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Role of the *blm5-1* mutation of *Saccharomyces cerevisiae* in DNA repair and meiosis and a potential requirement of vacuolar metabolism in the repair of bleomycin-induced double-strand breaks

By

Marcia T. C. Martinez

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

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ABSTRACT

Role of the *blm5-1* mutation of *Saccharomyces cerevisiae* in DNA repair and meiosis and a potential requirement of vacuolar metabolism in the repair of bleomycin-induced double-strand breaks.

by

Marcia T. C. Martinez.

Advisor: Professor Carol Wood Moore.

Mutational alteration of the *BLM5* gene of the model eukaryote, *Saccharomyces cerevisiae*, confers extreme hypersensitivity to lethal effects of ionizing radiation and anticancer bleomycins. Since bleomycin hypersensitivities in *blm5-1* mutant strains were unrelated to drug accumulations in the cells, a possible role in the repair of radiomimetic damage was proposed for the Blm5p. This hypothesis was examined in two parts: The global repair of chromosomal double-strand breaks (DSBs) and the isolation of genes that functionally complemented the bleomycin hypersensitivity conferred by the *blm5-1* mutation.

Pulsed field gel electrophoresis (PFGE) was used to examine the induction and repair of chromosomal DSBs in *BLM5/BLM5*, *BLM5/blm5-1* and *blm5-1/blm5-1* strains before and after bleomycin treatments and during post-treatment incubation in phosphate buffer. Analyses showed that DSBs were processed in cycles of chromosomal degradation and rejoining. Spontaneous DSBs accumulated and were repaired in the *BLM5/BLM5* and *BLM5/blm5-1* cells, but did not accumulate in *blm5-1/blm5-1* cells. Bleomycin-induced DSBs occurred in a dose-dependent manner and were repaired in all strains. However, repair was slowest in *blm5-1/blm5-1* cells,

suggesting rates of repair induction influenced bleomycin hypersensitivity. In addition to genotype, numbers of breaks and the temperature of post-treatment incubation affected repair rates.

The *VPS8* gene was shown to functionally complement the *blm5-1* mutation and the *VPS3* and *PEP7* genes were identified on two additional plasmids with *blm5-1* complementing activity. This result, together with the observation that *blm5-1* mutants display the reduced mitotic growth rate and sporulative abilities associated with vacuolar mutants, suggest that the *BLM5* gene could be either *VPS8, VPS3, PEP7* or another gene involved in vacuolar metabolism. The Vps8p, Vps3p and Pep7p function with other gene products to mediate vesicular docking and fusion onto the endosome at the intersection of the endocytic and the vacuolar biogenesis pathways in yeast. If a relationship is uncovered in the future between Blm5p and one or more of the gene products of vacuolar metabolism it would suggest an important relationship between vacuolar metabolism and the rate of repair of bleomycin-induced DSBs.

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DISCUSSION.

INTRODUCTION

I. The *blm5-1* mutation of *S. cerevisiae*.

The repair of double-strand breaks in DNA is necessary for the maintenance of normal DNA function and the survival of cells. Double-strand breaks may arise spontaneously within the cell or they may be induced by a number of DNA-damaging agents, including ionizing radiation and radiomimetic chemicals such as bleomycin and structurally related phleomycin. Cells employ a number of strategies to repair spontaneous and induced damage in chromosomal DNA. The current work utilized molecular and genetic characterizations of a mutation conferring bleomycin hypersensitivity (*blm5-1*) in the budding yeast, *Saccharomyces cerevisiae,* to elucidate cellular responses to spontaneous and induced double-strand breaks in chromosomes. The ultimate goal of this study was to identify a new gene required for the repair of double-strand breaks in chromosomes.

S. cerevisiae provides a model eukaryotic system to conduct these studies. This organism has stable haploid and diploid life cycles and a well-characterized genetic system. A large number of cloning vectors are available and since many unique genes are conserved across the evolutionary tree, homologues of any gene isolated may be identified in other organisms.

The *blm5-1* mutant was isolated on the basis of its hypersensitivity to killing by bleomycin and phleomycin, from among cells that were mutated with treatments of x-rays (Moore, 1980). The *blm5-1* mutation is recessive and bleomycin sensitivity segregates 2:2, indicating single gene or Mendelian inheritance. In addition to their elevated sensitivities to killing by bleomycin, *blm5-1* mutant strains show cross hypersensitivities to the lethal effects of ionizing radiation (Moore, 1991). The expression of sensitivities of *blm5-1* mutants to ionizing radiation, bleomycin and phleomycin was greater at 37°C than at 30°C or 23°C. The *blm5-1* mutants were also hypersensitive to hydrogen peroxide, but they are not hypersensitive to killing by ultraviolet (UV) light (Moore, 1991). Before this study, mitotic and meiotic defects had not been further investigated and the *BLM5* structural gene was not identified.

Yeast strains that are defective in the repair of DNA double-strand breaks are sensitive to the lethal effects of ionizing radiation and the bleomycin-phleomycin family of chemical congeners (Moore, 1978, 1982 [a and b]). The cross sensitivities of *blm5-1* mutant strains to ionizing radiation and radiomimetic bleomycin suggested the possibility of a defect in the repair of DNA double-strand breaks in these mutants. We therefore predicted a probable role for the Blm5p in the repair of double-strand breaks in DNA.

