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Blm10p of *Saccharomyces cerevisiae* is a Nuclear Protein that Functions in Relieving Cellular Stresses Caused by Bleomycin

by

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A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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Abstract

Blm10p of *Saccharomyces cerevisiae* is a Nuclear Protein that Functions in Relieving Cellular Stresses Caused by Bleomycin

by

Kevin Doherty

Advisor: Professor Carol Wood Moore

Blm10p has been one of many uncharacterized proteins of *Saccharomyces cerevisiae*. The work presented here provides an initial characterization of the protein. *BLM10* acts as a multi-copy suppressor of the hypersensitivities conferred by the *blm3-1* mutation to lethal effects of the bleomycin-phleomycin family of glycopeptides. Mutant *blm3-1/blm3-1* cells suffered extremely high DNA damage, assayed in pulsed field gel electrophoretic analyses, and killing. *BLM10* was found to be nonessential for viability, but essential for protection against lethal effects of the drug family. The 6432 bp gene (YFL007w) was sequenced in three strains, and was actually larger than originally predicted. The size was confirmed in northern analyses. Deletion of *BLM10* conferred complete growth inhibiton and high killing in the presence of 5 μg/ml phleomycin. Overexpression of Blm10p restored growth and survival.

A Blm10p-YFP fusion protein was created. It localized to nuclei throughout the cell cycle, and co-localized with Spc42p-CFP. After drug treatments, Blm10p also localized to nuclei, but did not form foci. Nuclei were fragmented after drug treatment. A truncated Blm10p-GST fusion, missing 340 amino acids at the Blm10p carboxy terminus, also localized to nuclei in stationary phase cells, but localized to bud necks in budded cells. This indicated the importance of the carboxyl terminal region of the protein for its retention in the nucleus. Blm10p was found highly conserved among seven lower and higher eukaryotes, and evaluation of each region of the proteins revealed five conserved regions in similar spacial arrangements. The conserved carboxyl-terminal region contained the strongest homology, further indicating the importance of this region. Only one homolog, human PA200, a 20S proteasomal activator, is characterized. Based on this knowedge, proteasomal function was investigated in *BLM10* and *blm10Δ* cells, but found to be equivalent in both genotypes for the degradation of whole proteins. Finally, extensive two hybrid screens were carried out to determine specific, direct interactions of Blm10p with other proteins, and should be informative when analyses are completed.

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Introduction

I. The Blm10p of *Saccharomyces cerevisiae*

The ability of a cell to relieve itself of cellular stresses is important to the survival of the organism. These stresses come in a variety of forms. These forms include but are not limited to oxidative, heat, osmotic, and a variety of organic molecules, just to name a few. Over thousands of years, organisms have evolved a number of strategies for dealing with these stresses. These strategies are very intricate and usually involve a number of different cellular components to function in a very specific manner(s). Understanding these cellular responses are of considerable biochemical and medical importance. The current work has utilized a number of genetic, molecular, and biochemical techniques in order to characterize a protein whose function is necessary for survival after the application of at least one type of cellular stress. The protein of interest has been Blm10p of the yeast *Saccharomyces cerevisiae*.

A. *Saccharomyces cerevisiae* as a model organism

Saccharomyces cerevisiae has come to be recognized as a model eukaryotic organism for these types of studies. The budding yeast can exist stably as either a haploid or diploid in its life cycle. Some other properties that have made this yeast particularly suitable for biological studies include rapid growth, dispersed cells, the ease of replica plating and mutant isolation, a well defined genetic system, a large number of vectors available and, a

highly versatile DNA transformation system. This yeast has been a model system for molecular, genetic, and biochemical research for more than three decades because many of its basic cellular mechanisms also exist in mammals. The yeast has a high degree of genetic conservation with higher eukaryotic organisms and numerous genes initially characterized in the budding yeast have expedited the functional identification of genes in eukaryotes.

B. The *blm3-1* mutation

The *BLM10* gene was cloned in an effort to elucidate genes which encode proteins that are involved in resistance to oxidative damage and/or DNA repair. In this effort a series of mutants were created in the laboratory of Dr. Carol Moore (Moore, 1980, 1991). These mutants were isolated based upon their elevated sensitivities to killing by bleomycin (and structurally related phleomycin), as well as an increased sensitivity to the lethal effects of X-rays, ultraviolet radiation or hydrogen peroxide. These mutants were then divided into complementation groups.

The *blm3-1* mutant fell into a unique complementation group (Moore, 1980, 1991). The mutant is unique in that it demonstrates codominant expression in combination with the wild-type gene such that heterozygous *BLM3/blm3-1* diploid strains exhibit drug hypersensitivities that are intermediate to those exhibited by homozygous normal and homozygous mutant diploids (Moore, 1991). The *blm3-1* mutation confers hypersensitivity

to killing by bleomycin (Figure 1), as well as phleomycin (a structural analog), hydrogen peroxide, and ionizing radiation (Moore, 1991). However, mutant strains are not hypersensitive to killing by ultraviolet radiation.

C. Cloning of *BLM10*

The *BLM10* gene was cloned by functional complementation (Febres *et. al.*, 2001). The multicopy plasmid, which functionally complemented the *blm3-1* mutation was found to contain an insert with the first approximately 50% of the *BLM10* gene (Figure 2). This portion of the gene was demonstrated to fully restore the cellular resistance to the drug (Figure 1; Febres *et. al.*, 2001). Genetic analysis of *blm3-1* and a *blm10Δ* revealed that *BLM10* is a multicopy suppressor of the *blm3-1* mutation. The identity of the gene was facilitated by the fact that the *Saccharomyces cerevisiae* genome had been completely sequenced (Goffeau *et. al.*, 1996). The open reading frame encoding the *BLM10* gene was determined to be on chromosome VI and had been notated YFL007w.

D. Computer analysis

A hydropathy analysis of the protein predicts the Blm10 protein to exist in a hydrophobic environment (Figure 3). The plot was done using the Kyte Doolittle method (Kyte and Doolittle, 1982) and predicts that the protein contains 10 transmembrane regions. Other hydropathy plots predict a

membrane protein with 7-10 transmembrane regions depending on the algorithm used and the number of amino acids spanning the membrane.

The hydrophilic areas, which are predicted to exist outside the membrane, contain numerous potential motifs (Bucher and Bairoch, 1994). The protein is predicted to contain a total of 95 putative motifs. These include 3 leucine zippers, 3 cAMP/cGMP-protein kinase phosphorylation sites, 5 tyrosine kinase phosphorylation sites, 13 n-myristoylation sites, 16 n-glycosylation sites, 21 protein kinase C phosphorylation sites, and 34 casein kinase II phosphorylation sites. Although all of these domains may not exist on the protein, they could be helpful in providing a reference for future studies on the Blm10p.

In an effort to try to better understand a potential relationship between the hydropathy analysis and the predicted potential motifs a hypothetical model was generated (Figure 4). The sites are predicted to occur throughout the protein, but most fall within the predicted hydrophilic regions.

Further computer aided analysis of the Blm10p has led to putative characterizations of the protein. In one study, computer aided analysis lead a group to suggest the Blm10p may be a new member of the major facilitator superfamily of permeases due to the presence of the 10 transmembrane regions, as well as the absence of homology to p-type transport ATPases or an ATP binding cassette (Nelissen *et. al.*, 1997). In another study, cells were treated with methylmethanesulfonate (MMS), a DNA alkylating agent, and the expression of genes was looked at using DNA microarray technology

(Jelinsky and Samson 1997). The results showed that the gene encoding the Blm10p was induced 5.6 fold after the MMS treatment. Another group (http://bioinfo.mbb.yale.edu/genome/yeast) performing a global analysis of the yeast genome looked at mRNA expression of the genes. This group predicts *BLM10* to have a very low expression at 0.8137 copies/cell. This is consistent with the prediction made by another group that Blm10p is present in the cell at a quantity of less than 1 copy per cell (Professor Leona Sampson, Harvard School of Public Health, personal communication).

II. Ionizing radiation

DNA double-stranded breaks are recognized as the major genotoxic lesion of ionizing radiation. The use of ionizing radiation in medical diagnostics and therapy mandates an exact understanding of the biological processes surrounding the formation of radiation damage, its recognition, its repair, and of other cellular responses (such as apoptosis) to double-strand breaks. To potentially enhance the efficacy of treatment and to minimize side affects it is crucial to understand the full range of cellular responses to this type of DNA damage. These double-strand break repair pathways are conserved in all eukaryotes. In fact, recombinational repair is conserved in all forms of life (Cromie *et. al.*, 2001). This high degree of conservation combined with these genetic and biochemical advantages of yeast have made the yeast a model organism for the study of these repair pathways.

III. Bleomycin

Since their discovery in 1962 the bleomycin family of antibiotics has gained much interest into their structure, function, potential research as well as medicinal applications. Bleomycin is a low molecular weight metalloglycopeptide (it has an approximate molecular weight of 1500 daltons). It is produced by *Streptomyces verticillus*, and is isolated from the culture medium as a copper complex (Umezawa et al., 1966, 1967, 1976). Bleomycins are DNA binding and cleaving agents in the presence of a metal ligand (usually iron). Its primary uses have been derived by the fact that the drug is radiomimetic and acts as a limited endonuclease, thereby producing a variety of lesions in nucleic acids.

A. Bleomycin structure

The bleomycin molecules are structurally complex molecules, which even after 37 years of research there are still many unanswered questions remaining. The structure of bleomycin and its analogs are depicted in figure 5. The bleomycin family contains at least three functional domains; the bithiazole moiety, the amine-pyrimidine-imidazole region, and the sugars gulose and mannose.

The bithiazole region (including C-terminal) is known to function in the DNA binding ability of the drug. This planar part of the molecule partially intercalates into DNA in the minor groove (Lehmann *et. al.,* 1997). Once intercalated, the activated drug then abstracts a proton from carbon 4 of the

deoxyribose sugar thereby generating free radicals (Povirk, 1996). Removal of the C-terminal substituent or introduction of a substituent that lacks a positive charge under conditions of DNA cleavage dramatically reduces the ability of the drug to cleave DNA (Berry *et. al.*, 1985).

The amine-pyrimidine-imidazole domain is responsible for the drug's ability to bind a redox-active transition metal. The ligand is coordinated by the beta-aminoalanine primary and secondary amines, the pyrimidine N5, the beta-hydrohistidine amide, and the imidizole N1. At times it has been found that the mannose carbamoyl nitrogen is substituted for the beta-aminoalanine primary amine (Burger, 1998). Divalent metal ions found coordinated include Fe(II), Co(II), Cu(II), Ni(II) and Zn(II). BLM-Co(II)-O_2 has been found to be the most stable complex (Stubbe and Kozarich, 1987). It is this domain which is responsible for O_2 activation and which must ultimately mediate the abstraction of H atoms from the DNA substrate.

The last domain contains the sugars gulose and mannose. This domain is the least well characterized of the functional domains of the drug. It is currently thought that this domain is involved in cell recognition by bleomycin and it is possible that it also is involved in cellular uptake and metal ion coordination (Natrajan and Hecht, 1994). This region has also been shown to play an important role as an environmental factor for the efficient dioxygen reduction and DNA cleavage (Sugiura *et. al.*, 1983).

B. Mechanism of action on nucleic acids

The best characterized property of bleomycin is its ability to degrade DNA substrates. In the presence of a divalent transition metal, bleomycin catalytically cleaves double-stranded, and single-stranded DNA, and RNA. These actions take place both *in vivo* and *in vitro*. When either DNA single- or double-stranded breaks are produced, both 5'-phosphate- and 3'-phosphoglycolate-termini are produced (Giloni *et. al.*, 1981). The cleavage of DNA is a two-step process. The first step involves the binding of the drug through its partial intercalation. The second step involves the abstraction of a proton from C-4' of the deoxyribose sugar.

The drug has been shown to have a sequence preference of GpC or GpT. The pyrimidines are released when they are located to the 3' side of guanosine, thereby leaving DNA alkali-labile (D'Andrea and Haseltine, 1978). This sequence specificity may result from the binding of the drug with enhanced efficiency to these sites, as well as to variations in DNA structure which render certain C-4' H atoms more amenable to abstraction (Hecht, 2000). Although the cleavage at the first site is G-Py-specific, the second nucleophilic attack by the activated drug complex on the opposite strand is a more opportunistic cleavage. This second cleavage is believed to be due to structural perturbations of the DNA at the first cleavage site (Steighner and Povirk, 1990). The drug's DNA degradation also produces 3-(pyrimidin-1'-yl)-2-propenal and 3-(purin-9'-yl)-2-propenal derivatives (Giloni *et. al.*, 1981).

Chromosomal cleavage usually takes place in the linker regions of chromatin between nucleosomes. This preferential cleavage is thought to be

due to availability of the DNA, not protected by nucleosomes, for bleomycin binding and intercalation. Bleomycin has also been shown to produce more damage to DNA of active genes (Kuo and Hsu, 1978).

Bleomycin has also been shown to cleave a variety of RNA's. The most studied RNA has been tRNAs. The RNA cleavage is mostly at a single site often at the junctions between single- and double-stranded regions in the substrate (sites common on tRNAs) (Holmes and Hecht, 1993).

C. Structurally related species

Bleomycin is part of a family of antibiotics. It has many structural analogs and different forms which have been isolated and at least partially characterized. These include the congeners bleomycin A_2 and B_2, phleomycin and tallysomycin.

Early studies showed that the bleomycins were biosynthesized as a mixture of congeners (Umezawa *et. al.*, 1966). These congeners were found to be separable by paper and ion exchange chromatography, and were named bleomycin A_2 and bleomycin B_2. These two congeners were shown to differ in structure exclusively at their C-terminus (Figure 5). It is in this region that the terminal amines of bleomycin A_2 and B_2 are dimethylsulfonium propylamine and agmatine, respectively. This difference does produce differences in the activity of the congeners. Bleomycin A_2 produces considerably fewer DNA breaks and less killing than bleomycin B_2 over a wide range of concentrations (Moore, 1990).

Before bleomycin was discovered, there was phleomycin. Although phleomycin was discovered before bleomycin, it was never used as a chemotheraputic agent. This was due to its very high activity. Phleomycin has been shown to be substantially more destructive to the cell than bleomycin on a per mole basis in yeast (Moore, 1982). Phleomycin differs from bleomycin in that phleomycin has a thiazinylthiazole moiety in lieu of the bithiazole moiety present in bleomycin. Although the activity of phleomycin is higher, both drugs cleave DNA *in vitro* at similar preferred sites with similar frequencies, and produce a similar number of breaks (Suzuki *et. al.,* 1969).

The third structural analog is tallysomycin. This analog differs from bleomycin in three ways. First is the absence of a α-CH_3 group in the valerate region of tallysomycin. Second is the presence of a talose sugar as part of a structurally unique glycosylcarbinolamide. The last difference is the presence of C-substituents, most of which are different from those observed in bleomycins and phleomycins (Kawaguchi *et. al.*, 1977).

D. Medicinal and research uses

The activities described thus far have lead to both research and medical uses of the glycometallopeptide. Medicinally, bleomycin has found use as an anticancer drug. It is used to treat several types of cancer, including malignancies of the head and neck, squamous cell carcinomas, testicular cancer, as well as Hodgkins and nonHodgkins lymphomas (Ferrando *et. al.*, 1996). The effectiveness of bleomycin in these treatments

comes from its ability to produce lesions in DNA. However, it should be noted that the use of the drugs does not come without a major side effect. Pulmonary fibrosis is a major side effect of susceptible patients who have received these treatments (Marshall, 1997).

The ability to create single- and double-stranded breaks in DNA has lead to the use of the drug as a research tool. The damage it causes to the DNA is radiomimetic in that it mimics damage caused by ionizing radiation. This radiomimetic property has made the drug an attractive chemical tool to study and understand how cells repair this kind of damge to its DNA.

E. Additional targets

DNA breaks have been shown not to be the only effect bleomycin has on cells. It has also been shown that the drug also damages the cell wall and plasma membrane in yeast (Moore *et al.*, 1992). It inflicts this damage by creating lesions in the cell wall that extend into the cell membrane. The damage to the plasma membrane also occurs in human cells (Moore *et al.*, 1985).

F. Cellular defenses

The toxicity of bleomycins are dealt with in many ways. As previously stated the drug is very toxic to all cells. The *Streptomyces,* which produces the glycopeptides, possesses a bleomycin acetyltransferase which inactivates the drug in the presence of acetyl coenzyme A. The enzyme acetylates the

β-aminoalanine moiety of bleomycin thereby deactivating the drug (Sugiyama *et. al.*, 1994). A mechanism involved in the detoxification of the drug within this producing strain is expected. However, other cells not producing the drug have evolved numerous ways of dealing with this drug by creating protective barriers the drug needs to overcome.

The first barrier bleomycin needs to overcome to get into the cell is the plasma membrane. The drug, however, is unable to diffuse through the plasma membrane in animal cells due to its hydrophilic nature. Bleomycin has been shown to be recognized at the cell surface of both Chinese hamster fibroblasts and human cells by an unidentified membrane-bound protein believed to be involved in the internalization and cytotoxicity of the molecule (Pron *et. al.*, 1993, and 1999). In Chinese hamster fibroblasts, a 250 kDa membrane protein has been located which binds bleomycin and has been associated with bleomycin toxicity (Pron *et. al.*, 1994). A protein of similar molecular weight was later found in human cells and shown to bind bleomycin molecules (Pron *et. al.*, 1999). A decrease in the number of bleomycin binding sites has been observed in bleomycin resistant cells compared to bleomycin sensitive cells. Pron and co-workers (1999) proposed a mechanism of receptor-mediated endocytosis involving the internalization of bleomycin in human cells. The membrane protein that specifically binds bleomycin is considered to be a receptor protein. The group hypothesized that after the binding of the bleomycin molecules to this possible membrane receptor, the drug is internalized by plasma membrane engulfment while the

cell is undergoing endocytotic activity. Once inside the cell in a vesicle the bleomycin molecule must have some way of escaping before it gets degraded by lysosomes.

Once inside the cell the cytotoxic molecule may still face destruction by another cellular defense. This time the defense is an enzyme called bleomycin hydrolase. Bleomycin hydrolase is a neutral cysteine protease, discovered by virtue of its ability to detoxify the bleomycin molecule (Akiyama S. *et. al.*, 1981). The enzyme is evolutionarily conserved, as homologous enzymes have been shown to exist in bacteria, yeast, reptiles, birds, and mammals (Takeda *et. al.*, 1996). Its enzymatic functions include acting as a carboxypeptidase, aminopeptidase, as well as a peptide ligase (specifically the yeast variant of the enzyme) (Zheng *et. al.*, 1998). The cysteine protease is ubiquitous in nature and has been shown to be expressed in most tissues and cell types, as well as overexpressed in some tumors (Ferrando *et. al.*, 1996). The enzyme has the ability to bind both single- and double-stranded DNA and RNA (all without sequence specificity). This DNA binding activity is only possible when there is a free end of the DNA to bind. This implies that the DNA binding activity is involved in protecting the cells from DNA cleaving agents like bleomycin (Zheng and Johnston, 1998). The enzyme deactivates bleomycin when it deamides the beta-aminoalanine moiety of the drug. This converts the carboxyamide of the beta-aminoalanine to a carboxylic acid, which in turn results in the alpha-amino group on the beta-aminoalanine becoming more basic. At physiological pH the free carboxylic acid has a

higher affinity for the metal ligand than the protonated amino group and therefore occupies the fifth coordination site of the bleomycin metal complex. This change in the coordination of the ligand results in an inactive form of bleomycin (Sebti and Lazo, 1988).

The next line of defense the cell has against the cytotoxic effects of the drug at the level of DNA. The drug has been shown to induce different pathways of repair. Drug treatments have been shown to induce error prone repair in yeast (Severgnini *et. al.*, 1991), long patch excision repair in human fibroblasts (DiGiuseppe and Dresler, 1989), and SOS repair in *E. coli* (Povirk *et. al.*, 1988). It has also been shown that several yeast mutants which display sensitivity to bleomycin exhibit a cross sensitivity to ionizing radiation (Moore, 1978, 1980, 1982, 1991). This suggests that the damage caused by both bleomycin and ionizing radiation are repaired by the same pathway(s). However, there is other evidence suggesting that the repair of damage caused by ionizing radiation and bleomycin do not involve exactly all the same processes (Jaffe *et. al.*, 1990)

Eukaryotic cells utilize a variety of mechanisms to repair broken DNA caused by the drug and ionizing radiation, not all of which maintain the original DNA sequence. A cell's ability to repair these double-strand breaks is critical, not only for the prevention of cancers, but also for the resistance of many tumors to current therapies. Double-strand breaks can be repaired without a loss of information by homologous recombination (Critchlow and Jackson, 1998). Recombination is the only inherently error-free pathway to

accomplish repair and it involves a complex series of events, which are just now beginning to be understood in eukaryotes. Other pathways involved in the repair of damage like non-homologous end joining (NHEJ) and break-induced replication (BIR) are error-prone, and lead to mutations. Many of the components involved in the different repair pathways are conserved between humans and yeast, including the *RAD52* epistasis group of genes (Morrison, and Takeda, 2000), the Ku70/Ku80 end-joining heterodimer (encoded by *HDF1* and *HDF2* in yeast) (Feldmann *et. al.*, 1996), DNA ligase IV (*LIG4*) (Ramos, 1998), and XRCC4 (*LIF1*) (Herrmann *et. al.*, 1998). This conservation of repair genes along with the previously mentioned genetic and biochemical advantages of yeast have lead to yeast as a model organism to study cellular processes in this investigation

IV. Yeast proteasomes

The 26S proteasome is an unusually large multisubunit proteolytic complex of about 2000 kDa that is responsible for the rapid degradation of ubiquitinated proteins. The 26S proteasome is composed of a catalytic core called the 20S proteasome and two ATP-dependent 19S caps, which are believed to unfold substrates and feed them to the actual protease, the 20S proteasome (Bochtler *et. al.*, 1999). The 20S proteasome is not able to degrade ubiquitinated substrates unless it is assembled with the 19S caps (Yen *et. al.*, 2003). The 26S proteasomes are required for the degradation of both short lived and unstable proteins (Sweder and Madura, 2002).

Proteasome dependent degradation of proteins is crucial in many different biological processes and regulatory pathways of the cell. The proteasomes have been shown to regulate cell cycle, DNA repair, differentation, signal transduction, and metabolic pathways. The complex regulates these pathways by degrading proteins after their function is complete (Hochstrasser *et. al.*, 1999). Proteasomes regulate the cell cycle by degrading cells cycle proteins (cyclins), thereby allowing transition from one cell cycle phase to another (Yew *et. al.*, 2001). In oxidative stress, the complex has been shown to respond by degrading damaged or misfolded proteins (Gerlinger *et. al.*, 1997). The proteasome is thought to regulate DNA repair by lowering the level of repair proteins back to a basal level after repair is complete, or alternatively it may act as a molecular chaperone which promotes disassembly of certain repair complexes (Sweder and Madura, 2002).

The proteasome is found in both the cytoplasm as well as the nucleus. In mammalian cells the localization seems to be predominantly cytoplasmic, but is observed in the nucleus periodically (Gordon *et al*, 2002). However, in yeast approximately 80% of proteasomes are localized in the nucleus (Gordon, 2002).

V. Major Findings

The findings presented in this thesis demonstrate that Blm10p is a novel 245.6 kDa nuclear protein with a significant role in relieving the cell of

the toxic effects conferred by bleomycin. At the beginning of this research Blm10p was one of a number of uncharacterized proteins in *Saccharomyces cerevisiae*. During this research, the ORF encoding *BLM10* was found to contain a mistake which when corrected merged YFL006w into YFL007w. The research clearly shows that Blm10p resides in the nucleus and functions there in a manner which protects cellular DNA. In growing cells, a truncated Blm10p was observed to localize to the bud neck. Although the exact protective functional role remains to be elucidated, this research has narrowed down the spectrum of possible functions.

