottlieb, R.A., Lau, L.F., and Karin, M. 1991. Rapid and preferential activation of *c-jun* gene during the mammalian UV response. Mol. Cell. Biol. 11: 2804-2811.

Devary, Y., Gottlieb, R.A., Smeal,T., and Karin, M. 1992. The mammalian ultraviolet response is triggered by activation of Src tyrosine kinases. Cell 71: 1081-1091.

Dizdaroglu, M. 1992. Measurement of radiation-induced damage to DNA at the molecular level. Int. J. Radiat. Biol. 61: 175-183.

Donahue, B. A., Yin, S., Taylor, J. S., Reines, D., and Hanawalt, P. C. 1994. Transcript cleavage by RNA polymerase II arrested by a cyclobutane pyrimidine dimer in the DNA template. Proc. Natl. Acad. Sci. USA 91: 8502-8506.

Donoviel, M. S. and Young, E. T. 1996. Isolation and identification of genes activating UAS2-dependent ADH2 expression in *Saccharomyces cerevisiae*. Genetics 143: 1137-1148.

Dulic, V., Kaufmann, W. K., Wilson, S. J., Tlsty, T. D., Lees, E., Harper, J. W., Elledge, S. J., and Reed, S. I. 1994. p53-depedent inhibition of cyclin-depedent kinase activities in human fibroblasts during radiation-induced G1 arrest. Cell 76:1013-1023.

Dujon, B. 1996. The yeast genome project: what did we learn? Trends Genet. 12: 263-270.

Elledge, S. J. 1996. Cell cycle checkpoints: Preventing an identity crisis. Science 274: 1664-1672.

Elledge, S. J., and Davis, R.W. 1989a. DNA damage induction of ribonucleotide reductase. Mol. Cell. Biol. 9: 4932-4940.

Elledge, S.J., and Davis, R.W. 1989b. Identification of the DNA-damage responsive elements of *RNR2* and evidence that four distinct cellular factors bind. Mol. Cell. Biol. 9:5373-5386.

Elledge, S. J, Mulligan, J. T., Ramer, S. W., Spottswood, M., Davis, R.W. 1991. λYES: A multifunctional cDNA expression vector for the isolation of genes by complementation of yeast and *Escherichia coli* mutations. Proc. Natl. Acad. Sci. USA 88:1731-1735.

Elledge, S. J., and Walker, G. C. 1983. Proteins required for ultraviolet light and chemical mutagenesis. Idnetification of the products of the umuC locus of *Escherichia coli*. J. Mol. Biol. 164: 175-192.

Engelberg, D., Klein,C., Martinetto, H., Struhl, K., and Karin, M. 1994. The UV response involving the Ras signaling pathway and AP-1 transcription factors is conserved between yeast and mammals. Cell 77: 381-390.

Engelward, B. P., Boosalis, M. S., Chen, B. J., Deng, Z., Siciliano, M. J., and Samson, L. 1993. Cloning and characterization of a mouse 3-methyladenine/7-methylguanine/3-methylguanine DNA glycosylase cDNA whose gene maps to chromosome 11. Carcinogenesis 14: 175-181.

Engelward, B. P., Dreslin, A., Christensen, J., Huszar, D., Kurahara, C., and Samson, L. 1996. Repair-deficient 3-methyladenine DNA glycosylase homozygous mutant mouse cells have increased sensitivity to alkylation-induced chromosome damage and cell killing. EMBO J. 15: 945-952.

Enoch, T., and Norbury. C. 1995. Cellular responses to DNA damage: cell cycle checkpoints, apoptosis, and the role of p53 and ATM. TiBS 20: 426-431.

Fargnoli, J., Holbrook, N. J., and Fornace, A. J. J. 1990. Low-ratio hybridization subtraction. Anal. Biochem. 187: 364-373.

Farr, S. B., and Kogoma, T. 1991. Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium*. Micro. Rev. 55: 561-585.

Feldmann, H., Driller, L., Meier, B., Mages, G., Kellermann, J., and Winnacker, E. L. 1996. HDF2, the second subunit of the Ku homologue from *Saccharomyces cerevesiae*. J. Biol. Chem. 271: 27765-27769.

Feldmann, H., and Winnacker, E. L. 1993. A putative homologue of the human autoantigen Ku from *Saccharomyces cerevisiae* . J. Biol. Chem. 268:12895-12900.

Fields, S., and Song, O. K. 1989. A novel genetic system to detect protein-protein interactions. Nature 340: 245-246.

Fire, A., Samuels, M., and Sharp, P. A. 1984. Interaction betwen RNA polymerase II, factors, and template leading to accurate transcription. J. Biol. Chem. 259:2509-2516.

Firmenich, A. A., Ellas-Arnaz, M., and Berg, P. 1995. A novel allele of *Saccharomyces cerevisiae RFA1* that is deficient in recombination and repair and suppressible by *RAD52*. Mol. Cell. Biol. 15: 1620-1631.

Fishel, R., and Kolodner, R. D. 1995. Identification of mismatch repair genes and their role in the development of cancer. Curr. Opin. Genet. Dev. 5: 382-395.

Fisher, P.P., and Clayton, D. A. 1988. Purification and characterization of human mitochondrial transcription factor 1. Mol. Cell. Biol. 8: 3496-3509.

Fitzgerald, M., and Shenk,T. 1981. The sequence 5'-AAUAAA-3' forms part of the recognition site for polyadenylation of late SV40 mRNAs. Cell 24: 251-260.

Fogel, S., Mortimer, R. K., and Lusnak, K. 1981. Mechanisms of meiotic gene conversion, or "Wanderings on a foreign strand". In *the molecular biology of the yeast Saccharomyces: Life cycle and inheritance.* Strathern, J. N. et al., ed. p289. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Fornace Jr., A. J. 1992. Mammalian genes induced by radiation: Activation of genes associated with growth control. Annu. Rev. Genet. 26: 507-526.

Friedberg, E. C. 1994. DNA repair: Looking back and peering forward. BioEssays 16: 645-649.

Friedberg, E. C., Siede,W., and Cooper, A.J. 1991. Cellular responses to DNA damage in yeast. In *The molecular and cellular biology of the yeast Saccharomyces*. Edited by Broach, J. R., Pringle, J. R., and Jones, E. W. Cold Spring Harbor Laboratory Press. 1991. Cold Spring Harbor, New York.

Friedberg, E. C., Walker, G.C., and Siede,W. 1995. DNA Repair and Mutagenesis. ASM Press. Washington D.C.