Two major approaches were used to examine this theory. In the first approach, pulsed field gel electrophoresis (PFGE; Schwartz and Cantor, 1984) was utilized to study the global repair of spontaneous and induced DNA damage in chromosomes. These studies were possible because yeast chromosomes can be separated in pulsed field gels by chromosomal length and molecular weight. In addition PFGE was shown to be an efficient and sensitive method for the detection of DNA double-strand breaks and the repair of these breaks on different chromosomes of yeast (Game et al. *1989*; Mortimer et al. *1990*; Geigl and Eckardt-Schupp, 1990 and 1991; Dardalhon et al. 1994).

The second approach used to determine if the Blm5p functioned in DNA repair processes in the cell was to isolate genes that functionally relieved the bleomycin hypersensitivity conveyed by the *blm5-1* mutation, and to identify the function of such *blm5-1* complementing genes in the cell. It was hoped that the *BLM5* structural gene would be identified among genes that complemented the mutation. To optimize the selection of *blm5-1* complementing genes, standard procedures in molecular biology were used to screen two genomic libraries of the yeast strain S288C. These genomic libraries were carried in the multiple-copy yeast episomal plasmid YEp24 (Botstein et al. 1979) or in the single-copy yeast centromeric plasmid YCp50 (Rose et al. 1987). Both plasmids are yeast/*E. coli* shuttle vectors and carry markers that allow them to be selected in both organisms. However, there are advantages and disadvantages associated with the use of each library. Larger numbers of yeast transformants are usually obtained with the YEp24 library, but there is a greater chance of isolating suppressors with this library than with the YCp50 library. Additionally, high copy number suppression that may affect the isolation of *blm5-1* complementing genes is not associated with the YCp50 library.

Genetic techniques were also used to aid in the identification of *blm5-1* complementing genes. First, a chromosomal mapping method developed by Wakem and Coworkers (1986) was adapted and used to identify a chromosome or chromosomes bearing *blm5-1* complementing activity in yeast. Next, standard procedures used to meiotically map yeast genes (Mortimer and Schild, 1981; Spencer and Spencer, 1996 and C. Saunders-Singer, 1996) were utilized to analyze the accuracy of the method and to determine if the *BLM5* structural gene was among *blm5-1* complementing genes that were identified.

II. Bleomycins:

A. Structure and mechanism of action. Bleomycin was used as a tool to aid in the characterization of the *blm5-1* mutation. Bleomycins are a group of basic, water-soluble, glycopeptidic antibiotics, that are isolated from the bacterium, *Streptomyces verticillus* (Umezawa et al. 1966), and used in the treatment of head and neck carcinomas, Hodgkin's and non-Hodgkin's lymphomas and testicular cancers (Umezawa et al. 1966 and 1972, and L. F. Povirk, 1983). They consist of approximately 200 structurally related molecules (Mir et al. 1996) that differ from each other only in their terminal amine moiety. The commercial product, Blenoxane, used in clinical cancer treatments consists of a mixture of eleven of the bleomycin molecules, predominantly bleomycin-A_2 and bleomycin-B_2. It is this product that was used in the characterization of the *blm5-1* mutation.

Functionally, the bleomycin molecule can be divided into two regions, a nucleic acid binding region that includes the terminal amine and bithiazole group, and a metal binding region that can bind metals such as Fe^{2+}, Co^{2+}, Zn^{2+}, Ni^{2+} and Cu^{2+}. The metal binding region of the molecule also functions in the recognition of specific DNA sequences (Mir et al. 1996).

The effectiveness of bleomycin as a chemotherapeutic agent is considered to result from its ability to introduce double-strand breaks in DNA (Umezawa et al. 1966, and 1972). It is often used experimentally as a tool to study the induction of DNA double-strand breaks in the cells of a variety of organisms, including mammalian and yeast cells. Bleomycin-induced DNA cleavage occurs preferentially at GC sites (D'Andrea and Haseltine, 1978) possibly through a method involving the formation of free radicals (Petering et al. 1990 and Steighner et al. 1990). DNA cleavage occurs after the formation and binding of a bleomycin-metal-O_2 complex to DNA. The most effective and well studied of such complexes is the bleomycin-Fe^{2+} -O_2 complex. When this complex binds to DNA, Fe^{2+} is oxidized to Fe^{3+} and the coordinated activity of the two functional regions of the bleomycin molecule produces single- and double-strand breaks in the DNA (Burger et al. 1981). Furthermore, Fe^{2+} is regenerated so that the bleomycin-Fe^{2+}-O_2 complex reforms and more breaks are introduced into the DNA. Thus, each molecule of bleomycin can produce between 8 and 10 DNA breaks (Povirk et al. 1989).

B. Additional molecular targets. DNA is usually considered to be the primary molecular target for bleomycin within the cell. However, reports now suggest that besides DNA double-strand breaks, other factors may be involved in the antiproliferative activity of bleomycin (Berry et al. 1986; Smith, 1987; Lazo et al. 1989; Templin et al. 1992). There is now evidence to show that the drug attacks other cellular molecules and structures producing a host of cellular effects, which together may contribute to the cytotoxicity of the drug. In 1992, Moore and co-workers demonstrated that bleomycin-induced membrane damage might contribute significantly to cell death among treated cells. Bleomycin treatment was also shown to cause damage to the cell walls of *S. cerevisiae* (Moore et al. 1992; Beaudouin et al. 1993 and Lim et al. 1995). Additionally, the bleomycin-Fe(II)-O_2 complex cleaves RNA (Holmes et al. 1993), inhibits DNA synthesis (Drewinko et al. 1981) and causes cell cycle delay at G2 (Kimler, 1979 and Scott et al. 1985).