Sequencing of YFL007w revealed a mistake in the published sequence (Goffeau *et. al.*, 1996) The mistake was a base missing near the 3' region of the gene which when put in place merged the ORFs YFL006w with YFL007w. This finding was confirmed by the sequencing consortium (Robben *et. al.*, 2002). The sequence throughout the rest of the *BLM10* gene, as well as upstream of the gene were shown to match the published sequence (Goffeau *et. al.*, 1996) . A northern analysis revealed that this gene is expressed and confirmed the new predicted size of YFL007w.

The research demonstrates that Blm10p plays an essential role in protecting cells from the lethal effects of bleomycin. When the *BLM10* gene was replaced by a *HIS3* cassette it was shown that there was an increase in sensitivity of the *blm10Δ::HIS3* strain to the effects of the drug. This sensitivity to the drug when the gene is absent indicates an important role in relieving the cells of these toxic effects.

The importance of Blm10p role in a protective function was demonstrated when the protein was expressed in a *blm10* deletion background. The expression of this protein clearly demonstrates that Blm10p confers resistance to the hypersensitive *blm10Δ* strains. This expression also established the ability of cells to withstand larger amounts of this protein without the protein itself becoming toxic to the cell.

Blm10p was determined to have a nuclear localization. To determine where the protein exists in the cell a Blm10p-YFP fusion was created. The fusion was made in the chromosome and therefore under the control of the *BLM10* endogenous promoter. The protein was observed to localize to the nucleus and remain there even after drug treatments. This localization also illustrated the low level of expression of the *BLM10* gene. In a separate localization experiment a truncated Blm10p (missing the last 340 amino acids) was fused to GST and put under control of the CUP1 promoter. In this experiment the fusion protein was found to localize to the nucleus in stationary phase cells and to the bud neck region in budded cells.

The research demonstrates that Blm10p is evolutionarily conserved. Searches for homologous proteins in higher eukaryotes found that homologues of Blm10p exist in many higher eukaryotes. The regions of conservation indicate areas of potential significance. The degree of conservation and the specific order in which these areas occur predict functional importance of these areas. Interestingly, the human homologue has been characterized as having a proteasomal function affecting the 20S

proteasome (Ustrell *et. al.*, 2002). The Blm10p has been found associated with subunits of the yeast 20S proteasome (Gavin *et. al.*, 2002). Future studies should be done to demonstrate the importance of these conserved areas.

Next, the research demonstrates that protection of chromosomal DNA in *blm3-1/blm3-1* cells is not functioning properly. Pulsed field gel electrophoresis was performed on both homozygous mutant and normal strains. These experiments illustrated the need for protection of cellular DNA from the effects of the drug family. These experiments showed that in *blm3-1/blm3-1* cells chromosomal DNAs sustain a substantial amount of double strand breaks when compared to *BLM3/BLM3* cells.

Materials and Methods

1. Strains and plasmids.

The yeast and bacterial strains used in this work are listed in Table 1.

2. Media and growth conditions.

Nonsynthetic, complete solid medium (YPAD) containing 2% glucose, 2% Bacto-peptone, (Difco, Laboratory, Detroit, Michigan), 1% Bacto-yeast extract (Difco), 2% Bacto-agar (Difco), and 0.16 mg/ml adenine sulfate was used for the nonselective growth of all yeast strains.

Solid synthetic media (SD) and solid synthetic complete media (SC) contained 0.2% Bacto-yeast nitrogen base (Difco), 0.5% ammonium sulfate, 2% glucose and 2.5% Bacto-agar, and were used for the selection of strains during screening steps. Specific nutritional requirements for each strain were added during the preparation of SD and SC medium (Kaiser *et al.*, 1994). Solid synthetic medium containing seven amino acids (SD+7) adenine sulfate (0.480 g/100 ml), uracil (0.2 g/ 100 ml), tryptophan (1 g/100 ml), histidine (1 g/100 ml), leucine (1 g/100 ml), isoleucine (1 g/100 ml), valine (3 g/100 ml) was used for selective growth.

YNB-glucose minimal medium contains 6.7 g of yeast nitrogen base (without amino acids) per liter and 2% glucose supplemented with 50 mg of the appropriate amino acids and uracil and 100 mg of adenine per liter (Turchini *et al.*, 2000).

SP1 sporulation media contains 0.25% yeast extract, 0.1% glucose, 0.98% potassium acetate, 20 g/l agar and 40 mg/ml uracil, tryptophan and leucine.

3. Bacterial strains, media, and transformations

All *Escherichia coli* (*E. coli*) strains used in this study are listed in Table 1. The chemical competent *E. coli* strain used was DH10B (Gibco BRL, Carlsbad, Ca.). All LB, TB and SOC media were prepared as described (Ausebel *et. al.* 1989) and supplemented with 100 μg/ml ampicillin. For transformation a 15 ml Falcon tube was placed on ice for 10 minutes to pre-cool. To each of the tubes, 100 μl of chemically competent cells was added, followed by the addition of 5 μl of DNA (1 μg). The tube was gently swirled to mix the components, then incubated on ice for 30 minutes. The reaction mixture was heat shocked for 45 seconds at 42°C. After the heat shock, the cells were immediately placed on ice for a 2 minute incubation period. Next, 900 μl of SOC media was added and the reaction mixture was incubated at 37°C with shaking (225 rpm) for 90 minutes. Cells were alloquoted 100 μl of cells/plate then spread.

4. Phleomycin preparations

The phleomycin stock solution was prepared by dissolving phleomycin (Bristol-Myers Squibb Company, Princeton, NJ) in sterile 50 mM Tris-Cl (pH 7.5) to obtain a final concentration of 10 mg/ml. Phleomycin medium was

always prepared within 24 hours prior to use. Deletion mutants and overexpression strains were inoculated in synthetic and nonsynthetic liquid media containing 0, 3, 5, 7,and 9 μg/ml of phleomycin. Concentrations of phleomycin were determined using the Beer-Lambert equation (OD_{245} /1.6x10^{-2}).

5. Yeast strains, and transformations

All strains used in this section have been listed in Table 1. For transformations a volume of 10 ml of YPAD was inoculated with a single yeast colony and grown overnight with vigorous shaking at 30°C to prepare a preculture. A flask containing 100 ml of YPAD was then inoculated with enough preculture to achieve an OD_{600} =0.2. Culture was grown at 30°C with vigorous shaking to an OD_{600} = 0.5. The cells were then harvested by centrifugation at 3000 rpm (Juan swinging bucket centrifuge) for 4 min in 2 x 50 ml aliquots. The pellet was then washed in 10 ml water and harvested again by centrifuging at 3000 rpm for 4 min. Cells were resuspended in 5 ml of TE-LiAc (10x TE and 1 M LiAc [filter sterilized]), and then reharvest by centrifuging at 3000 rpm for 4 minutes. The Pellet was then resuspended in 1 ml of TE-LiAc and stored on ice for a 10 minute incubation period. Next, in a 1.5 ml Eppendorf tube 50 μl of competent cells were combined with 2 μl of carrier DNA (salmon sperm DNA 10 mg/ml), 2-3 μl each DNA (1 μg in concentration), and 300 μl PEG-TE-LiAc (50% PEG3350 [filter sterilized through a 0.25 micron filter]). The tubes were then incubated for 30 minutes

at 30°C. Immediately after the 30 minute incubation period the tubes were transfered to a 42°C water bath for a 15 minute incubation. The cells were then harvested for 4 minutes at 2000 rpm. The pellet was then resuspended in 1 ml of water to wash them and then spun down by centrifugation for 4 minutes at 1500 rpm. The pellet was then resuspended in 100 μl of water and spread immediately on a plate. For library transformations, the protocol was scaled up 60 fold to achieve optimal representation of the genomic library.

6. DNA Sequencing

All sequencing of DNA was done using the method developed by Sanger (1977) . Genomic DNA used for sequencing was isolated using the Yeastar Genomic DNA isolation kit (Zymo Research, Orange, CA) according to manufacturer's instructions.

7. Liquid β-Galactosidase assay

A 5 ml culture of cells was grown overnight at 30°C in a selective media to mid log phase. Cells were spun down to the equivalent of 1 ml of cells at OD_{600} ≅0.5 (10^6-10^7 cells). The cells were then resuspended in 1 ml Z-buffer followed by the addition of 50 μl of $CHCl_3$ and 32 μl of 0.1% SDS using a Pasteur pipette. The cells were then vortexed at high speed for 10 seconds. A blank was next set up containing everything but the cells. The reaction mixture was preincubated at 28°C for 3 minutes. 0.2 ml of ONPG (O-nitrophenyl-β-D-galactopyranoside; 4 mg/ml in H_2O) was added to each

sample, noting the exact time, and shaking gently to mix. When a noticeable yellow color had developed, the blank was compared (which should remain clear), the reaction was then stopped by adding 0.5 ml of 1 M Na_2CO_3 solution. Cell debris were spun down for 5 minutes in a bench top centrifuge. Read the OD_{420} of the supernatant was read and calculations were performed.

8. Isolation of Plasmid DNA from Yeast

The cells were inoculated into 2 ml of SD omission media in a 13x100-mm sterile glass tube with a single yeast colony containing the plasmid of interest. The culture was grown overnight to stationary phase at 30°C in a shaking incubator. A 1.5 ml aliquot of the overnight culture was transferred to a microfuge tube and spun for 5 seconds at high speed, room temperature. The supernatant was poured off and the pellet was disrupted by vortexing briefly. The cells were then resuspended in 200 µl of breaking buffer (2% (v/v) Triton X-100, 1% (v/v) SDS, 100 mM NaCl, 10 mM Tris-cl pH 8.0, 1 mM EDTA pH 8.0). 0.3 g of glass beads (~200 µl volume) were added and followed by the addition of 200 µl of a phenol/chloroform/isoamyl alcohol mix. The mixture was vortexed for 2 minutes at highest speed. The sample was then centrifuged in a microcentrifuge for 5 minutes at a high speed, room temperature. Next competent *E. coli* cells were transformed with 1 µl or 2 µl of the aqueous layer. The cells were then plated on LB medium containing

the appropriate antibiotic to select for the drug resistance marker on the plasmid. The plasmids were isolated from the *E. coli* strain and sequenced.

9. Construction of the gene deletion.

Deletion of the chromosomal *BLM10* gene was carried out by the PCR deletion and replacement method described by Baudin *et al.* (1993) (Figure 6). The strategy took place in three separate steps: (1) PCR amplification of a gene replacement cassette that includes *HIS3* as the selectable marker; (2) transformation of yeast with the PCR product and selection of *HIS3*$^{+}$ recombinants; (3) verification by PCR of correct gene deletion using oligonucliotides complementary to the flanking sequences of the *BLM10* ORF. The plasmid pRS303 (provided from the laboratory of Dr. Susan Henry, Department of Biological Sciences, Carnegie Mellon University, Pittsburgh, PA) carries the *HIS3* replacement cassette.

In the first step, the *HIS3* replacement cassette was obtained through the use of PCR, using two chimeric primers and the plasmid, pRS303, as the PCR template (a bluescript-based plasmid). Each of the primers contains two regions; The first region was homologous to the target region of the locus of interest referred to as the deleting sequence, and the second region was homologous to the *HIS3* selectable marker. The 5' deleting sequence referred to as the oligopro and the 3' deleting sequence referred to as the oligoterm were flanking sequences of the *BLM10* gene. They were followed by 17 nucleotides homologous to the *HIS3* selectable marker for oligopro and for

oligoterm. PCR (Perkin Elmer 9600 DNA Thermocycler, PerkinElmer Life and Analytical Sciences, Boston, Ma.) amplification was performed in a total volume of 100 μl containing 0.25 μg of pRS303, 200 μM of each dNTP, 10 μl of 10x buffer (500 mM KCl; 100 mM Tris-HCl pH 8.8; 15 mM $MgCl_2$; 1% Triton X 100; 0.1% gelatin), 0.5 μM of each primer and 2.5 units of Pfu polymerase (Stratagene, La Jolla, CA). The reaction mix was then incubated for 5 minutes at 95°C and submitted to 2 cycles of PCR (30 sec at 94°C, 30 sec at 45°C and 2 min at 74°C) followed by 30 cycles (30 sec at 94°C, 30 sec at 50°C and 2 min at 74°C). In the final cycle, the extension step was for 5 min followed by a 4°C hold.

In the second step, one μg of the PCR product, 750 bases long, was directly transformed into the *BMA-8A* haploid *his3Δ200* strain (kindly donated by Dr. Agnes Baudin, Dept. of Molecular Genetics, Centre de Genetique Moleculaire, Cedex, France). This strain was necessary since all the regions of the *HIS3* gene, which are homologous to *HIS3* in the PCR product have been removed in this strain. This removal of the gene prevents gene conversion and homologous recombination at the *HIS3* locus.

In the last step, genomic DNA was isolated from selected transformants. PCR was again used to test for the replacement of the *BLM10* gene by the *HIS3* gene. Primers were designed from a flanking region of the *BLM10* ORF and a region from within the *HIS3* region of PCR product respectively. PCR amplification was performed in a total volume of 100 μl containing 0.15 μg of genomic DNA isolated using The YeaStar Genomic

DNA Kit (Zymo Research, Orange, Ca.) according to the instructions of the manufacturer. The reaction mix was incubated for 10 minutes at 94°C and submitted to 3 cycles of PCR (10 sec at 94°C, 30 sec at 55°C and 10 min at 68°C) followed by 22 cycles (10 sec at 94°C, 30 sec at 55°C and 8 min at 68°C), then followed by 3 cycles (10 min at 72°C). PCR products were then run on a 1% agarose gel to verify the deletion was complete (Figure 7).

10. DNA Repair

Cells were grown to stationary phase (approximately 2 X 10^8 cells/ml) in sterile liquid YPAD at 30°C from an initial innoculum of 1 X 10^4 cells/ml from a preculture grown overnight. Following growth, cells were harvested, washed three times in sterile deionized water, then held for 16 hours at 4°C in sterile deionized water. Cells were then collected by centrifugation, again washed twice in sterile deionized water, then resuspended in sterile deionized water at a final concentration of 1 X 10^9 cells/ml. Cells were next treated with phleomycin for 30 minutes with aeration at a cell density of 2 X 10^7 cells/ml in 20 ml of cold sterile deionized water. After phleomycin treatment cells were collected and resuspended in 20 ml of sterile 0.01M sodium phosphate (pH 6.5) and incubated with aeration at 30°C. Aliquots were removed at different time points (t=0 - t=48 hours) and plated on solid YPAD to determine cell survival and used in the preparation of low melting point agarose plugs for pulse field gel electrophoresis.

11. Cells lysis and pulsed field gel electrophoresis

After liquid holding, cells were recovered from sodium phosphate buffer by centrifugation at 3000 rpm for 10 minutes, followed by two washings with 5 ml of 50 mM EDTA, then resuspended in 350 µl 50 mM EDTA. Next, 200 µl of solution I (500 µl of 2-mercaptoethanol and 35 mg zymolase [100,00 U/g] was added to 10 ml of solution containing 1 M sorbitol, 100mM sodium citrate, and 10 mM EDTA added just prior to use) was added to the resuspended cells and 635 µl of warm (42°C) low melting point agarose (1.5%). The cells suspension (85 µl) was immediately transferred to each well of a mold and allowed to solidify at 4°C for 10 minutes. Agarose plugs were then removed from the molds and placed in a sterile 15 ml Falcon tube containing 10 ml of solution II (30 ml of 2-mercaptoethanol was added immediately prior to use to 400 ml of a solution containing 0.5 M EDTA and 100 mM Tris-HCL [pH 7.5]). The agarose plugs were incubated at 37°C for 24 hours. The plugs were then transferred into 10 ml of solution III (40 ml of a 10% solution of sodium lauryl sarkosinate and 400 mg of proteinase K added just prior to use to 364 ml of a solution containing 0.5 M EDTA and 10 mM Tris-HCL [pH 7.5]). The plugs were next incubated at 50°C for 24 hours. Solution III was then decanted and the plugs were incubated in 10 ml of 0.5 M EDTA for 2 hours at room temperature. The EDTA solution was then decanted and the plugs were stored in 10 ml of a fresh 0.5 M solution of EDTA at 4°C. Prior to PFGE, plugs were washed several times (5 ml/wash) by gently shaking in a running buffer (0.5 X TBE [pH 8.0]), which was made

by diluting a solution containing 45 mM Tris, 45 mM borate, and 1 mM EDTA vol/vol with distilled water) before being sealed into the wells of a 1% agarose gel. The gel was run submerged in 0.5 X TBE [pH 8.0] with a voltage gradient of 6 volts/cm for 15 hours in a Biorad PFGE Chef Mapper with a switch time of 60 seconds followed by 9 hours with a switch time of 90 seconds. The gel was then stained 30 minutes in a solution containing 5 μg/ml ethidium bromide followed by destaining in deioninized water for an additional 30 minutes.

12. Blm10p expression analysis

The fusion used for overexpression was provided as a pool of a mix of colonies containing both the fusion of interest and colonies containing no *BLM10* insert in the plasmid. Checking for a fusion containing the correct size insert in the correct orientation was done by isolating the plasmid from the yeast, transforming it into *E. coli*, and isolating and purifying the plasmid from *E. coli*. Then, a restriction analysis was used to determine if it was a plasmid of interest (Figure 8). DNA sequencing of these plasmids confirmed the insert was ligated with the correct reading frame maintained. Following sequencing, the plasmid was transformed into a *blm10Δ* strain. Strain CM1522-9B was grown in 10 ml of YPAD overnight. The cells were then inoculated in 10 ml of liquid YPAD at a cell density of 5 X 10^6 cells/ml. To tubes suppose to contain copper, the copper was added at a concentration of 50 μM. To those tubes that are to contain phleomycin, the drug was added at a concentration of 5

μg/ml. Cells were counted at 0, 2, 4, 8, 16, 24, and 36 hours using a hemocytometer.

13. YFP fusion

The amino terminal fusion of the Yellow fluorescent protein (YFP) to BLM10 was done as previously described by Prein *et al* (2000) (Figure 9). Step 1 involves PCR amplification of the fusion cassette. In step 2 the cassette was transformed into a *BLM10*, *ura3-52* strain (CM1452-98B) and followed by selection. The third step was to transform selected G418 resistant colonies with the plasmid pSH47, followed by a selection of the Ura+ transformants. In step 4 Cre recombinase was induced followed by PCR to verify correct insert.

In the first step, the *loxPkanMX4loxPyEYFP* cassette was obtained through the use of PCR, using two chimeric primers and the plasmid, pDH22 (Figure 10), as the PCR template. The chimeric primers for PCR mediated generation of the cassette consisted of 70 nucleotides 5' homologous to the region directly upstream of the *BLM10* ATG plus a 12 nucleotide sequence homologous to the cassette (forward primer) and a 70 nucleotide 5' homologous to the region directly downstream of and including ATG plus a 24 nucleotide sequence homologous to fusion cassette (reverse primer). The primer sequence for tagged *BLM10* was shown in table 3. PCR reaction consists of 0.5 μM each primer, 1 X Pwo DNA polymerase buffer (contains 2 mM $MgSO_4$, 200 μM dNTPs, 0.1 U/μl Pwo DNA polymerase 2 ng/μl pDH22

as the template. The PCR conditions were as follows: denaturation at 95°C for 3 minutes; 10 cycles at 94°C for 30 seconds, 52°C 30 seconds, 72°C for 2 minutes and 20 cycles at 94°C for 30 seconds, 72°C 150 seconds, followed by a final elongation step at 72°C for 10 minutes. The theoretical size of the PCR fragment harboring the cassette including the 70 bp homologous to theintegration site on either end is 2399 bp. This PCR product was then run on a 1% agarose gel to verify size. The band was then cut from the gel, purifed, and used for the next step.

In step 2, CM1452-98B was transformed with a linear PCR fragment and selected on YPAD+ 25 mg/l geneticin (G418) medium. Each of these colonies, was then replica plated onto plates containing increasing amounts of G418 (50 mg/l, 100 mg/l, 150 mg/l, 200 mg/l) and only those, that showed the greatest resistance (up to 200 mg/l) were selected. Genomic DNA was isolated and PCR was used to verify correct insertion into the chromosome (Figure 11, lane 7).

In the third step the geneticin resistant transformants from step 2 were transformed with the plasmid pSH47 (Figure 12). Transformants were selected on a SD+7 -ura medium.

In the last step the geneticin resistant/uracil prototrophic transformants were grown in a galactose medium for 6 hours to induce the Cre recombinase. Excision of the *loxPKanMX4loxP* fragment of the cassette was verified by diagnostic PCR followed by running of PCR product on a 1% agarose gel (Figure 11, lanes 2-5).

14. Immunoflorescence

Cells were grown in a 5 ml culture to early exponential phase (10^6- 10^7 cells/ml) in liquid YPAD containing copper (50 μM) to induce expression of fusion protein. Cells were then fixed by adding 1/10 volume formaldehyde directly to the medium (total formaldehyde concentration is 3.7%; stock solution is 37%). Cells were next incubated in formaldehyde for at least 1 hour. After 1 hour the cells were pelleted in 1 ml of 50 μl/ml zymolase 100T in 0.1 M potassium phosphate (pH 7.5) with 2 μl/ml 2-mercaptoethanol. To check for spheroplasting cells were followed by phase contrast microscopy. When cells were a dark translucent gray, sheroplasts were considered ready and were then pelleted in a low speed (3000 g) microfuge. Cells were then resuspended in 1 ml PBS buffer (phosphate buffered saline)(10 mM $NaHPO_4$, 2 mM KH_2PO_4, 0.15 M NaCl, 3 mM Kcl [pH 7.4]) . The slides were prepared by adding 25 μl of 1 mg/ml polylysine onto slide and letting them sit for 5 minutes. The slides were then washed with distilled water 3 times followed by air drying. A volume of 5 μl of fixed cells were placed onto each slide. The cells were then left to sit for a few minutes, followed by an aspiration and washing three times with PBS. The slides were checked under the microscope to ensure the cells were at a suitable density and not overly clumped. A volume of 50 μl of blocking buffer was added (PBS, and 3% BSA [bovine serum albumin]) onto cells and left to incubate for 45 minutes in a humid chamber. Blocking buffer was then removed by aspiratation. An Alexa

Fluor 488 anti-glutathione S-transferase (Molecular Probes, Eugene, OR) was diluted 1:200 in blocking buffer. A total of 50 μl of antibody/blocking buffer mix was then added to cells on slide and incubated for 1 hour in a humid chamber. Antibody was then aspirated off followed by three washes with blocking buffer. A volume of 25 μl of 1 μlg/ml DAPI (4, 6-Diamidino-2-phenylindole) was added to the wells and incubated for 5 minutes. The slides were washed three times with PBS and all PBS was removed and add a small drop of mounting medium (50mg ρ-phenylenediamine in 5 ml PBS, adjust to pH 9.0, add 45 ml of glycerol, stir until homogenious and add 2.5 μl DAPI [1mg/ml in water]) was added to cells. A coverslip was put on slides and sealed with clear nail polish.

15. Flourescence microscopy

Cells were imaged using a DeltaVision microscopy system from Applied Precision (Issaquah, WA). The system incorporates an Olympus IL-70 microscope, a u-plan-apo 100X oil objective (1.35NA), a CoolSnap HQ digital camera from Roper scientific (Tucson, AZ.), and optical filter sets from Omega Optical (Brattleboro, VT). Live cells were imaged on a thin pad of media containing 1% agarose (Hailey *et al*, 2002). Images were analyzed using SoftWoRx software. For the conversion of the image files to TIFF format, the output was 8-bit grayscale.

