The Identification of Proteins that Interact with Rad7: A Protein Involved in DNA Repair in *Saccharomyces cerevisiae*

by

Donald William Paetkau

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Department of Human Genetics

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THE IDENTIFICATION OF PROTEINS THAT INTERACT WITH RAD7: INVOLVED IN DNA REPAIR IN *Saccharomyces cerevisiae*

BY

DONALD WILLIAM PAETKAU

A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

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For Brenda and Dad

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Abbreviations

APS= ammonium persulfate
bp= base pair(s)
BSA= bovine serum albumin
DEPC= diethyl pyrocarbonate
DMF= dimethylformamide
EDTA= ethylene diamintetraacidic acid
EtBr= ethidium bromide
HEPES= N-[2-Hydroxyethyl]piperazine-N'-2-ethanesulfonic acid
IPTG= Isopropyl-1-thio-β-D-galactosidase
MOPS= 3-[N-Morpholino]propanesulfonic acid
ONPG= *ortho*-nitrophenyl galactoside
PBS= phosphate buffered saline
PEG= polyethylene glycol
PMSF= phenylmethylsulfonyl fluoride
SC= synthetic complete
SDS= sodium dodecyl sulphate
TCA= trichloroacetic acid
TE= 10 mM Tris-HCl pH 8.0; 1 mM EDTA
TEMED= *N,N,N',N'*-tetramethylethylene diamine
UV= ultraviolet radiation
X-gal= 5-bromo-4-chloro-3-indolyl-β-D-galactosidase

ABSTRACT

The yeast two-hybrid system was used to identify the proteins that interact with the products of the nucleotide excision repair gene *RAD7*. Three fusion gene libraries in the pGAD vectors pDP4, 7 and 12 were prepared, each with a complexity of greater than 1 x 10^6 clones. A *GAL4*-binding domain *RAD7* fusion plasmid was used to screen 400,000 of these clones, identifying three pGAD fusion plasmids each containing a putative Rad7-interacting protein. Two of the pGAD fusion plasmids that showed interaction with Rad7 contained inserts encoding the Sir3 protein, which is involved in transcriptional silencing at the *HM* mating loci and chromosome telomeres. A deletion of *SIR3* in a *rad7* deletion mutant reduces the killing effect of UV by one to two orders of magnitude, indicating that a functional interaction occurs between the products of these two genes. *In vitro* analysis of the Rad7 and Sir3 proteins produced in *E. coli*, and affinity purified using the pGEX system, indicated that the Rad7 and Sir3 proteins are able to interact directly. These results suggests that Rad7 and Sir3 interact *in vivo*. This interaction may be the mechanism that the DNA repair machinery uses to gain access to transcriptionally silenced DNA.

INTRODUCTION

In the face of constant bombardment from a myriad of agents that damage DNA, the cells of any living organism must be able to repair this damage to survive. DNA repair, together with the processes of replication and transcription, is essential to a cell's ability to maintain, interpret and transmit genetic information from one generation to the next. In humans, defects in DNA repair processes result in several debilitating genetic diseases such as xeroderma pigmentosum, Cockayne syndrome, PIBIDS (photosensitivity, ichthosis, brittle hair, impaired intelligence, decreased fertility, and short stature), ataxia-telangiectasia, Fanconi anemia and Bloom syndrome. These diseases cause, among other symptoms, an increased risk of developing specific types of cancer (reviewed in Hanawalt and Sarasin, 1986, Hoeijmakers, 1993b). Understanding how eukaryotic cells deal with DNA damage will provide insights into the deleterious effects of DNA-damaging agents such as ultraviolet radiation (UV), X and γ radiation, and genotoxic chemicals.

There is increasing evidence that the process of DNA repair is conserved between higher and lower eukaryotes. The single-celled eukaryote *Saccharomyces cerevisiae*, with its extensive genetic characterization and haploid-diploid life cycle, is an ideal model system to study the process of DNA repair (Sherman, 1991). In addition, the process of homologous recombination, which allows the simple deletion or addition of sequences at specific sites in the genome, provides a powerful tool for genetic studies (Rothstein, 1991). To date, human homologs corresponding to about one half of the yeast nucleotide excision repair genes and the postreplication repair gene *RAD6* have been identified (reviewed in Friedberg, 1991; Hoeijmakers, 1993b). Homologies to rodent and

Drosophila melanogaster repair genes have also been discovered (see Table 1).

i. DNA Repair

Spontaneous DNA damage can occur as the result of errors during DNA replication; the loss of bases; alterations in the chemistry of DNA bases, including tautomeric shifts and deamination; and DNA strand breakage. These modifications to the DNA molecule may introduce mutations which can result in cell death if not repaired (reviewed in Friedberg, 1985). DNA damage can also be caused by chemical and physical environmental agents. Types of chemical damage include the alkylation of bases, DNA-DNA and DNA-protein crosslinks, the covalent binding of numerous bulky adducts and the insertion of base analogs such as 5-bromouracil and 2-aminopurine. Physical DNA-damaging agents, such as UV and ionizing radiation, cause pyrimidine dimers, pyrimidine-pyrimidine adducts, pyrimidine hydrates, thymine glycols and DNA strand breaks (reviewed in Friedberg, 1985).

Several mechanisms have evolved to repair different types of DNA damage. These mechanisms can be grouped into two classes: (a) lesion specific strategies which involve the direct reversal of DNA damage and (b) mechanisms involved in the repair of a more general range of DNA damage. The first class includes the re-ligation of strand breaks, insertion of purines at apurinic sites, photoenzymatic cleavage of the bond between thymine dimers by photolyase and the removal of certain bulky adducts such as 0^6-alkylguanine by specific methyl transferases. Alkylated bases are also removed in some cells by the specific removal of the base and subsequent cleavage of the remaining apurinic or apyrimidinic (AP) site catalyzed by DNA glycosylases and AP endonucleases (reviewed in Friedberg, 1985). The second class of mechanisms tend to be more complex and

Table 1. Conserved eukaryotic DNA repair genes.

Yeast	Drosophila	Rodent	Human
RAD2		*ERCC5*	*ERCC5*
RAD3		*ERCC2*	*ERCC2*
RAD4			*XPC*
RAD6	*Dhr6*		*UBE2A, UBE2B*
RAD7			
RAD10		*ERCC1*	*ERCC1*
RAD14			*XPA*
RAD16		*ERCC6*	*ERCC6*
RAD18			
RAD23			*RAD23A, RAD23B*
RAD25	*haywire*	*ERCC3*	*ERCC3*

XPC=xeroderma pigmentosum complementation group C*; XPA=xeroderma pigmentosum complementation group A*
*not standard gene symbols
(Masutani *et al.*, 1994; 1993b; Koken et al., 1991a; Koken et al., 1991b; reviewed in Hoeijmakers; Gene symbols as described by McAlpine *et al.*, 1993 and McAlpine, personal communication)

requires the action of more proteins than the mechanisms dealing with specific types of DNA damage. The three pathways found in both prokaryotes and eukaryotes which handle a wide range of DNA lesions are nucleotide excision repair, postreplication repair and double strand break repair (reviewed in Haynes and Kunz, 1981; Friedberg 1985).

Nucleotide excision repair removes bulky damage from the DNA. The recognition of a distortion in the DNA duplex is followed by the removal of a section of the DNA strand containing the damage. The resulting gap is filled in by a DNA polymerase (reviewed in Friedberg 1988; Van Houten, 1990). Postreplication repair does not repair the DNA but rather allows the cell to tolerate the lesion until it can be corrected. DNA replication stalls at sites of DNA damage. For replication to continue past a lesion, one of two mechanisms is used; trans-lesion synthesis or re-initiation of DNA replication. Trans-lesion synthesis replicates the damaged region but inserts random bases; this mechanism is error-prone. Re-initiation of DNA replication past the lesion leaves a gap in the replicated DNA which is later corrected through a combination of sister chromatid recombination and nucleotide excision repair. This mechanism is error-free (Howard-Flanders and West, 1983). Double strand break repair is involved in the repair of DNA strand breaks caused by ionizing radiation. The proteins involved in this process are also involved in recombination and may be important in error-free postreplication repair (reviewed in Haynes and Kunz, 1981).

Although the mechanisms of DNA repair have been extensively studied in the bacterium *Escherichia coli* (reviewed in Howard-Flanders, 1981; Friedberg, 1985; Van Houten, 1990), less is known about these processes in eukaryotic cells. Mutants of the yeast *Saccharomyces cerevisiae* that exhibit sensitivity to UV or other DNA damaging

agents have allowed the identification of a large number of genes involved in these repair pathways (reviewed in Haynes and Kunz, 1981; Friedberg, 1988). Pairwise analysis of mutants assigned to specific genes has led to the recognition of three epistasis groups (reviewed in Haynes and Kunz, 1981), which define three functionally related DNA repair systems (Friedberg, 1988).

Several of the genes within these epistasis groups function in other cellular processes. *RAD3* (Prakash *et al.*, 1993) and *RAD25* (Park *et al.*, 1992), which are involved in nucleotide excision repair, are also important for cell viability. *RAD3* is essential for RNA polymerase function (Guzder *et al.*, 1994). Based on the function of its human homolog, *ERCC3, RAD25* is likely a part of a transcription initiation factor (Schaeffer *et al.*, 1993). The postreplication repair gene, *RAD6*, is involved in cell growth and sporulation (Siede, 1988). The interacting proteins encoded by the *RAD1* and *RAD10* genes are involved in mitotic recombination as well as nucleotide excision repair (Fishman-Lobell and Haber, 1992). *CDC9*, the gene coding for DNA ligase, is required for all three of these repair mechanisms, and there is evidence that DNA photolyase, the product of the *PHR1* gene, may have an accessory role in nucleotide excision repair (Sancar and Smith, 1989).

Determining the biochemical properties and functions of the proteins involved in DNA repair is critical. Towards this end, several groups have developed cell-free systems from mammalian cells (Wood *et al.*, 1988; Hoeijmakers *et al.*, 1990; Masutani *et al.*, 1993; Sugasawa *et al.*, 1993) and *Saccharomyces cerevisiae* (Wang *et al.*, 1993) that catalyze nucleotide excision repair.

ii. Nucleotide Excision Repair

Nucleotide excision repair is responsible for the repair of an array of DNA lesions including UV-induced photoproducts, chemical adducts and certain crosslinks. The five basic steps involved in nucleotide excision repair are: 1) recognition of the DNA damage; 2) incision on either side of the damage; 3) excision of the oligonucleotide containing the damage; 4) DNA synthesis to fill the gap; and 5) ligation of the DNA backbone (reviewed in Haynes and Kunz, 1981; Friedberg, 1985; Van Houten, 1990; Hoeijmakers, 1993a).

Nucleotide excision in *E. coli* involves the proteins UvrA, UvrB, UvrC UvrD, DNA polymerase I and DNA ligase. These proteins have been well characterized. The UvrA protein has two nucleotide triphosphate (NTP) binding sites; two zinc fingers and one putative helix-turn-helix motif implicated in DNA binding. Recognition and binding to a site of DNA damage occurs when UvrA dimerizes and forms a complex with one UvrB molecule, which has 5'-3' DNA helicase activity. A UvrC dimer creates an incision in the DNA seven nucleotides upstream and three to four nucleotides downstream of the lesion. UvrD, which has 1 NTP binding site and a 3'-5' DNA or RNA/DNA helicase activity removes the oligonucleotide containing the damage. DNA polymerase I fills the gap and DNA ligase seals the nick. There is also evidence that several other proteins, including photolyase (Sancar and Smith, 1989) may be involved in the *in vivo* modulation of the nucleotide excision repair process (reviewed in Van Houten, 1990; Hoeijmakers, 1991).

The mechanism of eukaryotic nucleotide excision repair is less well understood. In *Saccharomyces cerevisiae* at least eleven genes which encode products involved in nucleotide excision repair have been identified. Seven of these genes, *RAD1*, *RAD2*,

RAD3, *RAD4*, *RAD10*, *RAD14* and *RAD25*, encode products that are essential for the incision step of nucleotide excision repair (Reynolds *et al.*, 1980; Bankman *et al.*, 1992; Park *et al.*, 1992). Yeast strains carrying mutations in any of these seven genes are very sensitive to the damaging effects of UV. The *RAD7*, *RAD16*, *RAD23*, and *RAD24* genes are not involved in the incision step of nucleotide excision repair and are less sensitive to UV (Friedberg, 1988; Bang *et al.*, 1992; Hoeijmakers, 1993a). They are thought to encode accessory proteins which enhance the efficiency of nucleotide excision repair. The *RAD24* gene also functions in postreplication repair (Eckardt-Schupp *et al.*, 1987).

Biochemical analyses and amino acid sequence comparison to proteins of known function have suggested functions for several of the yeast proteins involved in nucleotide excision repair (summarized in Table 2). The Rad1-Rad10 complex is involved in a mitotic recombination pathway (Bailly *et al.*, 1992; Bardwell *et al.*, 1992). The Rad3 protein has been shown to be a DNA-dependent ATPase with both DNA-DNA and DNA-RNA helicase activity and arrests on the DNA at sites of damage. This activity suggests a possible role in damage recognition (Naegeli *et al.*, 1992). The Rad25 human homolog, ERCC3, is a subunit of the BTF2 basic transcription factor which has DNA dependent ATPase activity and is involved in the initiation of transcription (Schaeffer *et al.*, 1993). The *RAD3* and *RAD25* genes are also vital for the viability of the yeast cell. Rad4 has possible chromatin and DNA binding activity (Hoeijmakers, 1993a) and Rad14 has a DNA binding zinc finger motif which may be involved in DNA damage recognition (Bankmann *et al.*, 1992). Finally, it has been suggested that Rad7 and Rad16 may be involved in making transcriptionally silenced chromatin accessible to the nucleotide excision repair complex (Bang *et al.*, 1992).

Table 2. Yeast Genes Involved in Nucleotide Excision Repair

Gene	Sensitivity to UV	Human Homolog	Protein Function
RAD1	Extreme		mitotic recombination; binds Rad10; endonuclease activity
RAD2	Extreme	*ERCC5*	
RAD3	Extreme	*ERCC2*	cell viability; RNA polymerase function; DNA-DNA and DNA-RNA dependent helicase; arrest at DNA damage sites
RAD4	Extreme	*XPC*	chromatin and DNA binding activity?
RAD7	Moderate		differential repair; nuclear membrane protein?; Rad23 interaction?
RAD10	Extreme	*ERCC1*	mitotic recombination; binds Rad1; single-stranded DNA binding activity?
RAD14	Extreme	*XPA*	Zn finger?; DNA binding?; DNA damage recognition?
RAD16	Moderate	*ERCC6*	accessability to transcriptionally silent chromatin?; differential repair?; helicase?
RAD23	Moderate	*RAD23A* *RAD23B*	Rad7 interaction?
RAD24	Moderate		
RAD25	Extreme	*ERCC3*	cell viability; transcription initiation factor?; DNA dependent ATPase activity?; helicase?

(Tomkinson *et al.*, 1993; Naegeli *et al.*, 1992: Perrozzi and Prakash, 1986; Bang *et al.*, 1992; Bardwell *et al.*, 1992; Bailley *et al.*, 1992;Bankmann *et al.*, 1992; Schaeffer *etal.*, 1993; reviewed in Hoeijmakers, 1993a)

ERCC2 and *ERCC3* exhibit homology to the *RAD3* and *RAD25* genes of *Saccharomyces cerevisiae* respectively (Weber *et al.*, 1990; Park *et al.*, 1992) and *RAD16* has partial homology to *ERCC6* (Troelstra *et al.*, 1992). Amino acid sequence analyses indicate that the ERCC2, ERCC3 and ERCC6 human proteins encode putative DNA helicases. The homology displayed between the human *ERCC1* and the yeast *RAD10* genes (van Duin *et al.*, 1986) suggest that like the Rad10 protein, ERCC1 may be involved in the binding of the repair complex to single stranded DNA (Sung *et al.*, 1992). The Rad10 protein is able to form a stable complex with the yeast Rad1 protein (Bardwell *et al.*, 1992; Bailly *et al.*, 1992). Rad1 is similar to UvrC in that they both have endonuclease activity (Tomkinson *et al.*, 1993). These functional homologies between prokaryotes and eukaryotes are expected since the steps of nucleotide excision repair are the same in both types of cells. (Hoeijmakers, 1991) Understanding more about the roles of the proteins involved this process in eukaryotes will provide us with the answer to why so many more proteins seem to be needed in eukaryotic nucleotide excision repair in comparison to prokaryotes (Hoeijmakers, 1991).

iii. *RAD7*

The *RAD7* gene encodes a protein that is not essential for the incision step of nucleotide excision repair but is thought to have an accessory role in this process. The protein has a molecular weight of approximately 63.7 kDa and contains 565 amino acids (Perozzi and Prakash, 1986). The Rad7 protein is highly charged, containing a highly hydrophillic amino terminus and hydrophobic carboxy terminus. Two possible membrane spanning domains around amino acids 425 and 475 have implicated Rad7 as a nuclear membrane associated protein (Perozzi and Prakash, 1986). This protein also contains

twelve tandem leucine rich motifs between amino acids 228-555, which are thought to be involved in protein-protein interactions (Schneider and Schweiger, 1991).

A *rad7* deletion mutant is partially defective in excision of UV-induced thymine dimers and psoralen plus light induced DNA interstrand crosslinks (Miller *et al.*, 1982). Based on the moderate sensitivity of a *rad7* mutant to UV it was proposed that the Rad7 protein may be involved in access of the nucleotide excision repair complex to pyrimidine dimers in transcriptionally silenced chromatin (Miller *et al.*, 1982; Perozzi and Prakash, 1986, Schiestl and Prakash, 1989) Deletions of *RAD7* can be complemented by a truncated Rad7 protein lacking the first 99 amino acids, provided that the truncated Rad7 is expressed from a multicopy vector. This complementation will not occur if the Rad23 protein is missing, suggesting either a common function for the Rad7 and Rad23 proteins, or a functional interaction between them (Perozzi and Prakash, 1986).

RAD7 is found in a gene cluster on chromosome X (*RAD7*, *OSM1*, and *CYC1*) which resembles another cluster containing the *RAD23* gene on chromosome V (*RAD23*, *ANP1*, and *CYC7*) (McKnight *et al.*, 1981). Both clusters contains genes involved in DNA repair (*RAD7* and *RAD23*), osmotic sensitivity (*OSM1* and *ANP1*) and cytochrome c production (*CYC1* and *CYC7*). The similarity between the two clusters has led to the hypothesis that they are the result of an ancient duplication event . The evolutionary similarity between the Rad7 and Rad23 proteins may also explain their suggested functional similarity. However, overproduction of Rad23 does not complement a *rad7* deletion, and overexpression of Rad7 will not complement a *rad23* deletion, indicating that each protein must have a unique function or functions (Perozzi and Prakash, 1986). Mutants of the *RAD7* gene were also found to be defective in repair of the

transcriptionally silent mating-type loci *HML α*.

The *HML α*, *HMRa* and *MAT* mating-type loci are involved in the determination of mating type of haploid *Saccharomyces cerevisiae* cells (reviewed in Haber, 1992; Watson *et al.*, 1987). The mating type information is expressed from *MAT*. *HML α* and *HMRa* contain transcriptionally silent copies of the a and *α* mating cassettes which can be transposed into the *MAT* locus. This occurs only in haploid *HO* yeast strains during cell growth, and allows the efficient conversion of daughter cells to the opposite mating type. The transcriptional inactivation of the *HML* and *HMR* loci can be reversed by the removal of the *E* and *I cis*-acting silencing sites at *HML*, removal of the *E* site at *HMR*, or by the mutation of at least 7 other genes (reviewed in Haber, 1992; Laurenson and Rine, 1992). These genes include the *SIR1*, *SIR2*, *SIR3*, and *SIR4* genes (SIR=silent information regulator), two genes encoding the subunits of an N-terminal acetyl transferase (*ARD1*, *NAT1*) and the histone H4 gene (*HHF2*).

Terleth *et al.* (1989) have used the silent mating type phenomenon to study differential repair. UV-induced thymine dimers were shown to be preferentially removed from the transcriptionally active *MAT α* locus in comparison to the transcriptionally silent *HML α* locus. This differential repair is abolished in a *sir3* mutant strain in which the *HM* loci are transcriptionally active. Further studies (Terleth *et al.*, 1990a, 1990b) also showed that four *rad* mutants (*rad7*, *rad9*, *rad16* and *rad24*) were defective in the removal of thymine dimers from *HML α*, but not from *MAT α*. *rad24*, and *rad9* were shown to be partially defective in *HML α* repair, *rad16* was shown to be completely defective in *HML α* repair and slower in the repair of *MAT α*, and *rad7* was shown to be completely defective in *HML α* repair, but normal in the repair of *MAT α* (Bang *et al.*,

1992).

Transcriptional inactivation of genes near telomeres seems to occur through a similar mechanism to that seen at the *HM* loci. Mutants of the *SIR1*, *SIR2*, *SIR3*, *SIR4*, *ARD1*, *NAT1*, and *HHF2* trans-acting genes involved in *HM* loci transcriptional inactivation are also sufficient for the reversal of transcriptional inactivation of genes near telomeres (Aparicio *et al.*, 1991). The similarity in these two processes suggests that the *rad7*, *rad9*, *rad16* and *rad24* mutants may also be defective in the repair of transcriptionally silent genes located near telomeres.

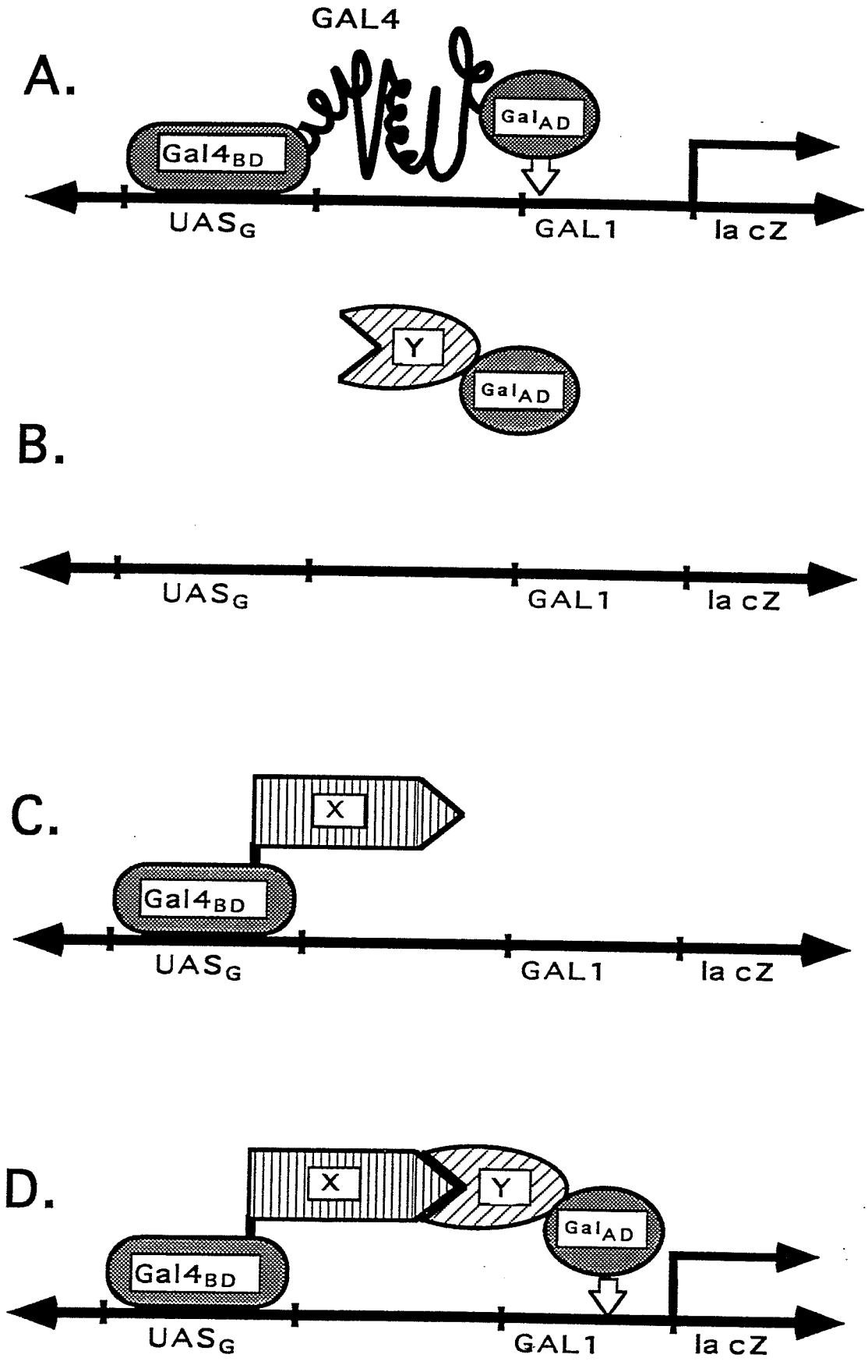
The inefficient repair of transcriptionally inactive DNA seen in *rad7* and *rad16* mutants resembles that seen in the fibroblasts of patients with xeroderma pigmentosum complementation group C (Venema *et al.*, 1990). This suggests that these yeast genes may have functional human homologs.

iv. Two-hybrid System for the Detection of Protein-Protein Interactions

The two-hybrid system, developed by Fields and Song (1989), uses the reconstitution of the Gal4 transcription activator as a basis for detecting protein-protein interactions *in vivo*. As is shown in Figure 1A, the Gal4 transcription activator is able to bind to the *GAL4* upstream activating sequence (UAS_{GAL}) and activate transcription of the *lacZ* reporter from the *GAL1* promoter. Protein fusion experiments showed that the domain of Gal4 responsible for binding to the DNA and the domain responsible for activating transcription were able to function independently (Keegan *et al.*, 1986). Fusion of a protein X to the fragment of the Gal4 (aa 1-147) responsible for DNA binding ($GAL4_{BD}$) will produce a fusion protein able to bind the UAS_{GAL}, but not activate transcription of the *GAL1* yeast promoter (see Figure 1C). Fusion of a gene Y to the

Figure 1. The two-hybrid system.

The Gal4 transcriptional activator, containing a DNA binding domain and transcription activation domain, binds to the *GAL4* upstream activating sequence (UAS_G) and activates transcription of the β-galactosidase reporter gene from the *GAL1* promoter (A). The Gal4 transcription activating domain fused to Protein Y (B) or the Gal4 DNA binding domain fused to Protein X (C) alone are insufficient for the activation of transcription of the β-galactosidase reporter gene from the *GAL1* promoter. When the two Gal4 domains are brought together by the interaction of Protein X and Protein Y, transcription of the reporter gene is activated from the *GAL1* promoter (D).