III. Control of cellular cytotoxicity of bleomycin molecules.

The factors affecting bleomycin cytotoxicity in *blm5-1* mutants were considered during the course of this study because of the apparent participation of several cellular molecules and structures in the drug's cytotoxicity. Bleomycin cytotoxicity appears to be controlled by three major mechanisms in intact cells. These include drug accumulations within the cell, the action of the cysteine protease, bleomycin hydrolase, and cellular repair mechanisms (Mir et al. 1996).

A. Entry of bleomycin into the cell.

The entry of bleomycin into the cell seems to be limited by the plasma membrane. The association of ^{14}C-labelled bleomycin to animal cells was shown to be less than 0.1% of the bleomycin added to the medium (Roy and Horwitz, 1984). Despite this, it was initially suggested that the main pathway used by bleomycin entering the cell was by diffusion through the plasma membrane (Roy and Horwitz, 1984 and Lyman et al. 1986). Poddevin and co-workers (1991) demonstrated that once internalized into bleomycin-resistent strains of Chinese hamster fibroblasts, the drug does not leak into the surrounding medium. They therefore suggested that simple diffusion does not explain the entry of bleomycin into the cell. It is now known that bleomycin is recognized at the cell surface of both Chinese hamster fibroblasts and human cells by an as yet unidentified membrane-bound protein believed to be involved in the internalization and eventual cytotoxicity of the molecule (Pron et al. 1993, 1994 and 1999). However, once internalized, the processes involved in the movement of bleomycin molecules to the nuclear DNA are still unclear.

B. Bleomycin hydrolase.

Cells possessing high levels of the enzyme bleomycin hydrolase appear to be extremely adept at controlling bleomycin cytotoxicity since, unlike cells with a paucity of the enzyme, they can sustain exposure to relatively high drug doses (Jani et al. 1992 and Morris et al. 1992). Bleomycin hydrolase is found in both animal (Sebti et al. 1989) and yeast cells (Kambouris et al. 1992). In yeast, the enzyme is encoded by a gene, *BLH1*, that was isolated using four functionally unrelated approaches (Kambouris et al. 1992; Enenkel and Wolf 1993; Magdolen et al. 1993; Xu and Johnston 1994; and Joshua-Tor et al. 1995).

The immunolocalization studies of Zheng and Johnston (1998) suggested that the intracellular location of the bleomycin hydrolase Blh1p was neither vacuolar nor nuclear, but primarily within the cytoplasm. These researchers also believed that despite their inability to locate the Blh1p in the nucleus, the requirement of the protein's DNA binding properties for bleomycin detoxification suggested that Blh1p may be temporarily located in the nucleus (Zheng and Johnston, 1998). Another group of researchers (Magdolen et al. 1993 and Niemer et al. 1997) suggested that the Blh1p may be located in the plasma membrane although it possessed no predicted membrane-bound sequence, because Blh1p co-precipitates with the cAMP-binding plasma membrane-bound protein, Gce1p. Yet a third group of scientists gave evidence to support a vacuolar location for Blh1p. Matsumoto and coworkers (1989) showed that vacuolar degeneration is one of the first changes observed among proliferating tumor cells treated with bleomycin. Enlarged vacuoles have also been observed in *S. cerevisiae* following bleomycin treatment (Moore et al. 1992). Lazo and coworkers (1990) demonstrated that bleomycin cytotoxicity increased in cells that had been treated with pharmacological agents known to disrupt vesicular function. They did not observe similar increases in bleomycin cytotoxicity among cells treated with agents that did not alter the acidity of vacuolar organelles. They therefore suggested that the endocytic pathway and acidic organelles of the cellular vacuolar system participate in controlling bleomycin cytotoxicity in the cell (Lazo et al. 1990).

C. DNA repair pathways induced by bleomycin damage.

Bleomycin-induced DNA damage is considered to be radiomimetic because of its similarity to the damage caused by ionizing radiation (Umezawa et al. 1966; Povirk 1983; Moore et al. 1985). The genes of several repair pathways appear to have functional roles in the repair of bleomycin-induced damage. Bleomycin damage has been shown to induce error-prone repair in yeast (Severgnini et al. 1991), SOS repair in *E. coli* (Povirk et al. 1988) and long patch excision repair in human fibroblasts (DiGiuseppe and Dresler, 1989). Moore (1978, 1982 [a] and 1991) showed that several radiation-sensitive mutants of *S. cerevisiae* were also sensitive to the lethal effects of bleomycin and structurally-related phleomycin, and that yeast mutants hypersensitive to these antibiotics bear cross sensitivities to x-rays. These results suggested that there might be similarities in the introduction and processing of DNA lesions caused by ionizing radiation and bleomycin treatment. In fact, the effect of bleomycin on the growth and survival of single, double and triple radiation sensitive (*rad*) mutants of *S. cerevisiae* further suggested that genes involved in recombination, excision and postreplication repair interact to repair bleomycin-induced DNA lesions in this organism (Keszenman et al. 1992). The drug was therefore considered to be an effective tool for use in the induction of pathways leading to the global repair of chromosomal double-strand breaks.