16. Northern Blot analysis

Total RNA was isolated from the strains CM1469-5A, CM1469-5C, and BMA-8A strains using the Qiagen Oligotex mRNA mini kit according to manufacturers instructions (Qiagen Inc., Santa Clarita, CA). Analyses of RNA by northern blot were accomplished using the NorthernMax kit according to manufacturers instructions (Ambion Inc., Austin, TX). A Probe was created using PCR by combining 5μl DNA template (2 μg genomic DNA isolated from CM1469-5A as previously described) 2μl (10mM) dATP, 2μl (10mM) dGTP, 2μl (10mM) dTTP, 5 μl ^{32}P dCTP, 2.5 μl (1μM) primer L1F, 2.5 μl (1μM) primer L2R, 10μl 10X PCR buffer (contains $MgCl_2$) (Roche-Boehringer Mannheim, Indianapolis, IN), 1 μl (3.5U) Taq polymerase (Roche-Boehringer Mannheim, Indianapolis, IN), and 68 μl H_2O. PCR thermocycling was accomplished by an initial denaturation step of 95°C for 5 minutes, followed by 35 cycles of 94°C for 90 seconds, 55°C for 60 seconds, and 67°C for 3 minutes. A final extention of 72°C was done for 7 mintues and then a 4°C hold. Purification of the probe was accomplished using Microspin G-50 Columns (Amersham Phamacia Biotech Inc., Piscataway, NJ) according to the manufacturers instructions. Probe was then hybridized to membrane using the NorthenMax kit according to the manufacturer's instructions. Film was exposed for 4 days at –20°C then developed.

Results

I. YFL007w is larger than predicted

Strain to strain sequence variations occur as strains become more distant relatives (Wicksteed *et. al.*, 1994). Although sequence variations may not affect the function of a protein they may provide valuable information about the functional significance of an area of the protein. To examine how close the sequence of YFL007w in our strains is to the published sequence (Goffeau *et. al.*, 1996), YFL007w was sequenced in three different strains and compared to the published sequence.

Sequencing was accomplished using numerous primers (Table 3) which were designed so sequence trials would contain overlapping regions. Each region was sequenced at least three times and compared to the published sequence. This sequencing revealed an additional guanine in all three strains, approximately fifteen bases before the predicted stop codon of YFL007w. When the base was added to the sequence, it produced a different reading frame for the last portion of the gene. This stop codon was eliminated in new reading frame, thereby merging YFL006w with YFL007w. The new open reading frame predicted a protein containing 2143 amino acids with a molecular weight of 246.5 kDa. The sequence throughout the rest of the *BLM10* gene, as well as 600 bp upstream of the gene in the three different strains matched the published sequence (Goffeau *et. al.*, 1996).

II. Northern analyses of *BLM10* confirm new size of ORF

Since sequencing results predicted a larger ORF, we sought to examine the expression of this new ORF in northern analyses. This approach would also allow us to verify the correct size of the gene.

For the northern analyses RNA was isolated from *BLM10* and *blm10Δ* cells (Figure 13A). The two strongest bands in all lanes represent the 28S and 18S ribosomal bands. These strong bands indicate the quality of the RNA is high, since the bands become less intense when the quality of the RNA diminishes. These bands also verify that equivalent amounts of RNA were loaded from each of the three strains. The RNA was then transferred to a membrane. After the transfer a radiolabeled probe was created by PCR. The probe was approximately 800 bases in length and was obtained using primers which amplify a region from within the *BLM10* gene (L1F and L2R: Table 3). The PCR product was cleaned so as to be free from nucleotides, and then hydridized to the membrane (Figure 13B). Hybridization revealed two bands of equal intensity (Figure 13). These bands represent hybridization of the probes to RNA from *BLM10* cells. The probe hybridized to a band which had migrated to an area which indicated the RNA was about 6400 bases long. This result confirmed both that the size of YFL007w is as predicted by our sequencing and shows that this ORF is expressed. These results also confirmed the deletion of *BLM10* since the probe did not hybridize to RNA isolated from the deletion strain (Figure 13B).

III. Construction of the full null mutant since *BLM10* comprises both YFL007W and YFL006W

The deletion of a gene from a genome can result in very strong phenotypes. These phenotypes may provide evidence as to the function of the deleted gene (Lorenz *et at.*, 1995). The easiest way to accomplish deleting a gene is to make use of the ability of cells to homologously recombine DNA when there are areas of homology between a PCR product and genomic DNA (Ivic *et. al.*, 2000). Since creating the deletion of the original YFL007w (Febres *et. al.*, 2001), our sequencing revealed a missing base in the published sequence. As described above, the correction of the sequencing mistake merged the orignial YFL007w with YFL006w. This new information meant that the first deletion created was not a complete deletion, but instead a partial disruption of the gene where the first 84% of the gene was deleted. Thus, it was important to delete the entire *BLM10* gene to determine the null phenotype.

To study if the region previously known as YFL006w, which was left in the genome in the deletion of YFL007w (Febres *et. al.*, 2001), may have contained important activity, a new full length deletion was created. The construction of the complete null was a multistep procedure (Figure 6). In the first step the plasmid pRS303 (Baudin *et. al.*, 1993) was used as a template for PCR. The primers used in this step were homologous to both *BLM10* and *HIS3* (Table 3: deletion primer 1 and deletion primer 2) The PCR product was then used to transform a strain with *HIS3* deleted (Table 1: BMA-8A).

This was followed by selection on synthetic media lacking histidine. Colonies that grew were selected and genomic DNA was isolated from them and used as template for PCR with verification primers. PCR was then used to confirm the creation of the null (Figure 7).

Phenotypes of strains bearing the new full deletion were examined. The strains were tested for hypersensitivities to phleomycin (5 μg/ml), and the full length deletion was found to confer a hypersensitivity to phleomycin (Figure 14). The growth of the deletion strain was found to be completely inhibited in the presence of the drug (5 μg/ml). The deletion of YFL007w was also found to exhibit a very high amount of killing in the presence of the drug (Figure 14).

IV. *BLM10* expression restores growth and viability.

Since initial reports of *BLM10* expression predicted a gene with very low expression (TRIPLES database, Yale Genome Analysis Center, New Haven, CT; Dr. Leona Samson, Harvard University, Boston, MA, personal communication) we sought to study what happens when the protein is overexpressed in *blm10Δ* cells. Overexpression of a protein can cause numerous defects. An overexpressed protein may overload a particular cellular function thereby causing a malfunction in the cellular process, increase the efficiency of a cellular process, or mislocalize and result in strong phenotypes. To determine any additional effects of Blm10p on the cell, Blm10p was overexpressed in a *blm10* deletion strain. The protein was

expressed from a plasmid. This plasmid was kindly provided by Dr. Eric Phizicky (Martzen *et. al.*, 1999). The plasmid (a slightly modified form of pYEX4t-1) was used since it puts the gene under the control of an inducible promoter (CUP1p). The use of this plasmid allows control of the expression level of the gene by adjusting the amount of copper added to the medium (Mascorro-Gallardo *et. al.*, 1996).

The fusion was provided as a pool of a mix of colonies containing both the fusion of interest and colonies containing no *BLM10* insert in the plasmid. Checking for a fusion containing the correct size insert in the correct orientation was done by isolating the plasmid from yeast, transforming it into *E. coli*, and isolating and purifying the plasmid from *E. coli*. Then, a restriction analysis was used to determine if it was the plasmid of interest (Figure 8). DNA sequencing of these plasmids confirmed the insert was ligated with the correct reading frame maintained. Following sequencing, the plasmid was transformed into a *blm10Δ* strain. A total protein extraction followed by a western blotting showed that the protein is expressed and stably expressed (Figure 15).

Cells containing Blm10p-GST were grown in the presence of 5 μg/ml of phleomycin and 50 μM copper, and the cell growth and killing were monitored at different time points for a 35-hour period (Figure 16). Expression of the Blm10p restored drug resistance. The deletion strain in which the protein was not overexpressed exhibited normal growth, and similar growth was observed when the protein was overexpressed after the addition of copper to media

(Figure 16, Panel A). In the presence of phleomycin (5 μg/ml) the cells showed no signs of growth (Figure 16, Panel A). However, when the protein was overexpressed, by the addition of copper, the cells grew in the presence of phleomycin (Figure 16, Panel A). These data indicate the cells are not able to grow in the presence of the drug unless Blm10p is overexpressed. The corresponding survival data (Figure 16, Panel B) indicate the killing of the cells in the presence of 5 μg/ml phleomycin is high unless the protein is overexpressed.

To determine if the parent plasmid (pYEX4-T1; no *BLM10* gene) may have an effect on cell growth and survival, cells with the parental plasmid were grown under the same conditions as used in Figure 16 Panel A. The results revealed that in the presence of drug and copper growth of the cells was completely inhibited and cell survival was extremely diminished in the absence of Blm10p (Figure 16, Panel C and D).

The overexpression of Blm10p demonstrated that the protein is functional when produced as a fusion, and is able to rescue the sensitivity at higher drug concentrations than had been previously used (Febres *et. al.*, 2001). This ability to protect in the presence of higher drug concentrations when the protein is expressed indicates a major role for the Blm10p in the relief of the toxic effects of the drug. The overexpression of Blm10p did not appear to produce any additional phenotypes.

V. Pulsed field gel electrophoretic analyses indicate *blm3-1* mutant cells suffer extreme DNA damage and killing was also extreme

In two separate proteomic studies, using systematic mass spectrometry, Blm10p was found to associate with two proteins involved in chromosomal integrity. One of these proteins is Sir4p (Gavin *et al.*, 2002). The Sir4p, upon DNA damage, has been shown to leave telomeres and relocate at double-strand breaks, where it binds yKu70, a well-established component of a double-strand break repair mechanism (Martin *et. al.*, 1999). In the other study, Blm10p was found associated in a complex pulled down by a tagged Zds2p (Gavin *et al.*, 2002). *ZDS2* was recently found in a search for genes which confer resistance to the anticancer DNA damaging drug cisplatin (Burger *et. al.*, 2000). Other screens in which *ZDS2* had been identified include those designed to suppress defects resulting from histone mutations (Ma *et. al.*, 1996), and to suppress mutations in *CDC20*, a gene required for chromosome segregation. Since both *blm10Δ* and *blm3-1* cells exhibit hypersensitivities to the bleomycin-phleomycin family of drugs, and these drugs have a direct effect on DNA we sought to study the quality of the chromosomal DNAs in both strains.

To examine the quality of the DNA and the possible role of Blm3p and Blm10p in DNA maintenance under stress conditions, pulsed field gel electrophoresis was used. In pulsed field gels, chromosomal degradation is characterized by a reduction in the intensity, or disappearance, of chromosomal bands and by an accumulation of the degraded chromosomes

at the bottom of the gel. As double-strand breaks in chromosomal DNA increase the chromosomal band intensities decrease and chromosomes degrade. As double-strand breaks are repaired, chromosomes can be reconstructed, and the intensity of the banding increases.

Although studies of chromosomal integrity in *blm10Δ/blm10Δ* strains are not yet complete, studies of the production of double-strand chromosomal breaks investigated in both *BLM10/BLM10 BLM3/BLM3* and *BLM10/BLM10 blm3-1/blm3-1* strains are reported here. In the absence of phleomycin, the chromosomes of each strain used in this study consistently produced strong bands after PFGE (Figure 17, lanes 1, 2, and 3). The bands in the gel on the right in Figure 17 appear lighter than those on the left but in prior experiments the bands were found to be equivalent to wildtype. In the presence of drug, the *BLM10/BLM10* cells were able to protect their chromosomal DNA as indicated by the presence of a strong banding pattern after 0.1 μg/ml and 0.25 μg/ml (Figure 17, lanes 4-9) and limited degradation after the highest drug concentration of 0.35 μg/ml (Figure 17, lanes 10-12). However, after the same drug treatments, chromosomes in the *blm3-1/blm3-1* cells were degraded, indicating a marked deficiency in their capacity to protect the chromosomal DNA at these drug concentrations (Figure 17, lanes 4-12).

To determine if DNA repair took place, *blm3-1/blm3-1* cells were treated with extremely low drug concentrations. Extremely low phleomycin doses of 0.0001 μg/ml, and 0.01 μg/ml had to be used in order to observe any intact chromosomes on the pulsed field gels (Figure 18, middle gel and far

right gel). With the phleomycin dose of 0.0001 µg/ml, banding was observed at the end of a 30 minute treatment period. After 24 hours post treatment incubation, chromosomes were degraded (Figure 18, middle gel, 24 hours) and chromosomes were reconstructed after 48 hours posttreatment incubation as indicated by the reappearance of chromosomal bands (Figure 18, middle gel, 48 hours). This pattern of processing and repair was reported previously after bleomycin treatments in wild type cells, and in mutant cells deficient in the repair of chromosomal double strand breaks (Moore *et. al.*, 2000).

After the drug concentration of 0.01 µg/ml, there was a significant amount of damage to the DNA as indicated by the lack of any strong bands (Figure 18 right gel; 0, and 24 hours). By 48 hours, double-strand breaks in DNA have been repaired as indicated by the reappearance of chromosomal bands.

VI. Computer analyses reveal conserved regions in homologous proteins, particularly at the carboxyl-terminus

When studying a novel protein such as Blm10p extensive computational analyses can provide insight into function. When this project began, there were a limited number of databases available. As time progressed more and more genomes of these organisms were sequenced and databases of the respective genomes became available for public use. With this knowledge, every few weeks the large *BLM10* gene or regions of it

were used for BLAST (Basic Local Alignment Search Tool) searches against the databases.

Recently a BLAST search of the whole Blm10p sequence resulted in strong homology in certain areas of the protein with a human, mouse, rat, plant, and fungal protein. The strongest region of homology was found to occur near the carboxyl-terminus of the proteins, the region missing in YFL007w (before the sequencing mistake was dicovered). The homology occured in different regions of the proteins, but when they were aligned in a multiple alignment using Block Multiple alignment processor which multiply aligns ungapped segments corresponding to the most highly conserved regions of proteins (Henikoff *et al*, 2000) (http://blocks.fhcrc.org/blocks/) the result (Figure 19) showed there are five conserved regions that occur in a very specific order. The order of these color coded conserved regions (Figure 19) in all organisms is maroon, blue, red, yellow, then green. The exact sequences of each conserved region in Figure 19 are shown in Figure 20.

The carboxyl-terminus has the highest degree of conservation among these proteins. This region, omitted in the protein predicted from the original YFL007w, appears to have the same three blocks of sequences all with about the same spacing between them. The end of the yellow block is always 10-12 amino acids from the green block and the end of the red block is always 41-52 amino acids for the beginning of the green block. Although the first two regions of homology do not have a specific spacing beween them,

they do always have the very specific order of the maroon region occuring closer to the amino-terminus than the blue region.

The proteins found to have homology to Blm10p also have other features in common. All of the large proteins (with the exception of the mouse) are all larger proteins. Each of the proteins has around 2000 amino acids. One of these homologous proteins (human, PA200) was found to be a proteasomal activator (Ustrell *et. al.*, 2002). Blm10p has been shown in previous studies to associate with proteins known to have proteasomal function (Gavin *et. al.* 2002).

VII. Blm10p is a nuclear protein

To better understand the type of environment in which Blm10p exists in the cell, protein localization studies were undertaken. These localization studies used live cells so conditions could be varied to examine any change in localization or signal strength. To accomplish this, an amino-terminal yellow fluorescent protein (YFP) fusion was made. This fusion was made in the chromosome so that the fusion protein would be under the control of the *BLM10* endogenous promoter. In summary, the purpose of this fusion was to determine the localization of the protein in living cells, produced at its endogenous level, and study the localization under various cell conditions.

A GFP carboxyl-fusion had previously been made (Febres, 2001). Looking at cells expressing this chromosomal fusion, it appeared that there was no signal. Numerous attempts were made to increase the expression

level of the fusion protein, but no definitive localization was found. We later discovered that this fusion was not in frame because the full YFL007w sequence was not originally known.

The results of the localization studies are shown in Figure 21A. The localization shows the protein to be in the nucleus throughout the cell cycle. The microscopy also demonstrated that the protein level remains constant throughout the cell cycle. To verify this nuclear localization, the Blm10p-YFP strain was crossed with a Spc42p-CFP strain (Cyan fluorescent protein). *SPC42* is an essential gene, which encodes one of the major components of the spindle pole body (Ishihara *et. al.,* 2001). The spindle pole body remains embedded in the nuclear envelope throughout the cell cycle so it was used as a marker of the nucleus (Donaldson and Kilmartin, 1996). The results show that in a strain expressing both tagged proteins that Blm10p clearly is inside the nucleus (Figure 21B). The protein was found to be expressed at a very low level, in agreement with a prior prediction (Professor Leona Samson, Harvard School of Public Health, personal communication).

Based on the sensitivities to bleomycin-phleomycin conferred by the *blm10Δ* , the nuclear localization of Blm10p, and the associations of Blm10p with DNA repair proteins in complexes, it seemed like a strong possibility that the Blm10p may function in DNA repair. Tagged proteins with a direct involvement in repairing DNA breaks can form foci when breaks in DNA are induced (Tashiro *et. al.*, 2000; Du *et. al.*, 2003). To determine if Blm10p may form foci, cells containing the tagged protein were also treated with varying

amounts of bleomycin. The Blm10p remained in the nucleus, but appeared not to form foci after bleomycin treatments (Figure 22). Interestingly, after bleomycin treatments the nuclear localization was still observed even after nuclear fragmentation in response to drug treatment (Figure 22).

The images clearly in Figure 21 and 22 show that the Blm10p-YFP fusion localizes to the nucleus, consistent with our prediction of a role for Blm10p in protection of DNA. The localization strength appears not to change over the course of the cell cycle, and is about twice as intense in budded cells before the nucleus migrates to the bud (Figure 21A). The full images show that the intense nuclear localization signal is consistent in all cells. Blm10p does not localize in any membranes, in spite of the thought originally that it might be a membrane protein (Febres *et. al.,* 2001) These results demonstrate that it is not a membrane protein because nuclear membrane proteins have an intense signal at the nuclear periphery, rather than inside the nucleus, and the signal in nuclear membrane proteins appears as a ring at the nuclear periphery. The latter is certainly not the case for nuclear Blm10p in these images.

VIII. Without its C-terminus, Blm10p localizes to nuclei in stationary phase cells and to the bud neck in budded cells

The Blm10p-GST was utilized to examine the localization of Blm10p when the conserved C-terminal region was absent. The Blm10p-GST was expressed using the plasmid discussed in the overexpression section (section

IV. of results). This plasmid was useful for localization since the amount of protein produced from the plasmid could be controlled by varying the amount of copper in the medium. The plasmid also allowed the protein to be produced as a fusion to the glutathione-S-transferase protein (from *Schistosoma japonicum*). The use of this fusion protein made it possible to localize the protein using immunofluorescent techniques, since antibodies to GST are readily available.

To localize the protein, cells were grown in a selection medium and the protein was induced with 50 μM copper since this amount of copper was shown to produce a stable protein (Figure 15) and was shown to be functional in the relief of drug sensitivity (Figure 16). Cells were grown, converted to spheroplasts, and permeabilized. They then were treated with an anti-GST alexa 488 conjugate antibody (Molecular Probes, Portland OR). The cells were then observed under a Zeiss fluorescent microscope using a DAPI filter and a FITC filter at 100X.

The tagged Blm10p-GST localized in stationary phase cells in the nucleus of the cell (Figure 23, column A) This nuclear localization was confirmed by staining of the DNA with DAPI (Figure 23, column A). In budded cells, the truncated Blm10p-GST fusion localized to the bud neck of the cell (Figure 23, column B and C). The area of localization was right between the mother and daughter. This localization appeared to have an arch, which follows the contour of the mother cell rather than the daughter. The protein localized there through the S, G2, and M phases of the cell cycle as indicated

by the staining of the DNA by DAPI (Figure 23, columns B and C). To verify that the localization is not due to the GST the plasmid carrying only GST was examined. The GST expressed alone revealed a cytoplasmic diffuse staining, thereby eliminating GST as the reason for the Blm10p localization (Figure 22, column D). Cells were also examined with no staining to verify no autofluorescence was occurring. These cells show no fluorescence (Figure 23, column E).

VIIII. Blm10p does not activate the proteasome to degrade whole proteins

Recently, Blm10p was found to be associated with proteins in a proteasomal complex in two separate analyses utlilizing systematic mass spectrometry (Gavin *et. al.*, 2002). In both studies Blm10p was pulled down with subunits of the 20S proteasome. Also recently, the human protein with homology to Blm10p (Figures 19 and 20) was found to be a proteasomal activator (Ustrell *et. al.*, 2002). To determine if Blm10p has a role in proteasomal function experiments were carried out to determine the activity of proteasomes in *BLM10*, *blm3-1* and *blm10Δ* cells.

To determine proteasomal function in live cells, the plasmid pUB23 was obtained (donated by Dr. Alfred Goldberg, Harvard University, Boston, MA). The plasmid pUB23 contains a ubiquitin-*lacZ* gene fusion (Bachmair *et. al.*, 1986). The fusion gene is under the control of a GAL promoter which allows gene expression to be controlled by the amount of galactose in the

media. After induction of the fusion protein, a ubiquitinated β-galactosidase is produced. The ubiquitin tag automatically targets this fusion protein for degradation by proteasomes (Bachmair *et. al.*, 1986). The degradation is then measured by comparisons of enzymatic activity in the three strains.

BLM10, blm3-1 and *blm10Δ* cells were all transformed with the plasmid. Cells were then grown in media lacking uracil (all strains are auxotrophic for uracil and the plasmid carries the *URA3* gene that allows growth on media lacking uracil). Once cells were grown to an OD_{660} 0.8 they were washed and the plasmid was induced in a medium in which galactose was substituted for glucose. The cells were assayed for β-galactosidase activity at different time points.

The results indicate that all three strains produced the same amount of β-galactosidase (Figure 24). At time zero, very little enzymatic activity was detected. After the first hour there was a large increase in the enzyme activity. This activity increased for two additional hours, but then appeared to reach a plateau. Because the β-galactosidase is ubiquitinated it is automatically targeted for destruction by the proteasome. A strain deficient or diminished in proteasome activity should show an increase in the amount of enzyme over time due to the diminished proteasomal activity (Bachmair *et. al.*, 1986). However, all three of the strains used in this study exhibited fully functional 26S proteasomes.

Discussion

The results presented in this thesis have demonstrated that *BLM10* is larger than predicted. This larger Blm10p is a novel nuclear protein with a significant role in relieving cells of the toxic effects conferred by the bleomycin-phleomycin family of antibiotics. Before this research began Blm10p was one of a number of uncharacterized proteins in *Saccharomyces cerevisiae*. The research in this thesis has demonstrated the importance of the protein in providing cells protection from the drug. The research clearly shows that Blm10p resides in the nucleus and functions there in a manner which protects cellular DNA. However, truncated Blm10p localized to nuclei in stationary phase cells, but localized to the bud neck in growing cells. Blm10p was determined to have a high degree of conservation in specific regions of the protein. The protein was also shown not to affect proteasomal degradation of folded proteins, although a proteasomal function still seems likely. Even though the exact protective functional role remains to be elucidated, this research has narrowed down the spectrum of possible functions.

I. *BLM10* is larger than predicted

This research demonstrated than *BLM10* is larger than predicted. Sequencing revealed that a base was missed when the yeast genome was sequenced (Goffeau et. al., 1996). The correction of this mistake merged YFL006w into YFL007w. The size of this new ORF was verified by a northern

analysis of the gene. Although this missing base has been verified by two other laboratories (Robben *et. al.*, 2002; Ustrell *et. al.*, 2002), the expression observed in this report is the first time the new size has been verified experimentally.

II. The *BLM10* gene encodes a protein involved in the relief of bleomycin-phleomycin hypersensitivity

The *BLM10* gene is required for the relief of stresses such as those conferred by the bleomycin-phleomycin family of glycopeptides. This relief was first observed when *BLM10* in a multicopy plasmid was found to suppress the hypersensitivity to bleomycin conferred by the *blm3-1* mutation (Febres *et. al.*, 2001). In the current studies, hypersensitivity to the drug family was also conferred when the full length gene was deleted (Figure 14). However expression of Blm10p in these strains revealed a restoration of cellular resistance to phleomycin indicating an important role of Blm10p in the relief of the drug sensitivity.

A. Deletion of *BLM10* confers growth inhibition and high amount amount of killing in the presence of phleomycin

Deletion of the full length *BLM10* was shown to confer a severe growth inhibition and high amount of killing in the presence of the bleomycin-phleomycin family of chemical congeners. This was not surprising since *BLM10* was previously shown to suppress the drug hypersensitivity in the

blm3-1 mutant cells (Febres *et. al.*, 2001). This hypersensitivity in cells missing the gene taken together with the fact it can compensate for another gene involved in cellular resistance to the drug indicate an import role for Blm10p in the relief of these hypersensitivities.