Geever, R. F., Huiet, L., Baum, J.A., Tyler, B.M., Patel, U.B., Rutledge, B.J., Case, M.E., and Giles, N.H. 1989. DNA sequence, organization and regulation of the *qa* gene cluster in *Neurospora crassa*. J. Mol. Biol. 207: 15-34.

George, L., Miklos, G., and Rubin, G. M. 1996. The role of the genome project in determining gene function: Insights from model organisms. Cell 86: 521-529.

Gietz, R.D., and Sugino, A. 1988. New yeast-*Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. Gene 74: 527-534.

Gill, G., Pascal, E., Tseng, Z. H., and Tijian, R. 1994. A glutamine-rich hydrophobic patch in transcription factor Sp1 contacts the dTAF$_{II}$110 component of the *Drosophila* TFIID complex and mediates transcriptional activation. Proc. Natl. Acad. Sci. USA 91: 192-196.

Goodrich, J. A., Cutler, G., and Tijian, R. 1996. Contacts in context: Promoter specificity and macromolecular interactions in transcription. Cell 84: 825-830.

Guarente, L. 1983. Yeast promoter and *lacZ* fusions designed to study expression of cloned genes in yeast. Methods in Enzymol. 101:181-191.

Guarente, L. 1984. Yeast promoters: Positive and negative elemetns. Cell 36: 799-800.

Guarente, L. 1988. UASs and Enhancers: Common mechanisms of transcriptional activation in yeast and mammals. Cell 52: 303-305.

Guarente, L., and Hoar, E. 1984. Upstream activation sites of the *CYC1* gene of *Saccharomyces cerevisiae* are active when inverted but not when placed downstream of the "TATA box". Proc. Natl. Acad. Sci. USA 81: 7860-7864

Guarente, L., Lalonde, B., Gifford, P., and Alani, E. 1984. Distinctly regulated tandem upstream activation sites mediate catabolite repression of the *CYC1* gene of *S. cerevisiae*. Cell 36: 503-511.

Guarente, L., and Ptashne, M. 1981. Fusion of *Escherichia coli lacZ* to the cytochrome c gene of *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA 78: 2199-2203.

Guarente, L., Yocum, R. R., and Giffold, P. 1982. A *GAL10-CYC1* hybrid yeast promoter identifies the GAL4 regulatory region as an upstream site. Proc. Natl. Acad. Sci. USA 79: 7410.

Guha, A., Saturen,Y., and Szybalski, W. 1971. Divergent orientation of transcription from the biotin locus of *Escherichia coli*. J. Mol. Biol. 56:53-62.

Hahn, S., Hoar, E., Guarente, L. 1985. Each of three "TATA elements" specifies a subset of the transcription initiation sites at the *CYC1* promoter of *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA 82:8562

Hanawalt, P., and Mellon, I. 1993. Stranded in an active gene. Curr. Biol 3: 67-69.

Hartwell, L. H., and Weinert, T. A. 1989. Checkpoints: controls that ensure the order of cell cycle events. Science 246: 629-634.

Hatahet, Z., Kow, Y. W., Parmal, A. A., Cunningham, R. P., and Wallace, S. S. 1994. New substrates for old enzymes. 5-hydroxy-2'-deoxycytidine and 5-hydroxy-2'-deoxyuridine are substrates for *Escherichia coli* endonuclease III and formamidopyrimidine DNA N-glycosylase, while 5-hydroxy-2'-deoxyuridine is a substrate for uracil DNA N-glycosylase. J. Biol. Chem. 269: 18814-18820.

Henning, K. A. Li, L., Iyer, N., McDaniel, L. D., Reagan, M. S. et al., 1995. The Cockayne syndrome group A gene encodes a WD repeat protein that interacts with CSB protein and a subunit of RNA polymerase II TFIIH. Cell 82: 555-564.

Herschbach, B. M., and Johnson, A.D. 1993. Transcriptional repression in eukaryotes. Annu. Rev. Cell. Biol. 9: 479-509.

Heyer, W.-D., Rao, M.R.S., Erdile, L.F., Kelly, T.J., and Kolodner, R.D. 1990. An essential *Saccharomyces cerevisiae* single-stranded DNA binding protein is homologous to the large subunit of human RP-A. EMBO J. 9: 2321-2329.

Hoeijmakers, J. H. J. 1993. Nucleotide excision repair. I. From *E. coli* to yeast. II. From yeast to mammals. Trends Genet. 9: 173-181, 211-217.

Hoffman, C. S., and Winston, F. 1987. A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. Gene 57: 267-272.

Hollingsworth, N. M., Ponte, L., and Halsey, C. 1995. MSH5, a novel MutS homolog, facilitates meiotic reciprocal recombination between homologs in *Sccharomyces cerevisiae* but not mismatch repair. Genes. Dev. 9: 1728-1739.

Huang, J. C., Hsu, D. S., Kazantsev, A., and Sancar, A. 1994. Substrate spectrum of human excinuclease: repair of abasic sites, methylated bases, mismatches, and bulky adducts. Proc. Natl. Acad. Sci. USA 91: 12213-12217.

Hurd, H.K., and Roberts, J.W. 1989. Upstream regulatory sequences of the yeast *RNR2* gene include a repression sequence and an activation site that binds the RPA1 protein. Mol. Cell. Biol. 9: 5359-5372.

Ito, H., Fukuda, Y., Murata, K., and Kimura, A. 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. 153:163-168.

Iyer, V. and Struhl, K. 1995. Poly(dA:dT), a ubiquitous promoter element that stimulates transcription via its intrinsic DNA structure. EMBO J. 14: 2570-2579.

Jayaraman, L., and Prives, C. 1995. Activation of p53 sequence-specific DNA binding by short single strands of DNA requires the p53 C-terminus. Cell 81:1021-1029.

Johnson, A. D. 1995. The price of repression. Cell 81: 655-658.

Jones, J.S., and Prakash, L. 1991. Transcript levels of the *Saccharomyces cerevisiae* DNA repair gene *RAD18* increase in UV irradiated cells and during meiosis but not during the mitotic cell cycle. Nucleic Acids Res. 19: 893-898.

Johnson, A. W., Demple, B. 1988. Yeast DNA diesterase for 3'-fragments of deoxyribose: Purification and physical properties of a repair enzyme for oxidative DNA damage. J. Biol. Chem. 263: 18009-18016.