D. The repair of DNA double-strand breaks in yeast.

The global repair processes considered in this study encompass all repair pathways in which DNA double-strand breaks are repaired in yeast. Double-strand breaks in DNA are processed in a variety of ways in eukaryotic organisms, including methods that involve recombination (Resnick, 1976; Szostak et al. 1983; Petrini et al. 1997; Osman and Subramani, 1998 and Eckardt-Schupp and Klaus, 1999;), single-strand annealing (Lin et al. 1984), and the end-to-end joining of nonhomologous DNA ends (Wilson et al. 1982; Thode et al. 1990; Goedecke et al. 1992 and 1994; Kramer et al. 1994 and Pfeiffer et al. 1994). The repair of DNA double-strand breaks in *S. cerevisiae* has been extensively reviewed (Friedberg, 1988; Prakash et al. 1993 and Moore and Haber, 1996) and occurs primarily through processes involving homologous recombination. The products of several genes mediate homologous recombination and repair processes in yeast, including the *RAD* genes (Reviewed by Friedberg, 1988; Friedberg et al. 1991; Game, 1993 and Prakash et al. 1993). These genes include the *RAD52* epistasis group, which comprises of the *RAD50* through *RAD58* genes, as well as the *RAD6*, *RAD9* and *RAD18* genes.

The repair of DNA double-strand breaks by the joining of nonhomologous ends has also been described in *S. cerevisiae* (Reviewed by Mezard and Nicolas, 1994). Unlike mammalian cells, nonhomologous repair or end joining occurs at a lower frequency in yeast than homologous repair (Schiestl and Petes, 1991 and Schiestl et al. 1993), and is therefore considered to be a minor repair pathway in the latter organism. The Rad52p, an essential protein in homologous repair, is not required for nonhomologous repair in yeast.

IV. Major findings.

The studies conducted here revealed that the bleomycin hypersensitivity of the *blm5-1* mutant did not result from high accumulations of the drug within the cell. Additionally, the *blm5-1* mutant demonstrated a surprising ability to repair double-strand breaks induced by bleomycin in whole chromosomes. However, the rate at which repair processes occurred was significantly slower in the mutant than in normal strains. This reduced repair rate appeared to contribute to the sensitivity of the *blm5-1* mutant to bleomycin treatment.

The studies also demonstrated that global repair processes did not end when chromosomal bands were regenerated. Instead, regenerated chromosomes were degraded and regenerated one or more times during the experimental period, suggesting that global repair processes did not occur as isolated events, but through a series of cycles. Cell survival appeared to be linked to the chromosomal degradation/regeneration repair cycles, since survival decreased when chromosomes were degraded and increased when they were regenerated.

The gene, *VPS8*, was shown to convey *blm5-1* complementing activity in homozygous mutant diploids. This gene functions in the docking and fusion of vesicles onto the endosome at the junction of the endocytic and vacuole biogenesis pathways in yeast. Other *blm5-1* complementing DNA fragments isolated from yeast genetic libraries all carried genes or gene fragments that functioned at the same step as the *VPS8* gene in the endocytic/vacuole biogenesis pathways in yeast. These genes included the *VPS3* and the *PEP7* genes. The *blm5-1* mutant was also found to display phenotypic characteristics commonly observed in mutants of the vacuole biosynthetic pathway. These characteristics included reductions in mitotic growth rate and a decreased ability to sporulate.

MATERIALS AND METHODS

1. Strains and plasmids.

Yeast and bacterial strains used in this study are listed in **Tables 1** and **2.**

2. Media and growth conditions.

Non-synthetic complete solid medium containing 2% glucose, 2% Bacto-peptone (Difco Lab., Detroit, Michigan), 1% Bacto-yeast extract (Difco), 2% Bacto-agar (Difco) and 0.16 mg/ml adenine sulfate (YPAD) was used for the non-selective mitotic growth of all yeast strains. If liquid YPAD medium was required, Bacto-agar was not included in the preparation. Selective (synthetic) media (SD) containing 2% glucose, 0.17% yeast nitrogen base (Difco), 0.5% ammonium sulfate and the specific nutritional requirements of individual strains were used for the selective growth of yeast strains. In addition to SD, solid media containing 5-

Fluoroorotic acid (5-FOA) were used for the selective growth of certain yeast strains bearing mutations in the *URA3* gene. This media was made by dissolving 5-FOA powder (Sigma Chemical Company, St. Louis, Missouri.) in SD media immediately after the media was autoclaved (Schatz et al. 1988). Unless otherwise specified, for mitotic growth strains cultured on solid or liquid media were incubated at 30°C in a Psycrotherm incubator (Model No. G-26 from New Brunswick Scientific Company, Edison, New Jersey) with aeration.

For meiotic growth, stationary phase yeast cells from fresh cultures were inoculated at a density of 1000 cells/ml into liquid presporulation media 5 (0.8% Bacto-yeast extract, 0.3% Bacto-peptone and 10% glucose supplemented with 0.16 mg/ml adenine sulfate) and grown at 23°C to mid-exponential growth (1 x 10^7 to 5 x 10^7 cells/ml). Cells were harvested, washed two times in sterile distilled water, then transferred to liquid minimal sporulation media 2 (0.5% potassium acetate supplemented with the nutritional requirements of sporulating strains). To obtain optimum sporulation, strains were always grown and sporulated at 23°C as described by Codon and coworkers (1995), unless otherwise specified.

Bacterial strains were grown in Luria (LB) or YT broth (Maniatis et al. 1982), with the addition of antibiotics (ampicillin or tetracycline) when selective bacterial growth was required.

3. Bleomycin treatments.

Bleomycin stock solutions (2 mg/ml) were prepared by dissolving bleomycin sulfate (Bristol-Myers Squibb, Syracuse, N.Y. and Evansville, IN) in sterile deionized water. The concentration of bleomycin in the stock solution was determined using the formula $OD_{292}/9.36 \times 10^{-3}$. For bleomycin screening procedures, solid SD media containing 2% agarose and bleomycin sulfate were prepared as described by Pramanik et al. (1997). For DNA repair studies, cells were always treated for 30 minutes with aeration at a cell density of 2×10^7 cells/ml in 20 ml of cold (4°C) sterile deionized water. Immediately after treatment, bleomycin activity was minimized (Burger, 1998) by the addition of 0.5 M EDTA (pH 8) to give a final concentration of 0.025 M EDTA to each treatment tube.