A.
GAL4
Gal4BD
GalAD
UASG
GAL1
la cZ
Y
GalAD
B.
UASG
GAL1
la cZ
C.
X
Gal4BD
UASG
GAL1
la cZ
D.
X
Y
GalAD
Gal4BD
UASG
GAL1
la cZ

fragment of Gal4 (aa 768-881) that is responsible for transcription activation ($GAL4_{AD}$), produces a fusion protein that is unable to bind the *GAL1* yeast promoter and therefore is also unable activate transcription (see Figure 1B). Interaction between protein X and Y, however, will reconstitute the functional Gal4 protein, and thereby activate transcription of the *lacZ* reporter gene from the GAL1 promoter (see Figure 1D). The yeast Snf1 and Snf4 proteins were used to show that this reconstitution of the functional Gal4 protein, using interacting fusion proteins, was possible (Fields and Song 1989).

Dr. S. Fields realized that detecting the interaction between $Gal4_{BD}$ and $Gal4_{AD}$ fusion proteins could be used as a molecular genetic screen for proteins that interact with a protein of interest. In this two-hybrid system screen the two *GAL4* domains, $GAL4_{BD}$ and $GAL4_{AD}$, are supplied on two different plasmids. The protein of interest (X) can be cloned into the plasmid carrying the $GAL4_{BD}$ to produce a fusion protein which is able to bind the UAS_{GAL} DNA sequence (Figure 1B). A library of DNA sequences (Y), genomic (yeast) or cDNA (mammalian), can be cloned into the plasmid carrying the $GAL4_{AD}$ to produce fusion proteins able to activate transcription, but not bind DNA (Figure 1C). The library of activation domain fusion genes can be screened by transformation into a yeast strain containing the $GAL4_{BD}$-X fusion plasmid. If the two fusion proteins produced from the $GAL4_{AD}$-Y library plasmid and $GAL4_{BD}$ plasmid interact, then they will activate transcription of the *lacZ* reporter gene from the *GAL1* promoter, producing β-galactosidase in that particular yeast colony. β-galactosidase gives rise to a blue yeast colony on medium containing the chromogen X-gal. An advantage of the two-hybrid system is that the gene coding for the interacting $GAL4_{AD}$ library fusion protein can be isolated from the $GAL4_{AD}$ plasmid found in the yeast colony.

Since its introduction the two-hybrid system has been used to confirm or discover novel protein:protein interactions; examples include interactions between two copies of the yeast Sir4 protein (Chien *et al.*,1991), the yeast Rap1 and Rif1 proteins (Hardy *et al.*, 1992), the yeast Snf1 and Sip1 proteins (Yang *et al.*, 1992), the human hSos1 and GRB2 proteins (Chardin *et al.*, 1993), the human $hTAF_{II}250$ and tata binding protein (Ruppert *et al.*, 1993), the HIV IN and IN proteins (Kalpana and Goff, 1993), and the amino and carboxy domains of the human Rb protein (Hensey *et al.*, 1994). Several of these studies have used deletion analysis of the fusion genes in this system to define the specific regions responsible for the interaction.

A modification of the original two-hybrid system, described by Zervos *et al.*, (1993), utilizes the *E. coli lexA* repressor in place of the $GAL4_{BD}$. This version requires the replacement of the UAS_{GAL} DNA sequence in the *GAL1* promoter with the *lexA* operator sequence. The $GAL4_{AD}$ libraries can be used interchangeably with both DNA binding domain plasmids.

v. Thesis Objectives

The objectives of this thesis were to identify proteins, using the yeast two-hybrid system, that interact with the product of the nucleotide excision repair gene *RAD7*, and determine the biological significance of these interactions.

MATERIALS AND METHODS

i. Bacterial Strains

The bacterial strains used in this study are listed in Table 3.

Table 3: Bacterial Strains

Strain	Genotype	Source	Reference
DH5α	F⁻, *recA*1, *endA*1, *gyrA*96, *thi*, *hsdR*17, *supE*44, *relA*1, Δ(*argF-lacZYA*) U169 (ϕ80d*lacZ*ΔM15)λ⁻	R.D. Gietz	Gibco BRL
JM109	*recA*1, *endA*1, *gyrA*96, *thi*, *hsdR*17, *supE*44, *relA*1, λ⁻, Δ(*lac-proAB*), [F', *traD*36, *proA*⁺*B*⁺, *lac*I^q^ZΔM15	R.D. Gietz	Yanisch-Perron *et al.*, 1985

Bacterial strains were stored at -70°C and streaked onto solid LB medium and grown overnight before use in experiments. Single colonies from these plates were used to inoculate liquid cultures.

ii. Yeast Strains

Yeast strains used in this study are listed in Table 4.

Table 4: Yeast Strains

Strain	Genotype	Source	Reference
GGY1::171	*leu2 his3 ura3 gal4Δ gal80Δ URA3::GAL1-lacZ*	S. Fields	Gill and Ptashne, 1987
CTY10-5D	*MAT*a *ade2 gal4 gal80 his3-Δ200 leu2-3, 112 trp1-Δ901 URA3::lexA op GAL1-lacZ*	S. Fields	Schiestl *et al.*, 1993
MKP°	*MAT*α *can1-100 ade2-1 lys2-1 ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901*	R.D. Gietz	Pierce *et al.*, 1987
LP2752-4B	*MAT*α *lys1-1 ura3-52 his4-864,1176* pBR313 *his4-260,39*	R.D. Gietz	Schiestl and Prakash., 1988
XS803-3A	*MAT*a *leu2-3, 112 ura3-52 his1-1 trp2*	R.D. Gietz	Schiestl *et al.*, 1990
RS185	*MAT*a *leu2-3,112 ura3-52 his1-1 can1^s trp2 HOMO3* *MAT*α *leu2-3,112 ura3-52 his1-7 can1^r TRP2 hom3-10*	R.D. Gietz	This study
RS195	*MAT*α *leu2-3,112 ura3-52 his1-7 can1^r ADE2 TRP1 hom3-10* *MAT*a *LEU2 ura3-52 his3-832 can1^s ade2-3 trp1-289 HOM3*	R.D. Gietz	This study

Yeast strains stored at -70°C, were streaked onto solid YPAD medium and grown for

two days.. Single colonies from these plates were used to inoculate liquid cultures.

iii. Plasmids.

Plasmids that were not created as part of this study are listed in Table 5.

Table 5: Plasmids

Plasmid	Genes	Source	Reference
pGAD1, 2, and 3	*LEU2*, $GAL4_{AD}$, Amp^R	S. Fields	Chien *et al.*, 1991
pBTM116	*TRP1*, $lexA_{BD}$, Amp^R	S. Fields	
pMA424	*HIS3*, $GAL4_{BD}$, Amp^R	S. Fields	
pMA424-*SNF1*	*HIS3*, $GAL4_{BD}$, Amp^R, *SNF1*	S. Fields	Chien *et al.*, 1991
pGAD-*SNF4*	*HIS3*, $GAL4_{BD}$, Amp^R, *SNF4*	S. Fields	Chien *et al.*, 1991
pMA424-*D12*	*HIS3*, $GAL4_{BD}$, Amp^R, *D12*	R.D. Gietz	
pBTM116-*RAD6*	*TRP1*, $lexA_{BD}$, Amp^R, *RAD6*	R.D. Gietz	
pBTM116-*RAD7*	*TRP1*, $lexA_{BD}$, Amp^R, *RAD7*	R.D. Gietz	
pBTM116-*RAD10*	*TRP1*, $lexA_{BD}$, Amp^R, *RAD10*	R.D. Gietz	
YEplac181	*LEU2*, Amp^R	R.D. Gietz	Gietz and Sugino, 1988
YCplac33	*URA3*, Amp^R	R.D. Gietz	Gietz and Sugino, 1988
YEplac112	*TRP1*, Amp^R	R.D. Gietz	Gietz and Sugino, 1988
pHR45-15	*LEU2*, *SIR3*, Amp^R	D.E. Gottschling	
pGEX-2T	Sj26(GST), Amp^R	M. Mowat	Smith and Johnson, 1988
pKM1310	*TRP1*, Amp^R, ha tag	K. Madura	
pDG18	Amp^R, *rad1Δ*, *URA3*	R.D. Gietz	Schiestl and Prakash, 1988
pDG47	Amp^R, *rad6Δ*, *URA3*	R.D. Gietz	
pDG79	Amp^R, *rad7Δ*, *URA3*	R.D. Gietz	Schiestl and Prakash, 1989
pDG323	Amp^R, *RAD18*	R.D. Gietz	
pDG649	*TRP1*, $lexA_{BD}$, Amp^R, *RAD7*	R.D. Gietz	
pCHS6.3	Amp^R	C. Sommers	

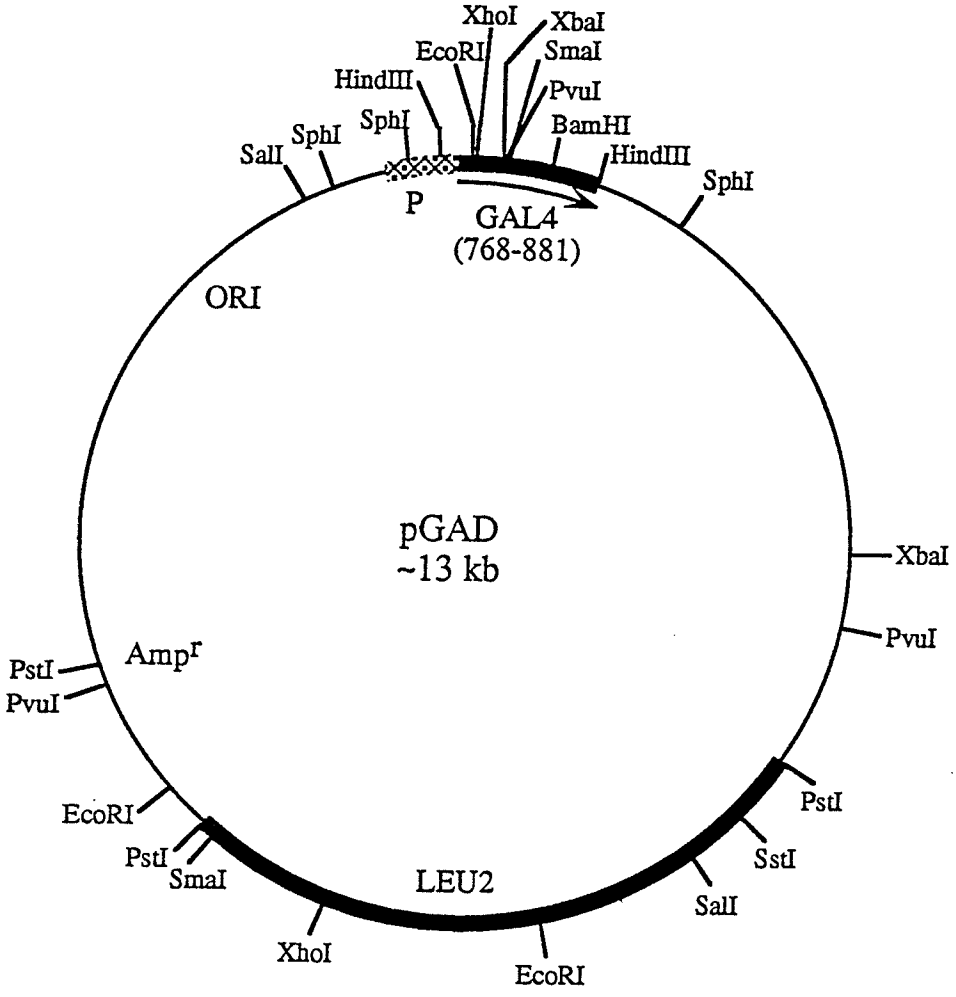
Plasmid DNA was stored at -20°C in TE buffer.

Figure 2. Two-hybrid System Plasmid Restriction Maps

A. pGAD: The three *LEU2* 2μm based pGAD plasmids contain the SV40 T antigen nuclear localization signal fused to the $GAL4_{AD}$, under the control of promoter P. The unique *Bam*H1 site, within the *Sph*I fragment, is located after codon 881 of *GAL4* in each plasmid. The reading frame of the *Bam*HI site in each vector, in respect to the *GAL4* gene, is shown below the plasmid (Chien et al., 1991).

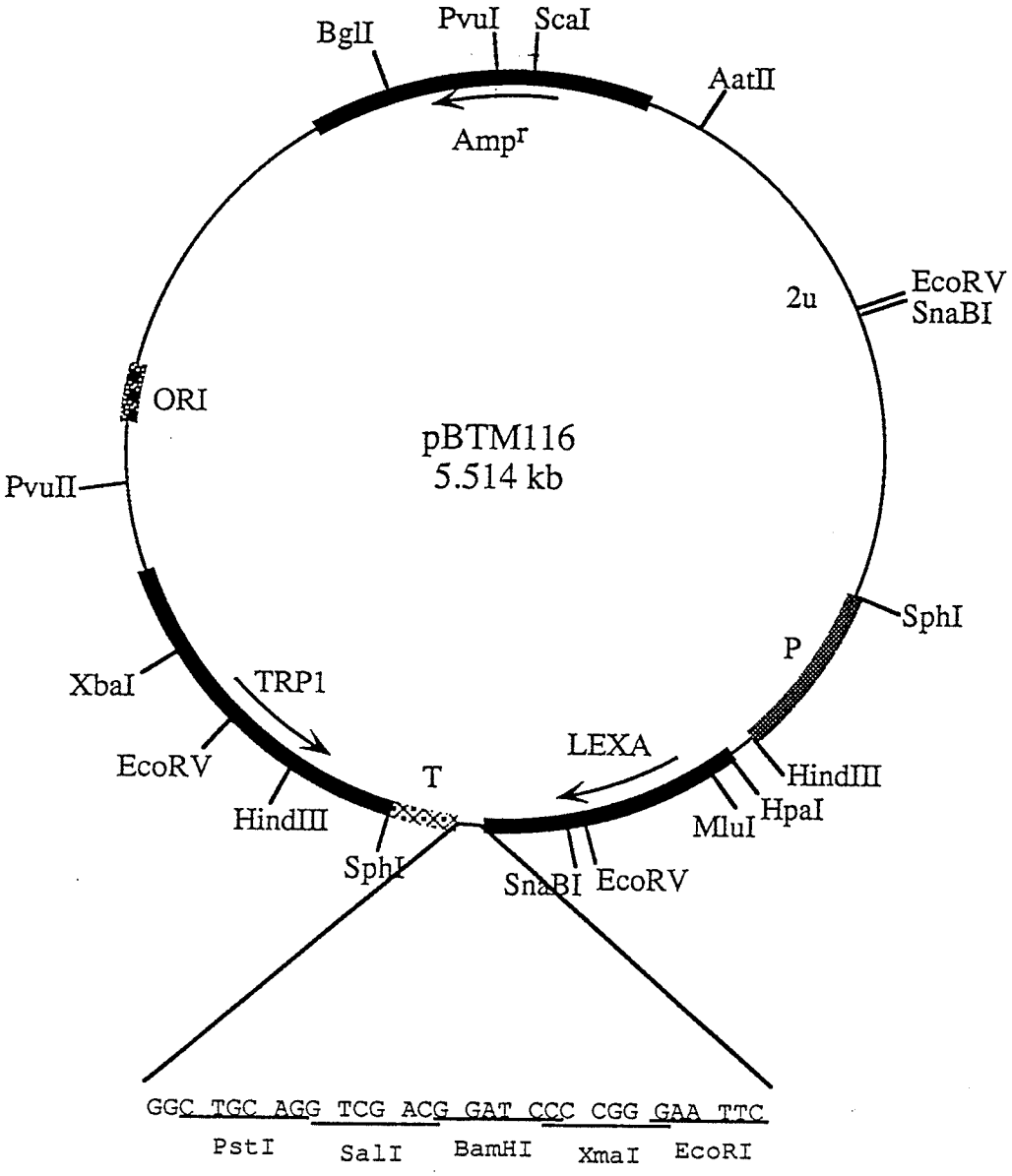
B. pBTM116: The *TRP1* 2μm based plasmid contains the *lexA* DNA binding domain under the control of an *ADH1* promoter. Specific genes can be cloned into the multicloning site to produce $lexA_{BD}$ fusion proteins (S. Fields, personal communication).

A.



Vector	Reading Frame of BamHI Site
pGAD1	GGA TCC
pGAD2	XGG ATC CXX
pGAD3	XXG GAT CCX

B.


BglII
PvuI
ScaI
AatII
Amp^r
EcoRV
SnaBI
2u
ORI
pBTM116
5.514 kb
PvuII
SphI
P
XbaI
TRP1
LEXA
EcoRV
T
HindIII
HindIII
HpaI
MluI
SphI
SnaBI
EcoRV
GGC TGC AGG TCG ACG GAT CCC CGG GAA TTC
PstI
SalI
BamHI
XmaI
EcoRI

iv. Two-hybrid System Vectors

Vectors used in the two-hybrid system were supplied by Dr. S. Fields and are shown in Figure 2. The structure of the pGAD1, 2, and 3 vectors, which contain the $GAL4_{AD}$ fused to a nuclear localization signal from SV40 T antigen, are shown in Figure 2A. The unique *Bam*HI site, found after codon 881 of *GAL4*, can be used as a cloning site to insert random yeast genomic DNA *Mbo*I fragments. In each of the three plasmids the reading frame of the *Bam*HI site differs with respect to that of *GAL4*. This ensures that any specific *Mbo*I fragment should be able to produce a fusion protein in the correct reading frame when cloned into one of the three pGAD vectors.

The structure of the pBTM116 vector, which contains the *lexA* DNA binding domain ($lexA_{BD}$) fused to a nuclear localization signal from SV40 T antigen, is shown in Figure 2B. A gene of interest can be cloned, in frame, into the multicloning site between these two domains to produce a fusion with the $lexA_{BD}$ and the nuclear localization signal. A pBTM116-*RAD7* vector, containing *RAD7* cloned into the *Eco*RI site of the multicloning site was created by Dr. R.D. Gietz.

v. Bacterial Media

Bacterial media was prepared as described in Maniatis *et al.*, 1982. All media were autoclaved for 20 min at 125°C. Bacto-agar was only added to solid media.

Luria Bertani (LB):

Bacto-yeast Extract	3 g
Bacto-tryptone	6 g
NaCl	6 g
Bacto-agar	9 g
pH	7.0
Water to	600 ml

LB + ampicillin contained 50 µg/ml ampicillin (Sigma) added after autoclaved medium had been cooled to 50°C.

SOC:

Bacto-yeast extract	12 g
Bacto-tryptone	3 g
NaCl	0.36 g
KCl	0.108 g
Dextrose	1.2 g
Water to	600 ml

6 ml of a filter sterilized solution of 1 M $MgCl_2$; 1 M $Mg(SO_4)_2$ was added after autoclaving.

M9 minimal medium:

Na_2HPO_4	3.6 g
KH_2PO_4	1.8 g
NaCl	0.3 g
NH_4Cl	0.6 g
Dextrose	1.2 g
$MgSO_4$	0.6 g
$CaCl_2 \cdot 2H_2O$	3.3 mg
Thiamine	1.2 mg
$FeCl_3$	0.32 mg
Each Supplement*	12 mg
Water to	600 ml

*Supplements: methionine, arginine, histidine, leucine, proline, threonine, tryptophan and uracil. M9 methionine medium was made by omitting the methionine.

via. Yeast Media

Yeast media was prepared as described in Sherman, 1991.
YPAD:

Bacto-yeast extract	6 g
Bacto-peptone	12 g
Dextrose	12 g
Adenine hemisulphate	60 mg
Bacto-agar	10 g
pH	6.0
Water to	600 ml

Synthetic Complete (SC):

Difco Yeast Nitrogen Base (w/o amino acids)	4 g
Dextrose	12 g
Amino acid Mix	0.4 g
Bacto-agar	10 g
pH	5.6
Water to	600 ml

Amino acid Mix

Supplement	Amount	Final Concentration
Adenine sulfate	1.8 g	30 mg/L
Arginine HCl	1.2 g	20 mg/L
Glutamic Acic	6.0 g	100 mg/L
Histidine HCl	1.2 g	20 mg/L
Inositol	2.0 g	33 mg/L
Isoleucine	1.8 g	30 mg/L
Leucine	1.8 g	30 mg/L
Lysine HCl	1.8 g	30 mg/L
Methionine	1.2 g	20 mg/L
p-Aminobenzoic acid	0.2 g	3 mg/L
Phenylalanine	3.0 g	50 mg/L
Homoserine	6.0 g	100 mg/L
Tryptophan	2.4 g	40 mg/L
Tyrosine	1.8 g	30 mg/L
Uracil	1.2 g	20 mg/L
Valine	9.0 g	150 mg/L

SC minus medium was prepared by omitting the appropriate amino acid supplement from the mix.

SC raffinose, SC galactose were prepared by substituting raffinose or galactose for dextrose in SC medium.

X-gal medium:
SC medium with the following changes: sucrose replaced dextrose as the carbon source; 35 ml of 1.0 M KPO_4 was added after autoclaving and cooling to 50°C; X-gal was added to a final concentration of 100 µg/ml from a stock solution of 60 µg/ml in DMF (Chien *et al.*, 1991).

vii. Electroporation of Bacteria Cells

Electrocompetent DH5α *E. coli* cells were prepared, and plasmid DNA electroporated into them, by the method described by Dower *et al.* (1988). Cultures were grown in 1 L of LB to an OD_{600} between 0.5 and 1.0. The cells were quick cooled in an ice-water bath to 0°C and harvested by centrifugation at 4000 x g for 15 min at 4°C. The cell pellet was resuspended in 1 L of sterile ice-cold nanopure water and collected by

centrifugation. The cells were washed once with 500 ml of ice-cold water, once with 20 ml of ice-cold 10% (v/v) glycerol, and resuspended in a final volume of 2 ml ice-cold 10% glycerol. The cells were dispensed into 50 µl aliquots in 0.5 ml microfuge tubes, frozen with liquid nitrogen and stored at -80°C. For electroporation, a 50 µl aliquot of competent cells was thawed, mixed with 1-2 µl of plasmid DNA (10-100 ng) and electroporated in a 0.1 cm electroporation cuvette (BioRad) using the BioRad Genepulser™ set to 1.25 kV and 25 µFD, with a pulse controller with a 400 Ω resistor in parallel with the sample. The pulse length varied from 7-9 milliseconds. Immediately after electroporation 1 ml of SOC medium was added to the cuvette and the cell suspension was transferred to a sterile culture tube and incubated at 37°C for 30 min before plating on LB + ampicillin plates.

viii. Isolation of Bacterial Plasmid DNA

Plasmid DNA was isolated from *E. coli* cells using the method of Birnboim and Doly (1979). Single bacterial colonies were inoculated in 2 ml of LB + ampicillin and incubated with shaking for 12-16 hr at 37°C. 1.5 ml of the culture was then transferred to a microfuge tube and centrifuged for 1 min at 12,000 x g. The pellet was resuspended in 100 µl of TGE (25 mM Tris-HCl pH 8.0; 1% (w/v) glucose; 50 mM EDTA pH 8.0) and lysed by the addition of 200 µl of lysis buffer (0.2 N NaOH; 1% SDS) and incubation on ice for 5 min. Potassium acetate (150 µl of 3 M, pH 4.8) was then added and mixed followed by an additional 5 min incubation on ice. The supernatant was collected after centrifugation at 12000 x g at 4°C for 5 min and removed to a fresh tube. One volume of TE-saturated phenol: chloroform (1:1 v/v) was added, the phases were mixed by vortexing for 1 min, and separated by centrifugation at 12,000 x g for 1 min. The aqueous phase was removed and the nucleic acids precipitated by the addition of 2.5 volumes of cold absolute ethanol. The

nucleic acids were collected by centrifugation at 12000 x g at 4°C for 10 min, the pellet rinsed with 70% EtOH, air dried for up to 10 min and finally dissolved in 50 µl TE (10 mM Tris-HCl pH 8.0; 1 mM EDTA).

ix. Restriction Endonuclease Digestion of DNA

Purified DNA was digested according to the specifications provided by the manufacturer. RNAse A was added to a final concentration of 40 µg/ml to restriction digests containing RNA. The digestions were incubated for 2-12 hr with a 2-10 fold excess of enzyme.

x. Agarose Gel Electrophoresis of DNA

Restriction enzyme digests of plasmid DNA were analyzed using agarose gel electrophoresis (Maniatis *et al*, 1982). Gels were made by dissolving ultraPURE agarose (Gibco BRL) at a number of concentrations ranging from 0.45% to 1% (w/v) in TAE buffer (40 mM Tris acetate pH 8.3, 2 mM EDTA). The molten agarose was cast in a horizontal gel apparatus after the addition of EtBr to 0.5 µg/ml. 0.1 volumes of 10 x loading dye containing 0.125% (w/v) bromophenol blue, 0.125% (w/v) xylene cyanol FF and 50% (v/v) glycerol was added to all samples. A 1 kb ladder (BRL) was used as a size standard. Restriction enzyme digests were visualized after electrophoresis by exposure to short range ultraviolet light (UV) using a hand held transilluminator (UVProducts). Gels were photographed with a Polaroid MP4 camera (f8, 10 sec exposure) using polaroid type 667 film and a Kodak #9 Wratten filter, while being illuminated using a Spectroline UV 254 nm transilluminator.

xi. DNA Fragment Isolation

DNA fragments were isolated from agarose gels using the paper:dialysis membrane

dam method of Girvitz *et al.* (1980) with minor modifications. Typically the restriction digest reaction containing up to 10 µg of plasmid DNA was loaded into two 50 µl wells in a 0.7% agarose minigel and electrophoresed until the DNA fragment desired had separated as observed by a short exposure to long range UV. An incision was made into the agarose gel in front of the DNA fragment to be isolated. If the gel contained a larger DNA fragment, it was removed by cutting the gel between it and the desired fragment. A large piece of 3MM Whatman paper backed with dialysis membrane was cut to size and inserted into the incision, creating a barrier to trap the migrating DNA. The DNA electrophoresis was continued until the entire DNA fragment had migrated into the paper:membrane dam. The paper:membrane insert was then removed and inserted into a decapitated 500 µl microfuge tube with an 18 gauge needle hole in the bottom nested inside a decapitated 1.5 ml microfuge tube. The DNA was eluted by the addition of 100 µl of Band Elution buffer (BEB) (50 mM Tris-HCl pH 7.6, 0.2 M NaCl, 1 mM EDTA, 0.1% SDS), followed by a 2 min incubation and a 700 x g centrifugation for 15 sec. The elution procedure was repeated three times and was followed by one 13000 x g centrifugation to remove all the liquid from the paper:membrane. The eluates from each spin were pooled, and extracted once with TE-saturated phenol:chloroform (1:1). The aqueous phase was removed and the DNA precipitated by the addition of 0.1 vol of 3.0 M sodium acetate, pH 6.0, and two volumes of 100% ethanol. The DNA was pelleted by centrifugation at 13,000 x g for 5 min, washed with room temperature 70% ethanol, dried briefly, and dissolved in 20-50 µl of TE. The concentration of the DNA was determined by comparison with standards after electrophoresis of a fraction of the sample on an agarose gel containing EtBr (0.5 µg/ml).

xii. Preparation of Vector DNA for Cloning

All vector DNA to be used for plasmid constructions was digested with an appropriate restriction enzyme(s), and then treated with 1-2 units of calf intestinal alkaline phosphatase (CIP; Boehringer Mannheim) for 30 to 45 min at 37°C, to prevent self ligation. CIP was added directly to the restriction digests, after they had been confirmed to be complete, of CsCl purified vector DNA that did not contain RNA (gift from Dr. R.D. Gietz). CIP was added to purified DNA fragments containing the *E. coli ori* and *β-lactamase* genes, purified from agarose gels as described above, after dissolving the DNA in TE, usually 43 µl, and adding 5 µl of 10 x dephosphorylation buffer (0.5 M Tris-HCl pH 8.5, 1 mM EDTA). Restriction enzyme cut plasmid DNA preparations that contained RNA oligomers, as a result of digestion with RNAse A, were mixed with 0.6 volumes of 2.5 M NaCl, 20% (w/v) PEG 8000 and incubated for at least 1 hr in an ice water bath. This treatment precipitates the large molecular weight DNA but leaves small molecules, such as RNA oligomers and small DNA fragments. in solution The plasmid DNA was pelleted by centrifuged at 13000 x g for 5 min at 4°C, rinsed in 70% EtOH and resuspended in 43 µl of TE. The PEG precipitated vector DNA was then treated with CIP after the addition of 5 µl of 10 x dephosphorylation buffer.