Johnson, R. E., Kovvali, G. K., Prakash, L., and Prakash, S. 1996. Requirement of the yeast *MSH3* and *MSH6* genes for *MSH2*-dependent genomic stability. J. Biol. Chem. 271: 7285-7288.

Johnston, M. 1987. A model fungal gene regulatory mechanism: the *GAL* genes of *Saccharomyces cerevisiae*. Microbiol. Rev. 51:458-476.

Johnston, M., Andrews, S., Brinkman, R., Cooper, J. et al. 1994. Complete nucleotide sequence of *Saccharomyces cerevisiae* chromosome VIII. Science 265: 2077-2082.

Johnston, M, and Davis, R. W. 1984. Sequences that regulate the divergent *GAL1-GAL10* promoter in *Saccharomyces cerevisiae*. Mol. Cell. Biol. 4: 1440-1447.

Karran, P., and Bignami, M. 1994. DNA damage tolerance, mismatch repair and genome instability. BioEssays 16: 833-839.

Kastan, M.B., Zhan, Q., El-Deiry, W.S., Carrier, F., Jacks, T., Walsh, W.V., Plunkett, B.S., Vogelstein, B., and Fornace, A.J.Jr. 1992. A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in Ataxia-telangiectasia. Cell 71: 587-597.

Keleher, C.A., Goutte, C., and Johnson, A.D. 1988. The yeast cell-type-specific repressor a2 acts cooperatively with a non-cell-type-specific protein. Cell 53: 927-936.

Kitagawa, Y., Akaboshi, E., Shinagawa, H., Horii, T., Ogawa, H., and Kato, T. 1985. Structural analysis of the umu operon required for inducible mutagenesis in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 82: 4336-4340.

Kolodner, R. D. 1995. Mismatch repair: Mechanisms and relationship to cancer susceptibility. Trends Biochem. 20: 397-402.

Kolodner, R. 1996. Biochemistry and genetics of eukaryotic mismatch repair. Genes and Dev. 10: 1433-1442.

Kullik, I., Stevens, J., Toledano, M. B., and Storz, G. 1995 a. Mutational analysis of the Redox-sensitive transcriptional regulator OxyR: Regions important for DNA binding and multimerization. J. Bacteriol. 177: 1285-1291.

Kullik, I., Toledano, M. B., Tartaglia, L. A., and Storz, G. 1995 b. Mutational analysis of the Redox-sensitive transcriptional regulator OxyR: Regions important for oxidation and transcriptional activation. J. Bacteriol. 177: 1275-1284.

Lane, D. P. 1992. p53, guardian of the genome. Nature 358: 15-16.

Larimer, F. W., Perry, J. R., and Hardigree, A. A. 1989. J. Bacteriol. 171: 230.

Larson, K., Sahm, J., Shenkar, R., and Strauss, B. 1985. Methylation-induced blocks to in vitro DNA replication. Mutation Res. 150: 77-84.

Lee, S., Elenbaas, B., Levine, A., and Griffith, J. 1995. p53 and its 14 kDa C-terminal domain recognize primary DNA damage in the form of insertion/deletion mismatches. Cell 81: 1013-1020.

Levin, J. D., Johnson, A. W., and Demple, B. 1991. Metalloenzymes in DNA repair. J. Biol. Chem. 266: 22893-22898.

Lewin, B. 1994. In *Gene V.* Oxford University Press, Walton Street, Oxford.

Li, J. J., and Herskowitz, I. 1993. Isolation of *ORC6*, a component of the yeast origin recognition complex by a One-Hybrid System. Science 262: 1870-1874.

Li, W., and Sherman, F. 1991. Two types of TATA elements for the *CYC1* gene of the yeast *Saccharomyces recevisiae.* Mol. Cell. Biol. 11: 666-676.

Lin, J., Chen, J., Elenbaas, B., and Levine, A. J. 1994. Several hydrophobic amino acids in the p53 amino-terminal domain are required for transcriptional activation, binding to mdm-2 and the adenovirus 5 E1B 55-kD protein. Genes and Dev. 8: 1235-1246.

Lindahl, T. 1976. New class of enzymes acting on damaged DNA . Nature 259: 64-66.

Lindahl, T. 1993. Instability and decay of the primary structure of DNA. Nature 362: 709-715.

Lindahl, T., Sedgwick, B., Sekiguchi, M., and Nakabeppu, Y. 1988. Regulation and expression of the adaptive response to alkylating agents. Annu. Rev. Biochem. 57: 133-157.

Loeb, L. A., and Preston,B. D. 1986. Mutagenesis by apurinic/apyrimidinic sites. Ann. Rev. Genet. 20:201-230.

Lu, X., and Lane, D.P. 1993. Differential induction of transcriptionally active p53 following UV or ionizing radiation: defects in chromosome instability syndromes? Cell 75: 765-778.

Madura, K., and Prakash, S. 1990. Transcript levels of the *Saccharomyces cerevisiae* DNA repair gene *RAD23* increase in response to UV light and in meiosis but remain constant in the mitotic cell cycle. Nucleic Acids Res. 18: 4737-4742.

Maga, J. A., McClanahan, T. A., and McEntee, K. 1986. Transcriptional regulation of DNA damage responsive (*DDR*) genes in different rad mutant strains of *Saccharomyces cerevisiae*. Mol. Gen. Genet. 205: 276-284.

Maniatis, T., Fritsch, E. F., and Sambrook, J. 1982. *Molecular cloning: A laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Marnett, L. J., and Burcham, P. C. 1993. Endogenous DNA adducts: potential and paradox. Chem. Res. Toxicol. 6: 771-785.

Marsischky, G. T., Filosi, N., Kane, M. F., and Kolodner, R. 1996. Redundancy of *Saccharomyces cerevisiae* MSH3 and MSH6 in MSH2-depencent mismatch repair. Genes and Dev. 10: 407-420.

Marx J. 1994. DNA repair comes into its own. Science 266: 728-730.

Matsunaga, T., Mu, D., Park, C. H., Reardon, J. T., and Sancar, A. 1995. Human DNA repair excision nuclease: analysis of the roles of the subunits involved in dual incisions and the order of incision by using anti-XPG and anti-ERCC1 antibodies. J. Biol. Chem. 270: 20862-20869.

McClanahan, T. and McEntee, K. 1984. Specific transcripts are elevated in *Saccharomyces cerevisiae* in response to DNA damage. Mol. Cell. Biol. 4: 2356-2363.