4. Identification of chromosomes with *blm5-1* complementing activity.

Standard mating procedures (Mortimer and Schild, 1981) were used to create cir^+/cir^o diploid strains. Each cir^+/cir^o diploid was grown for 25 generations (approximately 2×10^7 cells) from a single cell on solid YPAD media to allow chromosomal loss events to occur (Wakem et al. 1986). The appearance of auxotrophies to selected amino acids or the development of hypersensitivity to killing by bleomycin was used to identify monosomic diploids. After 25 generations of growth, diploid colonies were randomly selected and resuspended in sterile deionized water, plated for growth on YPAD (approximately 70 to 100 cells per plate), then velvet replica plated onto SD media with and without 50 μg/ml bleomycin. Cells with *URA3* auxotrophy were identified directly by plating onto 5-FOA media (Boeke et al. 1984). Monosomic diploids selected were rescreened by spot replica plating onto SD media with and without bleomycin (doses ranging from 0 μg/ml to 50 μg/ml) to verify their amino acid auxotrophies and bleomycin hypersensitivities.

For meiotic mapping procedures, sporulated diploids were washed two times in sterile distilled water then resuspended in a 1/10 dilution of the enzyme glusulase. Cells were incubated for 15 minutes at 23°C to allow ascus walls to digest. Only four-spored asci were selected and dissected on solid YPAD media using the Singer MS system series 200 complete workstation for micromanipulation in yeast as described by C. Saunders-Singer (1996). After dissection, plates were incubated at 30°C for 5 – 7 days for colony growth. Spores (also called segregants) were then screened for 2:2 segregation of all heterozygous markers in parental diploids. For each four-spored ascus dissected, only those where three or four segregants survived were used for genetic analyses.

5. Association and efflux of [S-*methyl*-^{3}H]bleomycin.

Stationary phase yeast cells were washed three times in sterile deionized water and pellets of approximately 1 x 10^7 cells were each resuspended in 200-µl of sterile deionized water. Resuspended cells were treated for 30 minutes at 30°C with different concentrations of bleomycin (0 µM – 67 µM). For drug association studies, cells were treated with a mixture of cold and [S-*methyl*-^{3}H]bleomycin in a ratio of 99.7% cold bleomycin to 0.3% [S-*methyl*-^{3}H]bleomycin, whereas for efflux studies cells were treated with a single concentration of 33.5 µM [S-*methyl*-^{3}H]bleomycin without cold drug. Following treatment, cells were collected by centrifugation and washed 3 times in 1 ml of sterile deionized water. The amount of radioactivity associated with treated cells was determined by adding cells to microfuge tubes containing 40 µl of 0.5 M NaOH overlaid with 400 µl of a mixture of silicone oil and mineral oil in a ratio of 84:16 respectively and centrifuging at 10,000 rpm for 30 seconds. The microfuge tubes were rapidly frozen in an acetone and dry ice bath, and each frozen cell pellet was cut off and placed in a scintillation tube containing 5 ml of Atom Light (Dupont Biotechnology Systems, Boston, Ma.). The radioactivity associated with each pellet was then determined in counts per minute (cpm) in a Scintillation counter.

To determine the quantity of effluxed ^3H, washed treated cells were incubated in 1 ml of sterile deionized water for 37 hours. The radioactivity associated with each wash, with 50 µl aliquots of the supernatant of suspended cells removed at various time points ($t = 0 - t = 37$ hours), and with the pelleted cells after 37 hours of incubation was determined by scintillation counts. The cytotoxicity of the efflux was also determined. This was done by treating 5×10^7 cells of a fresh culture of stationary phase A364A yeast cells for 45 minutes at 30°C with 200 µl of the effluxed material resulting from 37 hours of posttreatment incubation (Moore, 1982 [b]). The survival of treated cells was determined following growth on solid YPAD.

6. DNA repair.

Cells were grown to stationary phase (approximately 2×10^8 cells/ml) in sterile liquid YPAD at 30°C from an initial innoculum of 1×10^3 cells/ml from a fresh preculture. Following growth, cells were harvested, washed two times in sterile deionized water, then held for 16 hours at 4°C in sterile deionized water. Cells were collected by centrifugation, again washed twice in sterile deionized water, then resuspended in sterile deionized water to a final concentration of 1×10^9 cells/ml. Cells collected after bleomycin treatment were resuspended in 20 ml of sterile 0.01M sodium phosphate (pH 6.5) and incubated with aeration either at 30°C or at 37°C. Aliquots removed at different time points (t = 0 – t = 48 hours) were plated onto solid YPAD to determine cell survival and used in the preparation of LMP agarose plugs for pulsed field gel electrophoresis (PFGE).

7. Cell lysis and PFGE.

Cells were recovered from the sodium phosphate solution by centrifugation at 3000 rpm for 10 minutes, washed twice 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 of zymolyase [100,000 U/g] were added to 10 ml of solution containing 1 M sorbitol, 100 mM sodium citrate, 10 mM EDTA immediately prior to use) were added to the resuspended cells and 625 µl of warm (42°C) low melting point (LMP) agarose (1.5%). The cell 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 removed from the molds and placed in sterile 15-ml Falcon tubes 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 transferred into 10 ml of solution III (40 ml of a 10% solution of sodium lauryl sarkosinate and 400 mg of proteinase K added immediately 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 then incubated at 50°C for 24 hours. Solution III was removed and the plugs were incubated in 10 ml of 0.5 M EDTA for 2 hours at room temperature. The EDTA solution was removed 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 per wash) by gentle shaking in running buffer (0.5 X TBE [pH 8], 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] at 6 volts 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 stained for 30 minutes in a solution containing 5 µg/ml ethidium bromide (EtBr) then destained for 30 minutes in distilled water.