CIP was removed from all treated plasmid DNA preparations by extraction with 1 vol of TE-saturated phenol:chloroform (1:1 v/v). The DNA was precipitated and then collected by centrifugation at 13,000 x g for 5 min, washed with room temperature 70% ethanol, dried briefly, and the pellet dissolved in 50 µl of TE.

xiii. Ligation Reactions

Ligation reactions were prepared according to the method of Maniatis *et al.*, 1982.

a. Sticky-end ligations

5' or 3' sticky end ligation reactions contained approximately 200 ng of vector DNA; at least an equal molar amount of insert ends; 1 x Ligase buffer (10 x Ligase Buffer = 0.66 M Tris-HCl pH 7.5, 50 mM $MgCl_2$, 50 mM dithiothreitol, 10 mM ATP (Maniatis et al., 1982)), and 0.5-1.0 units T4 DNA ligase (Boehringer Mannheim), in a final volume of 20 µl. The reactions were incubated for 12-16 hr at 11-15°C to increase the half life of the T4 DNA ligase in the reaction.

b. Blunt-end ligations

Blunt end ligations, requiring the conversion of a 3' OH recessed end to a blunt end, contained 200 ng of vector; an up to 3 x molar excess of insert DNA; 1 x nick translation buffer (50 mM Tris HCl pH 7.2, 10 mM $MgCl_2$); 100 µM of each deoxyribonucleotide triphosphate (A, C, G, T); 2-5 units *E. coli* DNA polymerase I Klenow fragment (Pharmacia or Boehringer Mannheim). These reactions were incubated at room temperature for 30 min to allow for the Klenow polymerase to fill the ends. After this, dithiothreitol to 5 mM, ATP to 1 mM, and 2-5 units of T4 DNA ligase were added and the reaction incubated at 11°C for 12-16 hr.

The DNA in each ligation reaction was precipitated with the addition of Na acetate and ethanol, collected by centrifugation, rinsed in 70% ethanol and dissolved in 20 µl of TE in preparation for electroporation into *E. coli* cells.

xiv. Plasmid DNA Isolation from Yeast

Plasmid DNA to be used for electroporation into *E. coli* was isolated from yeast cultures using the method of Hoffman and Winston (1987). Liquid cultures (2 ml) were grown to saturation at 30°C in SC minus medium to select for the plasmid. The cells were

harvested by centrifugation for 1 min at 6000 x g after transfer into a 1.5 ml centrifuge tube. The cell pellet was resuspended in 200 µl of yeast cracking buffer (2% (v/v) Triton X-100, 1% (w/v) SDS, 100 mM NaCl, 10 mM Tris HCl pH 8.0 and 1 mM EDTA) and approximately 30 mg of acid-washed 40 nm diameter glass beads (Sigma) and 200 µl of TE-saturated phenol:chloroform (1:1 v:v) were added. The tube was vortexed for 2 min, centrifuged at 6000 x g for 1 min, the aqueous phase was recovered, and extracted again with 1 volume of TE-saturated phenol:chloroform (1:1). The nucleic acids were precipitated from the aqueous phase by the addition of 0.1 volumes 3.0 M Na Acetate, pH 6.0, and 2.5 volumes of ethanol, pelleted by centrifugation at 13,000 x g for 10 min and dissolved in 50 µl TE.

xv. High Molecular Weight Genomic DNA Isolation from Yeast

Genomic DNA was purified from the yeast strain GGY1::171 using the method of Cryer *et al.* (1975). Yeast cells were grown in 400 ml of YPAD to late log phase, harvested by centrifugation at 4000 x g for 5 min, washed with 10 ml of sterile water and pelleted again by centrifugation. The cell pellet was resuspended in 10 ml SCE (1 M sorbitol; 100 mM Na Citrate; 50 mM EDTA ph 7.5), containing 1 mg/ml of Zymolase (70,000) and 114 mM β-mercaptoethanol, and incubated for 1 - 2 hr at 37°C. The production of spheroplasts was monitored by observing cell lysis under a microscope after the addition of a small drop of 20% SDS to a corner of the coverslip. Spheroplasting was considered complete when at least 90% of the cells lysed upon addition of SDS. The spheroplasts were pelleted by centrifugation at 450 x g for 5 min and gently resuspended in 10 ml of 50 mM EDTA pH 8.0, 150 mM NaCl. Proteinase K was added to a final concentration of 100 µg/ml, followed by the addition of SDS to a final concentration of 1% (w/v) with gentle mixing to ensure

proper lysis. This viscous mixture was then incubated at 50°C for 1 hr.

The yeast cell lysate was then extracted with 1 volume of TE-saturated phenol and the phases separated by centrifugation at 2000 x g for 5 min. The aqueous layer was transferred to a fresh tube and extracted again with 1 volume of TE-saturated phenol:chloroform (1:1). The aqueous phase was transferred to a fresh tube and the nucleic acids were precipitated by the addition of 0.1 volumes 3.0 M Na Acetate pH 6.0 and 2 volumes ice-cold ethanol. The precipitated DNA was removed using a hooked glass rod, dried briefly by blotting on a paper towel and dissolved in 2 - 5 ml of TE by incubation at 4°C overnight. The concentration of the DNA was determined by comparison with 1 kb DNA standards (BRL) after electrophoresis of a fraction of the sample on an agarose gel containing EtBr (0.5 μg/ml).

xvi. High Efficiency Yeast Transformation

Plasmid DNA was transformed into yeast cells by the high efficiency transformation method of Gietz *et al.* (1992). A single yeast colony was inoculated into 10 ml of YPAD liquid medium and grown overnight at 30°C to a density of greater than 1 x 10^8 cells/ml. 100 ml of prewarmed YPAD was inoculated with the 10 ml culture and grown to a density of 2 x 10^7 cells/ml. The yeast cells were harvested by centrifugation for 5 min at 3000 x g, washed 1 to 3 times with sterile water, washed once with 1 ml of 100 mM LiAc or TE LiAc (10 mM Tris-HCl pH 7.5; 1 mM EDTA; 100 mM LiAc), resuspended in 1 ml of the same to give 2 x 10^9 cells/ml, and incubated at 30°C for 15 min. 50 μl of the cell suspension was added to 5 μl of plasmid DNA and 5 μl of single stranded carrier DNA (1 mg/ml). 300 μl of PEG/LiAc (40% PEG 3350 (Sigma); 100 mM LiAc) or PEG/TELiAc (40% PEG 3350 (Sigma), 10 mM Tris-HCl pH 7.5; 1 mM EDTA; 100 mM LiAc) were added, and this

solution was incubated for 30 min at 30°C, heat shocked for 20 min at 42°C, chilled briefly on ice, and centrifuged at 13,000 x g for 15 sec. After removal of the PEG/LiAc, the transformed yeast cells were resuspended in 1 ml of sterile water, and 200 µl aliquots were plated onto appropriate SC minus medium.

xvii. DNA Sequencing

Plasmid DNA was prepared and sequenced using the method of Hattori and Sakaki (1986) and the Sequenase kit version 2.0 from USB. Purified plasmid DNA was prepared from a 2 ml LB + ampicillin culture, as described, and dissolved in 40 ml of TE buffer. This DNA was denatured in preparation for sequencing by adding 18 μl of PEG purified DNA to 2 μl of 2.0 N NaOH and incubating at room temperature for 5 min. Following this, 8 μl of 5.0 M ammonium acetate and 100 μl of ethanol were added, the samples were incubated for 5 min at -70°C, and the precipitated DNA collected by centrifugation at 13,000 x g for 5 min at 4°C. The denatured plasmid was then sequenced according to the manufacturer's specifications. This method provides between 200 - 400 bp of sequence information from the primer site. The 18 bp *GAL4* activation domain primer (Research Genetics, Inc.) shown below was used to sequence the positive clones from the two-hybrid screens.

Primer Sequence: 5' GAA GAT ACC CCA CCA AAC 3'

xiii. ONPG β-galactosidase Assay

β-galactosidase activity was quantified using the method described by Miller (1972), with minor modifications. Yeast cells were inoculated into 2 ml of SC (raffinose) minus medium and grown for 2 days at 30°C. The cells were harvested by centrifugation at 2000 x g for 5 min and resuspended in 1 ml of Z buffer (100 mM KPO_4, 10 mM NaCl, 1 mM $MgSO_4$ and 39 mM β-mercaptoethanol). The OD_{600} of 0.5 ml of each cell suspension,

diluted in 0.5 ml of water, was read using 1 ml quartz cuvettes in a Gilford spectrophotometer. To ensure an accurate reading, cell suspensions were diluted and re-read if their OD_{600} reading was greater than 0.3. The remaining 0.5 ml of each cell suspension was used in the β-galactosidase assay. If the strain was known to have a high level of β-galactosidase activity, a smaller volume (0.025 ml), brought up to 0.5 ml with Z-buffer, was used to ensure that colour development did not occur immediately upon addition of ONPG, thereby providing an inaccurate time measurement. After the addition of 40 µl of 0.1% SDS, the samples were mixed thoroughly on a vortex mixer for 15 sec. 60 µl of chloroform was added and followed by an additional 15 sec mix. 100 µl of 4 mg/ml ONPG was added and the sample incubated at 30°C until a faint yellow colour was observed, or for 10 min if no change in colour was apparent. The reaction was stopped by the addition of 0.5 ml 1 M Na_2CO_3. The mixture was centrifuged for 1 min at 13000 x g and the absorbance at 420 nm (A_{420}) of the top 950 µl was determined. Three independent transformants for each strain tested were assayed in duplicate for β-galactosidase activity. These six readings were averaged to provide the final Miller units of β-galactosidase activity for each strain.

The activity in Miller Units of each sample was determined using the following equation:

$$\text{Miller Units} = \frac{(A_{420})(1000)}{(\text{volume})(\text{time})(OD_{600})}$$

volume = volume of cell suspension used in assay (ml)
time = time of incubation at 30°C (min)
OD_{600} = total OD of the original 1 ml cell suspension
(OD_{600} x 2 x dilution factor needed for a reading of < 0.3)

xix. Determination of Sensitivity to UV Damage

Two different methods were used to determine the sensitivity of yeast strains to 254

nm Ultra-violet radiation (UV); a) the spot test and b) the survival curve.

a). A qualitative assay (the spot test) was used to initially determine if a specific strain was sensitive to the DNA damage induced by UV in comparison to the RAD^+ wild type strain. Yeast colonies were patched to a 52 spot microtiter plate replicator grid on a 85 mm plate of appropriate medium and grown for about 2 days at 30°C. A row of RAD^+ colonies was also included on each plate for comparison. Cells from each patch were scraped and transferred, using a multiprong replicator, to a 96 well microtiter plate containing 150 µl of sterile water in each of the appropriate 52 wells. After mixing the inoculum well with the multiprong replicator, drops of the cell suspension were transferred to YPAD plates using the same multiprong replicator. The plates were dried for 5 min, giving rise to small circles of yeast inoculum, each about one cell layer thick. The plates were irradiated at various doses ranging from 5 to 60 Joules/m^2 with 254 nm UV, at a dose rate of 1-2 Joules/$m^2 \cdot sec^1$, wrapped in aluminum foil to avoid photoreactivation of pyrimidine dimers, and incubated overnight at 30°C. Cell growth in each patch was compared to the growth of the RAD^+ strain patches.

b). A quantitative test (the survival curve assay) was used to accurately determine a yeast strain's sensitivity to the DNA damage caused by exposure to UV. Liquid cultures of each strain were inoculated into YPAD or selective medium and incubated at 30°C overnight. The cell density was determined using a haemacytometer, and appropriate dilutions of cells, in water, where plated in duplicate onto YPAD plates to give 200 to 500 colonies per plate after irradiation. The dilution of cells plated was determined by estimating the expected number of surviving cells based on spot test or previous survival curve data. The maximum number of cells plated onto a single 85 mm plate was limited to 5×10^6 to avoid the

possibility of cells being shielded from the UV irradiation. Plates were irradiated in the dark using 254 nm UV, at a dose rate of 1-2 $J/m^2 \cdot sec^1$, wrapped in aluminum foil to avoid photoreactivation and incubated at 30°C. Strains highly sensitive to UV irradiation, such as *rad6* or *rad1* deletion mutants were irradiated at 5, 10 and 20 J/m^2. RAD^+ wild type strains or strains containing the *rad7* or *sir3* deletion mutants were irradiated at 10, 20, 40 and 60 J/m^2. The percent survivors of each yeast strain was determined at each dose, using the formula shown below, and plotted onto a semi-logarithmic graph to produce the survival curve.

Percent Survivors = # Col. at X J/m^2 x D./# Col. at 0 J/m^2 x D

# Col. at X J/m^2	= the average number of colonies on the two duplicate plates at a certain UV dose X.
# Col. at 0 J/m^2	= the average number of colonies on the two duplicate plates not treated with UV.
D	= dilution factor

xx. Purification of pGEX Fusion Proteins from *E. coli*

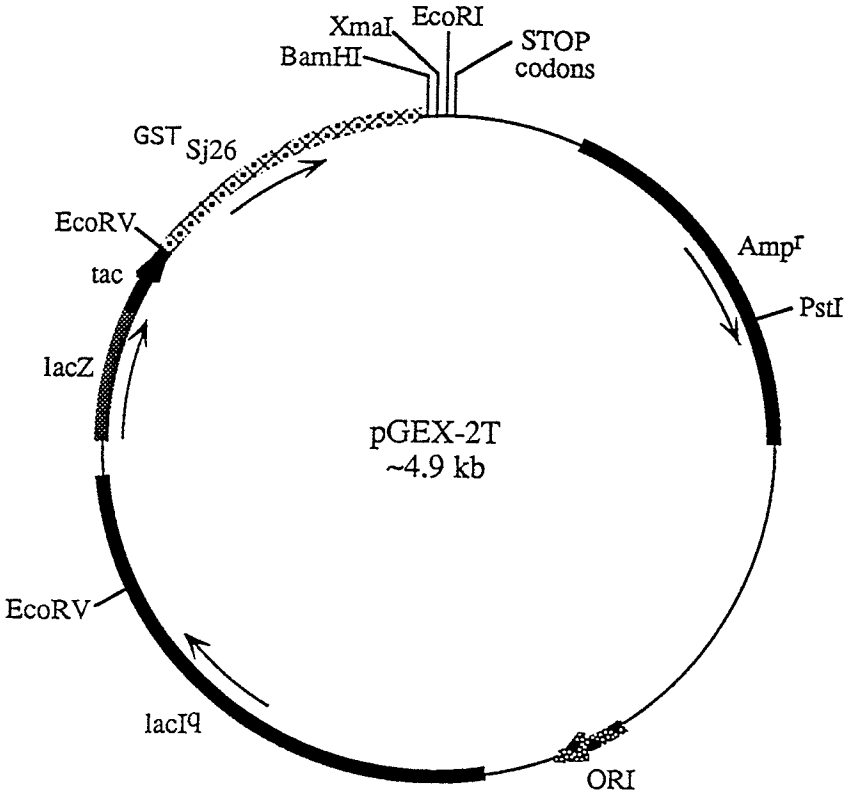
Glutathione-S-transferase (GST) fusion proteins were produced in *E. coli*, utilizing the pGEX-2T vector (Figure 3), following the methods described by Smith and Johnson (1988) and Oettinger *et al.* (1992). One hundred ml of LB + ampicillin medium, containing 1.0 M sorbitol and 2.5 mM betaine (Blackwell and Horgan, 1991) was inoculated with single colonies from each strain and incubated with shaking at 25°C until the culture reached an OD_{595} of 0.5. IPTG was then added to a final concentration of 0.5 mM and the cultures were incubated for an additional 2 hr at 25°C. The cells were harvested by centrifugation at 3000 x g for 5 min. The pellet was rinsed once with phosphate buffered saline (PBS) (20 mM Na phosphate buffer pH 7.4, 130 mM NaCl) and resuspended in 4.5 ml cold PBS containing 10 mM EDTA, 2 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin and 0.1%

Figure 3. The pGEX-2T vector.

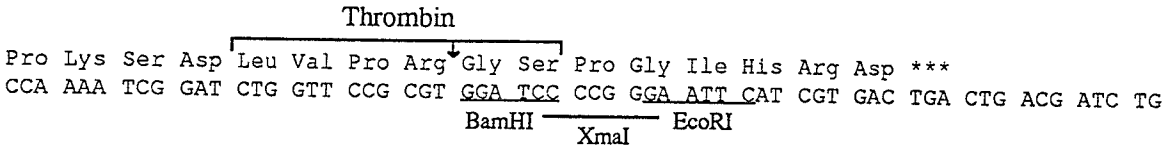
A. The pGEX-2T *E. coli* plasmid contains a copy of the gene encoding GST_{Sj26}, a 26 kDa glutathione-S-transferase (GST), fused to the multicloning site illustrated in (B). A gene can be cloned, into the unique *Bam*HI, *Sma*I or *Eco*RI sites, to produce an in frame fusion with GST. The expression of the GST fusion protein is under the control of the IPTG-inducible *tac* promoter.

B. The pGEX-2T multicloning site sequence showing the reading frame in respect to the GST protein. The thrombin cleavage-recognition site has been inserted between the C-terminus of the GST gene and the three cloning sites to allow removal of the GST portion from fusion proteins (Smith and Johnson, 1988).

A.


XmaI
EcoRI
BamHI
STOP
codons
GST
Sj26
EcoRV
tac
lacZ
Ampr
PstI
pGEX-2T
~4.9 kb
EcoRV
lacIq
ORI

B.


Thrombin
Pro Lys Ser Asp Leu Val Pro Arg Gly Ser Pro Gly Ile His Arg Asp ***
CCA AAA TCG GAT CTG GTT CCG CGT GGA TCC CCG GGA ATT CAT CGT GAC TGA CTG ACG ATC TG
BamHI
XmaI
EcoRI

(v/v) Triton X-100 and cooled in a ice water bath. The cells were sonicated for 15 sec and then cooled in an ice water bath; this was repeated 6 times. The lysates were spun at 13000 x g for 10 min at 4°C, the supernatant removed, and incubated with gentle rocking at 4°C for 20 min after the addition of 200 µl of a 1:1 slurry of glutathione-agarose beads (Sigma) in PBS. The beads were then centrifuged briefly, the supernatant was removed, and the beads rinsed 3 times in 500 µl PBS containing 10 mM EDTA, 2 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin and 0.1% (v/v) Triton X-100. The glutathione-agarose beads containing the bound GST fusion proteins were finally resuspended in one volume of the same buffer.

xxi. Purification of Radiolabelled pGEX Fusion Proteins

GST fusion proteins were metabolically labelled with [^{35}S]-methionine during production in *E. coli* (Ausubel, *et al.*, 1987). Cells were inoculated into thirty ml of LB + ampicillin medium containing 1 M sorbitol and 2.5 mM betaine and incubated with shaking at 25°C until the OD_{595} of the culture reached 0.5. The cells were pelleted and washed 3 times with 20 ml M9 minus methionine medium + 50 µg/ml ampicillin, 1 M sorbitol, 2.5 mM betaine and resuspended in 1 ml of the same. 1 mCi of [^{35}S]-methionine (Amersham) and IPTG to 0.5 mM was added and the cultures were incubated at 30°C for 2 hr. A cell extract was produced as described above.

The acid precipitable counts in each extract were determined using the method of Mans and Novelli (1961). A 10 µl radiolabelled sample was spotted onto a 1 cm^2 piece of 3MM Whatman and placed in a flask containing 100 ml of 10% (w/v) TCA. The flask containing the spotted filters was placed in a boiling water bath for 10 min, after which the hot TCA was replaced with ice-cold 10% TCA, and finally the filters were rinsed once in ice-cold 95% ethanol and air dried. The filters were placed in 1 ml of scintillation fluid and radioactivity

was determined using a Beckman LS1801 scintillation counter.

The radiolabelled GST fusion proteins were purified from the extract with 125 µl of a 1:1 slurry of glutathione-agarose beads, using the same procedure as for non-radiolabelled fusion proteins, described above. The purified radiolabelled fusion proteins were cleaved with the protease thrombin to release the yeast gene products from the GST portion of the fusion. The pelleted beads were washed once with PBS, once with PBS + 10 mg BSA and twice with thrombin cutting buffer (150 mM NaCl, 3 mM $CaCl_2$, 50 mM Tris-HCl pH 7.5). Two units of thrombin (Sigma) was added to each tube and incubated for 2 hours at room temperature. The reaction was terminated by the addition of EDTA and Triton X-100 to a final concentration of 20 mM and 1.0% (v/v), respectively. The supernatant and a 100 µl rinse (PBS, 20 mM EDTA, and 1.0% (v/v) Triton X-100) of the beads were removed and pooled.

xxii. SDS Polyacrylamide Gel Electrophoresis

Protein samples were analyzed by electrophoresis on SDS polyacrylamide slab gels using the discontinuous buffer system of Laemmli (1970) as modified by Kikuchi and King (1975). Protein samples were incubated at 90°C for 3 min with 1 volume of SDS loading buffer (3.05% SDS, 62.5 mM Tris-HCl pH 6.8, 0.72 M β-mercaptoethanol, 10% glycerol, 0.125% bromophenol blue) before electrophoresis on SDS polyacrylamide slab gels consisting of a upper stacking gel (4.5% 1:29 bis:acrylamide; 127 mM Tris-HCL, pH 6.8; 0.1% SDS; 1.3 mM APS; 9.1 mM TEMED) and a lower separating gel (10% 1:29 bis:acrylamide; 375 mM Tris-HCl, pH 8.8; 0.1% SDS; 2.2 mM APS; 3.9 mM TEMED). Gels were run at 30 mA for 3.5 hr. Nonradioactive protein bands were observed after staining with Coomassie Blue as described by Wilson (1983). Gels were submerged in stain (40% methanol; 10% acetic acid; 1 g/L Coomassie Brilliant Blue R250 (Sigma)) for 1 hr with gentle rocking, and then

destained in 10% methanol/10% acetic acid until bands were clearly visible.

xxiii. Fluorography

The method described by Chamberlain (1979) was used to fluorograph SDS polyacrylamide gels. Gels were fixed for 1 hr in 10% (v/v) acetic acid, washed with at least 20 volumes of water for 30 min to reduce acidity, and soaked in 1.0 M Na salicylate for 30 min. The gels were dried onto 3MM Whatman under vacuum for 2 hr at 70°C on a BioRad Model 583 gel dryer. Dried gels were exposed to Kodak AR X-ray film at -80°C and developed using Kodak HC-110 developer, stop and fixer according to the manufacturer's instructions.

xxiv. Radiolabelling of Yeast Proteins and Immunoprecipitation

Radiolabelling of yeast proteins and immunoprecipitation was done essentially as described by Kolodziej and Young (1991). Liquid 10 ml cultures of yeast were grown at 30°C in SC (galactose) minus medium of the appropriate type to an OD_{600} of approximately 1. The cells were harvested by centrifugation at 4000 x g for 5 min, washed twice in SC (methionine) minus galactose medium and resuspended in 1 ml of the same medium. After the addition of 150 μCi of [^{35}S]-methionine (Amersham), the samples were incubated for 1 hr at 30°C. Radiolabelled yeast cells were harvested by centrifugation at 13,000 x g for 5 sec and resuspended in 0.5 ml Maxi Buffer (10 mM Tris HCl pH 8.0; 0.14 M NaCl; 1 mg/ml BSA; 1 mM PMSF; 1% (v/v) Triton X-100; 0.2% Na deoxycholate; 0.1% SDS, 0.5% NP40; 50 mM β-mercaptoethanol) containing 20 μg/ml each of the following protease inhibitors; aprotinin, leupeptin, chymostatin, antipain, bestatin. Approximately 30 mg of acid washed 40 nm diameter glass beads (Sigma) were added to the tube, and the cells lysed by vortexing 5 times for 30 sec each followed by cooling for 30 sec in an ice water bath. The cellular

debris was pelleted by centrifugation at 13,000 x g for 10 min and the supernatant was removed to a fresh tube. The protein concentration in each extract was determined using a Bradford Protein Assay kit (BioRad). The incorporation of radiolabelled methionine into proteins was estimated by the TCA precipitable count as described above.

Proteins tagged with the 12 amino acid haemagglutinin sequence (ha) were immunoprecipitated from radiolabelled cell extracts after the addition of an approximate 10-fold excess of nonradioactive a yeast *rad7sir3* double deletion strain cell extract. The 12CA5 monoclonal antibody (Babco Co.) was added to the cell extracts and incubated on ice for at least 4 hr. The antibody complexes were harvested after the addition of 25 to 50 µl of Protein-A-Agarose beads (1:1 slurry in PBS)(Oncogene Science) and incubation at 4°C for 1 hr with rocking. The beads were harvested with 5 sec centrifugation at 13,000 x g, and were rinsed 3 times with 800 µl Maxi Buffer + protease inhibitors and once with 800 µl DB buffer (50 mM NaCl; 1 mM EDTA; 50 mM Hepes, pH 7.5). The beads were resuspended in an equal volume of SDS PAGE loading buffer, heated for 3 min at 90°C and electrophoresed on an SDS polyacrylamide gel as described above.

In one strain (MKP° *rad7Δsir3Δ*::pDP126, pDP113) the haemagglutinin tagged proteins from the SDS treated cell extract were boiled in Maxi buffer + 1% SDS, to dissociate bound proteins, and then diluted to 0.1% SDS in maxi buffer prior to addition of the antibody. The insoluble pellet from this same strain was boiled in 200 µl of maxi buffer + 1% SDS and diluted to 0.1% SDS in maxi buffer prior to addition of the antibody.

Each cell extract contained 4 to 6 x 10^6 cpm of radioactivity and approximately 2 mg of protein.