McClanahan, T. and McEntee, K. 1986. DNA damage and heat shock dually regulate genes in *Saccharomyces cerevisiae* . Mol. Cell. Biol. 6: 90-96.

Mellon, I., and Champe, G. N. 1996. Products of DNA mismatch repair genes mutS and mutL are required for transcription-coupled nucleotide-excision repair of the lactose operon in Escherichia coli. Proc. Natl. Acad. Sci. USA 93: 1292-1297

Meyn, M. S. 1995. Ataxia-Telangiectasia and cellular response to DNA damage. Cancer Res. 55: 5991-6001.

Miller, J. S., Paterson, B. M., Ricciardi, R. P., Cohen, L. and Roberts, B. E. 1983. Methods utilizing cell-free protein-synthesizing system for the identification of recombinant DNA molecules. Methods in Enzymol. 101: 650-674.

Milne, G. D., Ho, T., and Weaver, D. T. 1995. Modulation of *Saccharomyces cerevisiae* DNA double-stranded break repair by SRS2 and RAD51. Genetics 139: 1189-1199.

Milne, G. D., Jin, S., Shannon, K. B., and Weaver, D. T. 1996. Mutations in two Ku homologs defines a DNA end-joining repair pathway in *Saccharomyces cerevisiae* . Mol. Cell. Biol. 16: 4189-4198.

Miralles, V. J., and Serrano, R. 1996. A genomic locus in *Saccharomyces cerevisiae* with four genes up-regulated by osmotic stress. Mol. Microbiol. 17: 653-662.

Miret, J. J., Milla, M. G., and Lahue, R. S. 1993. J. Biol. Chem. 268: 3507-3513.

Miret, J. J., Parker, B. O., and Lahue, R. S. 1996. Recognition of DNA insertion/deletion mismatches by an activity in *Saccharomyces cerevisiae* Nucleic Acids Res. 24: 721-729.

Miyashita, T., Harigai, M., Hanada, M., and Reed, J.C. 1994. Tumor supressor p53 is a regulator of bcl-2 and bax gene expression *in vitro* and *in vivo*. Oncogene. 9: 1799-1805.

Miyashita, T., and Reed, J. C. 1995. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell 80: 293-299.

Modrich, P. 1987. DNA mismatch correction. Annu. Rev. Biochem 56: 435-466

Modrich, P. 1991. Mechanisms and biological effects of mismatch repair. Annu. Rev. Genet. 25; 229-253.

Modrich, P. 1994. Mismatch repair, genetic stability, and cancer. Science 266: 1959-1960

Modrich, P., and Lahue, R. 1996. Mismatch repair in replication fidelity, genetic recombination and cancer biology. Annu. Rev. Biochem. 65: 101-133.

Moradas, P., Costa, V., Piper, P., and Mager, W. 1996. The molecular defences against reactive oxygen species in yeast. Mol. Microbiol. 19: 651-658.

Mu, D., Park, C. H. ,Matsunaga, T., Hsu, D. S., Reardon, J. T., and Sancar, A. 1995. Reconstitution of human DNA repair excision nuclease in a highly defined system. J. Biol. Chem. 270: 2415-2418.

Mummenbrauer, T., Janus, F., Muller, B., Wiesmuller, L., Deppert, W., and Grosse, F. 1996. p53 protein exhibits 3'-to-5' exonuclease activity. Cell 85: 1089-1099.

Myers, A.M., Tzagoloff, A., Kinney, D.M., and Lusty, C.J. 1986. Yeast shuttle and integrative vectors with multiple cloning sites suitable for construction of *lacZ* fusions. Gene 45: 299-310.

Naegeli, H. 1994. Roadblocks and Detours during DNA replication: Mechanisms of mutagenesis in mammalian cells. BioEssays 16: 557-564.

Navas, T.A., Zhou, Z., and Elledge, S.J. 1995. DNA polymerase ε links the DNA replication machinery to S phase checkpoint. Cell 80: 29-39.

Nelson, J. R., Lawrence, C. W., and Hinkle, D. C. 1996. Thymine-thymine dimer bypass by yeast DNA polymerase ξ. Science 272: 1646-1649.

New, L., Liu, K., and Crouse, G. F. 1993. The yeast gene *MSH3* defines a new class of eukaryotic MutS homologues. Mol. Gen. Genet. 239: 97-108.

O'Connor, T.R. and Laval, F. 1990. Isolation and structure of a cDNA expressing a mammalian 3-methyladenine-DNA glycosylase. EMBO J. 9: 3337-3342.

O'Connor, T.R. and Laval, F. 1991. Human cDNA expressing a functional DNA glycosylase excising 3-methyladenine and 7-methylguanine. Biochem. Biophys. Res. Commun. 176: 1170-1177.

Olson, M.V. 1992. Genome structure and organization in *Saccharomyces cerevisiae*. In *The molecular and cellular biology of the yeast Saccharomyces* ". Edited by Jones, E. W., Broach, J. R., and Pringle, J. R. 1992. Cold Spring Harbor Laboratory Press.

Oltvai, Z., Lilliman, C. L., and Korsmeyer, S. J. 1993. Bcl-2 hetrodimerizes in vivo with a conserved homolog, Bax, that accelerates programed cell death. Cell 74: 609-619.

Osley, M. A., Gould, J., Kim, S., Kane, M., and Herford, L. 1986. Identification of sequences in a yeast histone promoter involved in periodic transcription. Cell 45: 537-544.

Palejwala, V. A., Pandya, G. A., Bhanot, O. S., Solomon, J. J., Murphy, H. S., Danman, P. M., and Humayun, M. Z. 1994. UVM, an ultraviolet-inducible RecA-independent mutagenic phenomenon in *Escherichia coli*. J. Biol. Chem. 269: 27433-27440.

Parker, B.O. and Marinus, M. G. 1992. Repair of DNA heteroduplexes containing small heterologous sequences in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 89: 1730-1734.

Passmore, S., Elble, R., and Tye, B.K. 1989. A protein involved in minichromosome maintenance in yeast binds a transcriptional enhancer conserved in eukaryotes. Genes & Dev. 3: 921-935.

Paulovich, A.G., and Hartwell, L. H. 1995. A ckeckpoint regulates the rate of progression through S phase in *S. cerevisiae* in response to DNA damage. Cell 82: 841-847.

Pegg, A. E. 1990. Mammalian O^6-alkylguanine-DNA-alkyltransferase: regulation and importance in response to alkylating carcinogenic and therapeutic agents. Cancer Res. 50: 6119-6129.