B. Overexpression of the Blm10p confers an increase in cellular resistance to the drug family

When Blm10p was overexpressed in the null background the overexpressed protein was able to overcome the cellular toxicity caused by the drug phleomycin (Figure 16). This relief of the cellular toxicity substantiates the role of Blm10p as a guardian against cellular stresses such as those caused by the drug family.

The overexpressed protein involved only the ORF originally thought to encode the *BLM10* gene (YFL007W). The portion which then was notated YFL006w was not part of the overexpressed protein. This demonstrates that the last 16% of the protein is not of extreme functional importance in the relief of the cellular toxicity. This was also the case observed in the initial cloning of the gene (Febres *et. al.*, 2001; Figure 2). The portion of the gene which was able to functionally complement the *blm3-1* mutation was within the first approximately 50% of the gene (Febres *et. al.*, 2001). Thus, the last part of the protein does not appear to be a absolute requirement for the function of Blm10p in the relief of cytotoxicity. It is possible that the carboxyl-terminal region is involved in an interaction(s) with another protein(s) but the first 84%

of the protein provides a strong enough interaction. While dispensible for protection against cytotoxicity the high amount of homology in this region (Figures 19 and 20) indicates that Blm10p may have more than one cellular function and the carboxyl-terminal region may be more important for some other cellular function(s).

III. The Blm10p is a nuclear protein

Two approaches were conducted in this study to localize Blm10p. Both studies revealed a distinct nuclear localization by the tagging of Blm10p. In one study Blm10p was fused to YFP and expressed from its endogenous promoter. This study revealed a distinct nuclear localization and was confirmed by a colocalization study. In the second study the localization was followed by immunoflourescence after expressing a truncated protein from an inducible promoter. This study showed a nuclear localization in unbudded cells and revealed a bud-neck localization in budded cells. The nuclear localization in stationary phase cells was consistent with the nuclear localization observed with the Blm10-YFP fusion, and with the colocalization of Blm10p-YFP with a nuclear protein.

It is not known why the truncated protein was nuclear in stationary phase cells yet it was not retained in the nucleus in growing cells. When the protein is observed at the bud neck there was no indication of any protein in the nucleus in contrast to the Blm10p-YFP studies where the protein remained in the nucleus whether in actively growing cells or stationary phase

cells. The Blm10p-GST was a truncated Blm10p, and this most likely accounts for why it is not retained in the nucleus in growing cells. The last 18% of the protein was not present, but may be important for retaining the protein in the nucleus. The homology results have indicated that the carboxyl-terminal region of the protein has been conserved through evolution. This type of conservation indicates an area of importance, and the inability of the truncated Blm10p to remain in the nucleus could indicate the importance of the carboxyl-terminal region in retaining Blm10p in the nucleus. In addition, all the proteins found to associate with Blm10p are also nuclear. Scl1p and Pre8p both were found in association with Blm10p in MALDI mass spectrometry experiments (Gavin *et. al.*, 2002). Both of the protein are subunits of the 20S proteasome. The 20S proteasome is found in the nucleus 80% of the time (Gordon, 2002). In other experiments Blm10p was found to associate with Sir4p (Ho *et. al.*, 2002) and Zds2p (Gavin *et. al.*, 2002). Both of these proteins are also known nuclear proteins

There are two differences between the Blm10p-YFP and the truncated Blm10p-GST studies. First, the Blm10p-GST fusion was expressed off a plasmid under control of an inducible promoter, in contrast to the Blm10p-YFP which was expressed from the *BLM10* promoter in the chromosome. A protein expressed under the control of its endogenous promoter is less likely to give a false localization pattern than one that is not (Koning *et. al.,* 1996). Second, Blm10p-GST was overexpressed and a protein expressed at higher levels than normal may cause it to mislocalize (Koning *et. al.*,1996).

IV. The Blm10p could have a proteasomal function

To test the proteasomal function in *BLM10*, and *blm10*Δ cells, a proteasomal assay was carried out as previously described (Bachmair *et. al.*, 1986). Results of the proteasomal assay indicated that the proteasome is fully functional in its ability to degrade whole proteins (Figure 24). These experiments were carried out since two prior reports indicated a possible proteasomal function for Blm10p (Ustrell *et. al.*, 2002, Gavin *et. al.*, 2002). The first of these reports found Blm10p associated with Pre8p and Scl1p, two subunits of the 20S proteasome. The second report determined Blm10p human homolgue PA200, to be a proteasome activator of the 20S proteasome.

Although the proteasomes were found to be fully functional in their ability to degrade whole proteins in the wild type and mutant strains that were tested, it still seems likely that Blm10p has a proteasomal function. First, Blm10p was pulled down in complexes in two separate experiments with two different proteasomal tagged proteins (Gavin et. al., 2002). This indicates that the association of Blm10p with proteins in the complexes is quite likely to be of functional significance since it occurred in separate experiments. Second, recent homology searches have turned up six new homologs (Figures 19 and 20). Although the homology is not strong, it is of significance that the three areas of homology lie in a very specific order close to the carboxyl-terminal region of Blm10p and the last three areas have very

specific spacing between them. One of these homologs , the human PA200, was shown to have proteasomal function as a proteasomal activator (Ustrell *et. al.*, 2002). Lastly, Blm10p also clearly localizes to the nucleus. Yeast proteasomes have been reported to be in the nucleus 80% of the time (Gordon, 2002). Moreover both Blm10p and its human homologue are in the nucleus (Ustrell *et. al.*, 2002).

Although Blm10p is not capable of activating the proteasome for degradation of whole proteins (Figure 24) the protein has not been tested for degradation of shorter peptides. It has also been determined that the human homolog of Blm10p is not capable of activating the 20S proteasome to degrade whole proteins (Ustrell *et. al.*, 2002). PA200 was found to only activate the 20S proteasome to degrade small flourogenic peptides *in vitro* (Ustrell *et. al.*, 2002). Although Blm10p has not yet been tested, it seems likely it could be found to also activate the 20S proteasomes for this type of degradation. The 26S proteasomes are composed of the 20S subunit and the 19S subunit. It is thought the 19S subunit unfolds the proteins and feeds them into the 20S subunit for degradation. Since Blm10p was found not to affect degradation of whole proteins it seems less likely it would be associated with the 26S proteasome but instead be associated with the 20S subunit alone. In this regard, the Blm10p would act like the 19S subunits to activate the 20S subunit to degrade additional substrates. These substrates may be partially degraded peptides, oxidatively damaged peptides or a class

of peptides not yet characterized as getting degraded by the 20S proteasomes.

V. Similar functions of proteasomes and bleomycin hydrolase to protect DNA

Why does bleomycin cause hypersensitivity to cells in which a protein which has a possible function in activating the proteasome is mutated or missing? The human 20S proteasome degrades small peptides. One function of this activity is to generate the antigen fragments for major histocompatibility complex (MHC) class I ligands. These class I ligands are small molecules (8-10 amino acids) which are presented at the cell surface by MHC class I molecules so the immune system may respond (Kessler et. al., 2002). Recently, it was determined that bleomycin hydrolase (a protease which previously was only known for the degradation and inactivation of bleomycin [Xu *et. al.*, 1994]) acts along with the proteasome in the preparation of MHC class I ligands (Stoltze *et. al.*, 2000). This indicates a cooperation and similar processing capabilities between the two proteases.

The cooperation of the 20S proteasomes and bleomycin hydrolase may well extend to eliminating the toxic effects of the bleomycin-phleomycin family. The main target of bleomycin is chromosomal DNA. Interestingly, the yeast bleomycin hydrolase is primarily cytosolic (Zheng and Johnston, 1998) whereas the yeast proteasomes are primarily nuclear (Gordon, 2002). In yeast it has been shown that cellular resistance to bleomycin is not affected

by increases in bleomycin hydrolase levels in hypersensitive mutant strains (Wang and Ramotar, 2002). There is also no increase in sensitivity in cells in which the enzyme is deleted (Wang and Ramotar, 2002), indicating that there is another means for relieving the cell of the toxic effects caused by the drug. The other mechanism may well involve the 20S proteasome.

VI. Blm10p and protection against DNA damage and killing

A second possiblity of the function of Blm10p in association with proteasomes is its involvement in the protection of DNA. Bleomycin generates reactive oxygen species which cause oxidative damage to DNA as well as nucleoproteins (Arnold *et. al.*, 2001). The 20S proteasome has been shown to be effective in the removal of oxidatively damaged proteins (Tsirigotis *et. al.*, 2001) . Recently it was shown that 20S proteasomes are effective in the removal of oxidatively damaged histones (Arnold *et. al.*, 2002). In addition, Blm10p has been shown to associate with the 20S proteasome as well as a complex with the Zds2p (Gavin *et al.*, 2002) and the Sir4p (Ho *et .al.*, 2002). The Sir4p upon DNA damage, has been shown to leave telomeres and relocate at double strand breaks, where it binds yKu70, a well-established component of double strand break repair (Martin *et. al.*, 1999). *ZDS2* was recently found in a search for genes which confer resistance to the anticancer DNA damaging drug cisplatin (Burger *et. al.*, 2000). It remains a possibility that the involvement of Blm10p with proteasomes is to activate the 20S proteasome for degradation of oxidatively damaged nucleoproteins, and

its associations with *ZDS2* and *SIR4* facilitate the release of these histones through chromatin reorganization. This chromatin reorganization also allows for an efficient repair of DNA damage.

The 20S proteasomes have been shown to have altered substrate specificity after activation by different activators (Harris *et. al.*, 2001). It has been found that cancer cells have a higher amount of 20S proteasomes and most of this "extra" proteasome is localized in the nucleus (Ullrich *et. al.*,1999). These same tumor cells also demonstrate an increase in cellular resistance to cancer drugs such as bleomycin (Ullrich *et. al.*, 1999) This opens up the possibility that 20S proteasomes may be involved in relieving cells of the cytotoxic effects of bleomycin. It seems very likely that Blm10p will be found to have a function of activating the proteasome for the degradation of small peptides.

VII. The *blm3-1/blm3-1* strains show an exceedingly diminished ability to protect their DNA in the presence of the bleomycin family of drugs

The *BLM3/BLM3*, and *blm3-1/blm3-1* strains displayed different susceptibilities to the lethal effects of bleomycin. After treaments with the low doses of 0.0001 and 0.01 µg/ml phleomycin, *blm3-1/blm3-1* cells still showed severe susceptiblilty to DNA damage and killing (Figures 17 and 18). The *BLM3/BLM3* cells displayed very little susceptibility (if any at all) to the drug at much higher concentrations of the drug (0.25 µg/ml).

At least two possibilities could account for the difference in resistance conferred by the two genotypes. The first possibility could be the differing abilities of the wild type and mutant strains to protect their DNA. A likely involvement of Blm3p is that the *blm3-1/blm3-1* mutant cells have a defect which leads to a decreased ability to detoxify the drug. Little is known about the mechanisms cells use to protect themselves from the toxic effects of bleomycin and phleomycin. The only well characterized protection mechanism in yeast is the detoxification of the drug by bleomycin hydrolase (Xu *et. al.*,1994). Other means of detoxification have been speculated (Pron *et. al.*, 1993, 1999) but none have been proven. It remains a possibility that the *BLM3/BLM3* cells are able to maintain the integrity of their chromosomal DNA by a process which directly detoxifies or removes the drug. The reduction in the amount of active drug thereby reduces the amount of damage to chromosomal DNA. The *blm3-1/blm3-1* strains appear to lack this ability to effectively deal with the drug thereby allowing the drug to inflict more double strand breaks to the chromosomal DNA.

A second possibility for the differences in susceptibility of the chromosomal DNA to damage by the drug in *BLM3/BLM3* and *blm3-1/blm3-1* strains is a difference in the ability of the two strains to repair the double-strand breaks caused by the drugs. The drug family has long been used as a tool to study DNA repair (Moore *et. al.*, 2000) due to its ability to induce pathways of repair (Moore, 1982; Severgnini et. al., 1991). The difference between the *blm3-1/blm3-1* cells and the *BLM3/BLM3* cells may be due to the

involvement of Blm3p in DNA repair. The lack of ability to repair cellular DNA's in the *blm3-1/blm3-1* cells may account for the tremendous increase in chromosomal damage even at extremely low doses.

Although the *blm10Δ/blm10Δ* studies have not been finished, the high amount of damage to chromosomal DNA in *blm3-1/blm3-1* cells open up the possibility for an equally exciting finding in the *blm10Δ/blm10Δ* studies. The ability of *BLM10* to suppress the *blm3-1* drug hypersensitivities along with the sensitivities conferred when Blm10p is not present suggests a deletion of this gene will leave chromosomal DNA unprotected. Pulsed field gel electrophoresis will reveal this vulnerability of cells lacking Blm10p.

Table 1: Yeast and bacterial Strains		
Saccharomyces cerevisiae		
Name of strain	Source	Genotype
BMA-1A	Baudin *et. al.* 1993	*MATa ura3-52 trp1Δ63 leu2Δ his3Δ200 gal2*
CM1452-98B	This laboratory	*MATα ura3-52 ade2-40 leu2-3 ilv1-92*
CM1469-5A	This laboratory	*MATα ade2-40* or *ade2-1 ilvl-92 his3-11* or *his3-15 leu2-3* and /or *leu2-112 trp1-1* or *trp5-12*
CM1469-5C	This laboratory	*MATa ade2-40* or*ade2-1 ura3-1 ilvl-92 trp1-1* or *trp5-12 ura3-1 blm3-1*
CM1522-9B	This laboratory	*MATa ura3-52 trp1Δ63 leu2Δ his3Δ200 blm3Δ::HIS3*
CM-1526	This laboratory	*MATa/MATα ade2-40/ade2-40 trp1-1/trp1-1 HIS3/his3-11 LEU2/leu2-3 ura3-1/ura3-1 ILV1/ilv1-92 blm3-1/blm3-1*
CM-1527	This laboratory	*MATa/MATα LEU2/leu2-3 ILV1/ilv1-92 HIS3/his3-11 ade2-40/ade2-40 TRP1/trp1-1*
CM-1528	This laboratory	*MATa/MATα LEU2/leu2-3 ade2-40/ade2-40 trp1-1/trp1-1 URA3/ura3-1 ILV1/ilv1-92 BLM3/blm3-1*
CM-1529	This laboratory	*MATa/MATα LEU2/leu2-3 URA3/ura3-1 ILV1/ilv1-92 ade2-40/ade2-40 trp1-1/trp1-1*
CM1530-1A	This laboratory	*MATa ura3-52 trp1Δ63 leu2Δ his3Δ200 gal2 blm3Δ::HIS3*
CM1531-1B	This laboratory	*MATα ura3-52 ade2-40 leu2-3 ilv1-92 BLM3::YFP*
EJ758	Martzen *et. al.*, 1999	*MATa his3-D200 leu2-3,112 ura3-52 pep4::URA3*

Bacterial strains		
Name of strain	Source	Genotype
DH10B	Gibco BRL	F- *mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80lacZΔM15 ΔlacX74 deoR recA1 endA1 araD139Δ(ara, leu)7697 galU galK λ-rpsL nupG*

Table 2: Plasmids used for these studies						
Plasmid Name	**Type of experiment**	**Parental Vector**	***E. coli* Selection**	**Yeast Selection**	**Tag**	**Insert**
pRS303	Deletion	pBluescript	Amp	*HIS3*	none	*HIS3*
pDH22	localization	unk	Amp	*Kan*^{r}	none	Kan and YFP
pSH47	localization	unk	Amp	*URA3*	none	Cre recombinase
pYEX 4T-1B	GST	pYEULC	Amp	*URA3*	GST	*BLM10*

Primer name	Primer use	Sequence 5'-3'
1	sequencing	ATATGCCGCAGACGGAAGAC
2	sequencing	ATATAAGACTGAAAGTCATG
3	sequencing	GCCTATCGTTACATCCGTTGT
4	sequencing	AGTAATTCGGTTTATTGTGAT
5	sequencing	CAAAGAACAAATCAAAAGA
6	sequencing	TCAGTGGCACGTACCTTCTA
7	sequencing	CTTCATTGACGTTGATTTCCT
8	sequencing	CAAAAAGAAAAAGCGTGAGTAC
9	sequencing	AAAGCTCAATTTACGTGAGAAT
10	sequencing	GTTGGTATTTGATCACCCATAC
11	sequencing	GTTCGTGCGGCATCCATTTTG
PA-05	Sequencing/deletion verification/YFP verification	GCGCGGTACCATTACGCAGAATAATCT ATG
YFP-up	YFP cassette	TTCAATTGGGATAAGGTCTTGTTAGTA ATGGGAAT GGGTGATTTGATATCATCGTCATTGTT AGCGGTCAT TTTGTACAATTCATCCATACCATG
YFP-down	YFP cassette	TTGCATACATAAACTTTATCATTGTTCG TTAGCTAG CTTTGCACATTAATTTTTCGATTTGTTA CCGCCACGG CCGCCAGGG
Deletion primer 1	Replacement cassette	ATGATCTCAAACTGCTTCTTAATATAG GCATCCAC CTTTTCTGGGACGCTTTTTACTCTTGG CCTCCTCTAG
Deletion primer 2	Replacement cassette	CAAATCTACATGTATATACAGATCTATA CAGCAA TTATAGGATATCTTTCGTTCAGAATGA CACG
HIS3 R	Verify deletion	CAGACAATCAACGTGGAGGGT
NF	Sequencing	ATTCCCATTACTAACAAGACCT

Primer name	Primer use	Sequence 5'-3'
NR	Sequencing	ATCGCAATATAAAGATTAACTA
L1F	Sequencing	AATCTTATATTGCGATCAGCTC
L1R	Sequencing	GATATGATAAGATAGGGCACAAC
L2F	Sequencing	GGGATTTTTACTGATGATCAAATG
L2R	Sequencing	GATATGATAATGATAGGGCACAAC
L3F	Sequencing	TGTTTAACTTCTTTTTGTCACGAA
L3R	Sequencing	GATAGGAATGAAAGCGGCTATAGA
L4F	Sequencing	AACCTCATCAACGGTATTGTATCT
L4R	Sequencing	TATTTCGGTTGTACATAGAGTTGC
L5F	Sequencing	ACTCTATGTACAACCGAAATAACTG
L5R	Sequencing	AAATATCAATCTGCCGATGTC
L6F	Sequencing	AGTGTATGTGTCATTTCCGATCAAG
L6R	Sequencing	CATATTCAGTTCGCAGAAACCAG
CF	Sequencing	TCATCTGGTTTCTGCGAACTGAAT
CR	Sequencing	GTTAGCGACAGCTGGCGAACCTGA

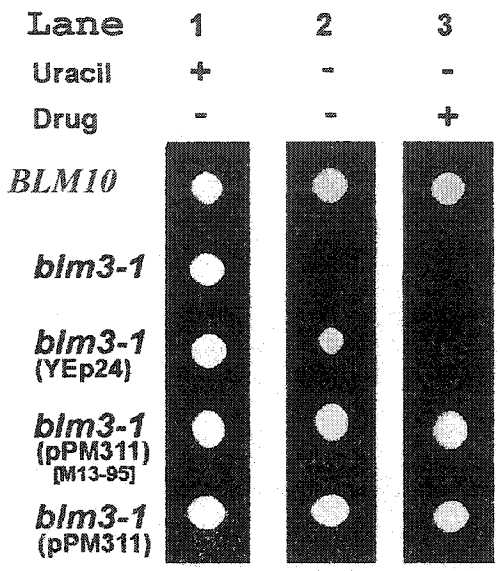

Figure 1 Complemtation of the *blm3-1* mutation. Growth of strains on the synthetic complete medium (SD+7, lane 1), SD+7 lacking uracil (-ura; lane 2), and SD+7 - ura + 7μg/ml of phleomycin (lane 3) are illustrated. **Row 1**: The *BLM10* control strain (CM1469-5B) grew on all three media. **Row 2**: The *blm3-1* mutant strain (CM1469-8B) is auxotrophic for uracil and thus grew on synthetic complete medium, but did not grow on SD+7-ura or SD+7-ura+ 7μg/ml. **Row 3**: The *blm3-1* mutant (CM1469-8B) transformed with the YEp24 vector that permitted growth on media lacking uracil and drug. **Row 4**: The (CM1469-8B) transformant (M13-95) containing pPM311 grew on SD+7μg/ml. **Row 5**: The plasmid pPM311 isolated from *E. coli* also complemented the *blm3-1* mutation on SD+7-ura+ 7μg/ml (CM1469-5C [pPM311]). Figure used with permission of authors (Febres *et. al.*, 2001)

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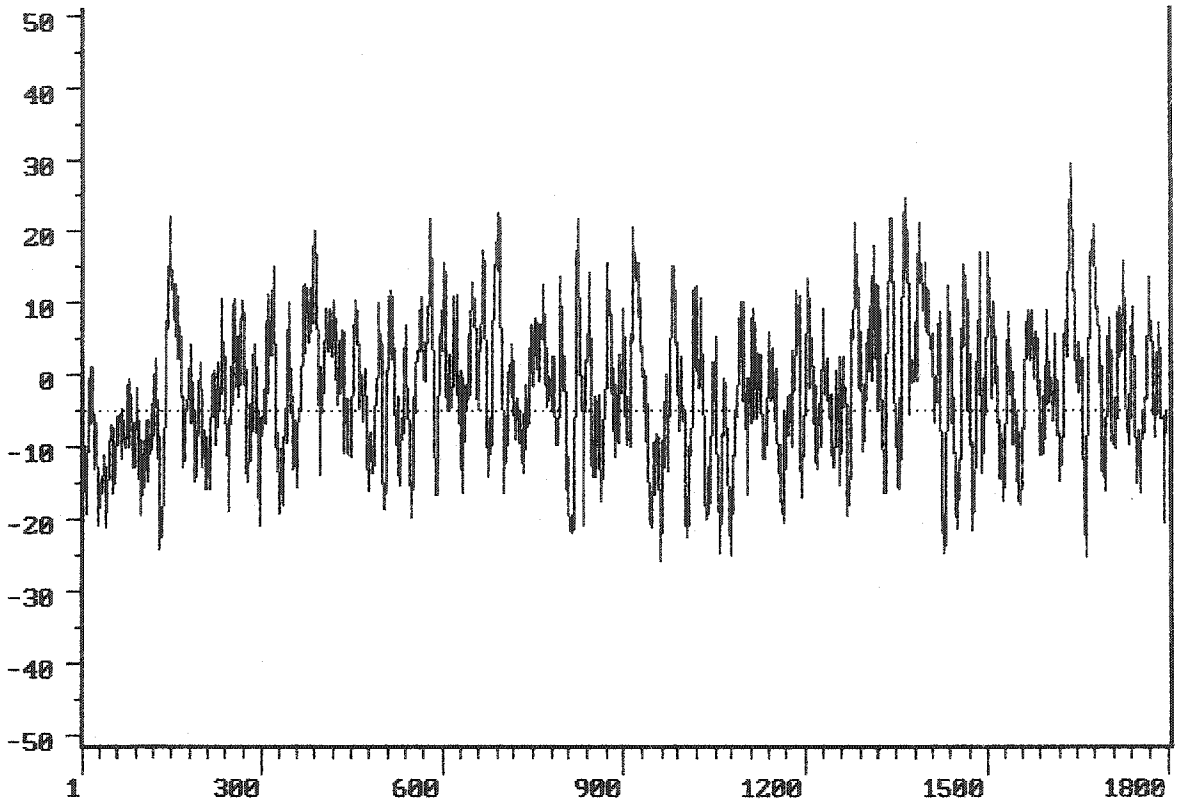

Figure 3 Hydropathy analyses of Blm10p reveal potential transmembrane domains. Hydropathy plot of the amino acid sequence for Blm10p was analyzed by the method of Kyte and Doolittle (Kyte and Doolittle, 1982). The plot predicts 10 transmembrane domains with at least 15 amino acids crossing the membrane. Figure used with permission of authors (Febres *et. al.*, 2001).