RESULTS

i. Two-hybrid pGAD Library Construction

In order to screen for proteins that interact with Rad7 in the two-hybrid system, pGAD libraries containing yeast genomic *Mbo*I DNA fragments were created. The creation of these libraries involved a) the re-construction of the pGAD vectors and b) the insertion of a library of yeast genomic DNA fragments into the new vectors.

a. pGAD Vectors

The pGAD vectors shown in Figure 2a were re-constructed in the plasmid YEplac181 (Gietz and Sugino, 1988) to improve their handling properties for transformation and subsequent analysis. This was accomplished by ligating the 1.5 kb *Sph*I fragment from each pGAD vector (1F, 2F, and 3F) into the *Sph*I site of a YEplac181 vector which has had its *Bam*HI site removed, creating vectors pDP4(1F), pDP7(2F) and pDP12(3F), shown in Figure 4. The *Sph*I fragment contains the $GAL4_{AD}$ expression constructs important for the production of $Gal4_{AD}$ fusion proteins. The reduction in size (13 kb to 7.2 kb) and the higher copy number in *E. coli* of the new pGAD like vectors makes them superior to the original pGAD vectors. The orientation of the *Sph*I pGAD fragment in each vector was determined by restriction enzyme digestion. To verify the reading frame of the *Bam*HI cloning site, each vector was sequenced using an oligonucleotide primer, which annealed upstream of the *Bam*HI site in the *GAL4* activation domain.

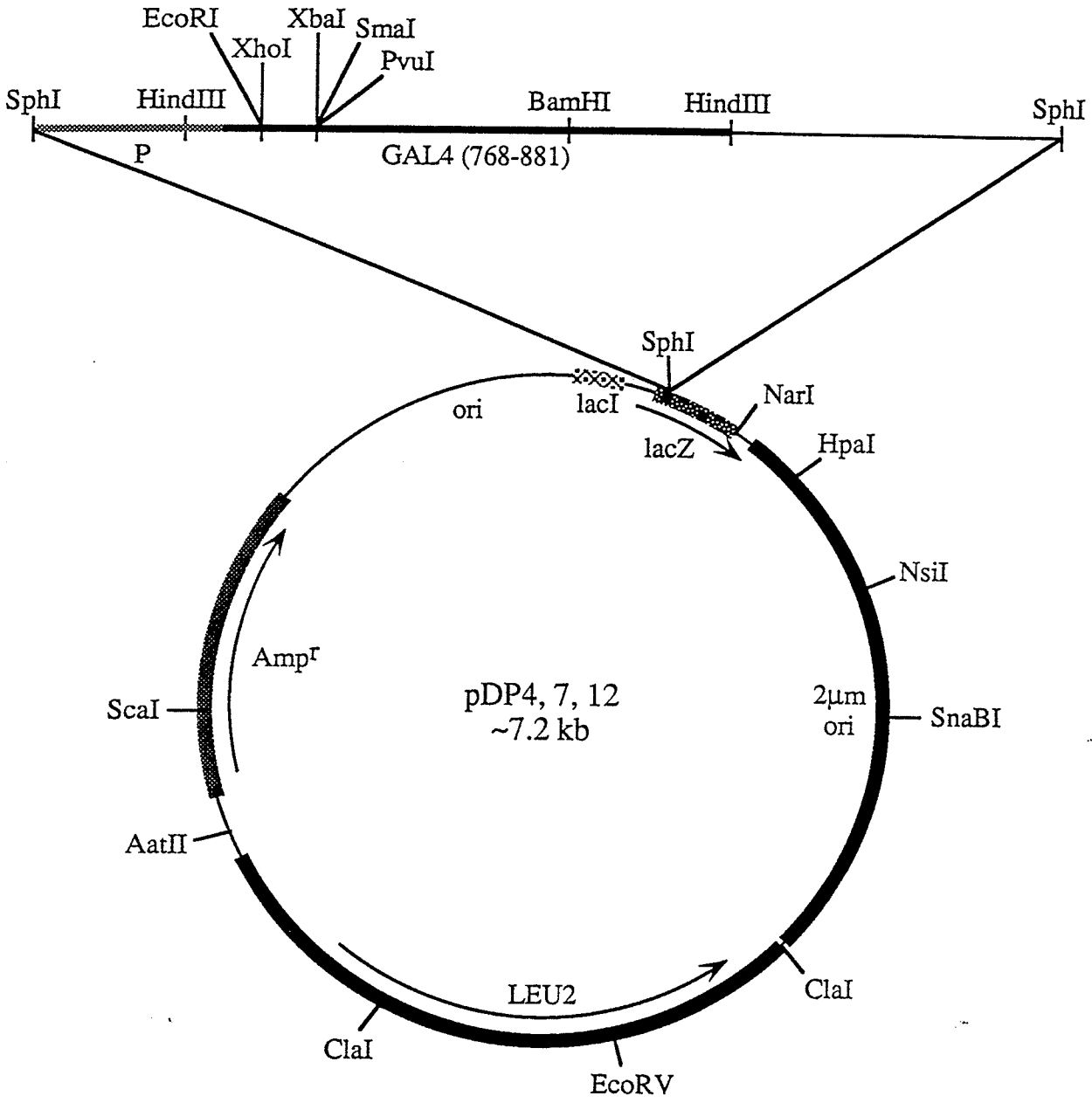
b. Library Construction

Three libraries containing 3-6 kb yeast genomic *Mbo*I DNA fragments, were constructed, one in each of the pDP vectors, as follows. Approximately 50 mg of

GGY1::171 yeast genomic DNA was partially digested with the restriction enzyme *Mbo*I. The partial digestion conditions used to produce unbiased digestion with DNA fragments in the 3-6 kb range were determined using the method described in Maniatis *et al.*(1982). The *Mbo*I partially digested DNA sample was loaded onto a 0.45% (w/v) agarose gel and electrophoresed for 450 V·hr at 4°C. The gel was stained with EtBr (0.5 µg/ml) in 1 x TAE for 30 min, the DNA size standards viewed under UV, and the position of the 3 kb band marked by inserting a toothpick into the gel. The portion of the gel containing the yeast genomic DNA was covered with a plexiglass shield to ensure that it was not exposed to UV at any time during the purification. An incision was made across the sample lanes at the position of the 3 kb marker and 4 overlapping 3MM Whatman paper/dialysis membrane dams were inserted into the incision, creating a barrier to all DNA fragments of larger size. The electrophoresis was continued until the 6 kb marker reached the paper:dialysis membrane dam (Figure 5A). The DNA was then purified from the paper:membrane pieces, as described in Materials and Methods, and re-dissolved in a total of 50 µl of TE. A 2.5 µl sample of the 3-6 kb purified DNA fragments was electrophoresed on a 0.7% agarose gel and the concentration was determined to be approximately 500 ng/ml (Figure 5B). These fragments were ligated into the *Bam*HI digested CIP treated pDP4, 7 and 12 vectors (pGAD1F, 2F and 3F, respectively). The ligation reaction was electroporated into DH5α cells by mixing 2 µl of each reaction with 50 µl of electrocompetent cells. Each electroporation reaction was plated onto 10 LB + ampicillin plates. This was repeated until the entire ligation reaction had been transformed into *E. coli*. When the colonies had grown sufficiently the plates were flooded with sterile 150 mM NaCl and then scraped using a sterile glass rod. The cell suspension was pooled and the plasmid DNA extracted using a

Figure 4. Construction of the Vectors pDP4, 7, and 12.

The pDP4, 7, and 12 vectors were constructed by cloning the 1.5 kb *Sph*I fragment, containing the *GAL4* transcription activation domain (amino acids 768-881), into the *Sph*I site of a *Bam*HI site minus YEplac181 vector. A library of *Mbo*I fragments can be inserted into the unique *Bam*HI site as indicated. The reading frame of the *Bam*HI site in each vector, in respect to the *GAL4* gene, is shown below the plasmid

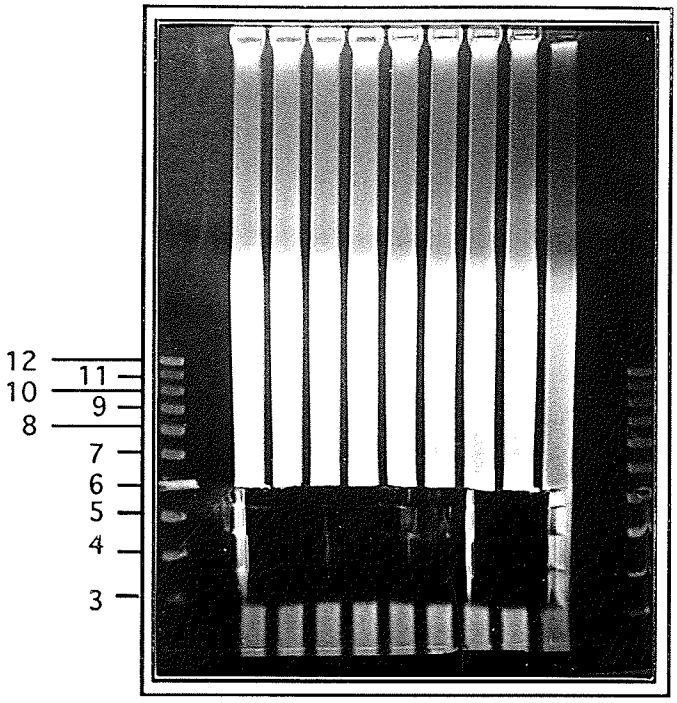


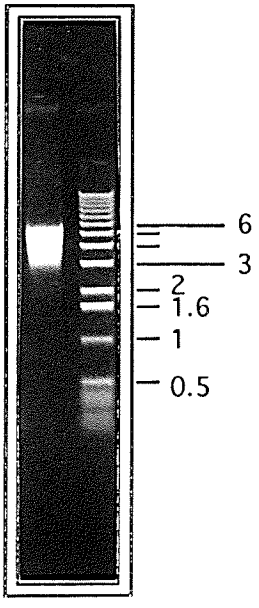
Vector	Reading Frame of BamHI Site
pDP4(1F)	GGA TCC
pDP7(2F)	XGG ATC CXX
pDP12(3F)	XXG GAT CCX

Figure 5. Purification of *Mbo*I digested yeast genomic DNA.

a. GGY1::171 genomic DNA was partially digested with *Mbo*I and electrophoresed beside 300 ng of BRL 1 kb molecular size standards (outside lanes) on a 0.45% agarose gel. The gel was run for 450 V·hr at 4°C and then stained with 0.5 μg/ml EtBr. The 3-6 kb *Mbo*I fragments were purified from the gel as described in Materials and Methods.

b. A 2.5 μl sample of purified 3-6 kb GGY1::171 *Mbo*I fragments was electrophoresed on a 0.7% agarose gel containing 0.5 μg/ml EtBr, with 1 kb DNA standards (300 ng) (BRL). The gel was run for ~75 V·hr at room temperature. The concentration of the DNA isolated was determined to be approximate 500 ng/ml.


12
11
10
9
8
7
6
5
4
3


6
3
2
1.6
1
0.5

scaled-up version of the procedure described in Materials and Methods.

To determine the percentage of library plasmids with yeast DNA inserts, between 10 and 40 *E. coli* transformants were analyzed. The insert frequency for the libraries was 90% for the pDP4 (1F) and pDP12 (3F) libraries and 60% for pDP7 (2F). Each library contained at least one million clones with inserts. According to Chien *et al.* (1991), 460,000 clones are necessary to give a probability of 0.99 that every yeast gene containing an appropriate *Mbo*I fusion site is represented in the library. The libraries constructed for this study should contain sufficient clones to represent the entire yeast genome 4.6 times. These libraries were used to screen for fusion proteins that interact with Rad7 using the two-hybrid system.

ii. The *RAD7* Two-hybrid Screen

The $GAL4_{AD}$ fusion libraries were screened for proteins that interact with Rad7 as follows. The yeast strain CTY10-5D was transformed with pBTM116-*RAD7* , and transformants were selected on SC-trp omission medium. The pDP4, pDP7 and pDP12 libraries were mixed in approximately equal proportions and transformed into the CTY10-5D strain containing the *lexA-RAD7* fusion plasmid. The transformation reactions were plated onto SC-trp-leu double omission medium and incubated 3 to 4 days at 30°C. Colonies were then replica plated using a sterile velveteen replicating system onto SC-trp-leu sucrose medium containing X-gal and incubated at 30°C for 1 - 4 days. Yeast colonies that turned blue were isolated from the original SC-trp-leu master plates and streaked onto the same medium to obtain single colonies. This plate was then replicated to X-gal medium to identify single colonies with the ability to turn blue.

In order to recover the pGAD library plasmid encoding the Rad7-interacting protein, the single blue colonies were inoculated into SC-leu liquid media and grown to saturation

to allow the loss of the pBTM plasmid. Yeast plasmids containing the 2μ origin of replication are lost from approximately 1 - 5% of all cells per generation (Gietz and Sugino, 1988). An appropriate dilution of a saturated liquid culture, usually 2 μl of a 10^{-1} dilution, was plated onto SC-leu plates and incubated until colonies appeared. The colonies were then replica-plated onto both SC-trp-leu plates and SC-leu X-gal plates. Yeast colonies that did not grow on SC-leu-trp plates, indicating they did not contain the pBTM plasmid, and did not turn blue on the SC-leu X-gal plates, indicating that the blue colour was dependent on the presence of both plasmids, were used to isolate the pDP-$GAL4_{AD}$ fusion plasmids.

These colonies, containing pDP-*GAL4* fusion plasmids producing proteins that interacted with the $lexA_{BD}$ - Rad7 fusion protein, were inoculated into 2 ml of SC-leu medium and grown at 30°C to saturation. The plasmid DNA was isolated from these cultures and transformed into DH5α cells by electroporation. The plasmids from 5 ampicillin resistant colonies were isolated as described above and characterized by digestion with *Eco*RI and electrophoresis on a 0.7% agarose gel. To confirm the ability of any particular plasmid to turn blue on X-gal medium it was re-transformed into CTY10-5D::pBTM116-*RAD7*. Those pDP-$GAL4_{AD}$ fusion plasmids that demonstrated that blue colour production was dependent on the presence of the pBTM116-*RAD7* fusion plasmid were studied further.

This screen, of approximately 400,000 library clones, produced 7 blue colonies. The results of tests carried out on them are listed in Table 6. Two of the seven purified library plasmids, pGAD7B11 and pGAD7B52, produced blue colonies in the absence of the plasmid pBTM116-*RAD7*, and were therefore considered false positives. Three hundred base pairs of each of the five remaining $GAL4_{AD}$ fusion plasmids inserts, spanning the *Bam*HI fusion

Table 6. Results of *RAD7* Two-hybrid Screen

Library Plasmid from screen	Colour in CTY10-5D	Colour in CTY10-5D:: pBTM116-*RAD7*	Gene that also interacted with plasmid	Results of DNA sequence databank search
7B5.1	white	blue		identical to *SIR3*
7B6	white	blue	*RAD18, RAD10*	no match
7B11	blue	blue		false positive
7B16	white	blue	*RAD6*	no match
7B34	white	blue		identical to *SIR3*
7B35	white	blue		no match
7B52	blue	blue		false positive

junction, were sequenced as described in Materials and Methods. Further analysis of these plasmids revealed that pGAD7B6 also interacted in the two-hybrid system with the pBTM116-*RAD18* and pBTM116-*RAD10* plasmids, and pGAD7B16 also interacted with pBTM116-*RAD6* plasmid. Since interaction with more than one two-hybrid binding domain fusion protein may indicate an ability to bind to the lexA DNA binding domain portion of the fusion protein (Bartel *et al.*, 1993), these plasmids were not pursued within the scope of this study. The pGAD7B34 and pGAD7B5.1 plasmids contained identical sequences from the *Bam*HI fusion junction. Two hundred base pairs of this DNA sequence was used to search the GenBank database, using the UWGCG FASTA program (Pearson and Lipman, 1988). The result of this search showed that the 7B34/7B5.1 inserts had perfect homology with the *SIR3* gene, beginning at base pair 977 of the open reading frame (ORF). This suggested that the Rad7 and Sir3 proteins interact, formally connecting DNA repair with chromatin structure. Since this result appeared to be the most interesting, the nature of this interaction was further characterized. Screening the GenBank and Swiss-Prot databases with the DNA and protein sequence of pGAD7B35 insert failed to detect any significant homologies.

iii. Quantitation of β-galactosidase Activity Induced by the Rad7 Sir3 Interaction

The interaction between the $Gal4_{BD}$-Rad7 and $Gal4_{AD}$-Sir3 fusion proteins results in the production of β-galactosidase, turning the yeast colony blue on medium containing X-gal. This qualitative result can be converted to a quantitative measurement by using ONPG to assay the β-galactosidase activity in a yeast culture. β-galactosidase activity in several yeast strains was determined according to the protocol outlined in Materials and Methods and the results are summarized in Table 7. A strain containing both the Rad6 and Sir3 fusion plasmids was assayed to show that Sir3 interacted specifically with Rad7 and not with other

Table 7. β-Galactosidase assays of colonies containing "Two-Hybrid System" fusion plasmids.

	DNA Binding Hybrid	Transcription Activating Hybrid	β-galactosidase Activity*	Colony Colour on X-Gal medium
1.	pBTM116-*RAD7*	-	<1	white
2.	-	pDP12-*SIR3*(307-978)	<1	white
3.	pBTM116-*RAD7*	pDP12-*SIR3*(307-978)	420+/-157	blue
4.	pBTM116-*RAD6*	pDP12-*SIR3*(307-978)	<1	white
5.	pMA424-*SNF1*	pGAD424-*SNF4*	29+/-5	pale blue

*Expressed in Miller Units.
Standard Deviations: rows 1, 2, 4 = < 1; row 3 = 157; row 5 = 8 n = 6

The plasmids in rows 1 to 4 are in the yeast strain CTY10-5D, and the plasmids in row 5 are in the GGY1::171 yeast strain. Three transformants were assayed in duplicate as described in Materials and Methods.

Rad proteins. The yeast strain GGY1::171, containing both the Snf1 and Snf4 fusion proteins, was used as a positive control (Fields and Song, 1989). The interaction of the Rad7 and Sir3 fusion proteins in strain CTY10-5D produced 420 Miller Units of β-galactosidase activity in comparison to the 29 Miller Units produced by the interaction of Snf1 and Snf4 in GGY1::171. This increased β-galactosidase activity may be an indication that the Rad7 Sir3 two-hybrid system interaction is stronger or more stable than the two-hybrid system interaction between Snf1 and Snf4.

iv. Phenotypic Analysis of Rad7 and Sir3 Deletion Mutants

The interaction between Rad7 and Sir3 in the two-hybrid system suggests that this interaction may be significant *in vivo*. If so, one might expect some effect on the phenotype of either single mutant strain when the other mutation is introduced. Gene deletions were introduced into isogenic yeast strains using the one step disruption method of Rothstein (1983). The *rad7*, *rad6*, and *rad1* deletions were introduced using plasmids pDG79 (Schiestl and Prakash, 1989), pDG47 (R.D. Gietz, personal communication), and pDG18 (a derivative of pRR27; Schiestl and Prakash, 1988), respectively (see Figure 6).

a. *RAD* Gene Deletions

The *rad7* deletion was created by transforming the *Eco*RI digested pDG79 plasmid DNA into the wild type yeast strain MKP° (Pierce *et al.*, 1987) and selecting for *URA3*$^+$ colonies. Transformants were tested for the UV sensitive phenotype of the *rad7* deletion using the spot test described in Materials and Methods. The *rad1* deletion was created in the strain MKP° by transformation with the *Bam*HI digested pDG18 plasmid DNA, selecting for *URA3*$^+$ colonies, and testing as described above. A *rad6* deletion in strain MKP° was obtained from Dr. R.D. Gietz.

Figure 6. Restriction maps of the deletion constructs.

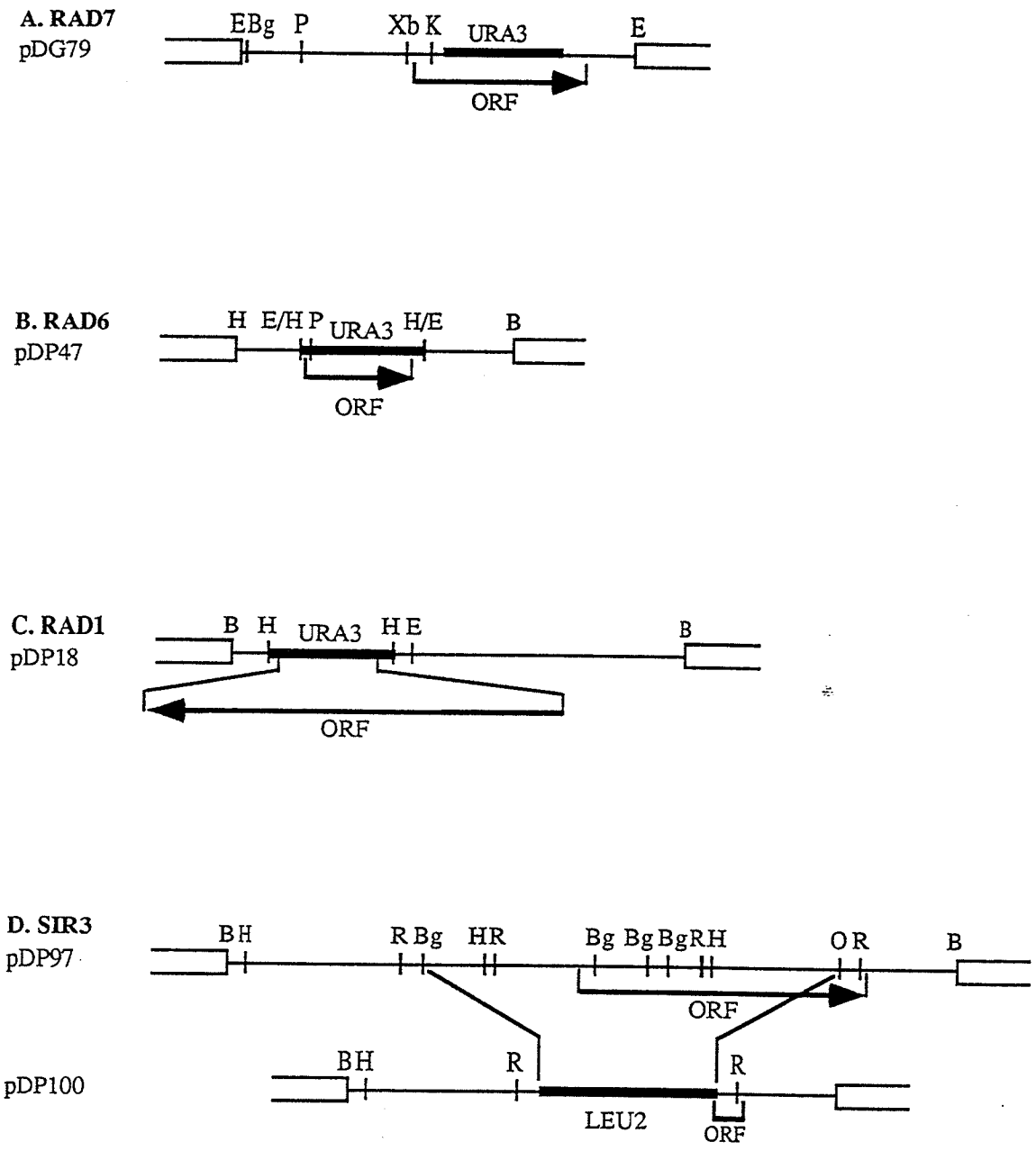
A. In pDG79, *Xho*I-*Hind*III fragment of *RAD7* was replaced with a 1.2 kb *Hind*III fragment containing the *URA3* gene, removing the 1241 bp between base pair +214 and +1455 of the *RAD7* open reading frame (ORF) (Schiestl and Prakash, 1989). The *Eco*RI fragment of pDG79 was used to replace the *RAD7* wild type gene in the yeast strain MKP°, creating the *rad7*Δ deletion mutant.

B. In pDG47, the 0.6 kb *Eco*RI fragment of *RAD6* was replaced with a 1.2 kb *Hind*III fragment containing the *URA3* gene, replacing 608 bp between base pair -43 and +565 of the *RAD6* ORF (Schiestl *et al.*, 1990). The *Hind*III/*Bam*HI fragment of pDG47 were used to replace the *RAD6* wild type gene in the yeast strain MKP°, creating the *rad6*Δ deletion mutant.

C. In pDG18, the 4.1 kb *Hind*III fragment of *RAD1* was replaced with a 1.2 kb *Hind*III fragment containing the *URA3* gene, replacing 4065 bp between base pair -212 and +3853 of the *RAD1* ORF (Schiestl and Prakash, 1988). The *Bam*HI fragment of pDG18 was used to replace the *RAD1* wild type gene in the yeast strain MKP°, creating the *rad1*Δ deletion mutant.

D. The 6.7 kb *Bam*HI fragment from plasmid pHR45-15, containing the *SIR3* gene, was cloned into the *Bam*HI site of pCHS6.3, creating pDP97. The 3.8 kb *Bgl*II and *Xho*I fragment of *SIR3* was then replaced by a 1.6 kb *Bam*HI-*Eco*RI fragment containing the *LEU2* gene, creating pDP100. This deletion removes all but the last 104 bp of the *SIR3* ORF. The *Eco*RI fragment of pDP100 was used to replace the *SIR3* wild type gene in the yeast strain MKP°, *rad7Δ*, *rad1Δ* and *rad6Δ*, creating the *sir3Δ*, *rad7Δsir3Δ*, *rad1Δsir3Δ*, and *rad6Δsir3Δ* deletion mutants, respectively.

Restriction Endonucleases: E=*Eco*RI; Bg=*Bgl*II; H=*Hind*III; K=*Kpn*I; O=*Xho*I; P=*Pst*I; Sc=*Sac*I; X=*Xba*I.