Perry, K. L., Elledge, S. J., Mitchell, B. B., Marsh, L., and Walker, G. C. 1985. *umuDC* and *mucAB* operons whose products are required for UV light- and chemical-induced

mutagenesis: UmuD, MucA, and LexA proteins share homology. Proc. Natl. Acad. Sci. USA 82:4331-4335.

Petes, T., Malone, R., and Symington, L. 1991. Recombination in yeast, p. 407-521. In *The molecular and cellular biology of the yeast Saccharomyces*. Broach, J., and Jones, E. (ed.). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y.

Pfeifer, K., Prezant, T., and Guarente, L. 1987. Yeast HAP1 activator binds to two upstream activation sites of different sequences. Cell 49: 19-27.

Polyak, K., Waldman, T., He, T-C., Kinzler, K. W., and Vogelstein, B. 1996. Genetic determinants of p53-induced apoptosis and growth arrest. Genes and Dev. 10: 1945-1952.

Popoff, S. C., Spira, A. I., Johnson, A. W., and Demple, B. 1990. Yeast structural gene (*APN1*) for the major apurinic endonuclease: Homolog to *Escherichia coli*. endonuclease IV. Proc. Natl. Acad. Sci. USA 87: 4193-4197.

Prakash, S, Sung P, and Prakash L. 1993. DNA repair genes and proteins of *Saccharomyces cerevisiae* . Annu. Rev. Genet. 27: 33-70.

Proffitt, J. H., Davie, J. R., Swinton, D., and Hattman, S. 1984. 5-Methylcytosine is not detectable in *Saccharomyces cerevisiae* . Mol. Cell. Biol. 4: 985-988.

Punt, P. J., Struss, J., Smit, R., Kinghorn, J.R., Van Den Hondel, C.M.J.J., and Scazzocchio, C. 1995. The intergenic region between the divergently transcribed *niiA* and *niaD* genes of *Aspergillus nidulans* contains multiple NirA binding sites which act bidirectionally. Mol. Cell. Biol. 15: 5688-5699.

Ramotar, D., Popoff, S. C., Gralla, E. B., and Demple, B. 1991. Cellular role of yeast Apn1 apurinic endonuclease/3'-diesterase: Repair of oxidative and alkylation DNA damage and control of spontaneous mutation. Mol. Cell. Biol. 11: 4537-4544.

Rebeck, G. W., Coons, S., Carroll, P., and Samson, L. 1988. A second DNA methyltransferase repair enzyme in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 85: 3039-3043.

Rebeck, G. W., and Samson, L. 1991. Increased spontaneous mutation and alkylation sensitivity of *Escherichia coli* strains lacking the ogt O^6-methylguanine DNA repair methyltransferase. J. Bacteriol. 173: 2068-2076.

Reenan, R. A., and Kolodner, R. D. 1992a. Isolation and characterization of two *Sccharomyces cerevisiae* genes encoding homologs of the bacterial HexA and MutS mismatch repair proteins. Genetics 132:963-973.

Reenan, R. A., and Kolodner, R. D. 1992b. Characterization of insertion mutations in the *Sccharomyces cerevisiae* MSH1 and MSH2 genes: Evidence for separate mitochondrial and nuclear functions, Genetics 132: 975-985.

Resnick, M. A. 1969. Genetic control of radiation sensitivity in *Saccharomyces cerevisiae*. Genetics 62: 519.

Resnick, M. A. and Setlow, J, K. 1972. Photoreactivation and gene dosage effect in yeast. J. Bacteriol. 109:1307.

Robson, C. N., and Hickson, I. D. 1991. Isolation of cDNA clones encoding a human apurinic/apyrimidinic endonuclease that corrects DNA repair and mutagenesis defects in *E. coli* xth (exonuclease III) mutants. Nucleic Acids Res. 19: 5519-5523.

Roeder, R. G. 1996. The role of general initiation factors in transcription by RNA polymerase II. TiBS 21: 327-335.

Ross-MacDonald, P., and Roeder, G. S. 1994. Mutation of a meiosis-specific MutS homolog decreases crossing over but not mismatch correction. Cell 79:1069-1080.

Rowan, S., Ludwig, R. L., Haupt, Y., Bates, S., Lu, X., Oren, M., and Vousden, K. H. 1996. Specific loss of apoptosis but not cell-cycle arrest function in a human tumor derived p53 mutant. EMBO J. 15: 827-838.

Ruby, S.W., and Szostak, J.W. 1985. Specific *Saccharomyces cerevisiae* genes are expressed in response to DNA-damaging agetns. Mol. Cell. Biol. 5: 75-84

Rydberg, B., and Lindahl, T. 1982. Nonenzymatic methylation of DNA by the intracellular methyl group donor s-adenosyl-L-methionine is a pontential mutagenic reaction. EMBO. J. 1: 211-216.

Sabbatini, P., Lin, J., Levine, A. J., and White, E. 1995. Essential role for p53-mediated transcription in apoptosis but not growth suppression. Genes and Dev. 9: 2184-2192.

Sachsenmaier, C., Radler-Pohl, A., Zinck, R., Nordheim, A., Herrich, P., and Rahmsdorf, H.J. 1994. Involvement of growth factor receptors in the mammalian UVC response. Cell 78: 963-972.

Saffhill, R., Margison, G. P., and O'Connor, P. J. 1985. Mechanisms of carcinogenesis induced by alkylating agents. Biochem. Biophys. Acta. 823: 111-145.

Sagher, D., and Strauss, B. 1983. Insertion of nucleotides opposite apurinic/apyrimidinic sites in deoxyribonucleic acid during in ivtro synthesis; Uniqueness of adenine nucleotides. Biochem. 22: 4518-4526.

Samson, L., and Cairns, J. 1977. A new pathway for DNA repair in Escherichia coli. Nature 267: 281-283.

Samson, L., Derfler, B., Boosalis, M., and Call, K. 1991. Cloning and characterization of a 3-methyladenine DNA glycosylase cDNA from human cells whose gene maps to chromosome 16. Proc. Natl. Acad. Sci. USA 88: 9127-9131.

Sancar A, 1994. Mechanisms of DNA excision repair. Science 266: 1954-1956.

Sancar A, 1995. DNA repair in humans. Annu. Rev. Genet. 29: 69-105.