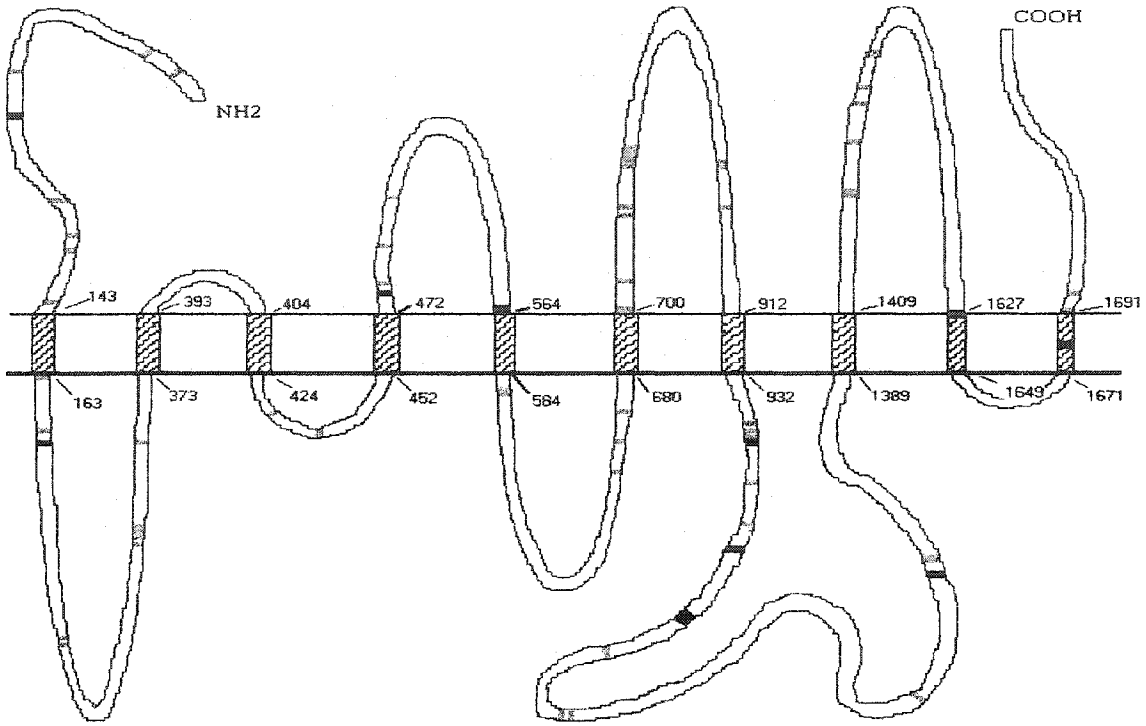

Figure 4 Blm10p is a predicted transmembrane protein with 10 possible transmembrane domains and numerous potential motifs. By combining the hydropathy plot and the predicted motifs, this hypothetical model was generated. Blm10p is predicted to cross the membrane 10 times, thereby generating 6 major loops and 3 minor loops. The possible motifs occur throughout the protein:

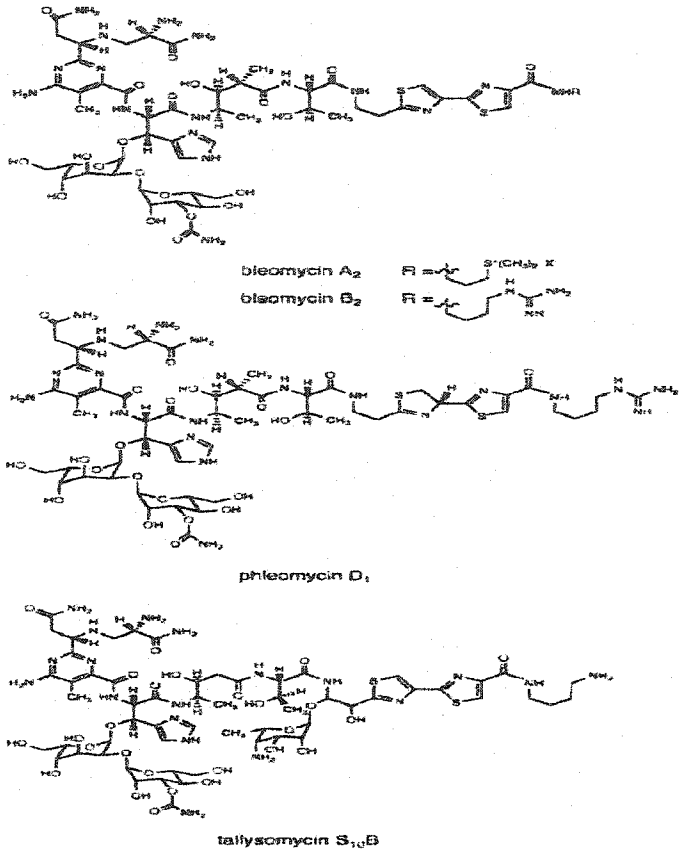

Figure 4 Structural formulas of bleomycin A_2 and B_2, phleomycin D_1, and tallysomycin $S_{10}B$.

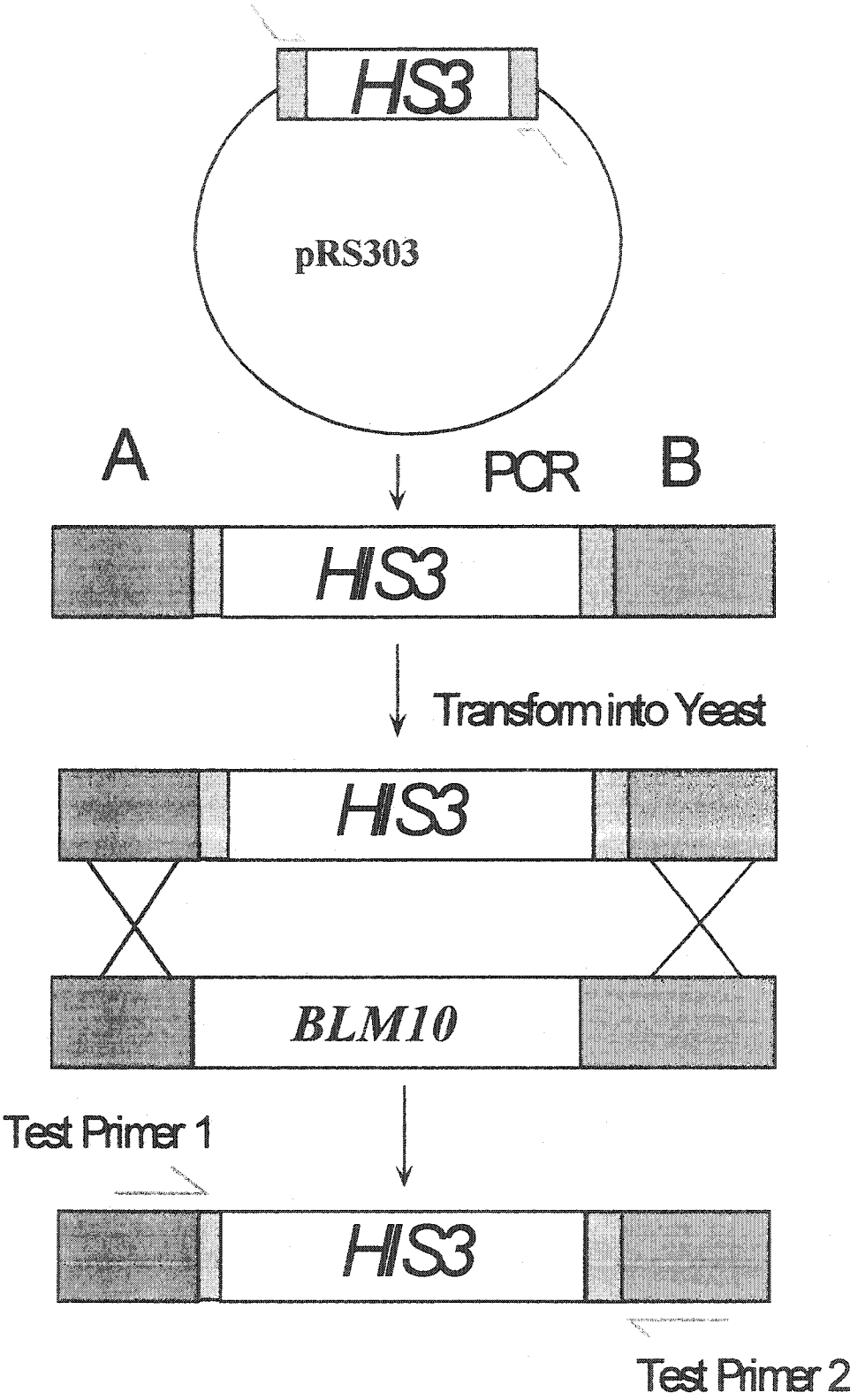

Figure 6 Schematic that summarizes the deletion of the chromosomal *BLM10* gene. 1) PCR amplification of gene replacement *HIS3* cassette; 2) transformation of yeast with PCR product; 3) verification by PCR of the correct gene deletion.

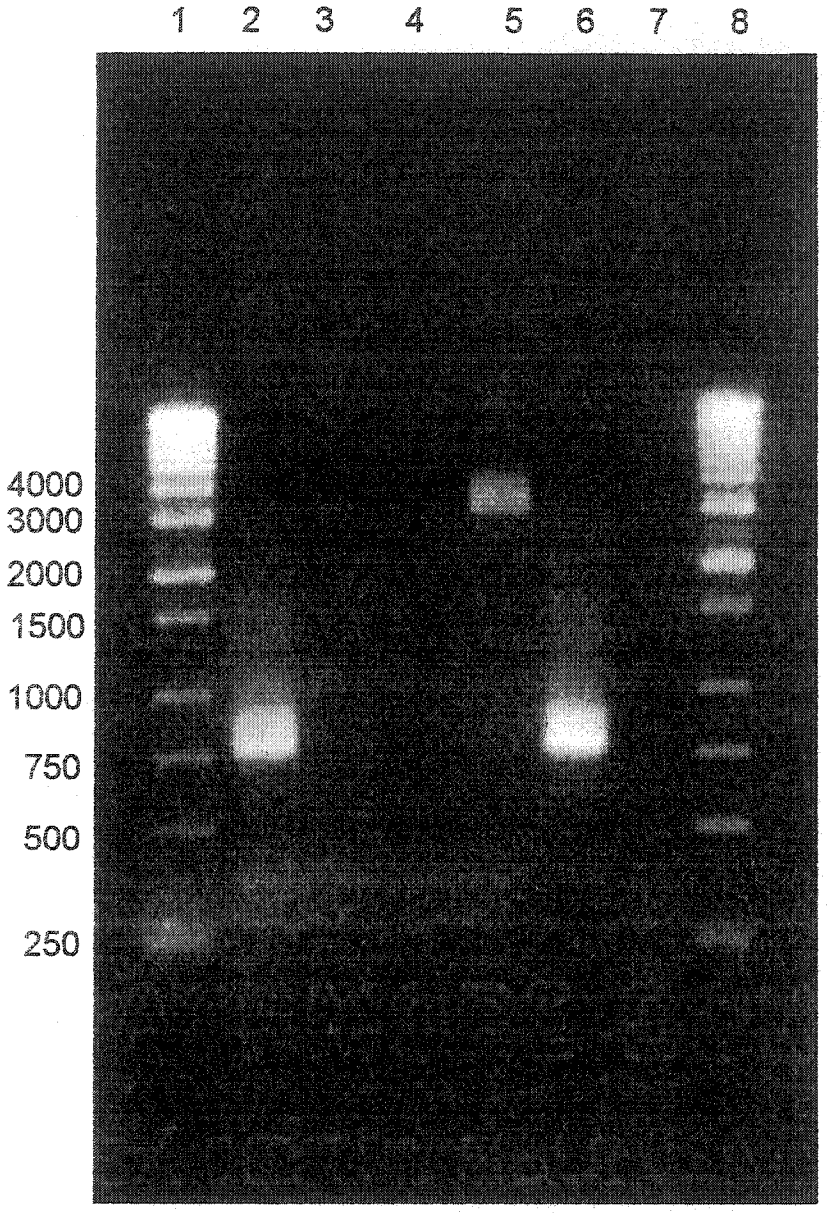

Figure 7 Confirmation of full *BLM10* deletion. Lane 1 and 8 Kb molecular weight standard (Stratagene, La Jolla, CA). Lane 2 colony 9 from deletion with primers PA05 and HIS3R, Lane 3 Colony 9 primers PA05 and L4, Lane 4 strain CM1469-5A primers PA05 and HIS3R Lane 5 CM1469-5A primers PA05 and L4, Lane 6 Colony 18 *BLM10* deletion primers PA05 and HIS3R, Lane 7 colony 18 primers PA05 and L4. Primer L4 is within the *BLM10,* PA05 is 200 bases upstream of the *BLM10* gene and primer *HIS3* is within the *HIS3* cassette which replaced the *BLM10* gene. PA05 is 750 bases upstream of HIS3 primer. PA05 is 3.2 Kb upstream of L4.

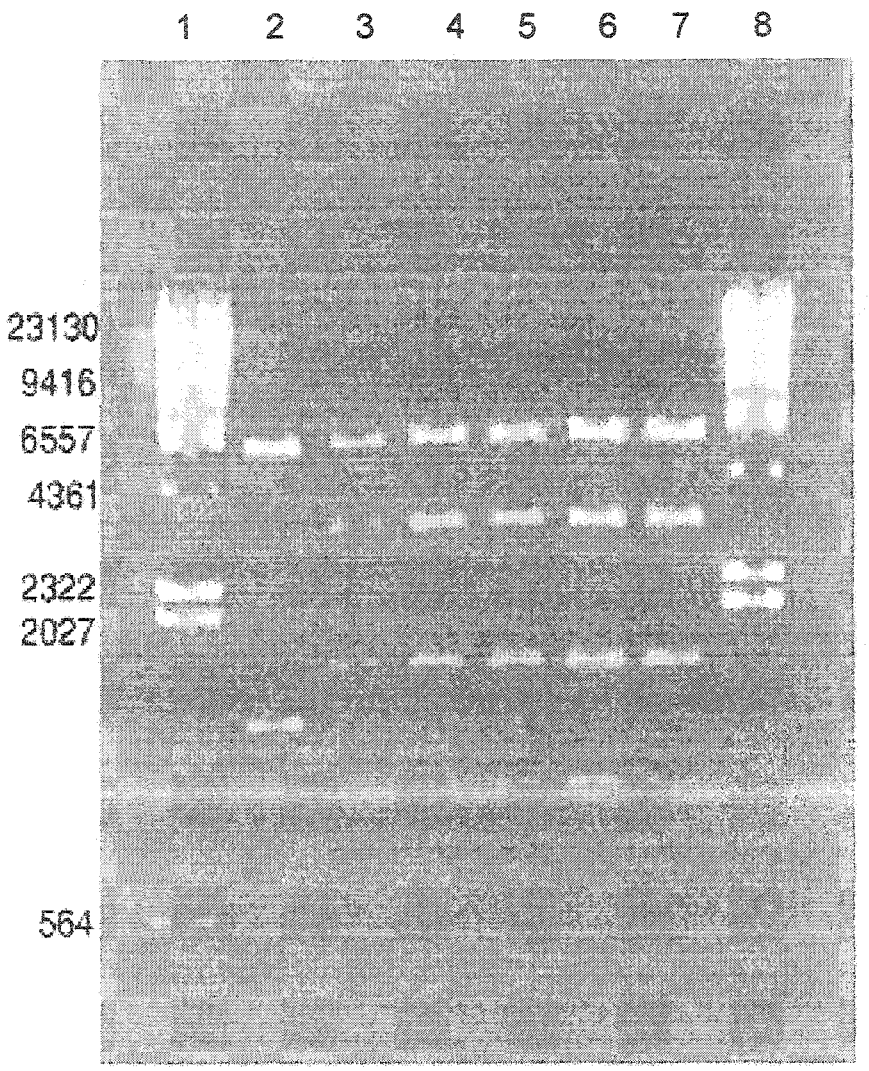

Figure 8 Verification that *BLM10* was inserted in plasmid pYEX4-T1. Lanes 1-8 (left to right) are as follows lanes 1 and 8 Hind III Lamda standard, lane 2 is pYEX4-T1 (no insert) after HindIII digest, lanes 3-7 plasmids isolated from *E.coli* which had exhibited ampicillian resistance. Each of the plasmids were digested by Hind III following isolation. *BLM10* in the correct orientation revealed bands at 6900 bp, 3300 bp, 1581 bp, and 950 bp indicating the *BLM10* insert was present . A digest by HindIII of pYEX4-T1 showed bands that migrated to 6550 and 1300 as expected. Sequencing was used to confirm presence of *BLM10*.

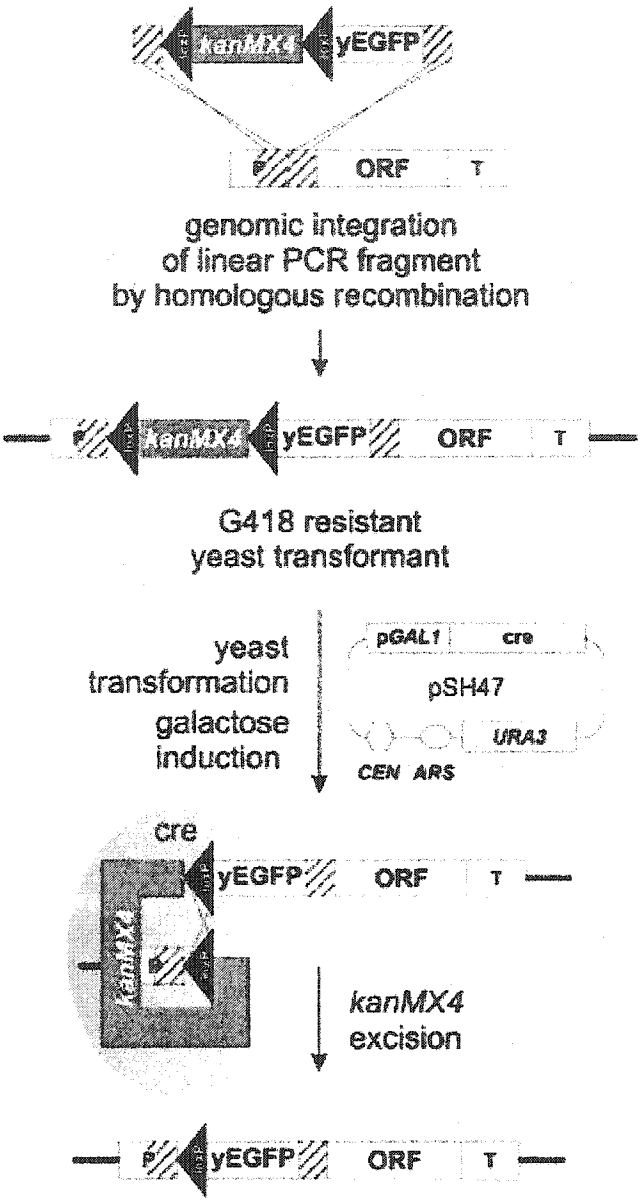

Figure 9 Method used to fuse YFP to the amino terminus of Blm10p. Primers were designed which contained regions of homology to *BLM10* (70 bases) and homology to the cassette from plasmid pDH22. PCR was used to amplify the product, which was verified on a gel, then cut out. The PCR product was then transformed into the strain CM1458-98B which would then integrate by homologous recombination and selected on media containing G418. Genomic DNA was isolated from G418 resistant transformants and PCR was used to check for the presence of the in cassette front of *BLM10*. G418 resistant transformants were then transformed with pSH47 (containing Cre recombinase) and transformants were selected on media lacking uracil. The selected transformants were then grown in media containing galactose (lacking glucose) to induce the Cre recombinase. Cre recombinase recognizes and cuts loxP sites which are present 5' and 3' to the kan resistance gene. Genomic DNA from the Cre induced cells was isolated and PCR was used verify the correct fusion was now present.

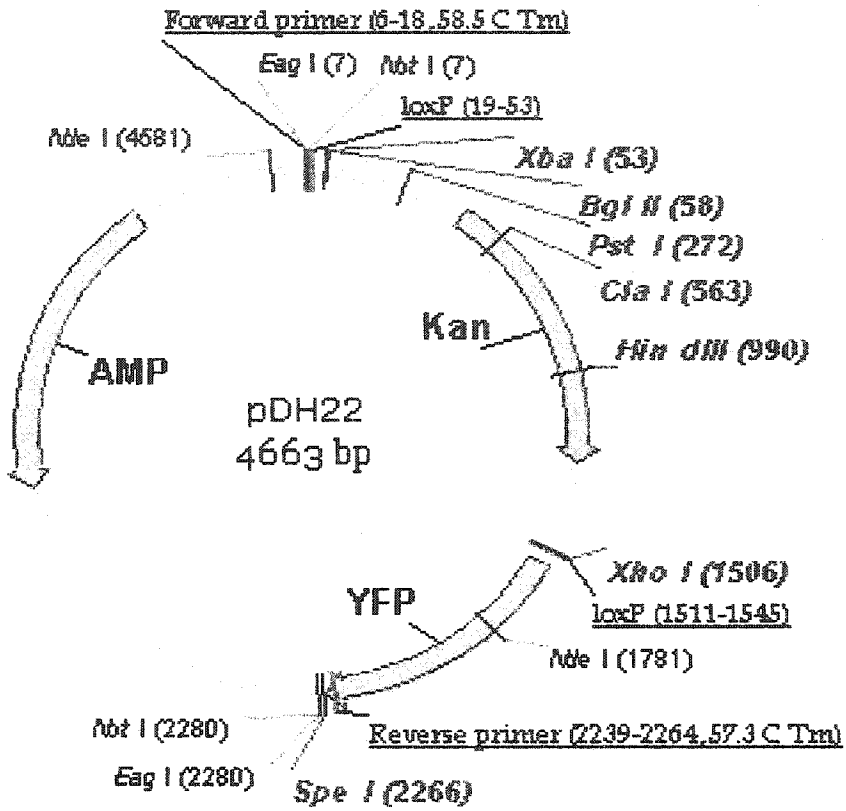

Figure 10 Plasmid pDH22. This plasmid was used as a template for PCR to produce a cassette which contained KanMX module, YFP, and regions of homology to *BLM10* on both sides of the cassette.