A. RAD7
pDG79
EBg P Xb K URA3 E
ORF
B. RAD6
pDP47
H E/H P URA3 H/E B
ORF
C. RAD1
pDP18
B H URA3 H E B
ORF
D. SIR3
pDP97
BH RBg HR Bg Bg BgRH OR B
ORF
pDP100
BH R R
LEU2
ORF
1 kb

b. Construction of the *sir3* Deletion Plasmid

As no suitable *sir3* deletion plasmid was available, it was constructed as described below (see Figure 6). The plasmid pDP97 was created by cloning the 6.7 kb *Bam*HI fragment containing the *SIR3* gene from plasmid pHR45-15 into the *Bam*HI site of pCHS6.3. The plasmid pDP100 was created by blunt end cloning the 1.6 kb *Bam*HI-*Eco*RI fragment containing the *LEU2* gene (Gietz and Sugino, 1988) between the outer *Bgl*II *Xho*I sites in the *SIR3* gene from pDP97. This deletion plasmid, pDP100, removes all but the last 104 bp of the *SIR3* open reading frame (see Figure 6B).

c. *sir3* Deletion

The *sir3* deletion was created by transforming the *Eco*RI digested pDP100 plasmid DNA into the wild type yeast strain MKP° (Pierce *et al.*, 1987) and selecting for *LEU2*+ colonies. This was also done for the strains MKP°*rad7Δ*, MKP°*rad1Δ* and MKP°*rad6Δ*, in order to obtain sir3/rad7, sir3/rad1 and sir3/rad6 double deletion mutants. Transformants were tested for their ability to mate with the strain XS803-3A, as *sir3* mutants are unable to mate with a yeast strain of the opposite mating type. The genotypes of the MKP° cells and the XS803-3A cells are complementary with respect to the *his* and *trp* mutations; MKP° is *his3-Δ200*; *trp1-Δ901* and XS803-3A is *his1-1*; *trp2*. This means a diploid formed by mating between these to cell strains will be able to grow on medium lacking tryptophan or medium lacking histidine. Four of the forty-four *LEU2*+ MKP° transformants tested failed to mate with XS803-3A cells, indicating that they contained *sir3* deletions. Five of forty-four *LEU2*+ MKP°*rad7Δ* transformants also showed the non-mating phenotype. *LEU2*+ transformants that showed the *sir3* deletion phenotype were also isolated for the MKP°*rad1Δ* and MKP°*rad6Δ* strains.

d. Quantitation of Yeast Strain UV Sensitivity

To determine the effect of the *sir3* deletion on the DNA repair phenotype of the *rad7* deletion, the sensitivity of each isogenic deletion mutant strain was measured using the survival curve assay (Figure 7) as described in Materials and Methods. Both the MKP° wild type strain and the *sir3Δ* strain show a limited sensitivity to the lethal effects of UV; 12% and 32% survival, respectively, at 60 J/m^2. The *sir3Δ* strain, however, seems to be slightly less sensitive to UV than the wild type. In comparison, the *rad7Δ* strain is quite sensitive to increasing doses of UV; 0.01% survival at 60 J/m^2. The yeast strain containing both the *rad7* and *sir3* deletion mutations shows an intermediate UV sensitivity; 0.8% survival at 60 J/m^2. This demonstrates that the *sir3* deletion mutation reduces the killing affect of UV by approximately one to two orders of magnitude. Transformation of the plasmid pDP113 (Figure 8), which contains the *SIR3* gene, into the *rad7Δsir3Δ* strain restores the UV sensitive phenotype of this strain to the level seen in the *rad7Δ* strain; 0.01% survival at 60 J/m^2.

Since the *sir3* deletion strain was less UV sensitive than wild type, other *sir3/rad* double and single deletion mutants were tested for their sensitivity to UV (Figure 7B and 7C). In Figure 7B, the *rad1* single mutant and the *rad1/sir3* double mutant were shown to have no obvious difference in their sensitivity to UV; 0.0003% and 0.0007% survival at 20 J/m^2. Similarly, the UV sensitivities of the *rad6* and *rad6/sir3* deletion mutants were indistinguishable; 0.0006% and 0.0003% survival at 20 J/m^2 (Figure 7C). This indicates that the effect of the *sir3* deletion mutation on UV sensitivity is specific for the *rad7* deletion mutant, suggesting that there is a functional interaction between the Sir3 and Rad7 proteins.

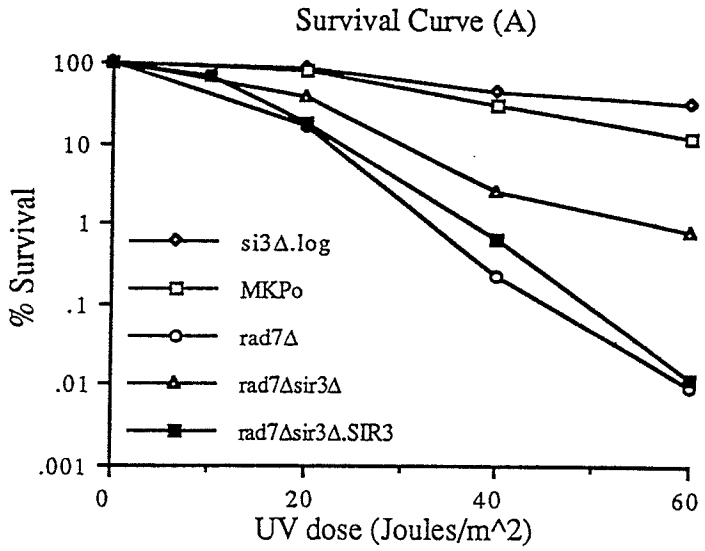
Figure 7. The Effect of the *sir3* Deletion on the UV Sensitivity of *RAD*$^+$, *rad7*Δ, *rad1*Δ, and *rad6*Δ.

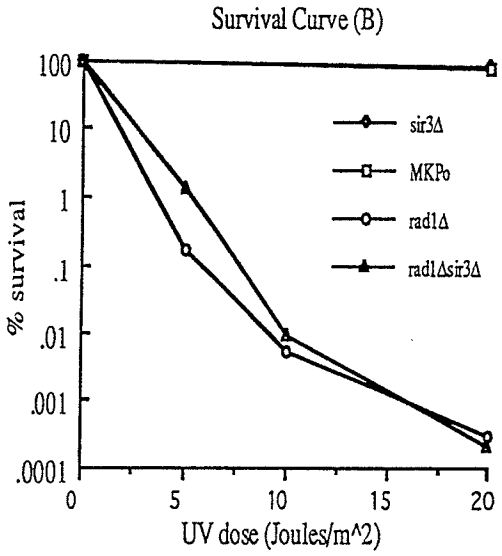
A: RAD$^+$ (MKP^o) (□), *rad7*Δ (○), *sir3*Δ (◇), *rad7*Δ*sir3*Δ (△), and *rad7*Δ*sir3*Δ::*SIR3* (▽).

B: RAD$^+$ (MKP^o) (□), *rad1*Δ (○), *sir3*Δ (◇), and *rad1*Δ*sir3*Δ (△).

C: RAD$^+$ (MKP^o) (□), *rad6*Δ (○), *sir3*Δ (◇), and *rad6*Δ*sir3*Δ (△).

Each point represents the mean value from duplicate experiments on 4 independent isolates of each genotype. The UV dose was delivered at a rate of 1 Joules/m^2/sec.


Survival Curve (A)
100
10
1
.1
.01
.001
% Survival
si3Δ.log
MKPo
rad7Δ
rad7Δsir3Δ
rad7Δsir3Δ.SIR3
0
20
40
60
UV dose (Joules/m^2)


Survival Curve (B)
100
10
1
.1
.01
.001
.0001
% survival
sir3Δ
MKPo
rad1Δ
rad1Δsir3Δ
0
5
10
15
20
UV dose (Joules/m^2)

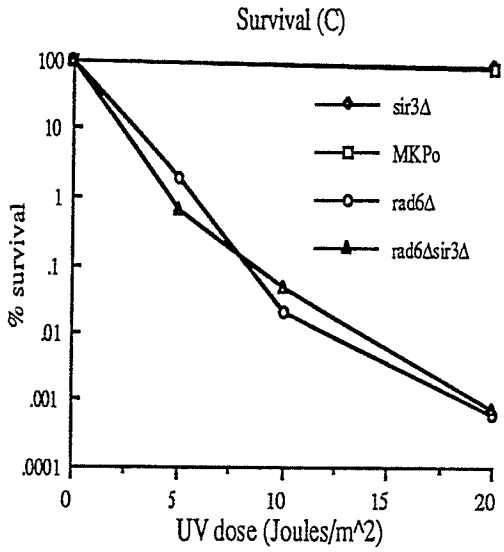
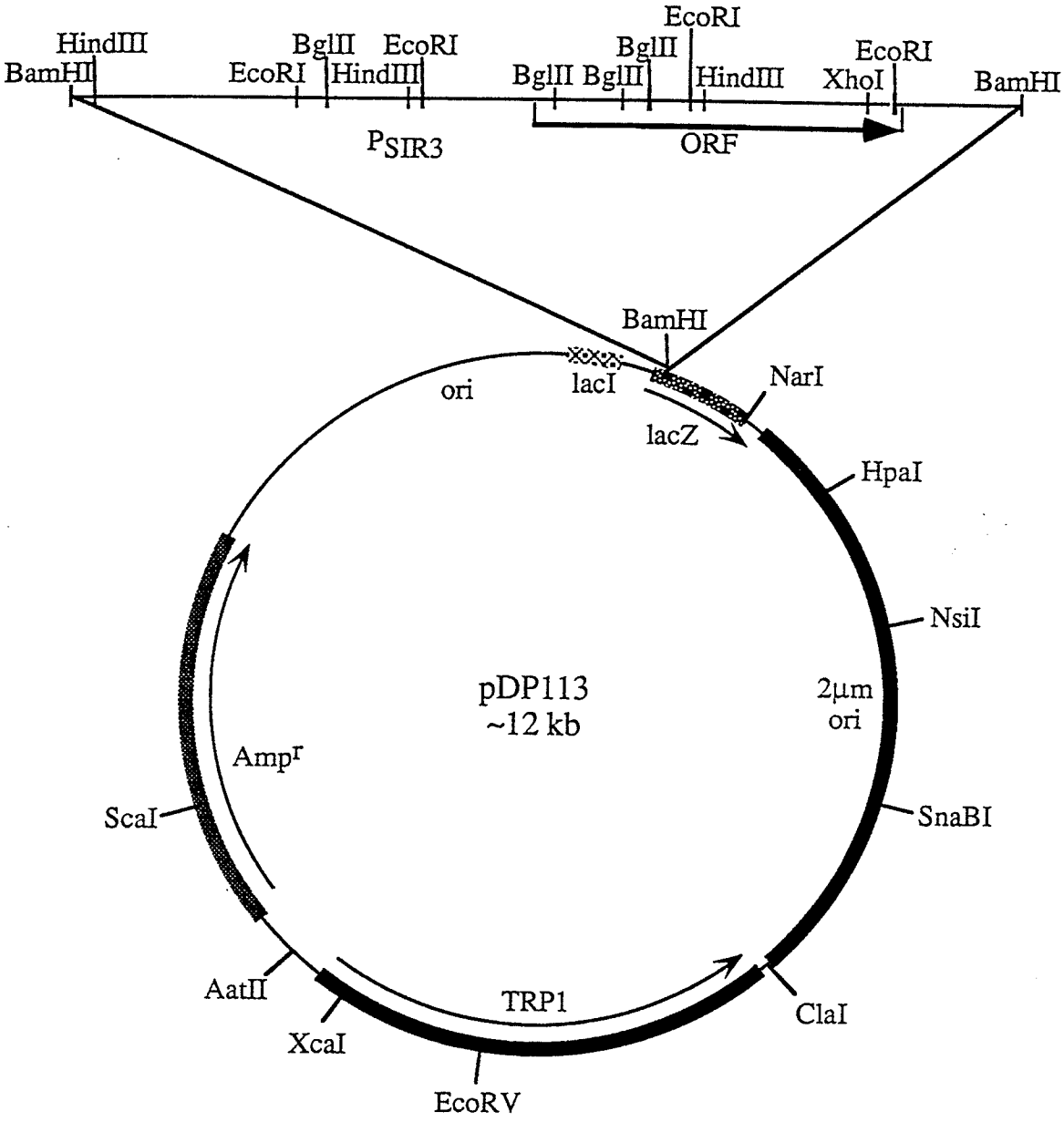

Survival (C)
100
10
1
.1
.01
.001
.0001
% survival
sir3Δ
MKPo
rad6Δ
rad6Δsir3Δ
0
5
10
15
20
UV dose (Joules/m^2)

Figure 8. Construction of the *SIR3* Expression Plasmid, pDP113.

The plasmid, pDP113, was constructed by cloning the 6.7 kb *SIR3* *Bam*HI fragment from plasmid pHR45-15 into the *Bam*HI site of Yeplac112. Transcription is controlled by the promoter P_{SIR3}.


HindIII
BamHI
BglII
EcoRI
EcoRI
HindIII
EcoRI
BglII
BglII
HindIII
EcoRI
XhoI
BamHI
P SIR3
ORF
BamHI
ori
lacI
NarI
lacZ
HpaI
NsiI
pDP113
~12 kb
2µm
ori
Amp r
ScaI
SnaBI
AatII
TRP1
ClaI
XcaI
EcoRV

v. Interaction of the Rad7 and Sir3 Proteins *in vitro*

To test if the Rad7 and Sir3 proteins interact directly *in vitro*, fusion gene constructs were made to glutathione-S-transferase (GST) in the pGEX vector (Figure 3) (Smith and Johnson, 1988). This allows these fusion proteins to be purified from *E.coli* extracts using glutathione-agarose affinity chromatography as described in Materials and Methods. The *RAD7* GST fusion plasmid, pDP104, was constructed by inserting a *RAD7 Eco*RI *PCR* fragment, obtained from Dr. R.D. Gietz, into the *Xma*I site of vector pGEX-2T by blunt end ligation (Figure 9). The *SIR3* GST fusion plasmid, pDP107, was constructed by inserting the *SIR3 Bgl*II-*Bam*HI fragment, coding for amino acids 307-978, into the *Xma*I site of vector pGEX-2T by blunt end ligation (Figure 10). These plasmids were each transformed into the *E. coli* strain JM109.

The scheme used for purification of the Rad7 and Sir3 proteins, and the detection of their *in vitro* interaction, is described in Figure 11. [^{35}S]-methionine labelled and unlabelled Rad7 and Sir3-GST fusion proteins were purified from the *E. coli* soluble protein lysate using glutathione-agarose beads as described in Materials and Methods. The radiolabelled Rad7 and Sir3 portions of these fusion proteins were cleaved from the glutathione-agarose bound GST portion by digestion with the protease thrombin. These two thrombin cut supernatants (TCS), containing either radiolabelled Rad7 (Figure 12, Lane F)or radiolabelled Sir3 (Lane A) protein fragments, were then mixed with the non-radiolabelled GST fusion protein of the opposite type bound to glutathione agarose. These beads were then washed exhaustively and the radiolabelled proteins that were still associated with the glutathione-agarose matrix were analyzed by SDS PAGE and fluorography (see Figure 12).

Lane C (Figure 12) should contain radiolabelled proteins that interact directly with

the glutathione-agarose bound GST-Rad7 fusion protein. A 76 kDa protein band, which corresponds to the predicted size of Sir3, is seen in this lane. Similarly, Lane D (Figure 12) should contain radiolabelled proteins that interact directly with the glutathione-agarose bound GST-Sir3 fusion protein. A 71 kDa radiolabelled protein band, corresponding to the predicted size of the Rad7 thrombin-cleaved peptide, is seen in this lane. The size of the Rad7 protein is 7 kDa larger than expected, however, this 7 kDa size increase was also consistently seen for all Rad7 fusion protein constructs electrophoresed on 10% SDS PAGE gels, in our laboratory. The apparent increase in size of the Rad7 protein is likely due to the hydrophobic nature of its C-terminus. The Sir3 TCS and Rad7 TCS were also mixed with clean glutathione agarose beads to ensure that the radiolabelled Rad7 and Sir3 fusion proteins did not bind to glutathione agarose (Lanes B and E, respectively). Neither of the two radiolabelled protein bands were seen in either lane. These results show that the Sir3 and Rad7 radiolabelled fusion proteins are only able to bind the glutathione agarose beads when GST-Rad7 and GST-Sir3, respectively, are present, *ie.* the Rad7 and Sir3 fusion proteins interact *in vitro*.

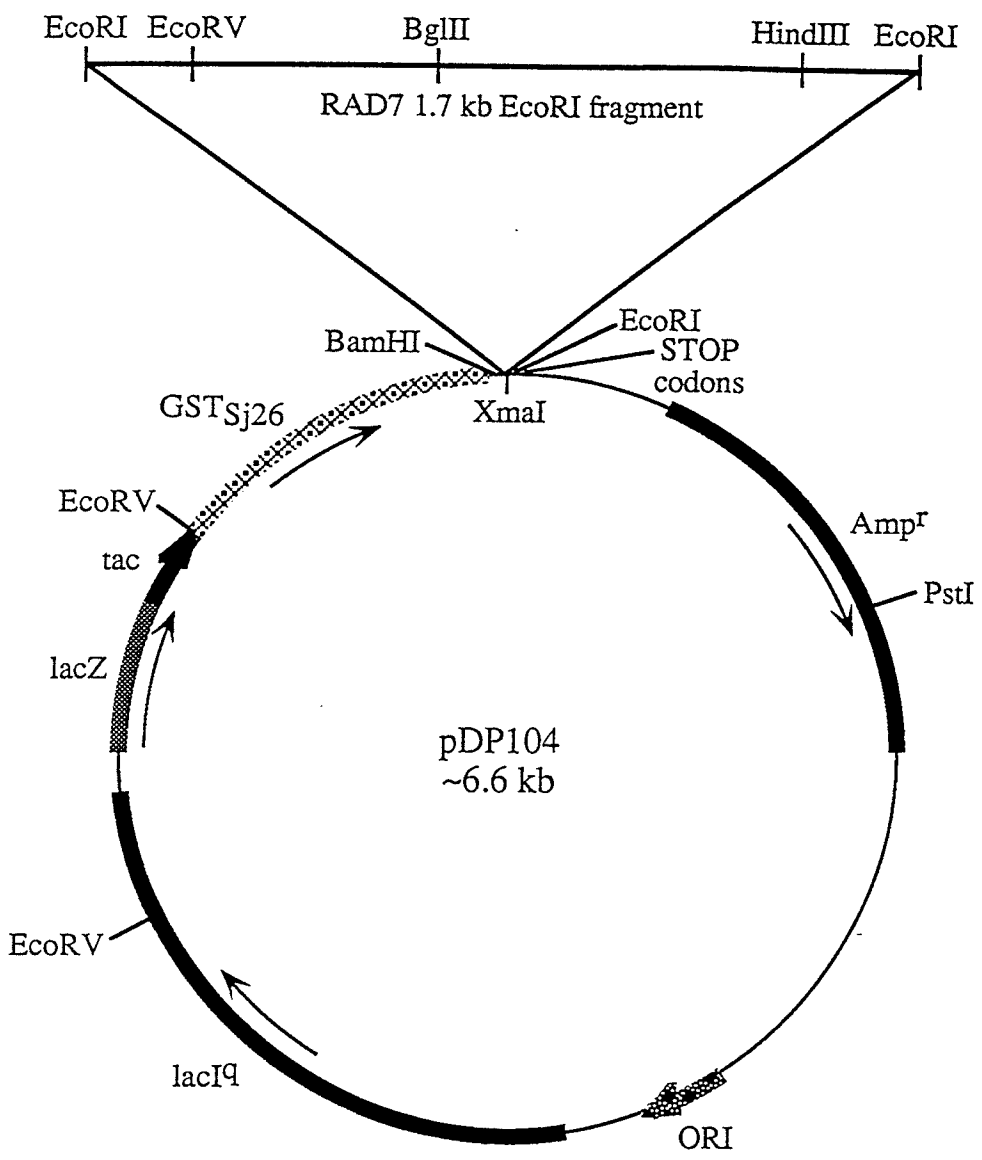
Small protein bands, seen at the bottom of Lanes B, C, D, and E (Figure 12), interacted with the glutathione agarose both in the presence and absence of GST-Rad7 or GST-Sir3 fusion proteins. The intensity of these protein bands, however, is higher in the lanes containing the fusion proteins, indicating that some of these small protein bands may be proteolytic products of Sir3 (Lane C) or Rad7 (Lane D) digestion that are able to specifically interact with GST-Rad7 and GST-Sir3, respectively.

Figure 9. Construction of the *RAD7*-GST Fusion Plasmid, pDP104.

A. The plasmid, pDP104, was constructed by blunt-end cloning the *RAD7* *Eco*RI *PCR* fragment into the *Xma*I site of vector pGEX-2T.

B. The DNA and inferred amino acid sequence of the GST-*RAD7* fusion junction. The *RAD7* gene has been fused in its proper reading frame. The *Xma*I site of GST was destroyed, but the *Eco*RI site of *RAD7* was regenerated in this blunt end cloning.

A.


EcoRI
EcoRV
BglII
HindIII
EcoRI
RAD7 1.7 kb EcoRI fragment
BamHI
EcoRI
STOP
codons
XmaI
GST Sj26
EcoRV
tac
lacZ
Amp r
PstI
pDP104
~6.6 kb
EcoRV
lacIq
ORI

B.

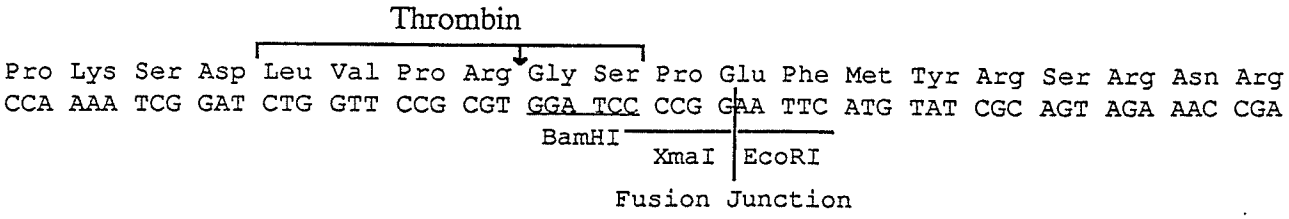
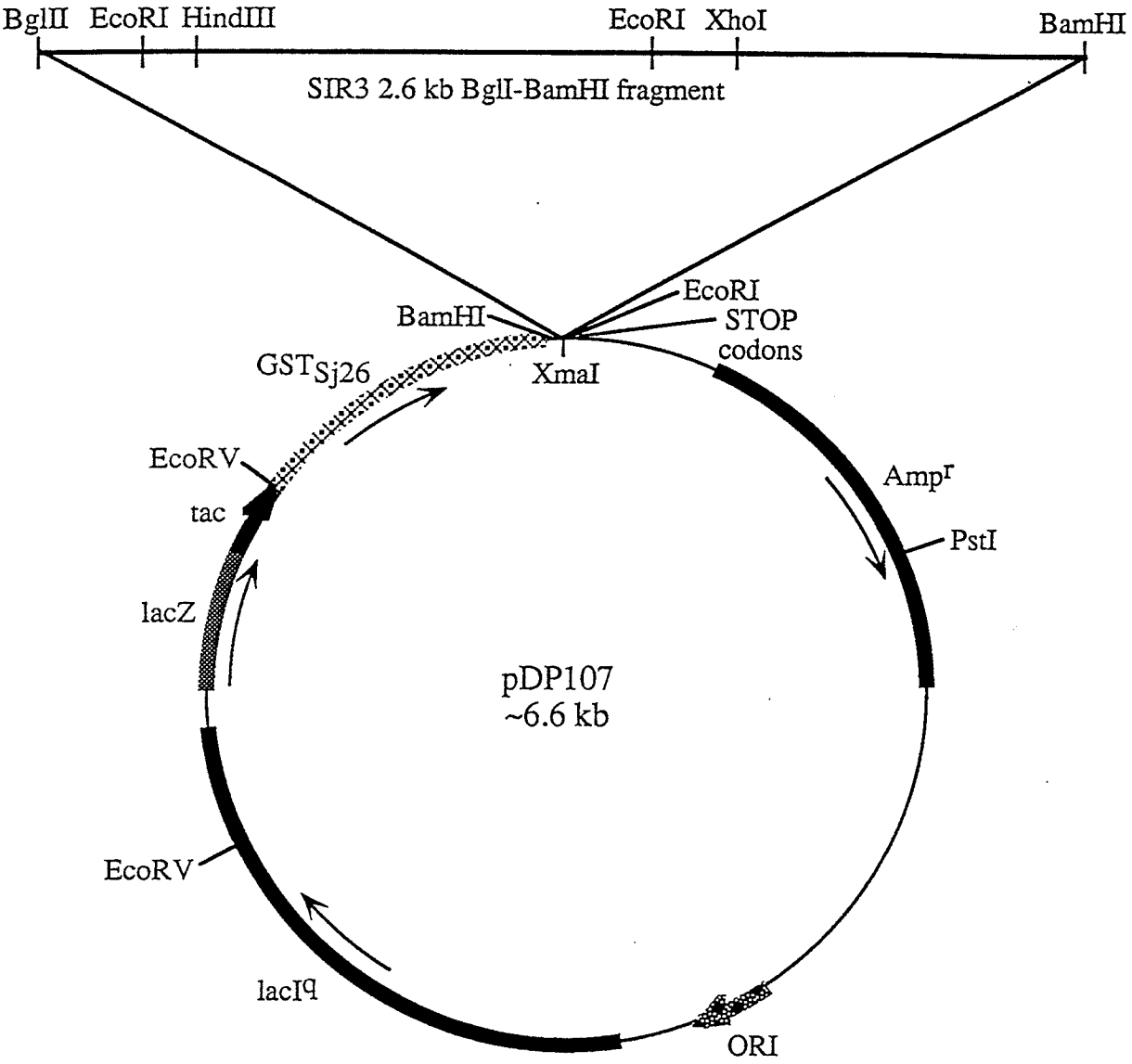

Thrombin
Pro Lys Ser Asp Leu Val Pro Arg Gly Ser Pro Glu Phe Met Tyr Arg Ser Arg Asn Arg
CCA AAA TCG GAT CTG GTT CCG CGT GGA TCC CCG GAA TTC ATG TAT CGC AGT AGA AAC CGA
BamHI
XmaI
EcoRI
Fusion Junction

Figure 10. Construction of the *SIR3*-GST Fusion Plasmid, pDP107.

A. The plasmid, pDP107, was constructed by blunt-end cloning the *SIR3* *Bgl*II-*Bam*HI fragment, coding for amino acids 307-978, into the *Xma*I site of vector pGEX-2T.

B. The DNA and inferred amino acid sequence of the GST-*SIR3* fusion junction. The *SIR3* gene has been fused in its proper reading frame. The *Xma*I site of GST was regenerated, but the *Bgl*II site of *SIR3* was destroyed in this blunt end cloning.

A.


BglII EcoRI HindIII
EcoRI XhoI
BamHI
SIR3 2.6 kb BglI-BamHI fragment
BamHI
EcoRI
STOP
codons
XmaI
GST Sj26
EcoRV
tac
lacZ
Amp r
PstI
pDP107
~6.6 kb
EcoRV
lacI q
ORI

B.

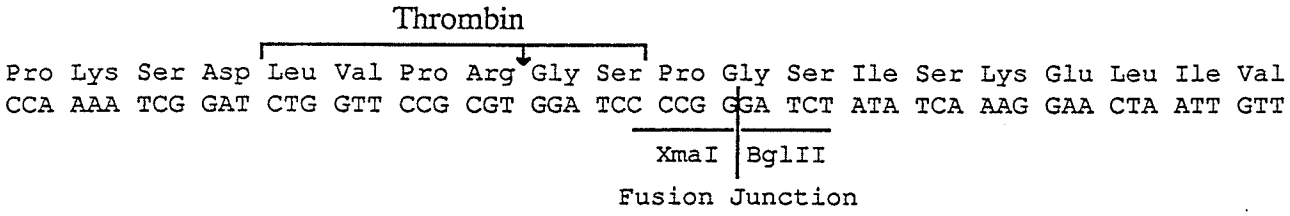

Thrombin
Pro Lys Ser Asp Leu Val Pro Arg Gly Ser Pro Gly Ser Ile Ser Lys Glu Leu Ile Val
CCA AAA TCG GAT CTG GTT CCG CGT GGA TCC CCG GGA TCT ATA TCA AAG GAA CTA ATT GTT
XmaI
BglII
Fusion Junction

Figure 11. Scheme for detecting the Rad7-Sir3 interaction *in vitro*.