8. Plasmid isolation.

The plasmids from yeast transformants were isolated by the method of Hoffman and Winston (1987). The method of Birnboim and Doly (1979) was used to isolate the plasmids from *E. coli* for transformation of yeast, digestion and preparation of probes. A mini alkaline-lysis/PEG precipitation procedure (Perkin-Elmer Applied Biosystems, Foster City, CA) was used to obtain sequencing grade DNA and for other DNA manipulations.

9. Yeast and *E. coli* transformations.

Several *blm5-1* haploid and diploid strains were transformed with genomic libraries of the yeast strain *S288C*. These libraries (kindly provided by Dr. Mark Rose, Princeton University, Princeton, N.J. and Dr. Marian Carlson, Columbia University, New York, N.Y.) were constructed by cloning *Sau*3A yeast chromosomal DNA fragments into the *Bam*HI site of either the centromeric plasmid shuttle vector YCp50 (Rose et al. 1987) or the yeast episomal plasmid shuttle vector YEp24 (Botstein et al. 1979). Transformation of yeast cells was carried out using the lithium acetate method of Ito and co-workers (1983) and as modified by Rose (1987) and Rose and Broach (1991).

Plasmids isolated from yeast or *E. coli* were used to transform *E. coli* cells made competent using the cold $CaCl_3$ method described by Sambrook et al. (1989a). Plasmids were also used to transform competent *E. coli* cells obtained commercially from Life Technologies, Inc (Frederick, MD).

10. Southern hybridization.

Plasmids were subjected to digestion by various restriction enzymes. Selected fragments were isolated by gel electrophoresis, purified and concentrated using either the ELUTIP-d (Schleicher & Schuell, Indianapolis, IN) or the GELase (Epicenter Technologies, Madison, WI) methods. Purified DNA fragments, intact recombinant plasmids and plasmid vectors were used as templates in the preparation of ^{32}P-labelled probes using a Random Primer DNA labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, IN). The protocol of Sambrook and co-workers (1989b) was used for the prehybridization and hybridization of ^{32}P-labelled probes to a yeast chromoblot (Clonetech, Palo Alto, CA) containing the sixteen chromosomes of *S. cerevisiae*.

A 751-bp fragment of the ampicillin gene was cut out of the YEp24 vector following digestion with the restriction enzymes *Pst*I and *Eco*RI. This fragment was purified, concentrated and used in the preparation of a ^{32}P-labelled probe as described above. Plasmid preparations isolated from Ura^+ transformants of the *blm5-1* haploid CM1403-12D were transferred to nitrocellulose filters (Brown,

1993) and hybridized to the ^{32}P-labelled probe of the ampicillin gene using the procedure already described.

11. DNA sequence analyses.

The Sanger-dideoxy sequencing method (Martin, 1993) was used to obtain approximately 500 bases of the DNA sequence on the left and right ends of each of the chromosomal inserts of recombinant plasmids. Customs made primers (obtained from National Biosciences, Plymouth, MN) from vector sequences flanking the chromosomal DNA insert of recombinant plasmids were used in sequencing reactions. Between 100 bases and 400 bases of the sequences obtained were used in computer sequence homology searches of yeast chromosomes reported in the *Saccharomyces* Genome Databases at Stanford University (Stanford, CA) and at NCBI.

RESULTS

Part 1: Growth of *blm5-1* mutant strains.

1. Mitotic growth.

For the purpose of this study it was considered important to determine if the growth of *blm5-1* mutants deviated significantly from that of normal strains and how any deviations might affect the results of the study. The mitotic growth of *blm5-1* haploid strains and homozygous mutant diploid strains was compared with the growth of normal and heterozygous strains at 30°C and 37°C. The growth at 30°C of *BLM5/BLM5* and *blm5-1/blm5-1* cells is compared in **Figure 1–1.** The growth rates of homozygous diploid mutant cells were found to range between 1.5 and 2 fold less than that of normal diploids during logarithmic growth. Haploid mutants also demonstrated a similar lag during logarithmic growth. All normal strains as well as heterozygous diploids showed strong growth after three days incubation at either 30°C or 37°C (**Figure 1-2**). Mutant haploids were also capable of growth at both 30°C and 37°C, but their growth was sometimes less than the growth of normal cells. Homozygous mutant diploids grew well at 30°C, but their growth was nearly completely inhibited at 37°C (**Figure 1-2**). This temperature-sensitive (ts) phenotype was somewhat variable among different *blm5-1/blm5-1* diploid strains in that the growth of some mutant diploids was more grossly inhibited at 37°C on solid growth media than the growth of others. In addition, the growth of all mutant diploids was completely inhibited when temperatures were further elevated to 39°C. All *blm5-1/blm5-1* strains grew normally when shifted down to 30°C from 37°C (data not shown). However, growth was not observed among homozygous mutant diploid strains that were shifted from 39°C to 30°C.

2. Meiotic growth.

S. cerevisiae is a budding yeast capable of existing in either a haploid or diploid state. Haploid cells come in either of two mating types, a or α, that can fuse to give rise to a/α diploid cells. Optimal growth of both haploid and diploid cells occurs at 30°C with aeration in a good nutritional environment. Under these growth conditions the mass of normal (wild type) cells will double approximately every 90 minutes. In a poor nutritional environment however, diploid cells may undergo meiotic division giving rise to four haploid progeny or spores encapsulated in thick cell-walled structures called asci.

a. Sporulation.