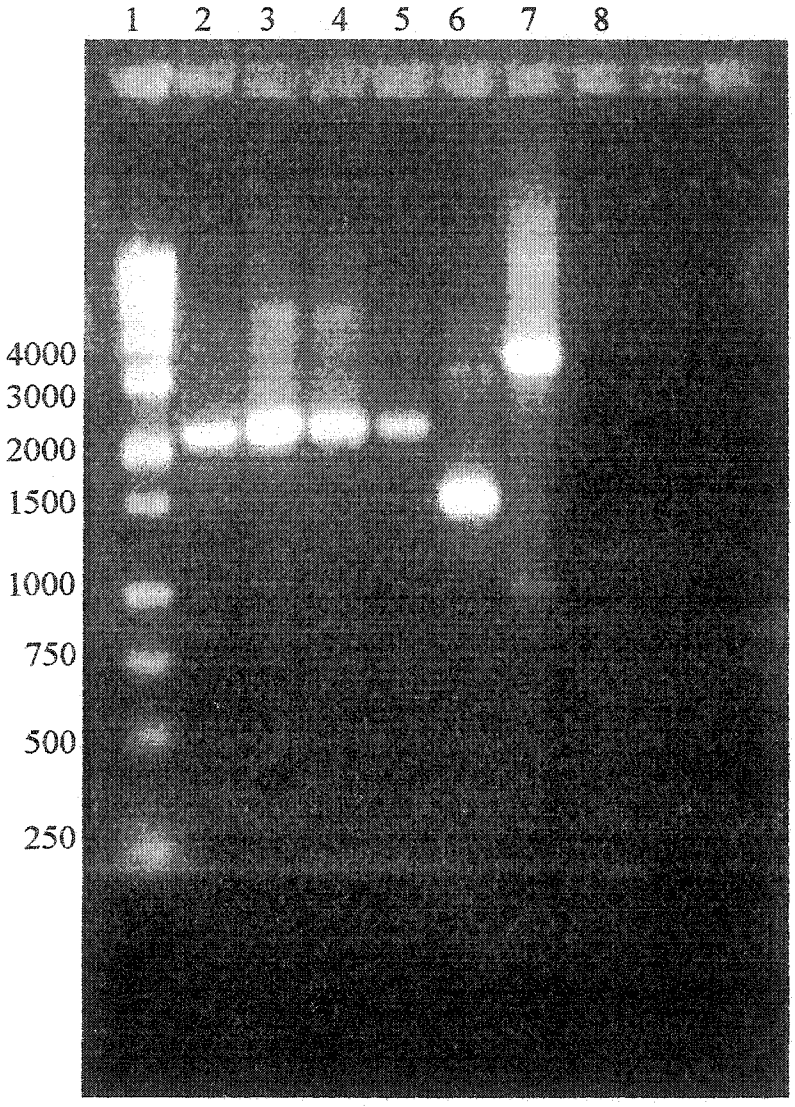

Figure 11 Confirmation of the YFP fusion and removal of the KanMX module. Lane 1 Kb molecular weight standard (Stratagene, La Jolla, CA). Lanes 2-5 PCR product from genomic DNA isolated from colony which had fusion with complete cassette (lane 7) after this colony was transformed with pSH47 and then had CRE recombinase expressed and time enough to remove the Kanomycin gene by excision at loxP sites. Genomic DNA was isolated and used as a template for PCR to check for removal of Kanomycin resistance gene (lanes 2-5). A band at 2.2 kb confirms YFP fusion. Lane 6 is the PCR product from genomic DNA isolated from CM1469-5A (no cassette inserted). A band at 1.5kb confirms PCR. Lane 7 shows verification of cassette insertion. A band of 3.8 kb was obtained after PCR of genomic DNA with primers PA05 and L1R indicating cassette inserted. Lane 8 is PCR negative control (no template DNA).

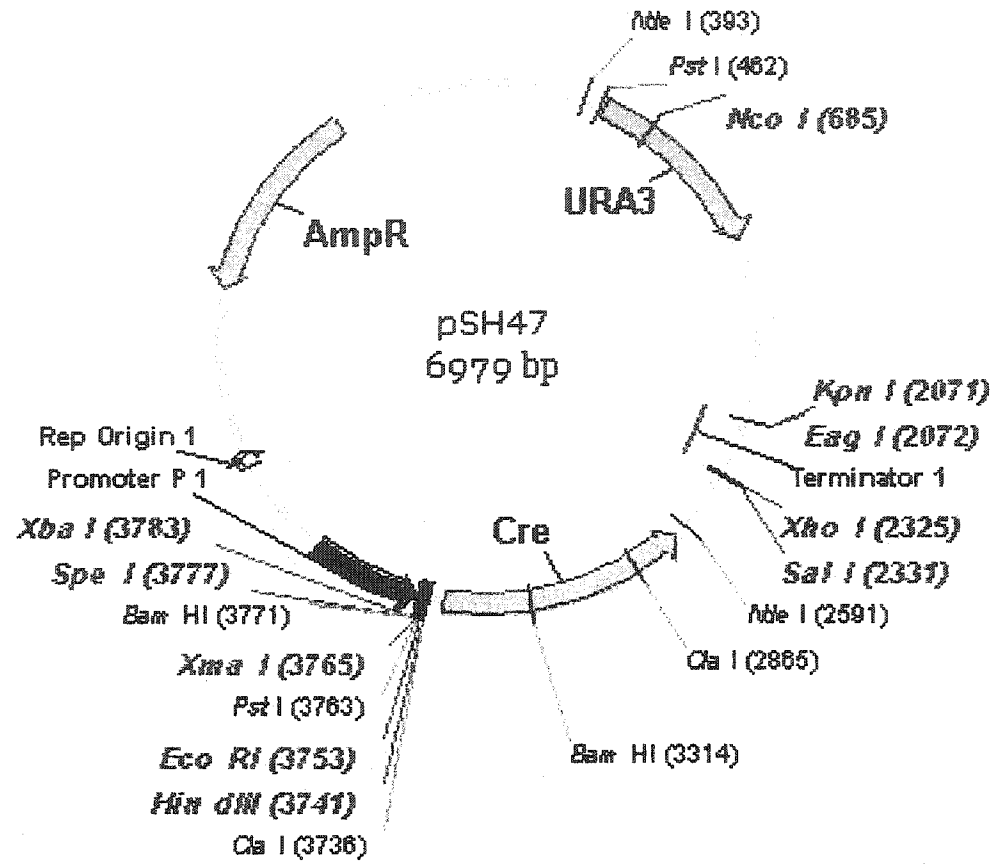

Figure 12 Plasmid pSH47. This plasmid was transformed into the yeast strain which contained the, YFP and kanMX module, cassette at the correct position in genome (just upstream *BLM10* start codon). The strain after seletion for the plasmid was then grown in galactose media to induce the Cre recombinase which cut at the loxp sites thereby leaving only YFP fused to *BLM10*

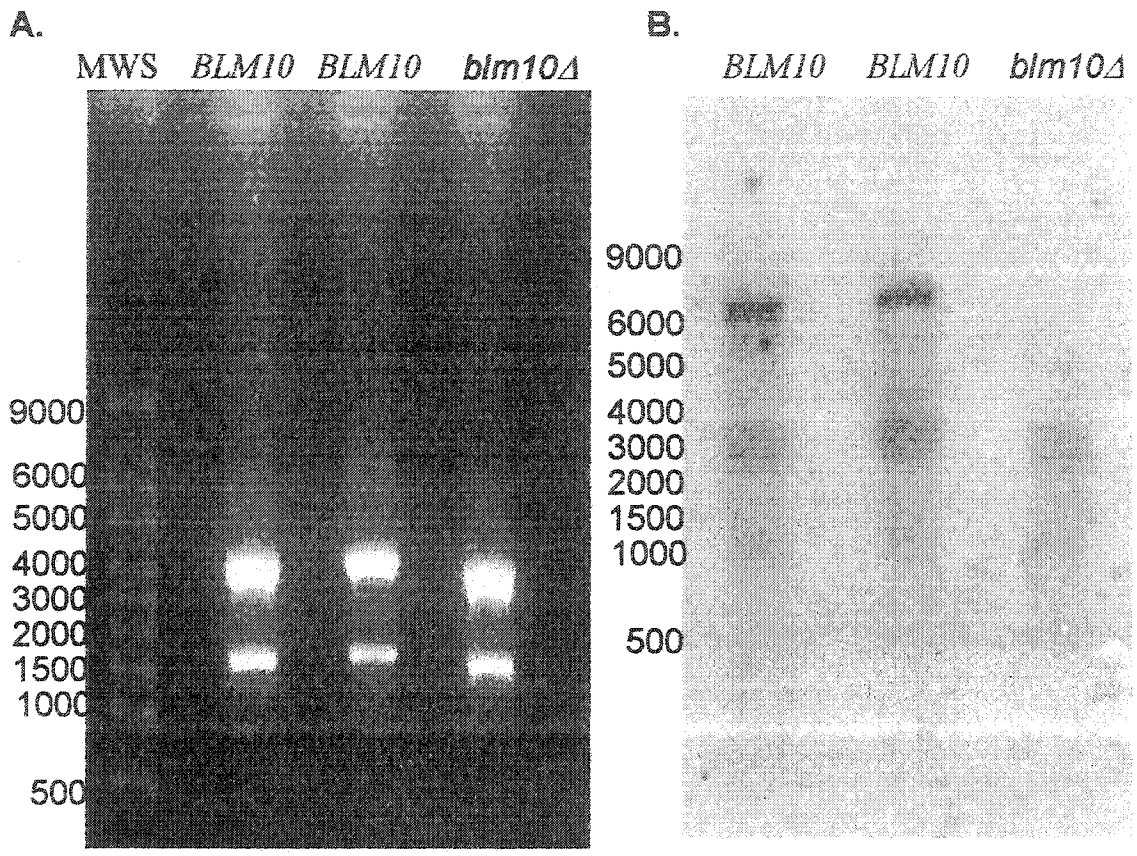

Figure 13 Expression analyses of *BLM10* From *BLM10* and *blm10Δ* cells. Results of two different *BLM10* strains are shown. In A., RNA was isolated from an overnight cultures of each strain, and run on a 1% agarose gel. A molecular weight marker was run. The other lanes show RNA isolated from *BLM10* and *blm10Δ* cells respectively. Section B shows the northern analysis of *BLM10* and *blm10Δ* strains. RNA was isolated from wild type and mutant strain, run on a gel, and transfered from the gel to a membrane. A radiolabeled single stranded DNA probe 800 bases long was created by PCR, and used to probe the membrane containing RNA. Following hybridization an autoradiogram was produced..

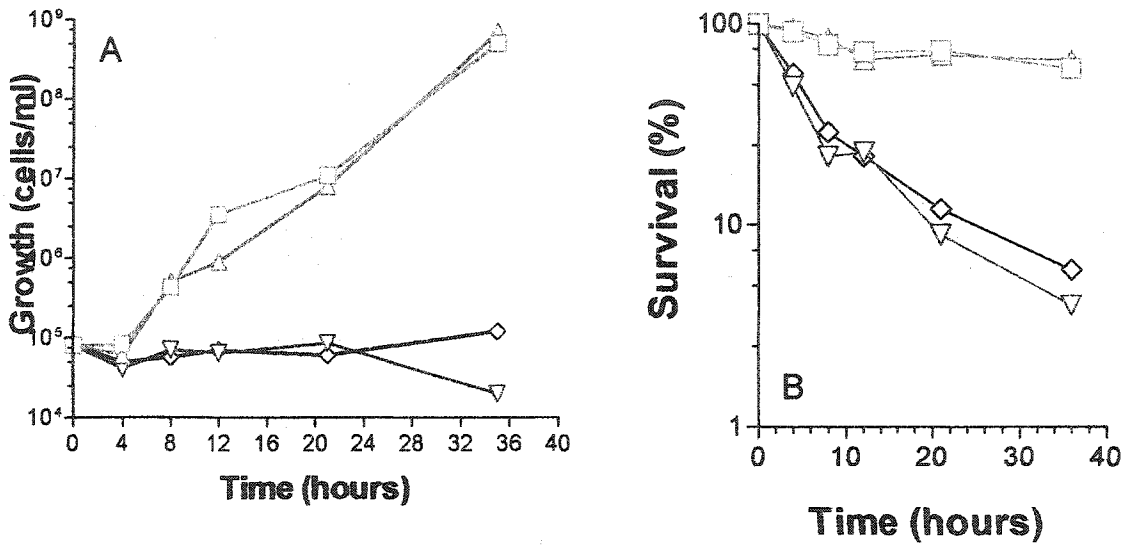

Figure 14 Deletion of YFL007w confers hypersensitivity to the bleomycin-phleomycin chemical congeners and a high degree of killing. Cells were grown in YPAD without phleomycin (□, Δ), and phleomycin (5 μg/ml; ∇, ◊). Replicated experiments are shown. Cell densities during growth are shown in panel A. Survival in the presence of drug are shown in panel B.

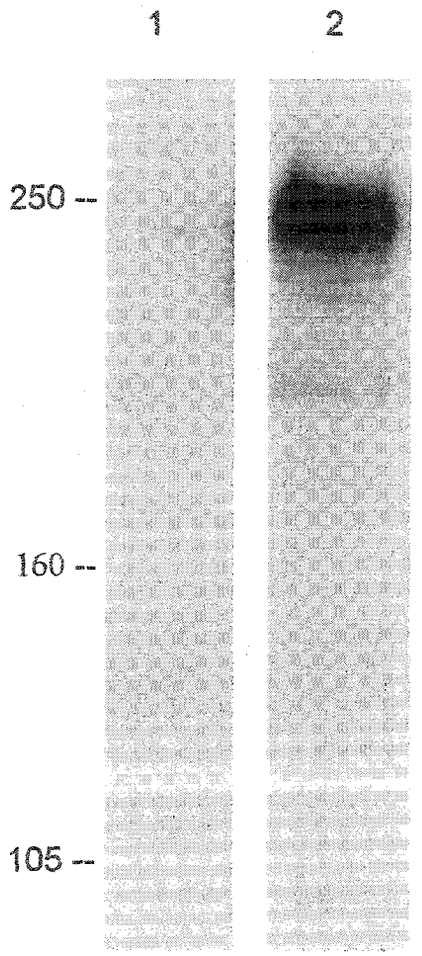

Figure 15 Bim10p is produced as a stable protein in *bim10Δ* cells. Whole cell extracts were prepared from *bim10Δ* cells expressing Bim10p from a plasmid. Extracts were run on a SDS page gel followed by a western analysis using an anti-GST antibody. Lane 1 is a whole cell extract from a strain grown without copper. Lane 2 is a whole cell protein extracts that was treated with 50 μM copper. Lane 2 reveals a strong band at 230 kDa, indicating the fusion protein is produced and is stable.

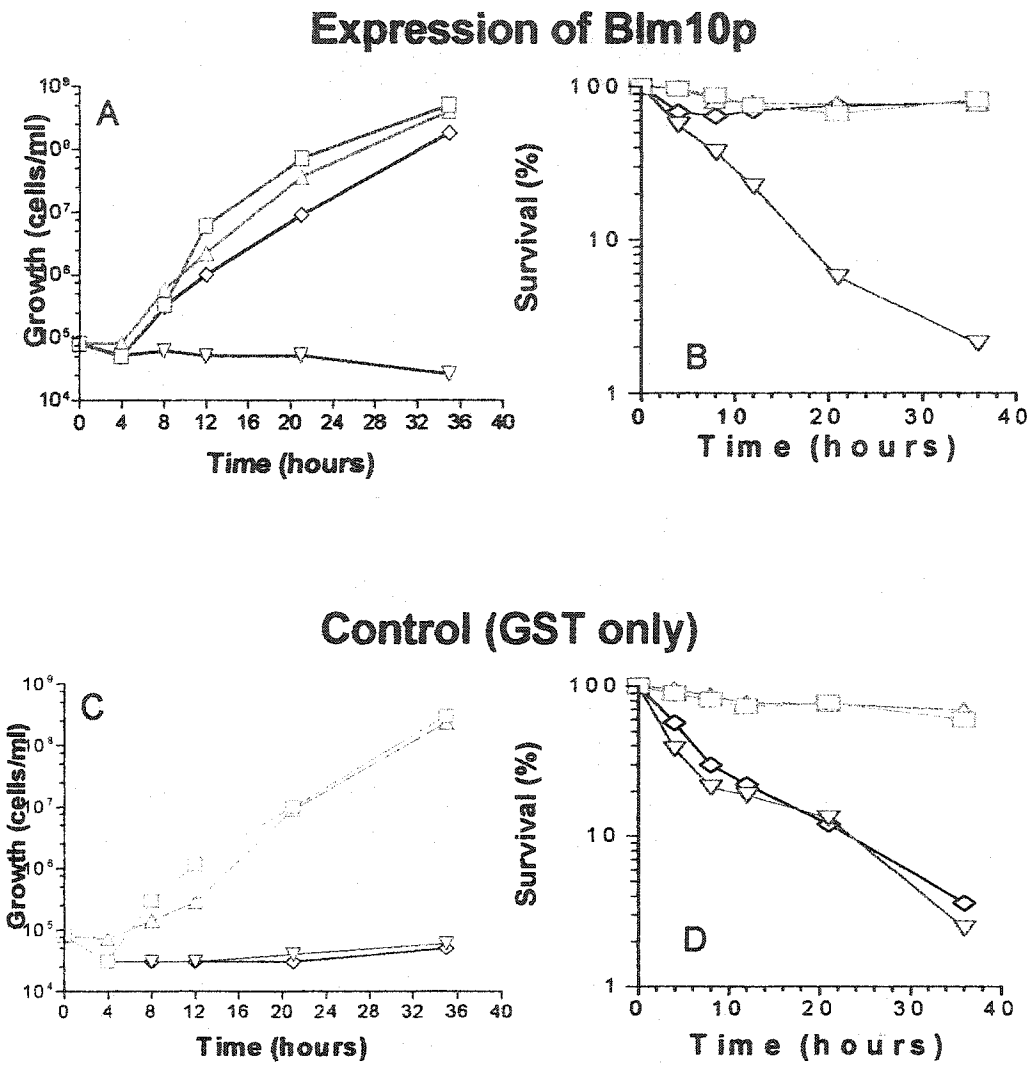

Figure 16 Expression of Blm10p restores resistance to *blm10Δ* cells. Cells were grown in YPAD without copper or phleomycin (□), with copper only (50 μM; Δ), with phleomycin only (5 μg/ml; ∇), or with both (◊). Cell densities during growth are shown in panels A, and C. Survival of the cells grown under the various conditions are shown in panels B, and D. Results for a *blm10Δ* strain containing the plasmid with the *BLM10* gene are shown in panels A and B. Results for the *blm10Δ* strain that contained the plasmid without the *BLM10* gene (only GST was expressed) are shown in panels C and D.

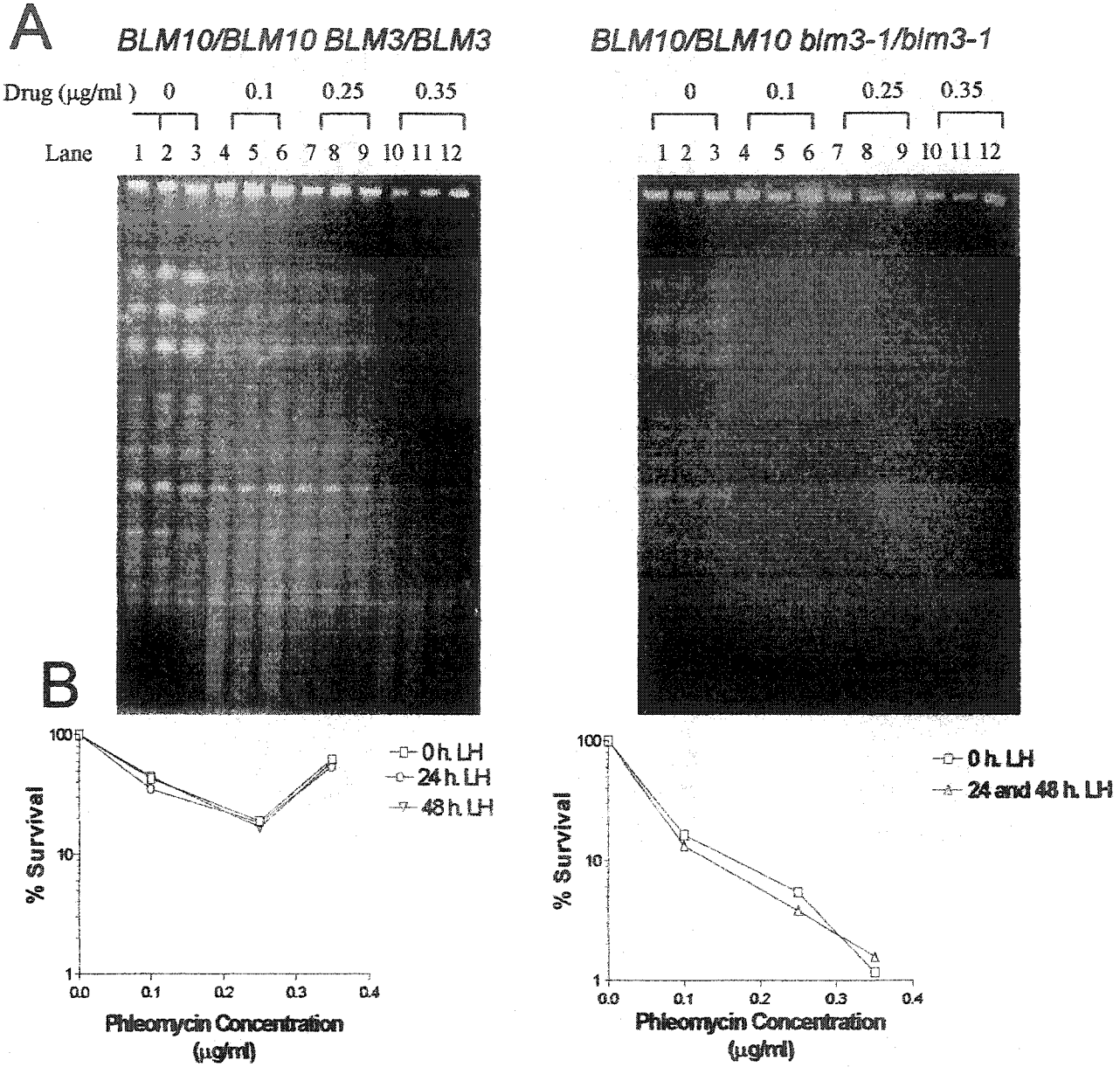

Figure 17 PFGE illustrating the excessive damage to chromosomal DNA in *BLM10/BLM10 blm3-1/blm3-1* strain in comparison to *BLM10/BLM10 BLM3/BLM3* after phleomycin treatment. Corresponding survival data are shown and indicate excessive killing of *BLM10/BLM10 blm3-1/blm3-1* mutant cells. A. Lanes 1, 4, 7, and 10 on both gels show DNA after a 30 minute treatment of cells with the drug. Lanes 2, 5, 8, and 11 on all gels show DNA after 24 hours posttreatment incubation (liquid holding [LH] in deionized water that does not affect survival of yeast cells [Moore *et. al.*, 2000]). Lanes 3, 6, 9 and 12 show DNA after 48 hours LH. Concentrations of the drug used in experiment are shown above each lane. B. Survival of the cells. The integrity of chromosomal DNA in replicate experiments was comparable for both strains.

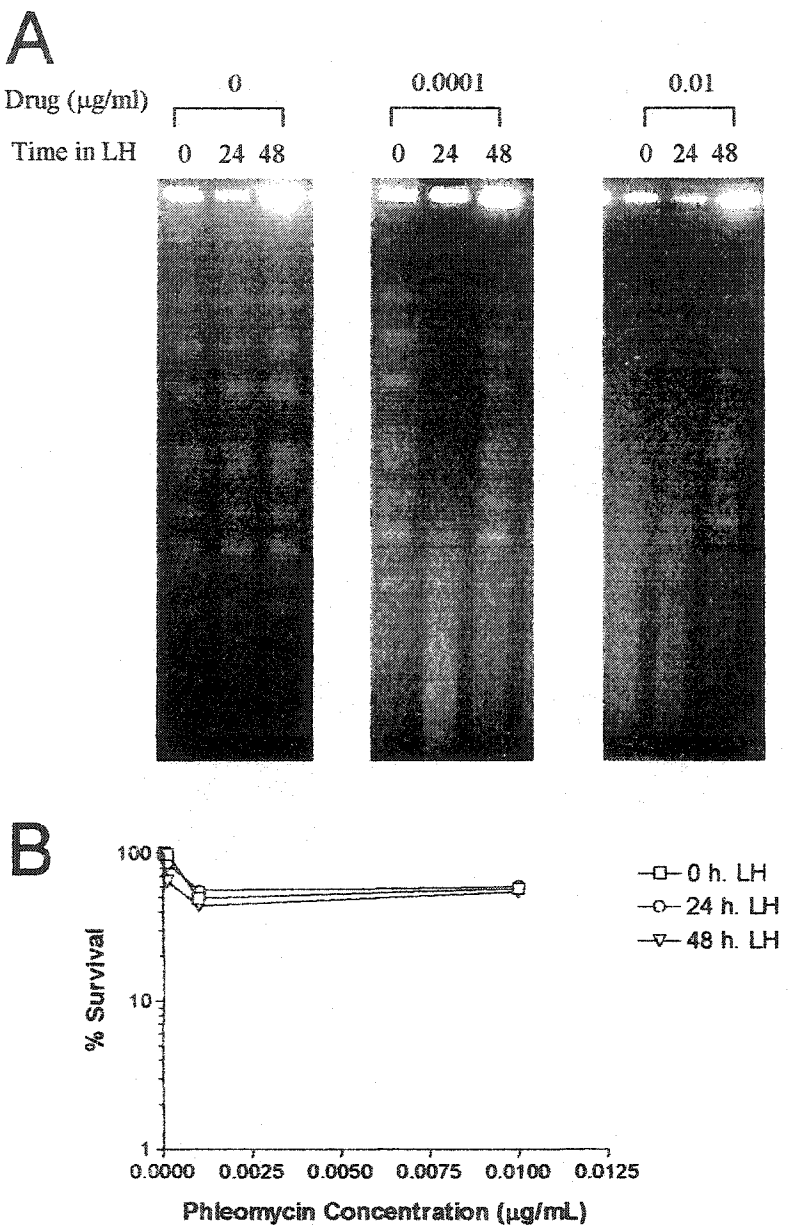

Figure 18 PFGE and survival illustrating ability of *BLM10/BLM10 blm3-1/blm3-1* cells to repair chromsomal DNA after extremely low doses of phleomycin. A. Lanes are labeled as time of posttreatment incubation. Time zero is after a 30 minute treatment of cells with the drug and no posttreatment incubation. B. Survival graph illustrating survival of *BLM10/BLM10 blm3-1/blm3-1* cells after treatment with 0.0001 and 0.01 μg/ml phleomycin.