Radiolabelled Rad7 and Sir3, and non-radiolabelled Rad7 and Sir3, fusion proteins were purified, mixed with each other or new glutathione agarose beads, and analyzed by SDS PAGE and fluorography. Radiolabelled proteins are marked with an asterisk.

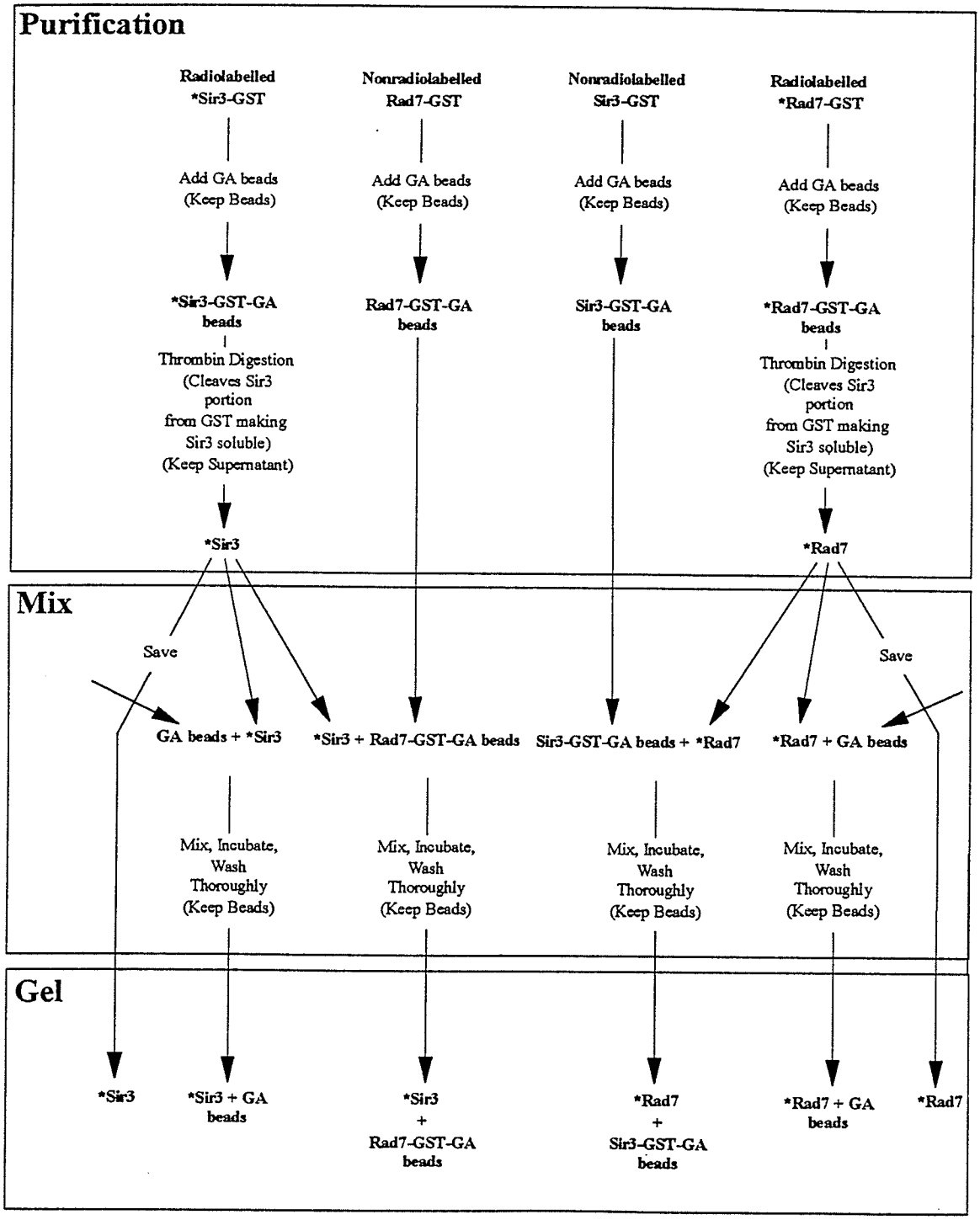

Purification
Radiolabelled *Sir3-GST
Nonradiolabelled Rad7-GST
Nonradiolabelled Sir3-GST
Radiolabelled *Rad7-GST
Add GA beads (Keep Beads)
Add GA beads (Keep Beads)
Add GA beads (Keep Beads)
Add GA beads (Keep Beads)
*Sir3-GST-GA beads
Rad7-GST-GA beads
Sir3-GST-GA beads
*Rad7-GST-GA beads
Thrombin Digestion (Cleaves Sir3 portion from GST making Sir3 soluble) (Keep Supernatant)
Thrombin Digestion (Cleaves Sir3 portion from GST making Sir3 soluble) (Keep Supernatant)
*Sir3
*Rad7
Mix
Save
Save
GA beads + *Sir3
*Sir3 + Rad7-GST-GA beads
Sir3-GST-GA beads + *Rad7
*Rad7 + GA beads
Mix, Incubate, Wash Thoroughly (Keep Beads)
Mix, Incubate, Wash Thoroughly (Keep Beads)
Mix, Incubate, Wash Thoroughly (Keep Beads)
Mix, Incubate, Wash Thoroughly (Keep Beads)
Gel
*Sir3
*Sir3 + GA beads
*Sir3 + Rad7-GST-GA beads
*Rad7 + Sir3-GST-GA beads
*Rad7 + GA beads
*Rad7

Figure 12. Evidence for an *in vitro* interaction of Rad7 and Sir3 GST fusion proteins. GST-Rad7 and GST-Sir3(aa 308-978) fusion proteins were radiolabelled, purified, and thrombin digested as described in Materials and Methods. These proteins were mixed with non-radiolabelled glutathione agarose bound GST fusion proteins of the opposite type. The radiolabelled proteins bound to the GST fusion proteins were analyzed on a 10% acrylamide SDS PAGE gel and fluorographed as described in Materials and Methods. The arrows indicate the positions of the Sir3 and Rad7 thrombin cleaved peptides.

Lane A: 5µl Sir3 TCS

Lane B: 25µl Sir3 TCS plus 25µl GA beads.

Lane C: 80µl Sir3 TCS plus 50µl GST-Rad7 beads

Lane D: 80µl Rad7 TCS plus 50µl GST-Sir3 beads

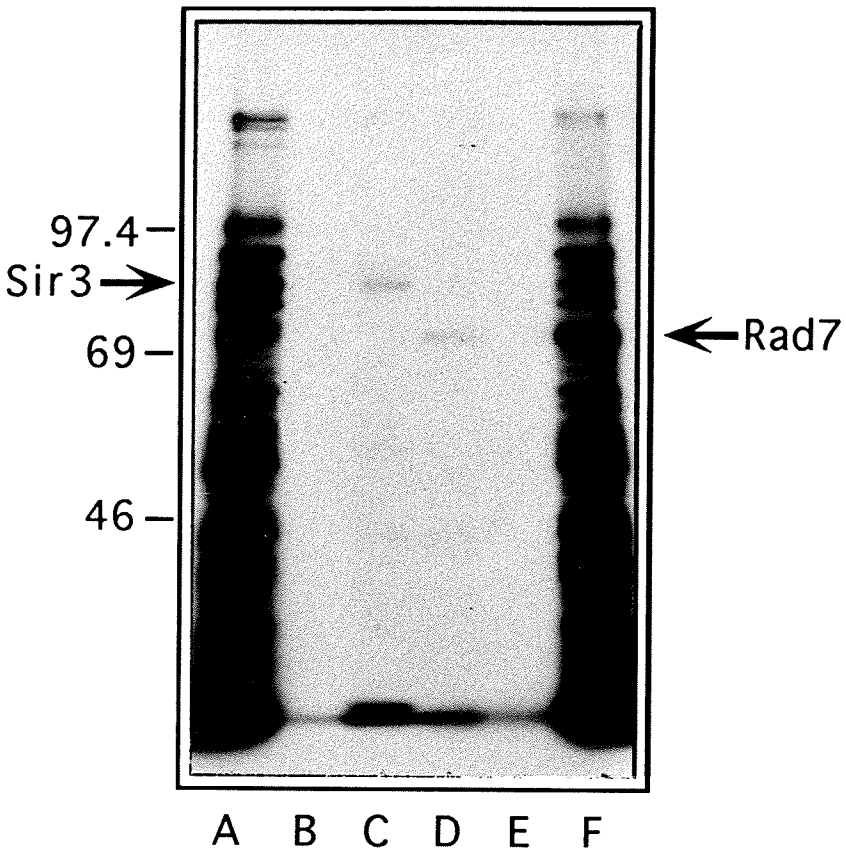
Lane E: 25µl Rad7 TCS plus 25µl GA beads.

Lane F: 5µl Rad7 TCS

GST-Rad7 beads = A 1:1 slurry of GST-Rad7 fusion protein attached to the glutathione agarose beads in PBS, 20 mM EDTA, 1 µg/ml BSA.

Sir3 TCS = Radiolabelled thrombin digested supernatant (TCS) from glutathione agarose beaded JM109::pGEX-2T-SIR3(aa 308-978).

GA beads = 1:1 slurry of glutathione agarose beads in PBS, 20 mM EDTA, 1 µg/ml BSA.


97.4
Sir3
69
46
Rad7
A B C D E F

vi. *In vivo* Analysis of the Rad7 Sir3 Interaction

Co-immunoprecipitation of Rad7 and Sir3 from yeast cells would indicate that these proteins interact *in vivo*. To test this, Rad7 was immunoprecipitated from [^{35}S]-methionine labelled yeast cells extracts from a *rad7*Δ*sir3*Δ double deletion yeast strain containing either the *RAD7* or the *SIR3* plasmid, or both plasmids. The presence of a specific Sir3-sized protein in the immunoprecipitate from the strain containing both plasmids could be taken as evidence of a specific interaction between these two proteins. either a plasmid that produced Sir3 protein.

Since an antibody for Rad7 was not available, it was made immunoreactive by addition of the 9 amino acid haemagglutinin (ha) tag recognized by the 12CA5 monoclonal anti-haemagglutinin antibody. This ha tag-Rad7 fusion protein was produced from the plasmid pDP126 (Figure 13). The plasmid pDP126 was constructed by first blunt end cloning the 1.7 kb *Eco*RI *RAD7* fragment, purified from pDG649, into the *Sma*I site of the vector pKM1310, creating pDP121. The 1.7 kb *Bam*HI - *Eco*RI fragment from pDP121, containing the ha tag-*RAD7* construct, was then cloned into the *Bam*HI - *Eco*RI sites of pDP122, which contains the galactose inducible *GAL1* promoter and the *HIS3* gene used for selection, creating pDP126.

The Sir3 protein was produced from the plasmid pDP113 (Figure 8). This plasmid containing the SIR3 gene and its promoter, was constructed by cloning the 7 kb *SIR3* *Bam*HI fragment from plasmid pHR45-15 into the *Bam*HI site of YEplac112. The Sir3 protein is produced from this high copy plasmid under the control of its own promoter.

The proteins in the MKP° *rad7*Δ*sir3*Δ double deletion strain containing either pDP113, pDP126, or both plasmids, were radiolabelled as described in Materials and

Methods. In addition, the double deletion strain containing neither plasmid was radiolabelled as a control. Extracts were immunoprecipitated with the 12CA5 antibody and analyzed by SDS PAGE and fluorography; the results are shown in Figure 14. The appearance of a 70 kDa protein, corresponding to the size of the ha tag-Rad7 protein was expected in protein extracts from cells containing the *RAD7* plasmid. A additional band of approximately 110 kDa, corresponding to the size of the Sir3 protein, was expected in extracts from cells containing both the *RAD7* and *SIR3* plasmids, but not in an extract from cells containing only the *RAD7* plasmids. Immunoprecipitation of the 70 kDa radiolabelled ha tag-Rad7 protein was seen in extracts from strains carrying the ha tag-*RAD7* plasmid (Lanes C, E, and I). This protein was not seen in the immunoprecipitates from the rad7Δsir3Δ control extract (Lane D) or those from the *SIR3* extract (Lane B). The 111 kDa protein band was not found in extracts from the strain containing both *RAD7* and *SIR3* plasmids (Lane D), indicating that under the conditions used the Rad7 Sir3 interaction was not detectable.

The role of Sir3 in chromatin structure may mean that most of the Sir3 in a yeast cell extract is associated with the DNA in the insoluble fraction.. The association of Sir3 with the DNA, and binding of Sir3 to the ha tag-Rad7 protein may, therefore, be sequestering a large portion of the ha tag-Rad7 protein to the insoluble fraction. To test this possibility, the pellet and the supernatant from the ha tag-*RAD7/SIR3* strain were heated in the presence of 1% SDS. This treatment should solubilize most proteins and allow immunoprecipitation after dilution of the SDS to 0.1%. Lanes G and H of Figure 14 contain the immunoprecipitates from the SDS treated supernatant and pellet, respectively. Both the pellet and the supernatant were shown to contain ha tag-Rad7 protein indicating that this treatment does not interfere significantly with the 12CA5 antibody. The supernatant

contained the majority of the immunoprecipitable product (Lane G), however some was found in the pellet (Lane H).

Figure 13. Construction of the *RAD7*-Haemagglutonin Tag Fusion Plasmid, pDP126.

(A) A two step process was used to construct the plasmid pDP126. 1) The 1.7 kb *Eco*RI *RAD7* fragment, purified from pDG649, was blunt-end cloned into the *Sma*I site of the vector pKM1310, creating pDP121. 2) The 1.7 kb *Bam*HI - *Eco*RI fragment from pDP121, containing the ha tag-*RAD7* construct, was blunt-end cloned into the *Bam*HI site of pDP122. The DNA and inferred protein sequence of the fusion junction between the 9 amino acid haemagglutinin tag and *RAD7* are shown to the left of pDP122. The sequence of the haemagglutinin tag is underlined. The start of the *RAD7* sequence is shown in capital letters. The right and left box indicate the *Sma*I-*Eco*RI fusion site of pDP121 and the *Bam*HI-*Eco*RI fusion site of pDP122, respectively.

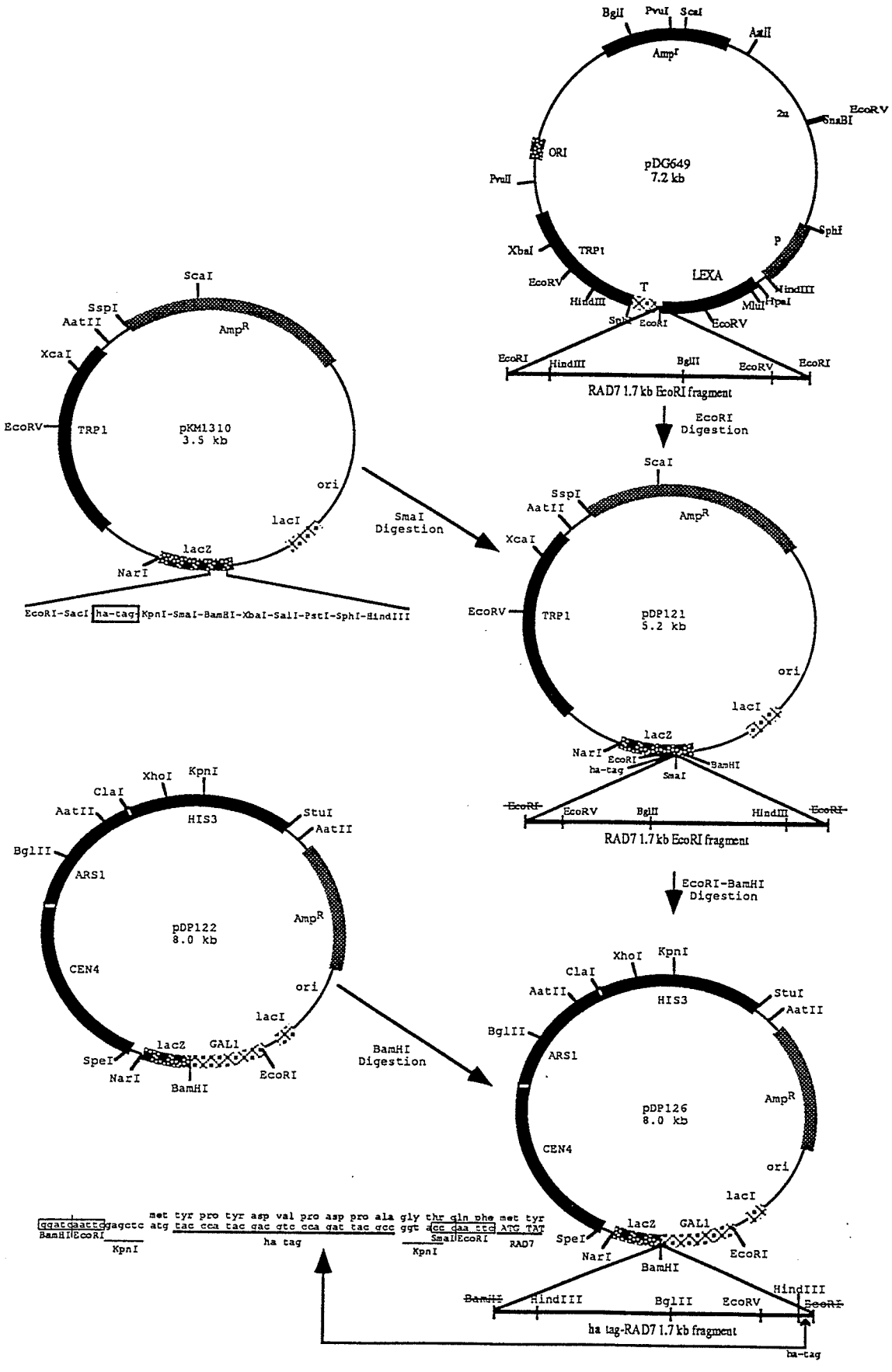

pDG649
7.2 kb
BglI
PvuI
ScaI
AatII
AmpR
2u
EcoRV
SnaBI
ORI
PvuII
XbaI
TRP1
P
SphI
LEXA
T
HindIII
MluI
HpaI
EcoRV
EcoRI
BglII
RAD7 1.7 kb EcoRI fragment
EcoRI Digestion
pKM1310
3.5 kb
ScaI
SspI
AatII
AmpR
XcaI
EcoRV
TRP1
ori
lacI
lacZ
NarI
EcoRI-SacI
ha-tag
KpnI-SmaI-BamHI-XbaI-SalI-PstI-SphI-HindIII
SmaI Digestion
pDP121
5.2 kb
RAD7 1.7kb EcoRI fragment
EcoRI-BamHI Digestion
pDP122
8.0 kb
XhoI
KpnI
ClaI
AatII
HIS3
StuI
BglII
ARS1
CEN4
lacZ
GAL1
SpeI
BamHI Digestion
pDP126
8.0 kb
met tyr pro tyr asp val pro asp pro ala gly thr gln phe met tyr
ha tag
RAD7
ha tag-RAD7 1.7 kb fragment

Figure 14. Immunoprecipitation of *in vivo* radiolabelled ha tag-Rad7.

The ha tag-Rad7 protein was immunoprecipitated from [^{35}S]-methionine *in vivo* labelled extracts produced from *rad7;sir3* double deletion yeast strains and analyzed by SDS PAGE and fluorography as described in Methods and Materials. The yeast strains contained the plasmids: pDP113 (Sir3)(B); pDP126 (ha-Rad7) (E and I); neither plasmid (D); or pDP126 and pDP113 (C). Lane G contains immunoprecipitates from the radiolabelled *rad7Δsir3Δ*::pDP126, pDP113 supernatant treated with SDS. Lane H contains immunoprecipitates from the SDS treated pellet from the same strain.

Lane A: C^{14} molecular weight markers.

Lane B: *rad7Δsir3Δ*::pDP113 (Sir3).

Lane C: *rad7Δsir3Δ*::pDP113, pDP126 (ha-Rad7).

Lane D: *rad7Δsir3Δ*.

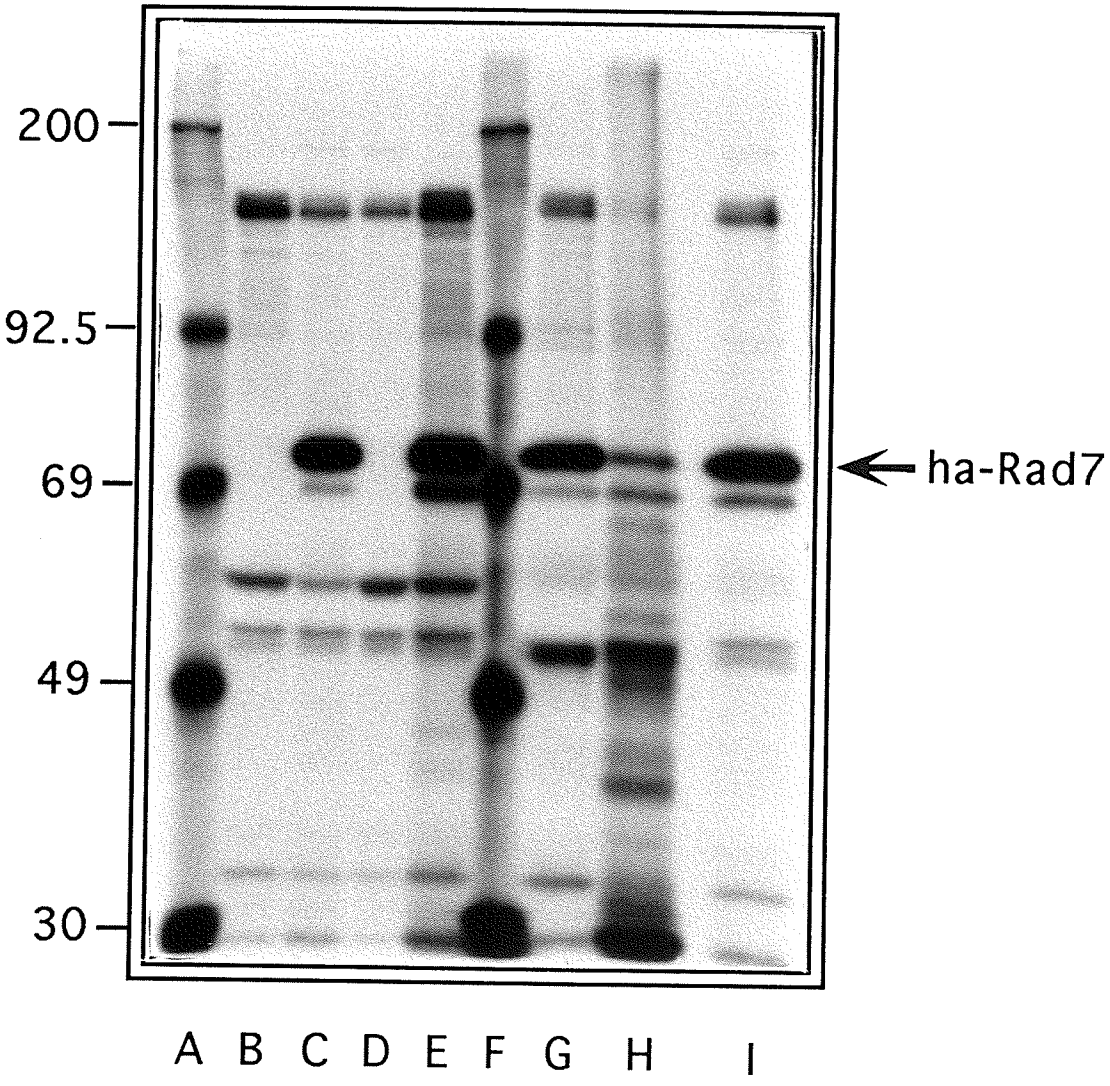
Lane E: *rad7Δsir3Δ*::pDP126.

Lane F: C^{14} molecular weight markers.

Lane G: *rad7Δsir3Δ*::pDP113, pDP126 SDS treated supernatant.

Lane H: *rad7Δsir3Δ*::pDP113, pDP126 SDS treated pellet.

Lane I: *rad7Δsir3Δ*::pDP126.


200
92.5
69
49
30
ha-Rad7
A B C D E F G H I

DISCUSSION

The two-hybrid system has provided a novel approach to identifying interacting proteins and new genes involved in specific biological processes (Fields and Song, 1989; Fritz and Green, 1992). This system provides a molecular genetic screen to detect interacting proteins by the fusion of each protein to one of the two separable domains of the Gal4 protein. In this study, the two-hybrid system was used to identify proteins that interact with the Rad7 DNA repair protein.

i. Two-hybrid System

Two improvements were made to the two-hybrid system: (1) construction of new pGAD vectors, and (2) production of libraries of yeast genomic DNA from a *gal4* deletion strain in the new pGAD vectors. These improvements did not affect the basic functions of the system (Fields and Song, 1989). The first involved the re-construction of the pGAD vectors, which was accomplished by inserting the core elements of the these vectors into the yeast-*E. coli* shuttle vector YEplac181 (Gietz and Sugino, 1988). This vector reconstruction reduced the size of the pGAD by 50%, improving yeast transformation efficiency, and changed the bacterial origin of replication, increasing the plasmid copy number in *E. coli* cultures. The second improvement involved the creation of pGAD fusion libraries in these vectors. Genomic DNA from a *gal4* deletion strain was used to eliminate false positives due to the reconstitution of the *GAL4* transcription activator gene. Chien *et al.*(1991) found that up to 85% of the positives found in screens using libraries made with genomic DNA from a *GAL4* yeast strain were of this type.

False positives were still detected in these *gal4Δ* pGAD libraries. Some were due

to the $GAL4_{AD}$ fusion protein activating transcription in the absence of the $GAL4_{BD}$ or $lexA_{BD}$ fusion. Bartel *et al.* (1993) suggest that these fusion proteins bind to the *GAL4* or *lexA* UAS regions and activate transcription, or to other proteins bound at this promoter region. A second type of false positive was able to activate transcription of β-galactosidase with numerous $GAL4_{BD}$ or $lexA_{BD}$ fusion proteins. This type of false positive can be recognized by testing for β-galactosidase production when they are combined with other unrelated $GAL4_{BD}$ or $lexA_{BD}$ fusion proteins (Bartel *et al.*, 1993). The positives which were detected with more than one *RAD* gene DNA binding domain plasmid may be of this type. An alternative explanation is that several *RAD* proteins may interact with a single protein in the repair complex. For example, if *RAD7* and *RAD6* have similar functions in their respective repair pathways, then one might expect them to both interact with the same protein. Conversely, if two repair genes function in the same repair complex they may both interact with a third protein within that complex. This may explain why both the Rad7 and Rad6 DNA binding domain fusion proteins seem to interact with the 7B16 fusion protein. This hypothesis could be tested by determining if the fusion proteins that activate transcription with more than one RAD DNA binding domain fusion protein are able to activate transcription with unrelated DNA binding domain fusion proteins.