Sancar, G.B., Ferris, R., Smith, F.W. and Vanderberg, B. 1995. Promoter elements of the *PHR1* gene of *Saccharomyces cerevisiae* and their roles in response to DNA damage. Nucleic Acid Res. 23: 4320-4328.

Sancar, G. B., Smith, F. W., and Heelis, P. F. 1987. Purification of yeast PHR1 photolyase from an *Escherichia coli* overproducing strain and characterization of the intrinsic chromophores of the enzyme. J. Biol. Chem. 262: 15457

Sanchez, Y., Desany, B. A., Jones, W. J., Liu, Q, Wang, B., and Elledge, S. J. 1996. Regulation of *RAD53* by their ATM-like kinasas *MEC1* and *TEL1* in yeast cell cycle checkpoint pathways. Science 271: 357-360.

Sanger, E., Nicklen, S., and Coulson, A.R. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74: 5463-5467.

Santerre, A and Britt, A.B. 1994. Cloning of a 3-methyladenine-DNA glycosylase from Arabidopsis thaliana, Proc. Natl. Acad. Sci. USA 91: 2240-2244.

Schild, D., Johnston, J., Chang, C., and Mortimer, R. K. 1984. Cloning and mapping of *Saccharomyces cerevisiae* photoreactivation gene *PHR1*. Mol. Cell. Biol 4: 1864

Schmitt, N. P. and McEntee, K. 1996. Msn2p, a zinc finger DNA-binding protein, is the transcriptional activator of the multistress response in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA 93: 5777-5782

Schwartz, J. L. 1989. Monofunctional alkylating agent-induced S-phase-dependent DNA damage. Mutation Res. 216: 111-118.

Sebastian, J., Kraus, B., and Sancar, G.B. 1990. Expression of the yeast *PHR1* gene is induced by DNA-damaging agents. Mol. Cell. Biol. 10: 4630-4637.

Sebastian, J., and Sancar, G.B. 1991. A damage-responsive DNA binding protein regulates transcription of the yeast DNA repair gene *PHR1*. Proc. Natl. Acad. Sci. USA 88: 11251-11255.

Seeberg, E., Eide, L., and Bjøras. 1995. The base excision repair pathway. Trends in Biochemical Sciences 20: 391-397.

Seki, S., Akiyama, K., Watanabe, S., Hatsushika, M., Ikeda, S., and Tsutsui, K. 1991. cDNA and deduced amino acid sequence of a mouse DNA repair enzyme (APE nuclease) with significant homology to *Escherichia coli* Exonuclease III*. J. Biol. Chem. 266: 20797-20802.

Selby, C. P., and Sancar, A. 1990. Transcription preferentially inhibits nucleotide excision repair of the template strand in vitro. J. Biol. Chem. 265: 21330-21336

Selby, C. P. and Sancar, A. 1993. Molecular mechanism of transcription repair coupling. Science 247:53-58.

Selby, C. P., Witkin, E. M., and Sancar, A. 1991. *Escherichia coli mfd* mutant deficient in "mutation frequency decline" lacks strand-specific repair: in vitro complementation with purified coupling factor. Proc. Natl. Acad. Sci. USA 88: 11574-11578

Service, R.F. 1994. Stalking the start of colon cancer. Science 263: 1559-1560.

Sharples, G. J., Chan, S. N., Mahdi, A. A., Whitby, M. C., and Lioyd, R. G. 1996. Processing of intermediates in recombination and DNA repair: identification of a new endonuclease that specifically cleaves Holliday junctions. EMBO J. 13: 6133-6142.

Sharples, G. J., and Leach, D. R. F. 1995. Structural and functional similarities between the sbcCD proteins of *Escherichia coli* and the RAD50 and MRE11 (RAD32) recombination and repair proteins of yeast. Mol. Microbiol. 17: 1215-1220.

Sheng, S., and Schuster, S.M. 1993. Purification and characterization of *Saccharomyces cerevisiae* DNA damage-responsive protein 48 (DDRP48). J. Biol. Chem. 268:4752-4758.

Sherman, F., Fink, R., and Ficks, E.B. 1983. *Methods in Yeast Genetics: a Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Shinohara, A., Ogawa, H., and Ogawa, T. 1992. Rad51 protein involved in repair and recombination in *S. cerevisiae* is a RecA-like protein. Cell 69: 457-470.

Shinya, E., and Shimada, T. 1994. Identification of two initiator elements in the bidirectional promoter of the human dihydrofolate reductase and mismatch repair protein 1 genes. Nucleic Acids Res. 22:2143-2149.

Shore, D., and Nasmyth, K. 1987. Purification and cloning of a DNA binding protein from yeast that binds to both silencer and activator elements. Cell 51: 721-732.

Siede, W. 1995. Cell cycle arrest in response to DNA damage: lessons from yeast. Mutation Res. 337: 73-84.

Siede, W., Allen, J. B., Elledge, S. J., and Friedberg, E. C. 1996. The *Saccharomyces cerevisiae MEC1* gene, which encodes a homolog of the human *ATM* gene product, is required for G1 arrest following radiation treatment. J. Bacteriol. 178: 5841-5843.

Siede, W., Friedberg, A. S., and Friedberg, E. C. 1993. *RAD9*-dependent G1 arrest defines a second checkpoint in the cell cycle of *Saccharomyces cerevisiae*.. Proc. Natl. Acad. Sci. USA 90: 7985-7989.

Siede, W., and Friedberg, E.C. 1992. Regulation of the yeast *RAD2* gene: DNA damage-dependent induction correlates with protein binding to regulatory sequences and their deletion influences survival. Mol. Gen. Genet. 232: 247-256.

Siede, W., Robinson, G.W., Kalainov, D., Malley, T., and Friedberg, E.C. 1989. Regulation of the *RAD2* gene of *Saccharomyces cerevisiae*. Mol. Microbiol. 3: 1697-1707.

Siliciano, P.G., and Tatchell, K. 1984. Transcription and regulatory signals at the mating type locus in yeast. Cell 37: 969-978

Singer, B. and Granberger, D. 1983. *Molecular Biology of Mutagens and Carcinogens.* Plenum, New York.

Singh, K.K., and Samson, L. 1995. Replication protein A binds to regulatory elements in yeast DNA repair and DNA metabolism genes. Proc. Natl. Acad. Sci. USA 92: 4907-4911

Smale, S. T., and Baltimore, D. 1989. The "inintiator" as transcription control element. Cell 57: 103-113.