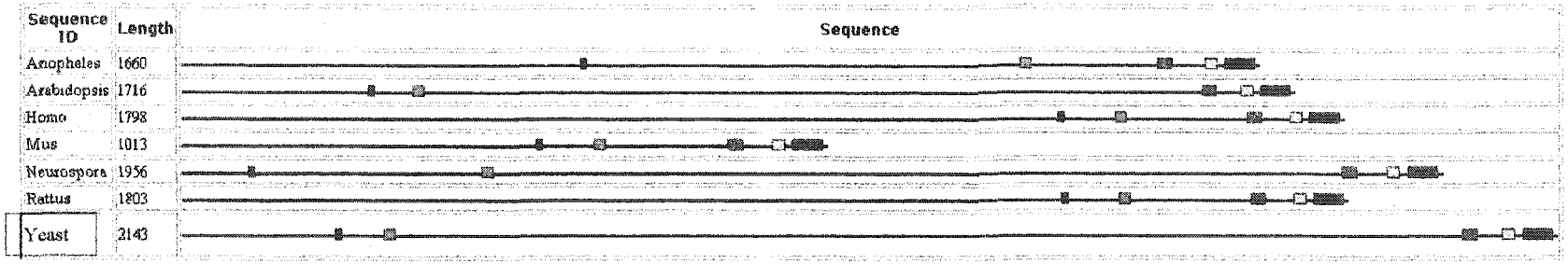

Figure 19 Blm10p has evolutionarily conserved regions. A multiple alignment was performed using BLOCKS with proteins found to have homology using the BLAST program (NCBI, protein-protein BLAST). Regions of homology were found to have a specific order with an aproximately similar spacing between them.

Figure 20 Blm10p sequence was compared to other organisms using the BLAST program (NCBI, protein-protein BLAST). Proteins with regions of homology were then run through a multiple alignment at CLASTALW. Amino acids are color coded according to the physiochemical criteria:
Red: small and hydrophobic (AVFPMILW)
Blue: acidic (DE)
Magenta: basic (RHK)
Green: Hydroxyl+ amine+ basic, and glutamine (STYHCNGQ)

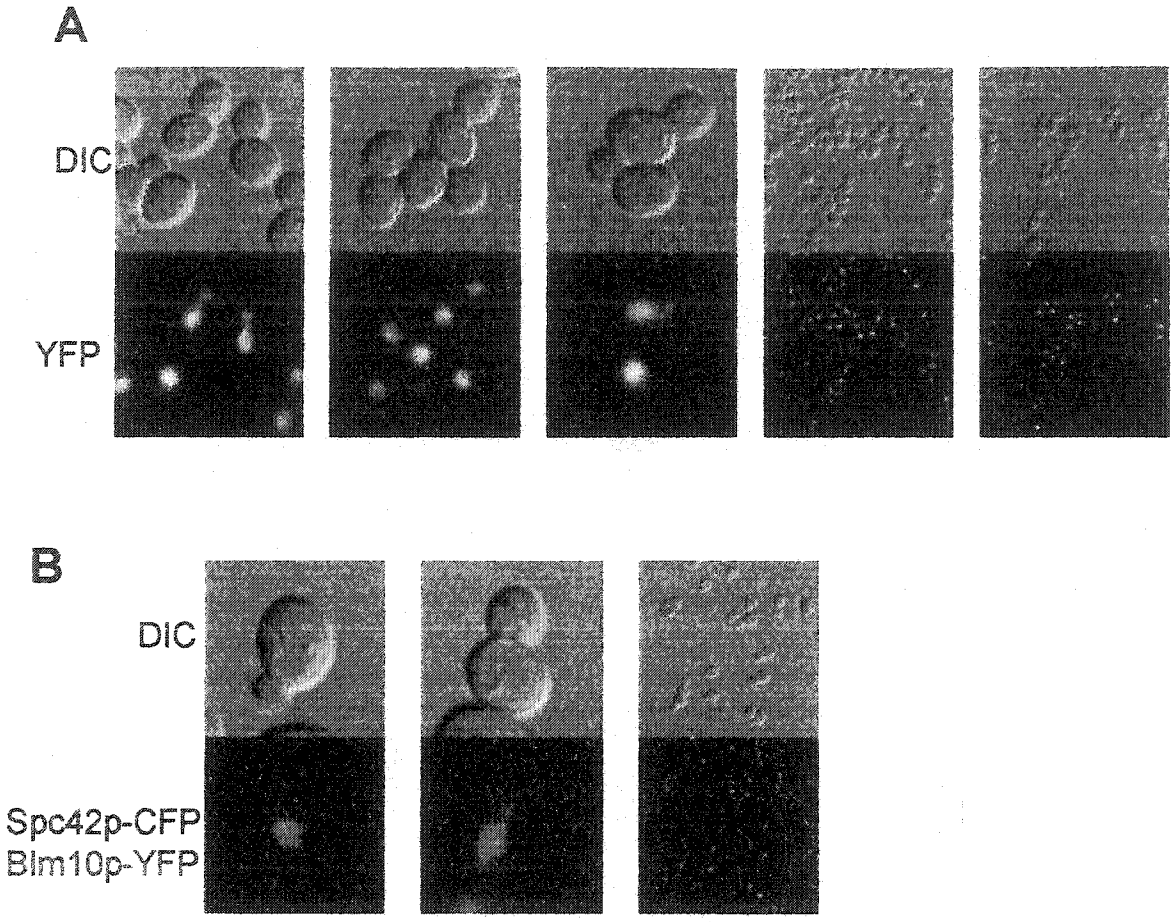

Figure 21 Nuclear localization of Bim10p. The top pictures in each group are differential interference contrast images and the YFP images are on the bottom. Cells were grown on nonsynthetic complete medium and imaged using a DeltaVision microscopy system from Applied Precision (Issaquah, WA). The system incorporates an Olympus IL-70 microscope, a u-plan-apo 100X oil objective (1.35NA), a CoolSnap HQ digital camera from Roper Scientific (Tucson, AZ) and optical filter sets from Omega Optical (Battleboro, VT). Live cells were imaged on a thin pad of media containing 1% agarose (Hailey *et. al.*, 2002) . Images were analyzed using SoftWoRx software. Section A shows a Bim10p-YFP localization. Section B shows a verification of the nuclear localization by a colocalization of Bim10p-YFP with Spc42p-CFP.

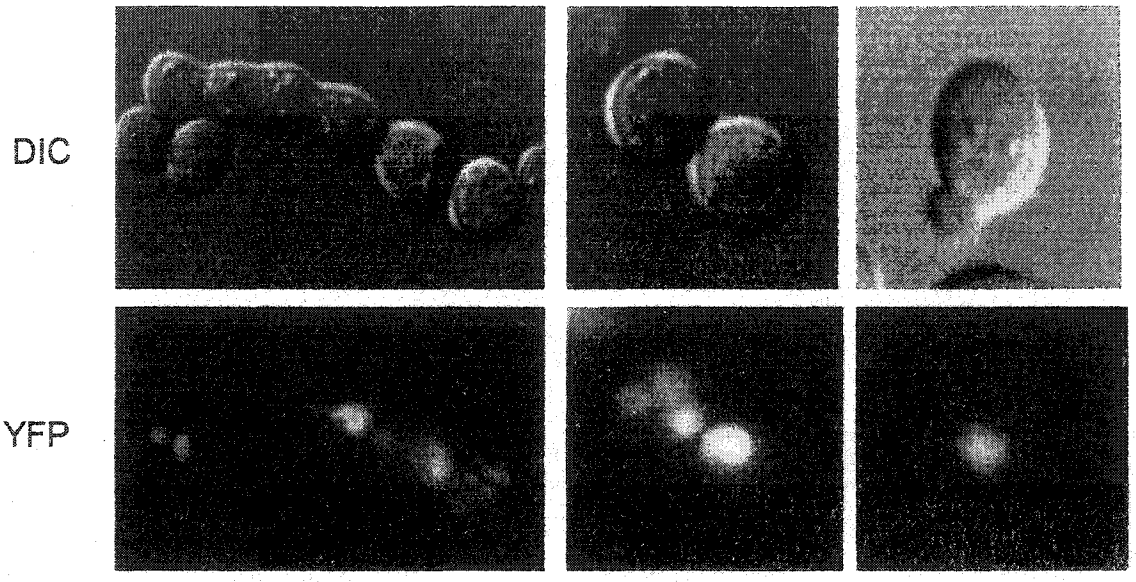

Figure 22 Blm10p does not form foci after DNA damage. The top pictures in each group are differential interference contrast images and the YFP images are on the bottom. Cells were grown on nonsynthetic complete medium suplemented with 20 μg/ml bleomycin, and imaged.

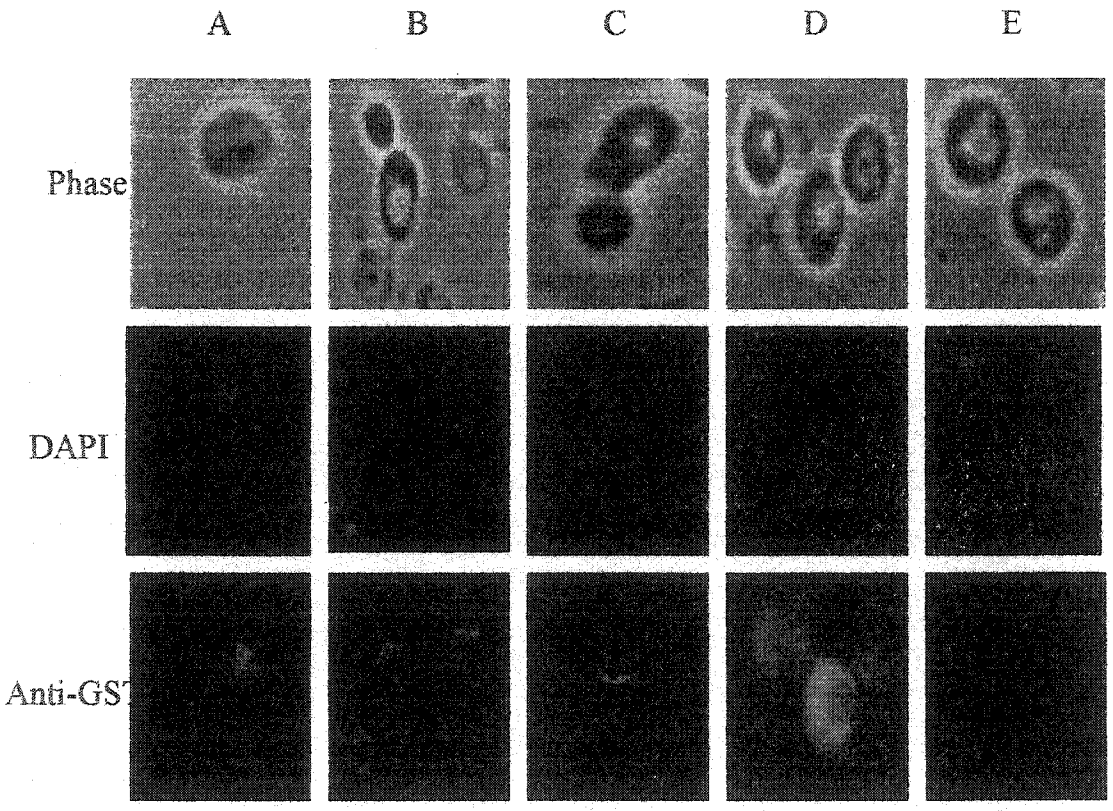

Figure 23 Bim10p-GST localizes to the nucleus and the bud neck when truncated. Cells were grown in the presence of 50 μM copper followed by fixation with PFA (paraformaldehyde). Cells were then converted to spheroplasts and then treated with anti-GST antibody and DAPI. The first row shows the cells as viewed in phase contrast, the second row shows the DNA after DAPI staining, and the third row shows the cells after antibody staining. Column A shows unbudded cells expressing Bim10p. Columns B and C are different populations of budded cells. Column D shows cells expressing GST only (no Bim10p). Column E shows the cells with no antibody treatment.

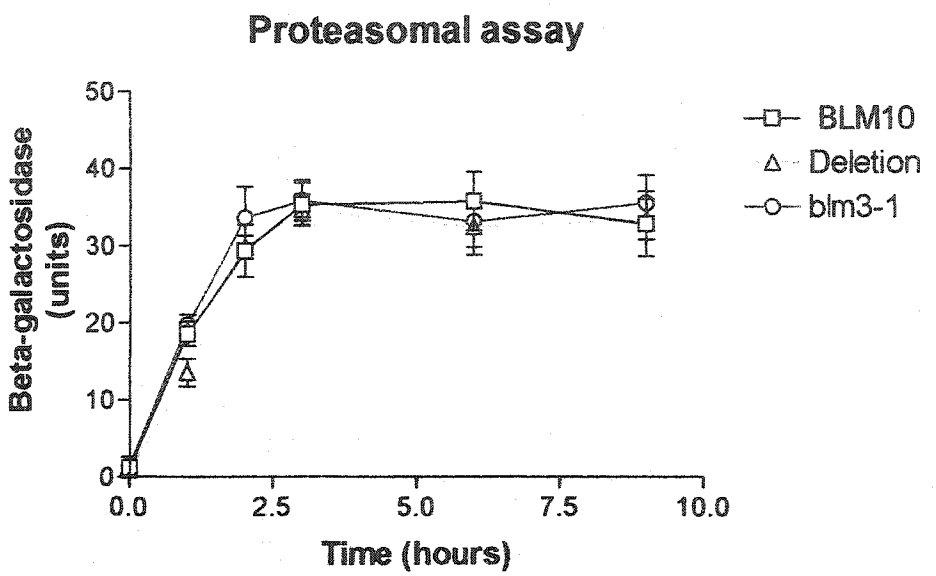

Figure 24 The degradation of whole proteins appears equivalent in wildtype and *blm10* deletion strain. Wildtype and mutant strains were all transformed with the ubiquitin-*lacZ* gene fusion containing plasmid pUB23 (Bachmair *et. al.*, 1986). After induction of the fusion protein by galactose, cells were collected at various time points and enzymatic activity was determined spectrophotometrically.

Appendix

Introduction

Two hybrid analyses are epected to reveal new protein interactions

The initial characterizations of the Blm10p gave no indication of a function. Computer analysis revealed a possible transmembrane protein, but aside from that there was no significant homology to any known protein in any of the organisms with a sequenced genome. Since the Blm10p is a very large protein with numerous potential domains it seemed likely it would have some directly interacting partners.

Protein-protein interactions *in vivo* are the basis of virtually every cellular process. These interactions have been shown to control processes such as DNA replication, transcription, translation, splicing, secretion, cell cycle control, signal transduction, and intermediate metabolism. One of the most powerful methods for studying protein-protein interaction is the yeast two-hybrid system developed by Stanley Fields and colleagues (Fields and Song, 1989). The system exploits the modular nature of a transcriptional activator, the Gal4 protein of yeast. This protein contains a DNA- binding domain (amino acids 1-147) and an activation domain (amino acids 768-881) that can be separated and still maintain activity. By creating genetic fusions of a gene of interest to one domain (normally the binding domain), and its

interacting partner to the other domain, interaction of the two hybrid proteins results in the reconstitution of its transcriptional activity (Figure A1).

MALDI mass spectrometry is now being applied to identify protein complexes in yeast and used to identify four proteins associated in complexes with Blm10p (Gaven *et. al.*, 2002; Ho *et. al.*, 2002). However the two hybrid analyses were used in the current study to indentify specific and direct protein interactions. The two hybrid system is used to identify direct interactions, is widely used, and has numerous advantages over previously used methods (Fields, and Song 1989). The yeast two-hybrid system is a method used to identify protein-protein interactions *in vivo*. The approach was chosen because of its advantage over other methods used to study protein-protein interactions. The first of these reasons is, since it is based on a powerful genetic selection scheme performed with a convenient microorganism, it allows very high numbers of potential coding sequences to be assayed. Second, it relies on an assay performed *in vivo* and thus it is not limited by the artificial conditions of *in vitro* assays. Third, the method identifies not only strong interactions but also weak and transient interactions. Finally, since it is based on a physical binding assay, a wide variety of protein-protein interactions can be detected and characterized following one single commonly used protocol (Vidal and Legrain, 1999).

Materials and Methods

1. Yeast strains and transformations

All strains used in this section have been listed in Table A1. For transformation a volume of 10 ml of YPAD was inoculated with a single yeast colony and grown overnight with vigorous shaking at 30°C to prepare a preculture. A flask containing 100 ml of YPAD was then inoculated with enough preculture to achieve an OD_{600} =0.2. Culture was grown at 30°C with vigorous shaking to an OD_{600} = 0.5. The cells were then harvested by centrifugation at 3000 rpm (Juan swinging bucket centrifuge) for 4 min in 2 x 50 ml aliquots. The pellet was then washed in 10 ml water and harvested again by centrifuging at 3000 rpm for 4 min. Cells were resuspended 5 ml of TE-LiAc (10x TE and 1 M LiAc [filter sterilized]), and then reharvest by centrifuging at 3000 rpm for 4 minutes. The Pellet was then resuspended in 1 ml of TE-LiAc and stored on ice for a 10 minute incubation period. Next, in a 1.5 ml Eppendorf tube 50 μl of competent cells were combined with 2 μl of carrier DNA (salmon sperm DNA 10 mg/ml), 2-3 μl each DNA (1 μg in concentration), and 300 μl PEG-TE-LiAc (50% PEG3350 [filter sterilized through a 0.25 micron filter]). The tubes were then incubated for 30 minutes at 30°C. Immediately after the 30 minute incubation period the tubes were transfered to a 42°C water bath for a 15 minute incubation. The cells were then harvested for 4 minutes at 2000 rpm. The pellet was then resuspended in 1 ml of water to wash them and then spun down by centrifugation for 4

minutes at 1500 rpm. The pellet was then resuspended in 100 μl of water and spread immediately on a plate. For library transformations, the protocol was scaled up 60 fold to achieve optimal representation of the genomic library.

2. **Plasmid constructs for two-hybrid study**

The polymerase chain reaction (PCR) was used to obtain the DNA to be used for the two-hybrid baits. The genomic DNA used as a template was isolated from the yeast strain CM1469-5A. The isolation was performed using a column purification protocol (Boehringer Mannheim, Indianapolis, IN). The oligonucleotide primer 5'-ATTCCCATTACTAACAAGACCT -3' was used as the amino terminal upper primer and the oligonucleotide 5'-ATCGCAATATAAAGATTAACTA-3' was used as the amino terminal lower primer to amplify the amino terminus region of the *BLM10* gene (all primers are listed in Table A3). The oligonucleotide 5'-AATCTTATATTGCGATCAGCTC-3' was used to amplify the Watson strand of loop 1 and the primer sequence 5'-GATATGATAAGATAGGGCACAAC-3' was used for the Crick strand amplification of the first hydrophilic loop of *BLM10* . Loop 2 was amplified using the primer 5'-GGGATTTTTACTGATGATCAAATG-3' for the Watson strand and the primer 5'-GATATGATAATGATAGGGCACAAC-3' was used for the Crick strand. The oligonucleotide sequence 5'- TGTTTAACTTCTTTTTGTCACGAA-3' was used as a primer for the Watson strand of the 3rd hydrophilic loop and 5'-GATAGGAATGAAAGCGGCTATAGA-3' was used for the Crick strand

amplification of the same region of the gene. Loop 4 amplification was done using the Watson primer 5'-AACCTCATCAACGGTATTGTATCT-3' and the Crick primer 5'-TATTTCGGTTGTACATAGAGTTGC-3'. The oligonucleotide 5'-ACTCTATGTACAACCGAAATAACTG-3' was used as a primer for the Watson strand of the region named loop 5 and the oligonucleotide 5'-AAATATCAATCTGCCGATGTC-3' was used as a primer for the Crick strand of loop 5. Loop 6 was amplified using the Watson strand primer 5'-AGTGTATGTGTCATTTCCGATCAAG-3' and the Crick primer 5-CATATTCAGTTCGCAGAAACCAG-3'. The carboxy terminal region of *BLM10* was amplified with the Watson primer 5'-TCATCTGGTTTCTGCGAACTGAAT-3' and the Crick primer 5'-GTTAGCGACAGCTGGCGAACCTGA-3'.

The DNA polymerase pfu (Stratagene, La Jolla, CA) was used for the PCR reactions because of its high fidelity and its ability to generate a blunt end on the PCR product. The specificity of PCR was checked on an agarose gel (the percent of agarose varies depending on the size of PCR product expected). The PCR product was cleaned up by a column purification method (Mobio, Carlsbad, CA). Phosphorylation of the PCR product DNA was done by combining DNA with 10 units of T4 polynucleotide kinase (New England Biolabs [NEB], Beverly, MA) and 1X T4 DNA ligase buffer (50 mM Tris-HCL (pH7.8), 10 mM $MgCl_2$, 10 mM dithiothreitol, and 1 mM ATP) and incubating the reaction mixture at 37°C for 4 hours. After the phosphorylation was complete the DNA was cleaned by the same method used to clean the PCR

product. The vector pGBD-C1 (see Table A2 for vector information) was linearized by combining 20 units of the restriction enzyme *Sma*I, DNA and NEB Buffer 4 (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, and 1 mM dithiothreitol [pH 7.9 @ 25°C] and incubating the reaction mixture for 3 hours at 25°C. This was followed by a ligation which was performed by combining the phosphorylated PCR product and the linearized vector in a 3:1 ratio (insert:vector determined by agarose gel electrophoresis), 60 units of *Sma*I, 60 units T4 DNA ligase, 1X NEB buffer 4, and supplementing the reaction mixture with 2 mM of ATP and incubating the reaction mixture at 18°C for 12-16 hours. The ligated DNA was next transformed into the *E. coli.* strain DH10B. Transformants were checked for vectors containing the correct clone by isolating the plasmid from the *E. coli.* using a DNA column purification kit (Mobio, Carlsbad, CA). The isolated plasmids were checked by restriction analysis to make the insert was in the correct orientation.

3. DNA sequencing

All sequencing of DNA was done using the method developed by Sanger (1977) . Genomic DNA used for sequencing was isolated using the Yeastar Genomic DNA isolation kit (Zymo Research, Orange, CA) according to manufacturers instructions.

4. β-Galactosidase filter assay

A Whatman 3 mm filter (Whatman, Clifton, NJ) is gently laid on an agar plate containing selective media. Each colony was streaked out on the filter followed by an overnight growth at 30°C. Buffer (Z) was prepared by combining 0.06 M Na_2HOPO_4, 0.04 M NaH_2PO_4, 0.01 M KCl, 0.001 M $MgSO_4$, and 0.05 M β-mercaptoethanol. The filter was gently lifted off the plate and placed in an empty petri dish cover, then put at -80°C for approximately 30 minutes. In an empty petri dish, enough thrombone was added(10 ml Z-buffer and 100 μl Xgal (100 mg/ml DMF [Dimethyl formamide]) (final concentration of 1 mg/ml) to just cover entire plate (approx. 6 ml). The filter with the cells was placed face up in the dish making sure not to leave any air bubbles. Any excess buffer was removed by pipetting. The plate was wrapped with parafilm and incubated at 30°C until a blue color developed (check every ten minutes until the negative control changed color then all assays were stopped).