Chien *et al.* (1991) stated that 460,000 $GAL4_{AD}$ fusion plasmids must be screened in each of the three fusion libraries to provide a 0.99 probability that all genes containing an appropriate *Mbo*I site have been screened. This number is determined using the following reasoning. The *Mbo*I restriction enzyme recognizes a 4 bp site which should occur randomly in the genome once every 256 bp. This would mean that there are

approximately 50,000 *Mbo*I sites in the 13.8 Mb yeast genome. Since each site can be cloned in two orientations, the frequency of cloning any one *Mbo*I DNA site in the correct orientation will be approximately 1/100,000. Using the formula N = *ln*(1-P) / *ln*(1-f) (Clarke and Carbon, 1976), where P = the probability of finding a particular insert, and f = the frequency of that insert, the number of clones (N) needed to generate a good library was calculated to be approximately 460,000 transformants at a probability of 0.99. Screening this number of clones from each library, or 1,380.000 clones from the three combined libraries, should generate 4.6 positives from the library with the particular site in the proper fusion frame. The insert frequency of the pGAD-2F and pGAD-1F/3F libraries was 60% and 90%, which increases the required number to 1,789,000 clones. Screening 389,000 transformants (1,789,000 / 4.6) from a mixture of the three libraries should give rise to one positive. The Rad7 screen of approximately 400 000 yeast transformants recovered two Sir3 clones, which is close to the number that would be expected from these calculations. Further screening of these libraries is necessary to ensure that all yeast *Mbo*I fragments have been tested for interaction with Rad7.

A perfectly random library of *Mbo*I fragments may not be sufficient to ensure that all genes are represented. Genes that do not contain an *Mbo*I site that can be fused to the $GAL4_{AD}$ to produce a functional fusion protein will not be found in this library. Consequently, very small genes may be impossible to detect in the two-hybrid system.

ii. The Rad7-Sir3 Interaction

a. The two-hybrid screen

The two-hybrid screen for proteins that interact with the Rad7 identified three classes of fusion proteins that interacted with Rad7. The first were produced from

plasmids pGAD-7B5.1 and pGAD-7B34 containing sequence identical to the known gene *SIR3*. In both plasmids the *SIR3* gene was fused to the $GAL4_{AD}$ at the same *SIR3* *Mbo*I site, however the downstream *Mbo*I site of this fragment differed, showing that they were independently derived. The second class, contained in plasmid pGAD-7B35, included a fusion protein from a unique gene fragment not found in GenBank. The function of this protein and the significance of its interaction with Rad7 remains to be determined. The third class, contained in plasmids pGAD-7B6 and pGAD-7B16, have also been identified in two-hybrid screens with other DNA binding domain fusion plasmids (*RAD18* and *RAD10,* and *RAD6*, respectively)(R.D. Gietz, personal communication). Due to their ability to activate β-galactosidase activity with more than one DNA binding domain fusion plasmid, these plasmids may be false positives, or they may encode proteins that interact with more than one DNA repair protein *in vivo*. Further analysis is needed to distinguish these two possibilities.

b. *SIR3*

SIR3 (silent information regulator 3 gene), together with *SIR1*, *SIR2*, *SIR4*, *histone H4*, *ARD1*, *NAT1*, *RAP1*, *RIF1* and *ORC2*, is one of at least ten genes involved in the transcriptional repression of the transcriptionally silent mating type loci (*HML α* and *HMRa*)(reviewed in Haber, 1992; Laurenson and Rine, 1992). The Sir3 protein is 978 amino acids in length, has a predicted molecular weight of 111 kDa (Shore *et al.* 1984), and contains a nuclear localization consensus sequence (Dingwall and Laskey, 1991). Loss of Sir3 protein function results in transcription at the normally cryptic *HML* and *HMR* loci, giving rise to the nonmating phenotype due to the expression of both a and α mating type information (Aparicio *et al.* 1991). *sir3* mutants also lack differential

DNA repair at the *HML* locus (Terleth. *et al.*, 1989). This is likely due to a change in chromatin structure at this locus associated with a *sir3* mutation (Terleth *et al.* 1989; Alberts and Sternglanz, 1990; Renauld *et al.*, 1993).

SIR3 is also involved in the transcriptional repression of genes located near the telomeres of *Saccharomyces cerevisiae* chromosomes. This is known as telomere position effect (TPE) (Aparicio *et al.*, 1991). The amount of Sir3 expressed in a yeast cell has also been found to modulate TPE, as overexpression of Sir3 increases the length of the transcriptionally silenced region extending from the telomere (Renauld *et al.*, 1993). This suggests that Sir3 is a structural component of transcriptionally silenced chromatin or is required for the assembly of this type of chromatin structure. However, other proteins must be involved, because there is no evidence to suggest that Sir3 interacts directly with DNA (Shore *et al.*, 1987; Haber 1992).

There is evidence that the products of several other genes interact with Sir3. Ivy *et al.* (1986) noted that overexpression of *SIR3* was able to suppress certain *sir4* mutants suggesting that Sir3 and Sir4 may interact. *SUM1-1*, a general suppressor of several mating type genes, also suppresses *sir3* mutants (Klar *et al.*, 1985; Livi *et al.*, 1990; Laurenson and Rine, 1991). The *sum2* and *sum3* mutants specifically suppress only *sir3* mutants (Lin *et al.*, 1990). The products of these three genes are thought to act downstream of Sir3 in the transcriptional silencing pathway. Overexpression of *SIR1* is also able to suppress certain *sir3* mutants, as well as *nat1*, *ard1* and histone *H4* mutants, suggesting functional interactions between the products of these genes (Stone *et al.*, 1991). Several *sir3* mutants are also able to suppress a mutation in the histone H4 N-terminal tail, but not a deletion of this tail, providing a link between a SIR protein and a

chromatin component (Johnson *et al.*, 1990). The ability of several *sir3* mutants to suppress one histone *H4* mutation has shed doubt on a direct interaction between these two proteins, yet a functional interaction seems to exist (Laurenson and Rine, 1992). Finally, there is recent evidence that Sir3 may interact with Rap1 (repressor/activator protein), which binds DNA at promoters, silencers and telomeres (Palladino *et al.*, 1993).

c. Further characterization of the Rad7-Sir3 interaction

The Rad7-Sir3 protein interaction which was detected using the two-hybrid system, was not found in the *SIR3* suppressor studies. The interaction occurs in the segment of Sir3 between amino acids 308 to 978. The mutations which suppress the histone *H4* mutants are found at amino acids 86 and 205 of *SIR3* (Johnson *et al.*, 1990), suggesting that separate regions of Sir3 are involved in the proposed interactions with histone H4 and Rad7.

A strain containing both the *rad7* and *sir3* deletions was between one and two orders of magnitude less sensitive to the killing effects of UV than a strain containing the *rad7* deletion alone. The *rad7* deletion strain level of UV sensitivity was regained when a *SIR3* plasmid was transformed into the double deletion strain, indicating that the reduced sensitivity to UV was due to the *sir3* deletion, and not an unknown mutation which occurred during the strain construction process. This suggests that the *RAD7* and *SIR3* gene products interact directly in the repair of transcriptionally silenced chromatin. Alternatively, the loss of transcriptionally silenced DNA in general, rather than the specific loss of the Sir3 protein, may cause the reduced requirement of Rad7 in DNA repair. If this is so, then the same effect should be seen when other genes involved in transcriptional silencing, such as *SIR1, 2* and *4*, are deleted in combination with *RAD7*.

These deletion constructs were not available to be tested.

The removal of functional Sir3 did not completely suppress the UV sensitivity of a *rad7* deletion. This suggests that Rad7 may either have a distinct role in DNA repair, separate from its interaction with Sir3, or that other proteins also interact with the Rad7 and Sir3 complex to provide access to DNA damage in transcriptionally silenced chromatin. Recent evidence suggests that a second role for Rad7, apart from its interaction with Sir3, may be in the repair of damage present in nontranscribed DNA strands (R. Verhage, personal communication).

Because the two-hybrid system is carried out in an essentially wild type yeast strain, it is possible that the Rad7-Sir3 interaction is mediated by another protein. The *in vitro* interaction demonstrated between the Rad7 and Sir3 GST fusion proteins in Figure 12, showed that no other yeast protein needs to be involved. Clearly the interaction between the Rad7 and Sir3 fusion proteins is specific, as the Rad7 and Sir3 GST fusion proteins bound to radiolabelled proteins of the appropriate molecular weight for Sir3 and Rad7, respectively. In addition, small peptides also bound to the GST fusion proteins. These may represent proteolytic fragments of the Rad7 and Sir3 proteins containing the interacting protein domains. The presence of these small peptides may explain the limited interaction observed between the radiolabelled Rad7 and Sir3 proteins with their GST counterparts. It is also possible that the affinity of Rad7 for Sir3 is not as strong as that seen between the subunits of a typical multimeric enzyme, such as casein kinase II (reviewed in Tuazon and Traugh, 1991; Litchfield and Lüscher, 1993). This may be due to the required reversible nature of this interaction. Alternatively, Rad7 and Sir3 may be complexed with other proteins that are required to stabilize their direct interaction. The

high production of β-galactosidase caused by the interaction between Rad7 and Sir3 fusion proteins in the two-hybrid system suggests that the interaction between Rad7 and Sir3 is stable in the absence of UV-induced DNA damage. However, β-galactosidase production is dependent on several unknown factors, including the proper folding and function of the $Gal4_{AD}$, $lexA_{BD}$, Rad7, and Sir3 domains. It is therefore not possible to determine the strength of the protein-protein interaction with this result. Further analysis with Rad7 and Sir3 proteins of greater purity is needed to further characterize this interaction.

The presence of multiple proteins in the radiolabelled supernatants (Figure 12) is likely due to a combination of thrombin-cleaved Rad7 or Sir3 peptides and co-purifying contaminating proteins that have been cleaved from the glutathione agarose by this thrombin treatment. The similarities between the protein bands in the thrombin-cleaved Rad7 and Sir3 radiolabelled supernatants provides further evidence that they are due to co-purifying contaminating proteins. The thrombin-cleaved co-purifying contaminating proteins do not bind either the glutathione beads, the GST-Rad7, or the GST-Sir3 loaded glutathione beads. This may occur because solubilization by thrombin cleavage removes the portion of these proteins that binds the glutathione-agarose beads.

Glutathione-agarose beads were used as a negative control in this experiment. A more appropriate control would have been glutathione-agarose beads bound with the GST protein. This raises the possibility that the Rad7 and Sir3 proteins interact nonspecifically with the GST portion of the fusion proteins and not with the Sir3 or Rad7 portions. If this were so, both the radiolabelled Rad7 and Sir3 thrombin-cleaved peptides would be required to interact with GST, while none of the other protein's thrombin-

cleaved supernatants did. Although it is unlikely that this occurs, the experiment utilizing GST-bound glutathione-agarose beads would provide more confidence in this result.

To further characterize the Rad7-Sir3 interaction, an attempt was made to identify this interaction *in vivo* by co-immunoprecipitation of radiolabelled yeast extracts. The results indicate that in this experiment the ha tag-Rad7 did not appear to bind the native Sir3 protein in a manner that allowed the co-immunoprecipitation of both proteins to be detected (Figure 14). Absence of a detectable interaction did not seem to be due to the high ionic strength of the maxi buffer (0.1% SDS), as the same buffer was used in the successful *in vitro* experiment. Renauld *et al.* (1993) suggest that the level of Sir3 protein in the yeast cell is autoregulated because the *SIR3* gene is located on chromosome XII near the telomere. When the Sir3 protein is overproduced, the spreading effect of TPE reduces transcription of the SIR3 gene, strictly regulating the level of this protein in the cell. During the short time of radiolabelling in this experiment, the amount of Sir3 protein produced may have been limited, resulting in an undetectable level of radiolabelled Sir3 on the fluorogram. The Sir3 protein may, however, be detectable on Western blots using an antibody which specifically recognizes it. Unfortunately, no such antibody was available. Another explanation for the absence of Sir3 from this fluorogram is that Sir3 may be bound in the insoluble fraction of the cell extract and not available for co-immunoprecipitation.

The evidence provided in this study suggests that the Rad7 and Sir3 proteins interact directly during DNA repair. This interaction may allow the nucleotide excision repair complex access to transcriptionally silenced chromatin. However, Rad7 and Sir3 proteins do not seem to be the only proteins involved in this process, as a *sir3* deletion

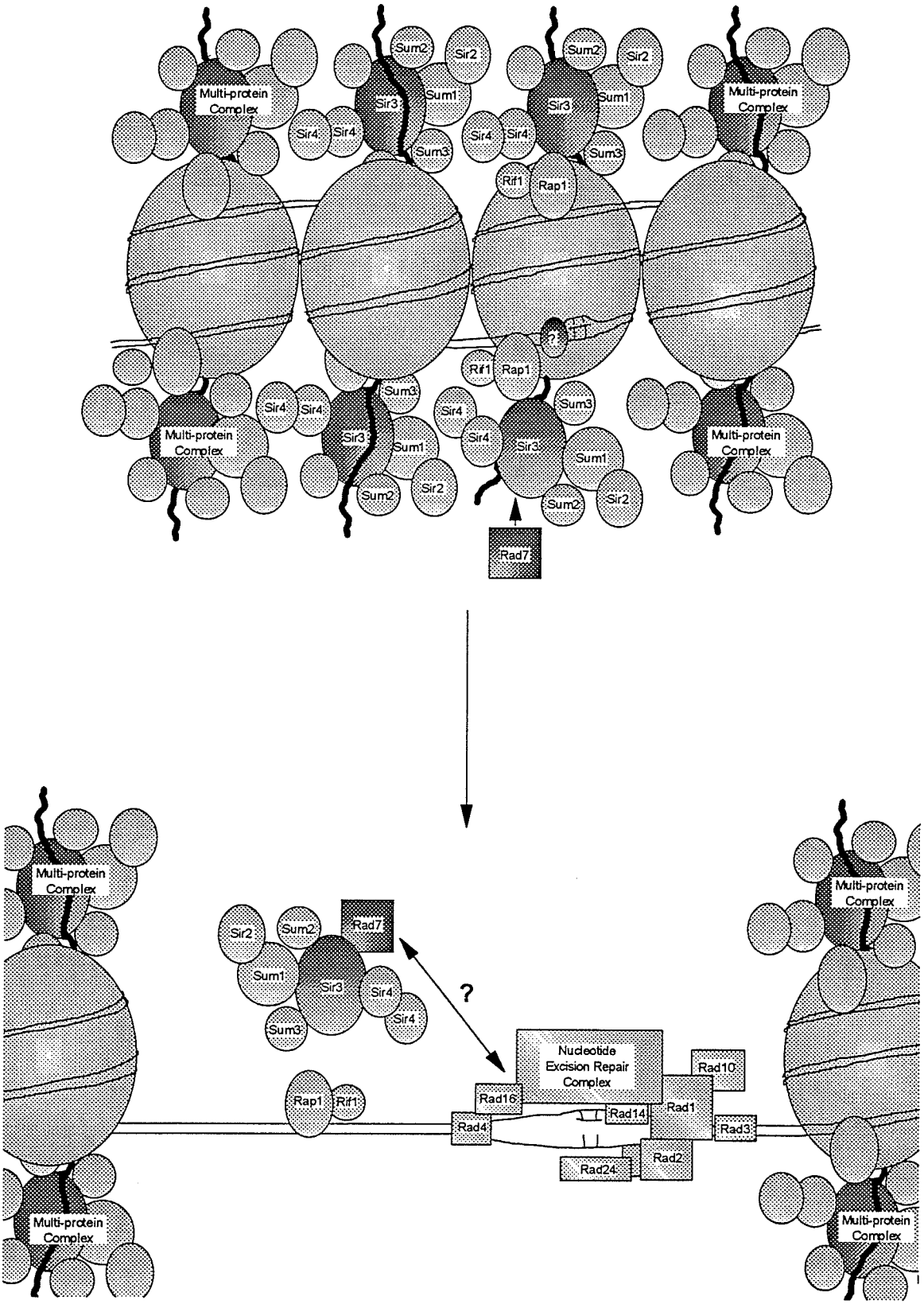
does not fully rescue the UV sensitivity phenotype of a *rad7* deletion mutant. Based on their proposed function in silenced chromatin structure or differential DNA repair, the Rap1, histone H4, Rad16, Rad23 and Sir proteins can be considered candidates for these other proteins (Palladino *et al.*, 1993; Johnson *et al.*, 1990; Bang et al., 1992; Schiestl and Prakash, 1989) . The Rap1 and histone H4 proteins may be involved in this process through their proposed interaction with Sir3. Similarly, Rad23 and the pGAD-7B35 proteins may be involved through an interaction with Rad7.

The interaction between Rad7 and Sir3 in the two-hybrid system did not require DNA damage. This suggests that Rad7 and Sir3 do not require DNA damage to interact *in vivo*. However, since the Rad7-Sir3 interaction was not seen *in vivo* using co-immunoprecipitation, this may not be the case. *RAD7* has been shown to not be involved in transcription silencing, as a *rad7* deletion mutant has no effect on the production of silenced chromatin at telomeres (Paetkau, D.W., J.A. Riese and R.D. Gietz. Interaction of the yeast *RAD7* and *SIR3* proteins; implications for DNA repair and chromatin structure. submitted). This also suggests that Rad7 and Sir3 do not interact under normal cellular conditions, but are stimulated to interact by DNA damage. The interaction of Rad7 and Sir3 in the absence of DNA damage seen in the two-hybrid system could be due to the overproduction of a fragment of Sir3 which titrates out another protein that is normally complexed with Sir3 in the absence of DNA damage.

Based on the data from this study and the literature, I have developed a model for the role of the Rad7-Sir3 interaction in DNA repair and chromatin silencing (see Figure 15). In the absence of DNA damage, Sir3 interacts with a number of proteins to condense the DNA in regions that are transcriptionally silenced. Several proteins,

Figure 15. Model of Rad7-Sir3 Interaction

In the absence of DNA damage, Sir3 interacts with histone H4 and a multiprotein complex to maintain the condensed DNA state. Protein interactions that occur in this complex may include Sir3-Rap1-Rif1, Sir3-Sir4-Sir4, SUM1-1-Sir2, SUM1-1-Sir3, SUM1-1-Sir2, Sir3-Sum2, Sir3-Sum3 and Sir3-histone H4. The TT represents a thymine dimer that alters the structure of the DNA, modifying the interaction between Sir3 and the other proteins mentioned above, and exposing a domain of Sir3 that can now bind Rad7. Rad7 binds to Sir3, causing local relaxation of the chromatin structure which allows the nucleotide excision repair complex to access this region and repair the damaged DNA. Rad7 may also interact with NER complex proteins. Upon completion of DNA repair the Rad7-Sir3 interaction is reversed, and Sir3 is able to interact with the transcriptional silencing proteins to re-establish the condensed chromatin state.


Multi-protein Complex
Sum2
Sir2
Sir3
Sum1
Sir4
Sum3
Rif1
Rap1
?
Rad7
Nucleotide Excision Repair Complex
Rad10
Rad16
Rad14
Rad1
Rad4
Rad3
Rad24
Rad2

including Rap1 (Palladino *et al.*, 1993), which binds DNA and interacts with Rif1 (Hardy *et al.*, 1992), Sir4, which dimerizes (Chien *et al.*, 1991), SUM1-1, which may also interact with Sir2 (Laurenson and Rine, 1991), Sum2, Sum3 (Lin *et al.*, 1990) and histone H4 (Laurenson and Rine, 1992), may be interacting with Sir3 to accomplish this task. Damage to the DNA in this region, such as a thymine dimer, alters the structure of the DNA. Either this alteration in DNA structure, or its recognition by some other protein, modifies the interactions between Sir3 and the other proteins involved in transcriptional silencing, exposing a domain of Sir3 that can now be bound by Rad7. Binding of Rad7, further alters the interactions between Sir3 and the other transcriptional silencing proteins, opening up the region of DNA surrounding the damage site. This change in chromatin structure allows the nucleotide excision repair complex to access this region and repair the damaged DNA. Interaction between Rad7 and proteins in the repair complex may also be necessary for repair of the damaged site. Once DNA repair is completed, the Rad7-Sir3 interaction is reversed, and Sir3 is able to interact with the transcriptional silencing proteins to re-establish the condensed chromatin state. Further analysis is needed to test this model.

iii. Concluding Remarks

The two-hybrid system has been used to identify proteins that interact with the Rad7 nucleotide excision repair and the Rad18 postreplication repair proteins. The Sir3 protein was detected using the two-hybrid system and shown to interact directly by other methods. These proteins were shown to be functionally related with respect to DNA repair and a model considering this interaction was presented. A direct interaction *in vivo* remains to be confirmed. This may be accomplished through the use of western blot

analysis with an antibody to detect Sir3 in immunoprecipitates. Alternatively, an inducible expression system can be used to produce high levels of Sir3 protein during radiolabelling.

The two-hybrid system has proved to be a powerful tool for the identification of protein interactions, *in vivo*. Further use of this system, by screening the two-hybrid system pGAD libraries with a GAL_{BD}-*SIR3* fusion plasmid, should identify additional genes involved in DNA repair of transcriptionally silenced chromatin.

APPENDIX A: INTERACTION BETWEEN *RAD18* AND 18B1

i. Summary

The yeast two-hybrid system was used to identify proteins that interact with the product of the postreplication repair gene *RAD18*. A total of 367,000 transformants from the pGAD fusion gene libraries were screened with a *GAL4-RAD18* fusion plasmid, allowing the identification of four clones encoding putative Rad18 interacting proteins. DNA sequence analysis of the plasmids that interact with pGAD-*RAD18* indicated that three of them contained inserts coding for an unknown protein designated 18B1. Approximately 2000 bp of the *18B1* gene were sequenced, including a potential stop codon. The size of the 18B1 mRNA was determined to be 3.3 kb, which suggests that approximately half of the gene has been sequenced. Gene knockout experiments failed to produce a recognisable phenotype for the *18B1* mutant.

ii. Introduction

The *RAD18* gene encodes a protein of 487 amino acids with a molecular weight of 55.5 kDa (Chanet *et al.*, 1988; Jones *et al.*, 1988). The function of the Rad18 protein has not been determined, but comparison of its protein sequence with proteins of known function has identified three putative zinc finger domains (amino acids 28-48, 51-65, 190-210) and a putative nucleotide-binding domain (amino acids 353-373) (Jones *et al.*, 1988; Chanet *et al.*, 1988). Zinc finger motifs are involved in binding to nucleic acids, providing evidence that Rad18 may be an ATP dependent DNA binding protein. Two possible functions have been suggested on the basis of these motifs. First, Rad18 may be a transcriptional activator of other DNA repair genes. Second, Rad18 may bind to damaged DNA sites and interfere with the mutagenic read-through repair process allowing the components of the error-free

process to operate instead (Jones *et al.*, 1988; Chanet *et al.*, 1988). In both cases, the binding and hydrolysis of ATP has been suggested as a mechanism which affects the affinity of the Rad18 protein for either the DNA regulatory sequences or the site of DNA damage (Jones and Prakash, 1991).

Deletion of the *RAD18* gene results in a similar phenotype as the *rad6* deletion: an increased sensitivity to UV; ionizing radiation; alkylating agents and other DNA damaging agents; and an elevation in the levels of spontaneous mitotic recombination and spontaneous mutations. However, the *rad18* deletion does not effect sporulation, spore viability, or levels of UV-induced mutagenesis. The lack of increase in UV-induced metagenesis, coupled with the involvement of *RAD18* in post-replication repair, has lead to the hypothesis that *RAD18* is involved in the error-free repair pathway. The function of *RAD18* in postreplication repair is still not clear. It is likely that the identification of proteins that interact with the product of *RAD18* will help to identify these functions.

iii. Methods

All methods not specific to this study were as described in Materials and Methods.

a. RNA Extraction

RNA was isolated from cells of the strain LP2752-4B according to the method of Jensen *et al.* (1983). A stationary phase liquid culture of LP2752-4B cells was diluted to 5 x 10^6 cells/ml in YPAD, grown at 30°C for one doubling, and harvested by centrifugation at 4000 x g for 5 min. The cell pellet was transferred to a microfuge tube, resuspended in 1 ml of water, centrifuged again for 1 min at 4000 x g, and resuspended in lysis buffer (0.5 M NaCl; 0.2 M Tris HCl pH 7.5; 0.01 M EDTA; 1% SDS) to a final volume of 500 µl. Approximately 30 mg of acid washed 40 nm diameter glass beads (Sigma) and 1 volume of

TE-saturated phenol:chloroform (1:1) were added and the tube was vortexed for 2.5 min. The aqueous phase was separated by centrifugation for 1 min at 13,000 x g, removed to a fresh tube, and extracted once again with one volume of TE-saturated phenol:chloroform (1:1).

The nucleic acids were precipitated by the addition of 0.1 vol of Na acetate pH 6.0 and 2.5 vol of ethanol, collected by centrifugation at 13,000 x g for 5 min at 4°C, and dissolved in 100 µl of sterile nanopure water. The concentration of RNA was estimated by determination of the absorbance at 260 nm (A_{260}) of a sample and using the following formula: A_{260}/ml x dilution factor x 40µg = µg/ml (Ausubel *et al.*, 1987).

b. Radiolabelling of DNA Probes

Purified DNA fragments to be used as probes for Southern or Northern gel blot analysis were radiolabelled by the method of Feinberg and Vogelstein (1983). The 25 µl radiolabelling reaction contained: 7.75 µl of DNA in sterile water (25-50 ng); 2.5 µl of [^{32}P]-cytosine triphosphate (25 µCi); 2.5 µl of [^{32}P]-adenine triphosphate (25 µCi); 1 µl (5 units) of DNA polymerase I Klenow fragment (Pharmacia); 11.75 µl of Klenow Reaction Buffer (0.5 M Hepes pH 6.6, 12.5 mM $MgCl_2$, 25 mM β-mercaptoethanol, 125 mM Tris HCl pH 8, 0.85 mg/ml BSA, 10.6 units/ml [$Pd(N)_6$] 6-mer Random Primers (Boehringer Manheim) and 0.25 mM each of dGTP and dTTP). The reaction was incubated for 50 min at 37°C, and then terminated by the addition of 175 µl of 15 mM EDTA. Klenow reactions used in the Northern blot procedure were run through a Pharmacia Sephadex G-50 Nick column according to the manufacturer's instructions. The approximate specific activity of the DNA in the column eluate was determined by counting 1 µl of the sample in 1 ml of scintillation fluid. The specific activities of the probes were between 10^8 and 10^9 cpm/µg of DNA.

c. Northern Blot Preparation

Yeast RNA was analyzed by Northern gel blot analysis following the method described in Maniatis *et al.*, 1982. The purified RNA was electrophoresed in a 1.2% (w/v) agarose gel containing 2.2 M formaldehyde and 1 x MOPS Gel Running Buffer (20 mM MOPS pH 7.0, 8 mM sodium acetate, 1 mM EDTA pH 8.0, filtered through a 0.2 μm filter) cast in a 14 cm x 15 cm BioRad gel box. The gel was electrophoresed for 5 min at 5 V/cm in 1 x Gel Running Buffer plus 2.2 M formaldehyde prior to loading the RNA samples.