Smith, M. L., Mitchell, P. J., and Crouse, G. F. 1990. Analysis of the mouse *Dhfr/Rep-3* major promoter region by using linker-scanning and internal deletion mutations and DNase I footprinting. Mol. Cell. Biol. 10: 6003-6012.

Stelow, R.B, and Carrier, W.L. 1964. The disappearence of thymine dimers from DNA: an error-correcting mechanism. Proc. Natl. Acad. Sci. USA 51: 226-231

Strauss, B. S. 1991. The 'A Rule' of mutagen specificity: a consequence of DNA polymerase bypass of non-instructional lesions? BioEssays 13:79-84.

Struhl, K, 1982. Regulatory sites for *his3* gene expression if yeast. Nature 300: 284-287.

Struhl, K. 1985. Naturally occurring poly(dA-aT) sequences are upstream promoter elements for constitutive transcription in yeast. Proc. Natl. Acad. Sci. USA 82: 8419-8423.

Struhl, K. 1986. Constitutive and inducible *Saccharomyces cerevisiae* promoters: evidence for two distinct molecular mechanisms. Mol. Cell. Biol. 6: 3847-3853.

Struhl, K. 1987. Promoters, activator proteins and the mechanism of transcriptional initiation in yeast. Cell 49:295-297.

Struhl, K. 1993. Yeast transcription factors. Curr. Opin. Cell. Biol. 5:513-520.

Struhl K. 1995. Yeast transcriptional regulatory mechanisms. Annu. Rev. Genet. 29: 651-674.

Sturzbecher, H-W., Donzelmann, B., Henning, W., Knippschild, U., and Buchhop, S. 1996. p53 is linked directly to homologous recombination processes via RAD51/RecA protein interaction. EMBO J. 15: 1992-2002.

Svejstrup, J. Q., Vichi, P., and Egly, J-M. 1996. The multiple roles of transcription/repair factor TFIIH. TiBS 21: 346-350.

Svejstrup, J. Q., Wang, Z., Feaver, W. J., Wu, X., Donahue, F., Friedberg, E. C., and Kornberg, R. D. 1995. Different forms of yeast RNA polymerase transcription factor IIH (TFIIH) for transcription and DNA repair: holo TFIIH and a nucleotide excision repairosome. Cell 80: 21-28

Sweder, K. S. 1994. Nucleotide excision repair in yeast. Curr. Genet 27: 1-16

Sweder, K.S., Rhoder, P.R., Campbell, J.L. 1988. Purification and characterization of proteins that bind to yeast ARSs. J. Biol. Chem. 263:17270-17277.

Sweder, K. S., Verhage, R. A., Crowley, D. J., Crouse, G. F., Brouwer, J., and Hanawalt, P. C. 1996. Mismatch repair mutants in yeast are not defective in transcription-coupled DNA repair of UV-induced DNA damage. Genetics 143: 1127-1135

Tang, C-M., Tomkinson, A. E., Lane, W. S., Wold, M. S., and Seto, E. 1996. Replication protein A is a component of a complex that binds the human metallothionein IIA gene trnascription start site. J. Biol. Chem. 271: 21637-21644.

Teo, I., Sedgwick, B., Kilpatrick, M. W., McCarthy, T. V., and Lindahl, T. 1986. The intracellular signal for induction of resistance to alkylating agents in *E. coli*. Cell 45:315-324

Thomas. D. C., Roberts, J. D., and Kunkel, T. A. 1991. Heteroduplex repair in extracts of human Hela cells. J. Biol. Chem. 266:3744-3751.

Tijian, R., and Maniatis, T. 1994. Transcriptional activation: A complex puzzle with few easy pieces. Cell 77: 5-8.

Torpey, L. E., Gibbs, P. E. M., Nelson, J. R., Lawrence, C. W. 1994. Cloning and sequence of REV7, a gene whose function is required for DNA damage-induced mutagenesis in *Saccharomyces cerevisiae*. Yeast 10: 1503-1509.

Treger, J. M., Heichman, K. A., and McEntee, K. 1988. Expression of the yeast UBI4 gene increases in response to DNA-damaging agents and in meiosis. Mol. Cell. Biol. 8: 1132-1136.

van Gool, A. J., Verhage, R., Swagermakers, S. M.A., van de Putte, P., Brouwer, J. et al, 1994. *RAD26*, the functional *S. cerevisiae* homolog of the Cockayne syndrome B gene *ERCC6*. EMBO J. 13: 5361-5369.

van Hoffen, A., Natarajan, A. T., Mayne, L. V., van Zeeland, A. A., Mullenders, L. H. F. et al. 1993. Deficient repair of the transcribed strand of active genes in Cockayne syndrome cells. Nucleic Acids Res. 21: 5890-5895

Venema, J., Mullenders, L. H. F., Natarajan, A. T., Van Zeeland, A. A., and Mayne, L. V. 1990. The genetic defect in Cockayne syndrome is associated with a defect in

repair of UV-induced DNA damage in transcriptionally active DNA. Proc. Natl. Acad. Sci. USA 87: 4707-4711.

Verhage, R., Gool, A. J. van., Groot, N. D., Hoeijmakers, J. H. J., Putte, P. V.D. et al., 1996. Double mutants of *Saccharomyces cerevisiae* with alterations in global genome and transcription-coupled repair. Mol. Cell. Biol. 16: 496-502.

Verhage, R., Zeeman, A. M., Groot, N. D., Gleig, F., Bang, D. D. et al., 1994. The *RAD7* and *RAD16* genes, which are essential for pyrimidine dimer removal from the silent mating type loci, are also required for repair of the nontranscribed strand of an active gene in *Saccharomyces cerevisiae*. Mol. Cell. Biol. 14: 6135-6142.

Verrijzer, C. P., and Tjian, R. 1996. TAFs mediate transcriptional activation and promoter selectivity. TiBS 21: 338-342.

Walker, G.C. 1984. Mutagenesis and inducible responses to deoxyribonucleic acid damage in *Escherichia coli* . Microbiol. Rev. 48: 60-93.

Walker, G.C. 1985. Inducible DNA repair systems. Annu. Rev. Biochem. 54: 425-457.

Walker, G. C. 1995. SOS-regulated proteins in translesion DNA synthesis and mutagenesis. Trends in Biochemical Sciences 20: 416-420.

Wang, G., and Humayun, M. Z. 1996. Induction of the *Escherichia coli* UVM response by oxidative stress. Mol. Gen. Genet. 251: 573-579.