The abilities of *BLM5/BLM5*, *BLM5/blm5-1* and *blm5-1/blm5-1* strains with similar genetic backgrounds to complete meiosis at both 30°C and 37°C were examined. Homozygous normal diploids had similar levels of sporulation at both temperatures (**Table 1-1**). This indicated that meiotic division is unaffected by the elevation of temperature in these strains. The levels of sporulation exhibited by heterozygous diploids at 30°C were comparable with those of normal strains at the same temperature (**Table 1-1**). However, whereas normal diploids sporulated at 37°C, heterozygous diploids were incapable of sporulating at this temperature. Homozygous mutant diploids consistently showed very low levels of sporulation at 30°C and an inability to sporulate at 37°C (**Table 1-1**). These results indicated that a single copy of the *blm5-1* mutation was sufficient to block sporulation at 37°C and two copies of the mutation resulted in significant reductions in the ability of strains to complete meiosis at 30°C.

Light micrographs of sporulated *BLM5/BLM5*, *BLM5/blm5-1* and *blm5-1/blm5-1* populations are shown on **Figure 1-3**. While no detectable differences were observed among populations of *BLM5/BLM5* cells sporulated at 30°C or 37°C or *BLM5/blm5-1* cells sporulated at 30°C, asci were not detected in *BLM5/blm5-1* cultures sporulated at 37°C. Very few asci were detected among *blm5-1/blm5-1* populations sporulated at 30°C, but an unexpected and unusual observation was the presence of enlarged cells in these cultures at high frequencies (**Figure 1-3**). Sporulated *blm5-1/blm5-1* cultures were stained with DAPI (4', 6-diamidino-2-phenylindole), a fluorescent dye that specifically stains DNA (Sherman et al. 1986).

The enlarged cells observed in such cultures sporulated at 30°C showed a single intensely stained mass that was significantly larger than those observed in similarly stained unsporulated *blm5-1/blm5-1* cells (data not shown). This result suggested that although DNA replication may have occurred in enlarged *blm5-1/blm5-1* cells, nuclear division did not occur. Asci were not observed in *blm5-1/blm5-1* populations sporulated at 37°C.

b. Spore viability.

The viability of segregants isolated from four-spored asci of each of the three genotypes was determined after dissection. Survival of *BLM5* and *blm5-1* segregants were equivalent (data not shown). However, there were differences in the numbers of survivors among the four segregants in the tetrads from each strain. A high level of survival of all four segregants was usually observed among the asci of sporulated *BLM5/BLM5* cells (**Table 1-2**). Survival was usually higher for one, two or three of the four segregants of the asci of *BLM/blm5-1* and *blm5-1/blm5-1* diploids rather than for all four segregants. The survival of one or three of the four segregants was usually highest among the asci of *blm5-1/blm5-1*.

PART 2: Bleomycin association and efflux in *BLM5* and *blm5-1* strains.

1. Association of [S-*methyl*-^{3}H]bleomycin with normal and *blm5-1* strains.

The likelihood that the elevated sensitivity of *blm5-1* strains to bleomycin treatment was due to the uptake of more drug by mutant strains than by *BLM5* strains was considered. Therefore, comparisons were made between the amount of radioactivity associated with *BLM5* and *blm5-1* strains following treatment with [S-

methyl-^{3}H]bleomycin. The amount of radioactivity associated with *BLM5* strains increased as drug doses were increased particularly at ≤ 33.3 μM. This is illustrated in **Figure 2–1** for one of the tetrads analyzed. Association was quite high and as high as 300 ρM – 400 ρM of drug per 10^8 cells for some *BLM5* segregants.

Surprisingly, *blm5-1* strains were shown to associate much less bleomycin than normal strains (**Figure 2-1**). The maximum amount of drug associated with bleomycin-treated *blm5-1* strains was sometimes as much as 1000 times less than the maximum amount of drug associated with treated normal cells. In addition, the amount of drug associated with treated *blm5-1* cells did not increase significantly as treatment doses were increased.

2. Efflux of ^{3}H from normal and *blm5-1* strains.

To examine the probability that the bleomycin hypersensitivity of *blm5-1* strains may be the result of the retention of drug by mutant strains following treatment, the efflux of radioactivity from [S-*methyl*-^{3}H]bleomycin-treated normal and *blm5-1* strains was monitored over a period of 37 hours and compared. This was done in spite of the fact that radioactivity associated with the material released from normal strains was always considerably higher than the amount of radioactivity associated with the effluxed material from *blm5-1* strains (**Table 2-1**). Since normal strains showed higher levels of associated [S-*methyl*-^{3}H]bleomycin than *blm5-1* strains after drug treatment, the radioactivity associated with the effluxed material from each strain was calculated as a fraction of the amount of [S-*methyl*-^{3}H]bleomycin initially associated with each strain. These calculations showed that the percent of associated radioactive material released from *blm5-1* strains within the first two hours after bleomycin treatment was approximately three times less than the percent of associated radioactive material released from normal strains during the same time period (**Table 2-1**). However, the percent of associated radioactive material released from each strain at the end of a 37-hour posttreatment incubation period was shown to be equivalent. Thus, although the initial rate of release of radioactive material from *blm5-1* strains was slower than in normal strains, the later release rates were similar for normal and mutant strains.