RNA samples were prepared by mixing 4.5 μl of RNA (1-10 μg) with 2 μl of 5 x Gel Running Buffer, 3.5 μl of 12.3 M formaldehyde and 10 μl of 100% (v/v) formamide, followed by incubation at 65°C for 5 min to denature the RNA. Loading buffer (50% glycerol, 1 mM EDTA ph 8.0, 0.25% bromophenol blue, 0.25% xylene cyanol) was added to 0.1 vol, and the samples were centrifuged at 13,000 x g. The prepared RNA samples were loaded on the gel and electrophoresed for 4 hr at 4 V/cm. After completion of the electrophoresis, the gel was rinsed in water three times for 15 min each with gentle shaking, stained for 20 min with 0.5 μg/ml EtBr in water, and destained for 45 min in 10 x SSC (150 mM NaCl, 15 mM sodium citrate) with gentle shaking.

The RNA was transferred to Zetaprobe membrane (BioRad) with 10 x SSC according to the manufacturers specifications. The positions of the 16S and 28S RNA bands were marked during illumination of the filter with 254 nm UV. The filter was then incubated in 150 μl/cm^2 of prehybridization solution (0.5 M Na_2HPO_4 pH 7.2; 0.5 M EDTA; 7% SDS) for 10 min at 65°C in a sealed plastic bag. Approximately 2.5 x 10^7 cpm of DNA probe and 5 x 10^5 cpm of radiolabelled lambda DNA were added to the bag and incubated for 16 hr at 65°C. The filter was washed once in Church Wash (0.04 M Na_2HPO4 pH 7.2; 1% SDS) for

15 min at room temperature, and twice for 40 min at 60°C. The filter was air dried and exposed to Kodak AR film at -80°C.

d. Southern Blot Preparation

Yeast genomic DNA samples digested with specific restriction enzymes were electrophoresed on a 0.7% (w/v) agarose gel. The gels were rinsed in water, treated with 0.2 N NaOH, 0.6 M NaCl, for 30 min to denature the DNA, rinsed again in water and then transferred to Zetaprobe membrane (BioRad) in 10 x SSC using the capillary blotting technique as specified by the manufacturer. Hybridization with radiolabelled probes was done as described for the Northern gel blots.

iv. RESULTS

a. The RAD18 Two-hybrid Screen

i. Construction of pMA424-*RAD18* and pBTM116-*RAD18*

The pMA424-*RAD18* vector was constructed by cloning the *Bam*HI *RAD18* PCR fragment into the *Bam*HI site of the pMA424 vector. The *Bam*HI *RAD18* PCR fragment was produced by PCR amplification of the *RAD18* gene from plasmid pDG323 with primers, shown below (DNA Laboratory, U. of Manitoba), that contained 5' terminal *Bam*HI sites.
Five Prime Oligonucleotide: 5'-CGCGGATCCGAATGGACCACCAAATAACCACT-3'
Three Prime Oligonucleotide: 5'-CGCGGATCCTTAATTGTTACCGGGTGGGTC-3',
This *RAD18* fragment was also cloned into the *Bam*HI site of the pBTM116 vector to create pBTM116-*RAD18*.

ii. RAD18 Two-hybrid Screen

The pMA424-*RAD18* and pBTM116-*RAD18* plasmids were used in the two-hybrid system to screen the pGAD libraries for Rad18 interacting proteins. The results of a screen

Table 8. Results of *RAD18* Two-hybrid Screens

Library Plasmid from screen	Colour in CTY10-5D	Colour in CTY10-5D-BTM116-*RAD18* or GGY1::171-pMA424-*RAD18*	Genes that also interacted with the plasmid	Identical DNA Sequences
Two-hybrid System Screen with pMA424-*RAD18* (287,000 clones)				
18B1	white	blue		MCM1 interacting protein
7 plasmids	blue	blue		false positive
Two-hybrid System Screen with pBTM116-*RAD18* (84,000 clones)				
18B2.2	white	blue		18B1
18B7.1	white	blue		18B1
18B17	white	blue		no match
18B19	white	blue		no match
18B1.1	white	blue	*RAD7, RAD10*	no match
18B2.1	white	blue	*RAD7, RAD10*	no match
18B12	blue	blue		false positive
18B15.1	white	blue	*RAD10*	no sequence
18B15.2	white	blue		no plasmid DNA

of 287,000 yeast library plasmids transformed into a strain containing the pMA424-*RAD18* plasmid and a screen of 84,000 plasmids transformed into a strain containing the pBTM116-*RAD18* plasmid are summarized in Table 8. The first screen produced 8 yeast colonies that turned blue on X-gal medium. Seven of these were false positives as they turned blue on X-gal medium when the pMA424-*RAD18* plasmid was lost. The remaining yeast isolate, 18B1, required the presence of the pMA424-*RAD18* plasmid for the induction of β-galactosidase. The second screen, using the pBTM116-*RAD18* plasmid, produced an additional 9 yeast colonies that turned blue on X-gal medium. Eight of them were dependent on the presence of the pBTM116-*RAD18* plasmid, for the induction of β-galactosidase. One, pGAD18B12, was found to be a false positive of the type described above.

The library plasmids from each blue yeast colony were recovered, transformed into *E. coli,* and the inserts were sequenced as described in Materials and Methods. Two of these plasmids, pGAD18B1, and pGAD18B2.2, proved to be identical. A third plasmid, pGAD18B7.1, was found to contain an insert from the same gene, but fused to $GAL4_{AD}$ at the next *Mbo*I site toward the 3' end. The fourth and fifth plasmids, pGAD18B17 and pGAD18B19, contained unique DNA sequence after the *Bam*HI fusion junction. The sixth and seventh plasmids, pGAD18B1.1 and pGAD18B2.1, both contained identical inserts also identified in a pBTM116:*RAD10* and a pBTM116:*RAD7* two-hybrid screen. The final plasmid pGAD18B15.1 did not yield DNA sequence, and no plasmid DNA was ever recovered from the yeast clone 18B15.2. A search for identical sequences in the GenBank database with the sequences of each insert was not successful.

Table 9. Colour of colonies containing RAD18 two-hybrid screen fusion protein plasmids.

	DNA Binding Hybrid	Transcription Activating Hybrid	Colony Colour*
1.	pMA424-*RAD18*	-	-
2.	-	pDP12-18B1	-
3.	pMA424-*RAD18*	pDP12-18B1	+++
4.	pBTM116-*RAD18*	pDP12-18B1	++++
5.	pMA424-*RAD6*	pDP12-18B1	-
6.	pMA424-*D12***	pDP12-18B1	-
7.	pMA424-*SNF1*	pGAD424-*SNF4*	+

*The blue colour of the colony is rated on a scale from white (-), to pale blue after 3 days growth (+), to dark blue after 1 day growth (++++).
**D12 refers to the vaccinia virus D12 ORF which encodes the small subunit of the virus-encoded mRNA capping enzyme(Carpenter and DeLange, 1991).

The plasmids in rows 1-3 and 5-7 are in the yeast strain GGY1::171, and the plasmids in row 4 are in the CTY10-5D yeast strain. Three transformants were assayed in duplicate as described in Materials and Methods.

b. Characterization of the 18B1 Library Plasmid

To determine if the 18B1 library plasmid was a true two-hybrid system positive it was transformed into yeast strains containing a number of different $GAL4_{BD}$ fusion plasmids. The results, summarized in Table 9, show that production of β-galactosidase only occurs in the presence of a DNA binding domain RAD18 fusion plasmid. This plasmid combination produced a more intense blue colour than the Snf1-Snf4 positive control. These results suggest that the Rad18 protein specifically interacts with the 18B1 protein.

Approximately 2000 bp of the 3.6 kb DNA insert from the 18B1 plasmid was sequenced (see Figure 16). The first stop codon in frame with the $GAL4_{AD}$ was found 1732 nucleotides from the fusion junction suggesting that the sequence coded for a polypeptide of at least 577 amino acids. A restriction map of the region surrounding the 18B1 genomic fragment was determined by digesting genomic DNA from the strain LP2752-4B with restriction enzymes (*Bam*HI, *Eco*RI, *Hind*III, *Kpn*I, *Pst*I, *Sac*I, *Sal*I, *Sma*I, *Xba*I, *Bam*HI/*Sac*I, *Bam*HI/*Kpn*I, *Bam*HI/*Pst*I, and *Pst*I/*Sac*I) followed by Southern blot analysis as described in Materials and Methods. The *Sal*I-*Xba*I fragment (Probe 1, Figure 17B) was used to probe the Southern blot. The restriction map generated by these experiments is shown in Figure 17A.

Northern gel blot analysis was used to determine if the 18B1 ORF region produces RNA in growing yeast cells. RNA from the strain LP2752-4B *(RAD*$^+$) was extracted, electrophoresed, and probed with Probe 1 (Figure 17B) as described in Materials and Methods. The results of this experiment, shown in Figure 18, indicate that RNA, about 3.3 kb in size, is expressed during the logarithmic growth phase of yeast. This suggests that the 18B1 ORF may produce a protein required during normal growth in yeast.

Figure 16. Nucleotide and predicted protein sequence of the 18B1 fragment.

The sequence of the *Mbo*I fusion site, and the first 1984 bp of the 18B1 insert fused to the $GAL4_{AD}$ at this site, are shown. The predicted 577 amino acid sequence of 18B1 ORF is given using the one letter code for amino acids.

MboI(Fusion Site)

```
CCGGATCAATGCCGTGATGTGTTGTGCCCACGCGAAAACTTACTAGCCATTAATAGAACTAATAATATAGAGAGTGTTGCT 81
 P  D  Q  C  R  D  V  L  C  P  R  E  N  L  L  A  I  N  R  T  N  N  I  E  S  V  A
ATACCGCGACAGAGATCGAGCAAGAACAAGAAACCCCATGAGCATACACAATCGCAGGTTCGATTTTCCATTCCAGATCCA 162
 I  P  R  Q  R  S  S  K  N  K  K  P  H  E  H  T  Q  S  Q  V  R  F  S  I  P  D  P
AATGAAATATCACAGGACTCCCCACTGAAAATTGTATTCCCCAAGAGTGGAAATGAAACCGAAAGAAGAATGTCTACTTCA 243
 N  E  I  S  Q  D  S  P  L  K  I  V  F  P  K  S  G  N  E  T  E  R  R  M  S  T  S
                  StyI        SalI
TCATTACTTATGAATAGCCATGGTCATTTAGTCGACATGCACTCTAAAATACTGGTCGATGTCCCTAAAGAAGTTTGGAAG 324
 S  L  L  M  N  S  H  G  H  L  V  D  M  H  S  K  I  L  V  D  V  P  K  E  V  W  K
TTTCATCATAACAGGAGAAAAAAATGTGAAAGCAGGCACCGTAAAACTCGTTCTGATGTTAGGTCCAACACAAGTAGTAGT 405
 F  H  H  N  R  R  K  K  C  E  S  R  H  R  K  T  R  S  D  V  R  S  N  T  S  S  S
               XbaI
GGAAAAGAACCAAACCATTCTAGATCAAAATCATTACAGTCGATCATTGTGGATACAATGAACACATATCGTGCAACCGAG 486
 G  K  E  P  N  H  S  R  S  K  S  L  Q  S  I  I  V  D  T  M  N  T  Y  R  A  T  E
                                                                 StuI
GTTGAGACCAGTATTAATGAGAATACTAGTAATATTAGCCAAGTATCTCCGCTGAATTTATCATTTGACAGGCCTCCTCCC 567
 V  E  T  S  I  N  E  N  T  S  N  I  S  Q  V  S  P  L  N  L  S  F  D  R  P  P  P
                                                 EcoRV
TTAACTCCCGAAAAGAATTTATATCTCACTCCTGAATCGCCATTGAACAGATATCATTTACCAGTTCCATTAGAAATATCT 648
 L  T  P  E  K  N  L  Y  L  T  P  E  S  P  L  N  R  Y  H  L  P  V  P  L  E  I  S
CTGCCGCCCTACTTATCTCCGCAAAACAAGGATAAAAAGAGGAGTAGCTTGGTTTACGATGGGGATGGCTATAGCCAGTTT 729
 L  P  P  Y  L  S  P  Q  N  K  D  K  K  R  S  S  L  V  Y  D  G  D  G  Y  S  Q  F
                              XbaI
CAAGAAGGTAACACCTCATCGTCAACTGAATCTTCTCTAGAACAGCCCTCATCGTCGTATTCAGATGAAGATGATTCTATT 810
 Q  E  G  N  T  S  S  S  T  E  S  S  L  E  Q  P  S  S  S  Y  S  D  E  D  D  S  I
                                                 HindIII
CCATATGCCCACCATGATGTGTCTTTCGAGTTAAATAATGCAGATGCTGATAAGCTTCTTGGAATTGATGAGAACGCCAAC 891
 P  Y  A  H  H  D  V  S  F  E  L  N  N  A  D  A  D  K  L  L  G  I  D  E  N  A  N
GTCAATCTGAAGATACAAAGGAGAAATCTCAAAAACCCACAACATATTAAGAGTAAAACAGATAGGGAATGTGAGGAAAAA 972
 V  N  L  K  I  Q  R  R  N  L  K  N  P  Q  H  I  K  S  K  T  D  R  E  C  E  E  K
AACACTGAAAAAAATGTTTCGTTAAAAATTCTGTCAACACCAAATAAACTGATAGACATCCCCGACTTAGAGCACATGAAA 1053
 N  T  E  K  N  V  S  L  K  I  L  S  T  P  N  K  L  I  D  I  P  D  L  E  H  M  K
TCCCCACCAAGCACAGGTTTAAATGGGACATTAAAATTTTTCCAGCAGTTTGAACCTAGCGAGGAGCCAACTTCTCCAACT 1134
 S  P  P  S  T  G  L  N  G  T  L  K  F  F  Q  Q  F  E  P  S  E  E  P  T  S  P  T
      HpaI
CGCCAAGTTAACCCTGAATCACTGGACAAACTCGATATGTCATTCAAATTTCCCTCCAGTACTACTAACAATAATGTGGAT 1215
 R  Q  V  N  P  E  S  L  D  K  L  D  M  S  F  K  F  P  S  S  T  T  N  N  N  V  D
AAGGTACATGAAAATAGAAACTCTGGAGACACGAACAATGAAGATTTCCTGAAAGTTGATACTTCGCCTGTGAATCAATCA 1296
 K  V  H  E  N  R  N  S  G  D  T  N  N  E  D  F  L  K  V  D  T  S  P  V  N  Q  S
TTTGAATCCAGAAGACAGATGTTAATAGACTTGCAAAAATCTCCGACGAATAATAATCCGAGGATACATAAACATAGGAGA 1377
 F  E  S  R  R  Q  M  L  I  D  L  Q  K  S  P  T  N  N  N  P  R  I  H  K  H  R  R
AGCAGGAGCGTTCATAATATTGATGACATTTCCTTAAATTTCGAGGCAACCTCCACACCACCAGCTCCGACCTCTGCTCCC 1458
 S  R  S  V  H  N  I  D  D  I  S  L  N  F  E  A  T  S  T  P  P  A  P  T  S  A  P
TCAATTCCTGTTGAACATTCTAACCCATGTACTTCCATTGAAATACCTAAGAGATCACCTTACGGTTCACGTCATCTCCAA 1539
 S  I  P  V  E  H  S  N  P  C  T  S  I  E  I  P  K  R  S  P  Y  G  S  R  H  L  Q
                                                                 KpnI
AAACATCTGATATTCCTCCTGAAGCTCAATCACCTAAAAATGGGTCCTTCCTCCAAGAAATCTCGGTACCTTCGATTCAAA 1620
 K  H  L  I  F  L  L  K  L  N  H  L  K  M  G  P  S  S  K  K  S  R  Y  L  R  F  K
                                                                       HindIII
TTATACCAGATGAAAGTATCTCTCATACAAGAGAACCATCACCATCACTTATTGAATGCCCAGAGGATGAAAACGAAGCTT 1701
 L  Y  Q  M  K  V  S  L  I  Q  E  N  H  H  H  H  L  L  N  A  Q  R  M  K  T  K  L
                              Stop                          ClaI
TTTCTACTGAAGTTGCTGACCATTCTATTGCAATAATTAGTGAAACAAAAAGTGTTCCATCGATAGAGCCATTCAAACCGC 1782
 F  L  L  K  L  L  T  I  L  L  Q
TATCTTCATTCAATTCATTTGGTCAAGAAATTCAAAACAAGGAACCAACTCCTTTGAACCAAACTCCTACAGACTTAATTG 1863
GTAAACAAAGAAACTGTGTGAATCCGCATTCAATTCCATTTAGTGTTTTATCTTCCAATCGCCAATCGCACAATCGGGTAG 1944
TAGTAAATCTTCTACAGGCTGAGTTTCTTCCATACTGCATTACGGTA                                    1991
```

Figure 17. 18B1 DNA restriction maps.

A. Restriction map of the 18B1 genomic DNA region. The sequenced region is indicated by the thick line below the restriction map.

B. Restriction map of the 1991 bp sequenced region of 18B1, and the pDP61 plasmid used to create an 18B1 deletion mutant. This deletion removed the 61 bp between the *Stu*I and *Eco*RV sites. The 1.7 kb *Xba*I fragment was used to create a mutation of the 18B1 ORF in the strain MKP°. The circled *Xba*I site is not cut in DNA from the dam$^+$ DH5α strain that was used for these constructions.

Probe 1 was used in the Northern blot analysis of the 18B1 mRNA and Southern blot analysis of 18B1 genomic region.

Probe 2 was used in the Southern blot analysis of the 18B1 deletion strains.

Restriction Endonucleases: K=*Kpn*I and *Asp*718; E=*Eco*RI; H=*Hind*III; S=*Sal*I; X=*Xba*I; Y=*Sty*I; U=*Stu*I; V=*Eco*RV; A=*Hpa*I; C=*Cla*I.

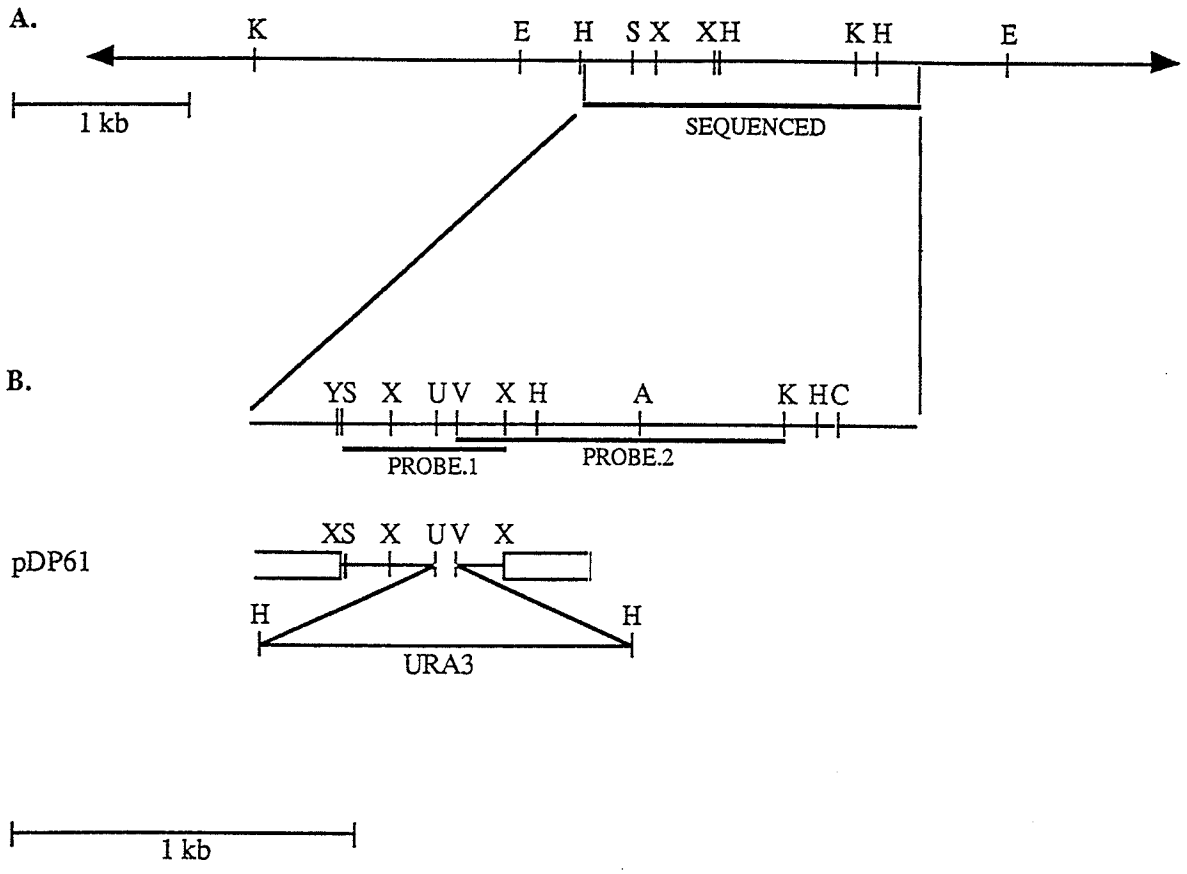
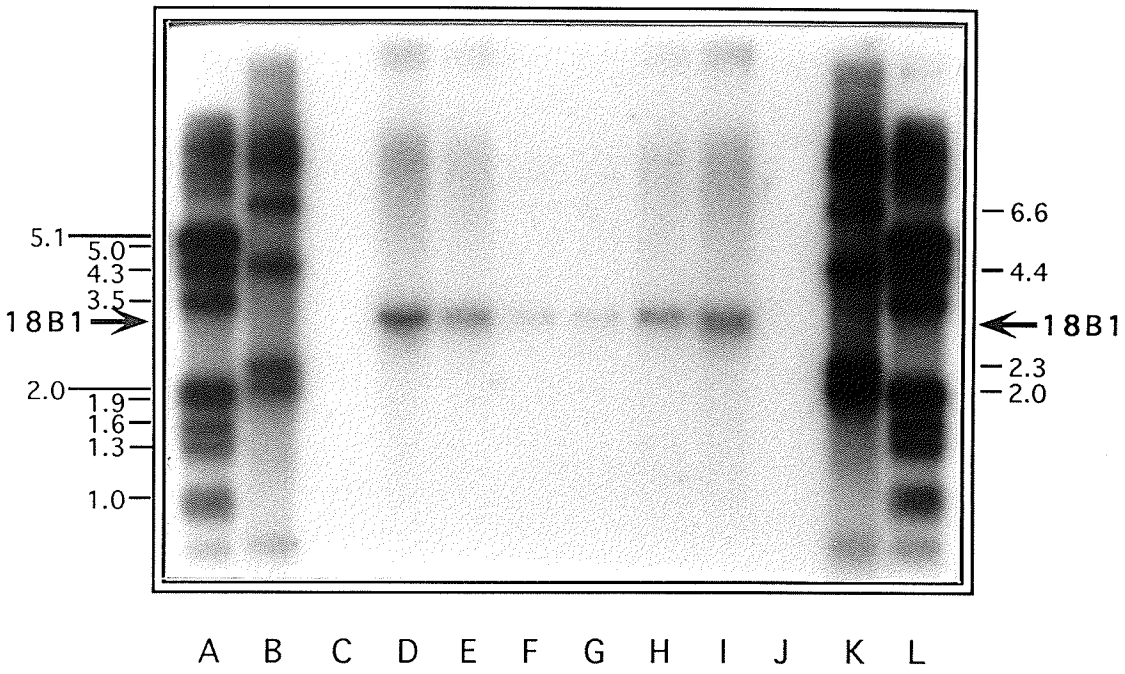

A.
K
E
H
S X
XH
K H
E
1 kb
SEQUENCED
B.
YS
X
U V
X H
A
K HC
PROBE.1
PROBE.2
pDP61
XS
X
U V
X
H
H
URA3
1 kb

Figure 18. Northern blot analysis of the 18B1 mRNA transcript.

Samples containing 10 μg (Lanes D and I), 5 μg (Lanes E and H) and 1 μg (Lanes F and G) of total RNA from the yeast strain LP2752-4B were purified, electrophoresed on a 1.2% (w/v) gel for 4 hr at 90 volts, blotted onto Zetaprobe membrane, hybridized to the Probe 1 DNA sequence, and exposed to X-ray film as described in Materials and Methods. *Hind*III (Lanes B and K) and *Hind*III/*Eco*RI (Lanes A and L) cut lambda DNA, hybridized to radiolabelled lambda DNA, were used as approximate size standards.


5.1
5.0
4.3
3.5
18B1
2.0
1.9
1.6
1.3
1.0
6.6
4.4
18B1
2.3
2.0
A B C D E F G H I J K L

Two deletion constructs were made in the *RAD*$^+$ yeast strain MKP$^\circ$ to determine if this expressed DNA sequence functions in postreplication repair. The first was constructed by transforming the strain MKP$^\circ$ with the 1.7 kb *Xba*I fragment of plasmid pDP61. Plasmid pDP61, was constructed by cloning the 490 bp *Xba*I fragment of pDP46 into the *Xba*I site of pUC19, and then replacing the 61 bp between the *Stu*I and *Eco*RV sites of 18B1 by blunt end cloning a 1.2 kb HindIII fragment, containing the *URA3* gene, into these sites. Plasmid pDP46 was created by cloning the 3.6 kb *Sal*I fragment containing the 18B1 ORF into the *Sal*I site of pUC19. The second deletion was made with PCR amplified DNA using 120 base oligonucleotide primers supplied by Dr. S. Weber (Kodak, Life Sciences Division, Rochester, NY). These primers were used to amplify the *URA3* gene flanked by 18B1 DNA at the 5' and 3' ends (bp 70 to 109, and bp 720 to 839, respectively). This construct was transformed into MKP$^\circ$ (haploid), RS185 (diploid) and RS195 (diploid) yeast cells resulting in a deletion of 611 bp between nucleotides 109 to 720 of the sequenced 18B1 ORF. Transformants containing the deletion construct were selected on medium lacking uracil and verified by Southern gel blot analysis using the *Eco*RV-*Kpn*I fragment (Probe 2, Figure 17B) (data not shown). One out of fifteen diploid and one out of two haploid *URA3*$^+$ transformants were confirmed as 18B1 deletions. Deletions in the 18B1 gene did not affect growth on minimal medium; sensitivity to UV (tested at 10, 20, 40 and 60 Joules/m^2, as described in Materials and Methods); mating ability (tested by mating the MKP$^\circ$-18B1 deletion strain to XS803-3A cells); or sensitivity to UV or gamma radiation. Plate assays for sensitivity to gamma radiation were done according to the procedure for UV plate assays given in Materials and Methods except that plates were exposed to 40 Krads of γ radiation and incubated with no regard to exposure to light.

The sequence of the pGAD18B1 ORF was sent to Dr. M. Goebl, (Indiana University, School of Medicine) who performed a search to determine if this sequence contained homology to known protein motifs. This search was unsuccessful, however the pGAD18B1 ORF was found to show identity with a segment of DNA that interacts with the yeast transcriptional activator MCM1 (minichromosome maintenance deficient 1, reviewed in Sprague, 1990) in a two-hybrid screen, sent to Dr. M. Goebl by Dr. G. Sprague Jr. (personal communication). The lack of a recognizable phenotype for the 18B1 deletion mutant caused this project to be discontinued so that more effort could be focused on the analysis of the Rad7-Sir3 interaction.

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