Wang. G., Palejwala, V. A., and Humayun, M. Z. 1995. Alkylating agents induce UVM, a recA-independent inducible mutagenic phenomenon in *Escherichia coli*. Genetics 141: 813-823.

Wang, M. M., and Reed, R. R. 1993. Molecular cloning of the olfactory neuronal transcription factor Olf-1 by genetic selection in yeast. Nature 364: 121-126.

Wang, Z., and Rossman, T. G. 1994. Isolation of DNA fragments from agarose gel by centrifugation. Nucleic Acids Res. 22: 14.

Waters, R., Zhang, R., and Jones, N. J. 1993. Inducible removal of UV-induced pyrimidine dimers from transcriptionally active and inactive genes of *Saccharomyces cerevisiae* . Mol. Gen. Genet. 239: 28-32.

Weinert, T., A., Kiser, G. l., and Hartwell, L. H. 1994. Mitotic checkpoint genes in budding yeast and the dependence of mitosis on DNA replication and repair. Genes and Dev. 8: 652-655.

Weis, L., and Reinberg, D. 1992. Transcription by RNA polymerase II: initiator-directed formation of transcription component complexes. FASEB J. 6: 3300-3309.

Wells, J., Held, P., Illenye, S., and Heintz, N. H. 1996. Protein-DNA interactions at the major and minor promoters of the divergently transcribed *dhfr* and *rep3* genes during the Chinese hamster ovary cell cycle. Mol. Cell. Biol. 16: 634-647.

West, Jr., R.W., Chen, S., Putz, H., Butler, G., and Banerjee, M. 1990. *GAL1-GAL10* divergent promoter region of *Saccharomyces cerevisiae* contains negative control elements in addition to functionally separate and possibly overlapping upstream activating sequences. Genes & Dev. 4:740-751.

West, R.W.Jr., Yocum, R.R., and Ptashne, M. 1984. *Saccharomyces cerevisiae* Gal1-Gal10 divergent promoter region: location and function of the upstream activating sequence UAS_G. Mol. Cell. Biol. 4: 2467-2478.

Winters, T. A., Henner, W. D., Russell, P. S., McCullough, A., and Jorgensen, T. J. 1994. Removal of 3'-phosphoglycolate from DNA strand-break damage in an oligonucleotide substrate by recombinant human apurinic/apyrimidinic endonuclease 1. Nucleic Acids Res. 22: 1866-1873.

Wolter, R., Siede, W., and Brendel, M. 1996. Regulation of *SNM1*, an inducible *Saccharomyces cerevisiae* gene required for repair of DNA cross-links. Mol. Gen. Genet. 250: 162-168.

Wu, J., and Weiss, B. 1992. Two-stage induction of the soxRS (Superoxide response) regulon of *Escherichia coli*. J. Bacteriol.174: 3915-3920.

Xanthoudakis, S., and Curran, T. 1992. Identification and characterization of Ref-1, a nuclear protein that facilitates AP-1 DNA-binding activity. EMBO J. 11: 653-656.

Xanthoudakis, S., Miao, G., Wang, F., Pan, Y. C., and Curran, T. 1992. Redox activation of Fos-Jun DNA binding activity is mediated by a DNA repair enzyme. EMBO J. 11: 3323-3335.

Xanthoudakis, S., Smeyne, R. J., Wallace, J. D., and Curran, T. 1996. The redox/DNA repair protein, Ref-1, is essential for early embryonic development in mice. Proc. Natl. Acad. Sci. USA 93: 8919-8923.

Xiao, W., Chow, B. L., and Rathgeber, L. 1996. The repair of DNA methylation damage in *Saccharomyces cerevisiae*. Curr. Genet. In press.

Xiao, W., Derfler, B., Chen, J., and Samson, L. 1991. Primary sequence and biological functions of a *Saccharomyces cerevisiae* O^6-methylguanine/O^4-methylthymine DNA repair methyltransferase gene. EMBO J. 10: 2179-2186.

Xiao, W., and Samson, L. 1992. The *Saccharomyces cerevisiae MGT1* DNA repair methyltransferase gene: its promoter and entire coding sequence, regulation and *in vivo* biological functions. Nucleic Acids Res. 20: 3599-3606.

Xiao, W., and Samson, L. 1993. *In vivo* evidence for endogenous DNA alkylation damage as a source of spontaneous mutation in eukaryotic cells. Proc. Natl. Acad. Sci. USA 90: 2117-2121.

Xiao, W., Singh, K.K., Chen, B., and Samson, L. 1993. A common element involved in transcriptional regulation of two DNA alkylation repair genes (*MAG1* and *MGT1*) of *Saccharomyces cerevisiae*. Mol. Cell. Biol. 13: 7213-7221.

Yagle, K., and Mcentee, K. 1990. The DNA damage-inducible gene *DIN1* of *Saccharomyces cerevisiae* encodes a regulatory subunit of ribonucleotide reductase and is identical to *RNR3*. Mol. Cell. Biol. 10: 5553-5557.

Yasui, A. and Chevallier, M. R. 1983. Cloning of photoreactivation repair gene and excision repair gene of the yeast Saccharomyces cerevisiae. Curr. Genet.7: 191.

Yao, B., Sollitti, P., Zhang, X., and Marmur, J. 1994. Shared control of maltose induction and catabolite repression of the *MAL* structural genes in *Saccharomyces* . Mol. Gen. Genet. 243: 622-630.

Yocum, R.R., Henley, S., West Jr., R., and Ptashne, M. 1984. Use of *lacZ* fusion to delimit regulatory elements of the inducible divergent *GAL1-GAL10* promoter in *Saccharomyces cerevisiae*. Mol. Cell. Biol. 4:1985 -1998.

Zawel, L., and Reinberg, D. 1993. Initiation of transcription by RNA polymerase II: A multi-step process. In *Progress in nucleic acid research and molecular biology* Vol 44. pp67-108. Academic Press Inc.

Zhang, J., and Jacob, S. T. 1990. Purification and characterization of a novel factor which stimulates rat ribosomal gene transcription *in vitro* by interacting with enhancer and core promoter elements. Mol. Cell. Biol. 10: 5177-5186.

Zhou, Z., and Elledge, S. J. 1992. Isolation of crt mutants constitutive for transcription of the DNA damage inducible gene *RNR3* in *Saccharomyces cerevisiae*. Genetics 131: 851-866.

Zhou, Z., and Elledge, S. J. 1993. *DUN1* encodes a protein kinase that controls the DNA damage response in yeast. Cell 75: 1119-1127.