5. Liquid β-Galactosidase assay

A 5 ml culture of cells were grown overnight at 30°C in a selective media to mid log phase. Cells were spun down to the equivalent of 1 ml of cells at $OD_{600} \cong 0.5$ (10^6-10^7 cells). The cells were resuspended in 1 ml Z-buffer followed by the addition of 50 μl of $CHCl_3$ and 32 μl of 0.1% SDS using a Pasteur pipette. The cells were vortexed at high speed for 10 seconds. A blank was set up containing everything but the cells. The reaction mixture was preincubated at 28°C for 3 minutes. 0.2 ml of ONPG (O-nitrophenyl-β-D-

galactopyranoside; 4 mg/ml in H_2O) was added to each sample, noting the exact time, and shaking gently to mix. When a noticeable yellow color had developed, the blank was compared (which should remain clear), the reaction was then stopped by adding 0.5 ml of 1 M Na_2CO_3 solution. Cell debris was spun down for 5 minutes in a bench top centrifuge. The OD_{420} of the supernatant was determined then the calculations were performed.

6. Isolation of plasmid DNA from yeast

The cells were inoculated into 2 ml of SD omission media in a 13x100-mm sterile glass tube with a single yeast colony containing the plasmid of interest. The culture was grown overnight to stationary phase at 30°C in a shaking incubator. A 1.5 ml aliquot of the overnight culture was transferred to a microfuge tube and spun for 5 seconds at high speed, room temperature. The supernatant was poured off and the pellet was disrupted by vortexing briefly. The cells were resuspended in 200 µl of breaking buffer (2% (v/v) Triton X-100, 1% (v/v) SDS, 100 mM NaCl, 10 mM Tris-cl pH 8.0, 1 mM EDTA pH 8.0). 0.3 g of glass beads (~200 µl volume) were added and followed by the addition of 200 µl of a phenol/chloroform/isoamyl alcohol mix. The mixture was vortexed for 2 minutes at highest speed. The sample was then centrifuged in a microcentrifuge for 5 minutes at a high speed, room temperature. Next competent *E. coli* cells were transformed with 1 µl or 2 µl of the aqueous layer. The cells were then plated on LB medium containing

the appropriate antibiotic to select for the drug resistance marker on the plasmid. The plasmids were isolated from the *E. coli* strain and sequenced.

Results

Two hybrid analyses of eight regions of Blm10p are likely to reveal proteins that directly interact with Blm10p

Our approach to the two-hybrid (Figure A2) was modified because of the predicted transmembrane domains in the Blm10p (Figures 3 and 4). The DNA for *BLM10* was cloned into the binding domain vectors in 8 fragments as shown in Figure A3. The reasoning behind this approach is that full length membrane proteins can be troublesome in a two-hybrid screen (Vidal 1997). This is due to the presence of a stronger membrane localization signal than that present on the Gal4p binding domain for its nuclear localization. To overcome this problem the approach taken was to use PCR to obtain individual regions of *BLM10* from yeast genomic DNA (strain CM1469-5A was used; see Table A1) and ligate each of these pieces of DNA to the Gal4p DNA-binding domain in the binding domain vector (pGBD-C2). The individual regions of *BLM10* were chosen because they have been predicted to be the hydrophilic loops of the protein and thus more likely to be involved in protein-protein interactions. This approach has been successfully used for a voltage gated potassium channel in an attempt to figure out the assembly of the subunits of the channel (Xu *et al.* 1995), as well as for a gastric H^+/K^+-ATPase (Melle-Milovanovic *et. al.*, 1998).

Once each of the fusions was constructed, sequenced, transformed into yeast, and checked for autoactivation of the markers. Then, three genomic libraries (containing Gal4p AD fusions) were used to screen for

interactions against the bait protein. These genomic libraries, were created and provided by Mr. Philip James of the University of Wisconsin (Madison, WI) (James *et. al.*, 1996). Each of the libraries are the same with the exception of the shift in the reading frame of the insert. The three vectors were created with either 0, 1, or 2 base insertions in the polylinker region, thereby generating a different reading frame, and ensuring each of the pieces of genomic DNA ligated into a vector should be in the correct reading frame in one of the three libraries. This increased the likelihood of every gene being represented in the library and also increased the chances of finding an interacting partner(s) of Blm10p.

Each of the plasmids containing a region of *BLM10* was transformed into PJ69-4a (Table A1). Each of these fusions was then checked for its ability to activate the reporter genes on its own. A β-galactosidase filter assay was used to verify that each fusion did not activate the reporter genes on its own. All fusions were unable to activate the reporter gene.

Each strain containing the regions of *BLM10* fused to the Gal4 binding domain was transformed with the three genomic library fusions. Transformants were then selected for the activation of the *HIS3* marker by plating on a synthetic media lacking histidine. The first 300 colonies to grow on media lacking histidine were then checked for their abilities to activate the *ADE2* reporter (a tighter selection). Each of the colonies that made it through the *ADE2* selection was then checked for checked for its ability to activate *LacZ* by β-galactosidase filter assay.

A liquid β-galactosidase was then used to quantitate the interaction. Thirty-nine potential interactors were selected for further characterization since their interaction appeared strong (Table A4). Plasmid loss experiments were carried out for some of these positives. The binding domain plasmid was lost and filter assays were performed to check for the ability of the fusion to the activation domain to bind the Gal promoter sequences and thereby activate the reporter gene without an interaction. Three of nine transformants which lost their plasmid were found not to activate transcription after plasmid loss. These three plasmids were isolated and sequenced. Two of these revealed out of frame sequences and the third encoded a mitochondrial membrane ATPase. The remaining presumptive positives have bee saved and the potential interactors will be identified in the future. It is expected that proteins that directly interact with Blm10p will be identified. The four identified recently in complexes are expected to be among those identified in the collection. Although, they may not be if those proteins do not interact directly with Blm10p.

Table A1: Yeast and bacterial Strains		
Yeast		
Name of strain	Source	Genotype
CM1469-5A	This laboratory	*MATα ade2-40* or *ade2-1 ilvl-92 his3-11* or *his3-15 leu2-3* and /or *leu2-112 trp1-1* or *trp5-12*
PJ69-4A	James *et al.* 1996	*MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ GAL2-ADE2 LYS2::GAL1-HIS3 met2::GAL7-lacZ*
PJ69-4α	James *et al.* 1996	*MATα trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ GAL2-ADE2 LYS2::GAL1-HIS3 met2::GAL7-lacZ*

Bacterial strains		
Name of strain	Source	Genotype
DH10B	Gibco BRL	F- *mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80lacZΔM15 ΔlacX74 deoR recA1 endA1 araD139Δ(ara, leu)7697 galU galK λ- rpsL nupG*

Table A2: Plasmids used two-hybrid studies

Plasmid Name	Type of experiment	Parental Vector	*E. coli* Selection	Yeast Selection	Tag	Insert
pGBD-C2NH	Two-hybrid	pGBD-C2	Amp	*TRP1*	GAL4BD	Amino Terminus
pGBD-C21	Two-hybrid	pGBD-C2	Amp	*TRP1*	GAL4BD	Loop1
pGBD-C22	Two-hybrid	pGBD-C2	Amp	*TRP1*	GAL4BD	Loop2
pGBD-C23	Two-hybrid	pGBD-C2	Amp	*TRP1*	GAL4BD	Loop3
pGBD-C24	Two-hybrid	pGBD-C2	Amp	*TRP1*	GAL4BD	Loop4
pGBD-C25	Two-hybrid	pGBD-C2	Amp	*TRP1*	GAL4BD	Loop5
pGBD-C26	Two-hybrid	pGBD-C2	Amp	*TRP1*	GAL4BD	Loop6
pGBD-C2C	Two-hybrid	pGBD-C2	Amp	*TRP1*	GAL4BD	Carboxy Terminus
pGBD-C1SNF6	Two-hybrid	pGBD-C1	Amp	*TRP1*	GAL4BD	*SNF6*
YL2H-C1	Two-hybrid	pGAD-C1	Amp	*LEU2*	GAL4AD	Genomic Library
YL2H-C2	Two-hybrid	pGAD-C2	Amp	*LEU2*	GAL4AD	Genomic Library
YL2H-C3	Two-hybrid	pGAD-C3	Amp	*LEU2*	GAL4AD	Genomic Library
pGBDU-CS	Two-hybrid	pGBDU-C1	Amp	*URA3*	GAL4BD	CS
pGAD-ES	Two-hybrid	pGAD-C1	Amp	*LEU2*	GAL4AD	ES

Table A3:Primers used for two hybrid studies			
Primer name	Primer use	length	Sequence 5'-3'
pGAD	Sequence verification two-hybrid	17 mer	TCGATGATGAAGATACC
PGBD-1	Sequence verification *insert for two-hydrid*	18 mer	AAGAGAGTAGTAACAAAG
PGBD-2	Sequence verification two-hybrid	18 mer	TGAAGTGAACTTGCGGGG
NR	Sequencing/two-hybrid	22 mer	ATCGCAATATAAAGATTAACTA
L1F	Sequencing/two-hybrid	22 mer	AATCTTATATTGCGATCAGCTC
L1R	Sequencing/two-hybrid	24 mer	GATATGATAAGATAGGGCACAAC
L2F	Sequencing/two-hybrid	24 mer	GGGATTTTTACTGATGATCAAATG
L2R	Sequencing/two-hybrid	25 mer	GATATGATAATGATAGGGCACAAC
L3F	Sequencing/two-hybrid	24 mer	TGTTTAACTTCTTTTTGTCACGAA
L3R	Sequencing/two-hybrid	25 mer	GATAGGAATGAAAGCGGCTATAGA
L4F	Sequencing/two-hybrid	24 mer	AACCTCATCAACGGTATTGTATCT
L4R	Sequencing/two-hybrid	24 mer	TATTTCGGTTGTACATAGAGTTGC
L5F	Sequencing/two-hybrid	25 mer	ACTCTATGTACAACCGAAATAACTG
L5R	Sequencing/two-hybrid	21 mer	AAATATCAATCTGCCGATGTC
L6F	Sequencing/two-hybrid	25 mer	AGTGTATGTGTCATTTCCGATCAAG
L6R	Sequencing/two-hybrid	23 mer	CATATTCAGTTCGCAGAAACCAG
CF	Sequencing/two-hybrid	24 mer	TCATCTGGTTTCTGCGAACTGAAT
CR	Sequencing/two-hybrid	25 mer	GTTAGCGACAGCTGGCGAACCTGA

Table A4: Two hybrid positives					
Region of protein	Library screened against	*HIS3* Selection	*ADE2* Selection	*LacZ* Filter Assay	Units of β-galactosidase activity(Miller units)
Loop1	Y2HL-C1	+	+	+	10.1 +/- 0.8
Loop1	Y2HL-C1	+	+	+	18.5 +/- 1.6
Loop1	Y2HL-C1	+	+	+	11.6 +/- 1.7.
Loop1	Y2HL-C1	+	+	+	63.6 +/- 2.7
Loop1	Y2HL-C1	+	+	+	15.9 +/- 0.7
Loop2	Y2HL-C3	+	+	+	46.6 +/- 5.2
Loop2	Y2HL-C3	+	+	+	19.0 +/- 1.8
Loop2	Y2HL-C3	+	+	+	26.9 +/- 3.6
Loop2	Y2HL-C3	+	+	+	17.9 +/- 2.8
Loop2	Y2HL-C3	+	+	+	20.9 +/- 1.7
Loop2	Y2HL-C3	+	+	+	21.0 +/- 3.2
Loop2	Y2HL-C3	+	+	+	28.7 +/- 1.9
Loop2	Y2HL-C3	+	+	+	16.6 +/- 2.9
Loop2	Y2HL-C3	+	+	+	18.5 +/- 1.2
Loop2	Y2HL-C3	+	+	+	17.6 +/- 2.6
Loop2	Y2HL-C3	+	+	+	31.3 +/- 4.1
Loop2	Y2HL-C3	+	+	+	340.0 +/- 18.4
Loop2	Y2HL-C3	+	+	+	24.0 +/- 2.2
Loop2	Y2HL-C3	+	+	+	21.0 +/- 3.3
Loop2	Y2HL-C3	+	+	+	27.8 +/- 2.5
Loop2	Y2HL-C3	+	+	+	18.7 +/- 1.6
Loop2	Y2HL-C3	+	+	+	17.3 +/- 4.3
Loop2	Y2HL-C3	+	+	+	15.3 +/- 1.7
Loop2	Y2HL-C3	+	+	+	16.1 +/- 2.2
Loop2	Y2HL-C3	+	+	+	15.4 +/- 2.5
Loop2	Y2HL-C3	+	+	+	39.8 +/- 3.9
Loop2	Y2HL-C3	+	+	+	34.3 +/- 5.2
Loop2	Y2HL-C3	+	+	+	12.0 +/- 1.6
Loop2	Y2HL-C3	+	+	+	27.0 +/- 2.6
Loop2	Y2HL-C3	+	+	+	15.4 +/- 2.6
Loop4	Y2HL-C1	+	+	+	24.7 +/- 4.2
Loop4	Y2HL-C1	+	+	+	15.9 +/- 1.5
Loop4	Y2HL-C1	+	+	+	24.7 +/- 3.3
Loop4	Y2HL-C1	+	+	+	11.7 +/- 1.4
Loop4	Y2HL-C1	+	+	+	23.9 +/- 0.9
Loop4	Y2HL-C1	+	+	+	21.8 +/- 0.6
Loop6	Y2HL-C1	+	+	+	485.7 +/- 27.6
Loop6	Y2HL-C1	+	+	+	399 +/- 18.4
Loop6	Y2HL-C1	+	+	+	15.8 +/- 1.5

Plasmids are listed by order they occur within Blm10p starting from amino-terminus

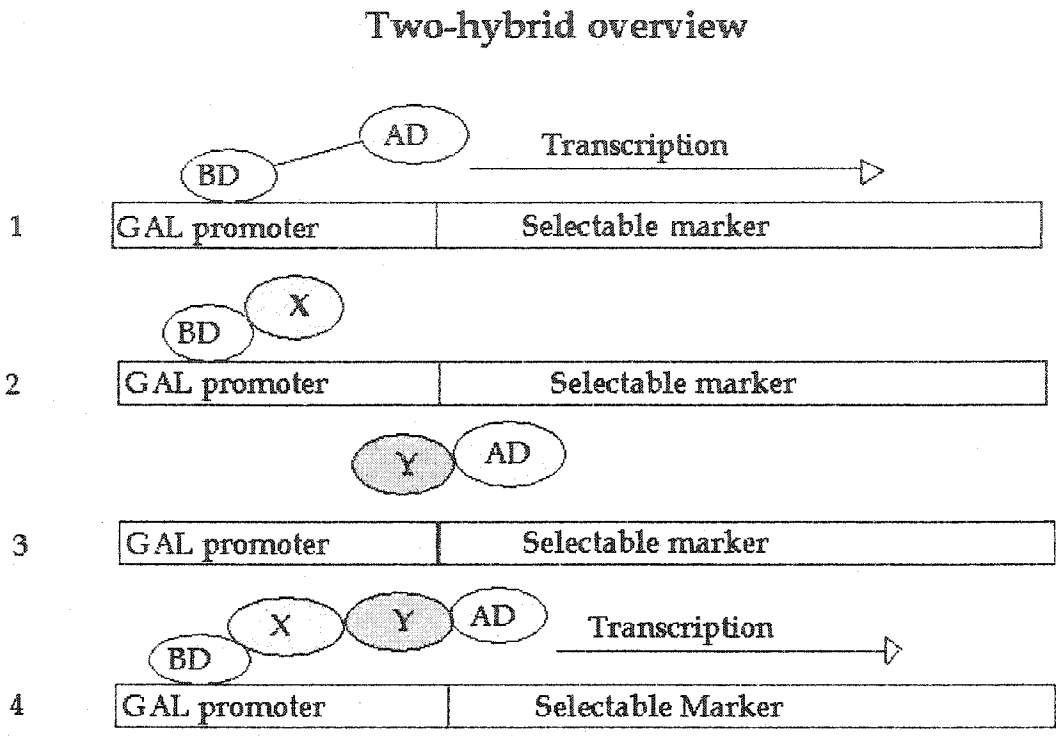

Figure A1 Overview of the two hybrid system. **1.** When the Gal4p binding domain (BD) and activation domain (AD) are in close proximity transcription of a marker occurs. **2.** When the binding domain is fused to protein x only, no transcription of the marker occurs. **3.** When the activation domain is fused to protein y only, no transcription occurs. **4.** When protein x interacts with protein y, there is a reconstitution of the Gal4p activity and transcription occurs.

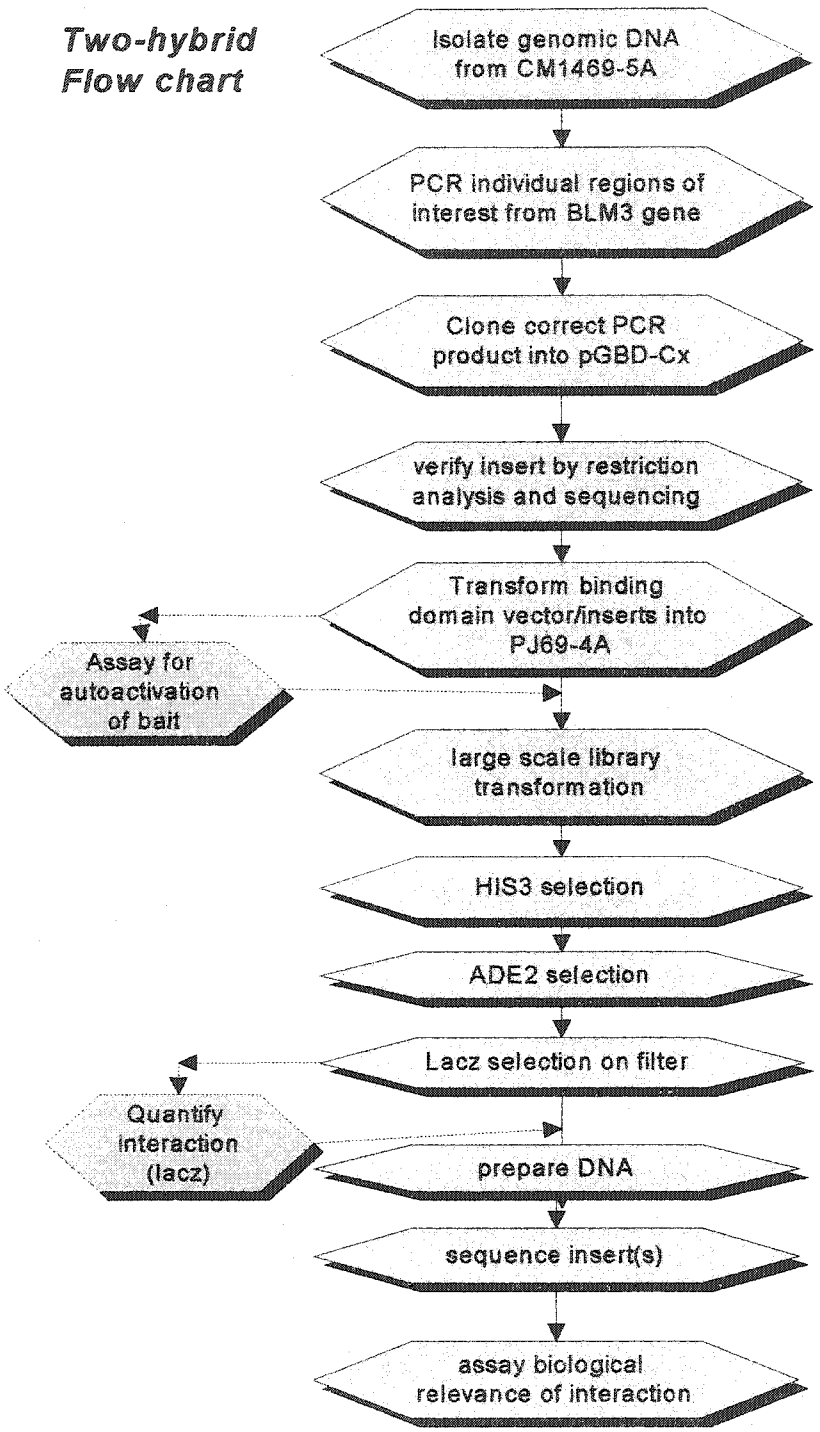

Figure A2 The two-hybrid flow chart. Those steps which were *required are depicted by a blue color. Those steps which were* optional are depicted in a orange color. All steps required and optional were performed in this study.

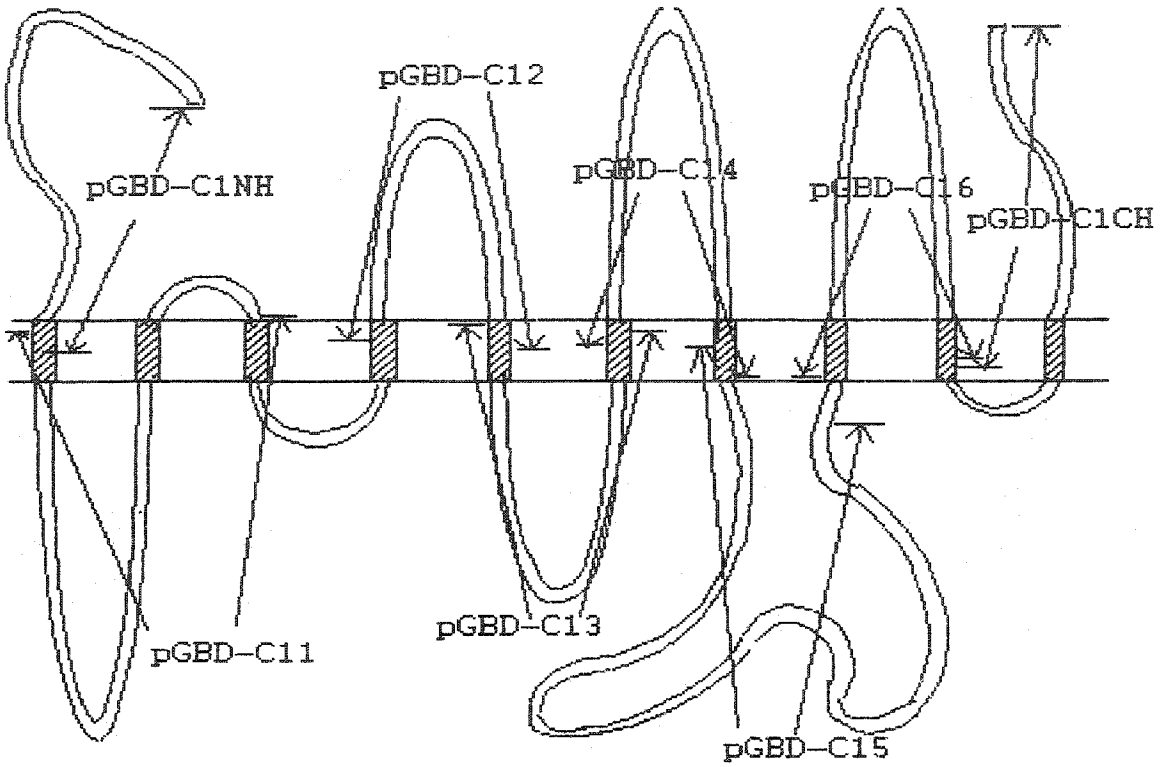

Figure A3 Dissection of Blm10p for use in two hybrid screen. Depicted are the proposed hydrophilic loops of the protein that were chosen to be cloned into the vector pGBD-C2 for use in the two-hybrid part of the project. PCR was used to obtain the DNA for each region. The DNA was then cloned into the vector. The new vector was named according to the number of the loop it contained.

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