3. Cytotoxicity of radioactive efflux from *blm5-1* strains.

Experiments conducted in this laboratory showed that material effluxed from bleomycin-treated normal yeast cells was cytotoxic (Moore, 1982 [b]) and had High Performance Liquid Chromatography (HPLC: Wilson and Schlabach, 1998) profiles that were different from those of bleomycin molecules (manuscript in preparation). These experiments suggested that bleomycin molecules might have been modified in the cell before being released. It was also shown that, when purified, a cytotoxic diffusible factor (CDF) was cytotoxic to yeast cells. To determine if the radioactive material effluxed from *blm5-1* strains was also cytotoxic, comparisons were made in the levels of survival of A364A yeast cells following treatments with the radioactive material released from normal and *blm5-1* strains. Cell survival was reduced significantly by the radioactive efflux from both normal and *blm5-1* strains (**Table 2-2**). However, the survival of cells treated with the radioactive efflux from normal cells was 3 – 4 times lower than the survival of cells treated with the radioactive material released from *blm5-1* strains (**Table 2-2**). Since the radioactive content of the efflux from *blm5-1* cells was much lower than the radioactive content of the efflux from *BLM5* cells, it is likely that the higher survival rates displayed by yeast cells treated with the radioactive efflux from the mutant strains resulted from its lower cytotoxic content than the efflux from *BLM5* strains.

PART 3: DNA Repair.

Cell cycle checkpoints respond to spontaneous and induced damage by causing delays in the cell cycle. In yeast, checkpoint genes function with cell division cycle (CDC) genes to cause cells to arrest in the S or G2 phase of the cell cycle in response to incomplete DNA replication and/or DNA damage (Weinert et al. 1994). Checkpoint genes may also have a functional role in meiotic recombination (Kato and Ogawa, 1994). Mutations in these genes lead to increased sensitivities to DNA damaging agents (Weinert and Hartwell, 1988). The discovery that *blm5-1* mutants, in addition to displaying elevated sensitivities to bleomycin treatment, also showed defects affecting both meiotic and mitotic division made it considerably important to maximize the opportunity for mutant cells to repair. The repair of spontaneous and induced chromosomal double-strand breaks was therefore investigated under non-growth conditions designed to impose a prolonged checkpoint delay (IPCD) in the cell. Under these conditions it was believed there was an increased likelihood that double-strand breaks in chromosomes would be repaired before the cells' progression from the G2 phase of the cell cycle and into the M phase.

PFGE analyses were used to examine chromosomal integrity, degradation and rejoining in strains at the normal growth temperature of 30°C and at the temperature restrictive for the growth of *blm5-1/blm5-1* strains, 37°C. In pulsed field gels, chromosomal degradation is characterized by a reduction in the intensity of chromosomal bands and by an accumulation of the product of chromosomal degradation at the bottom of the gel. The chromosomal degradation product appears as a migrating smear of DNA in the gel as chromosomal degradation and rejoining occurs. When double-strand breaks in chromosomal DNA increase in number, the DNA smear increases in intensity and migrates lower in the gel. As double-strand breaks are repaired, the smear of DNA decreases in intensity and moves upward until chromosomal bands begin to reappear.

1. Spontaneous production and repair of double-strand breaks in the chromosomal DNA of untreated *BLM5/BLM5*, *BLM5/blm5-1* and *blm5-1/blm5-1* diploids at 30°C and 37°C.

The spontaneous production of double-strand chromosomal breaks was investigated in *BLM5/BLM5*, *BLM5/blm5-1* and *blm5-1/blm5-1* strains. The purpose of this study was to determine if there were significant differences in the introduction and repair of spontaneous double-strand breaks in each of the three genotypes that would contribute to the interpretation of the repair of induced chromosomal breaks following bleomycin treatment. In the absence of bleomycin treatment, the chromosomes of each strain used in this study consistently produced strong bands after PFGE (**Figures 3-1, 3-2 and 3-3**). A low level of spontaneous double-strand breaks accumulated in the chromosomes of untreated *BLM5/BLM5* diploid cells after 30 minutes of incubation in phosphate buffer at 30°C (**Figure 3-1**). These double-strand breaks were repaired within two hours and appeared not to reoccur between the 2 hours and 48 hours of incubation at 30°C. Chromosomal breaks also accumulated in untreated *BLM5/BLM5* cells after 30 minutes of incubation in buffer at 37°C. The intensities of the DNA smears representing chromosomal degradation suggests that fewer breaks accumulated at 37°C than at 30°C. Accumulated breaks were also repaired in *BLM5/BLM5* cells within two hours at 37°C in non-growth conditions. However, in contrast to *BLM5/BLM5* cells held at 30°C, accumulations of breaks were again observed after 6 hours of incubation in non-growth media at 37°C. The reduction in the intensity of the DNA smear after 24 hours at 37°C suggests that these accumulated breaks were also repaired between the 6 hours and 24 hours of incubation at the elevated temperature.

Spontaneous double-strand breaks were also introduced and repaired in the chromosomes of untreated *BLM5/blm5-1* cells. Unlike *BLM5/BLM5* cells, breaks were detected at different times during incubation in phosphate buffer not only at 37°C, but also at 30°C (**Figure 3-2**). Like *BLM5/BLM5*, more spontaneous chromosomal breaks accumulated in *BLM5/blm5-1 cells* at 30°C than at 37°C. These breaks were initially detected in untreated *BLM5/blm5-1* cells prior to incubation in buffer, and again after 2 hours of incubation at 30°C and 4 hours at 37°C.

High levels of detectable spontaneous double-strand breaks did not accumulate in the chromosomes of the *blm5-1/blm5-1* cells during the first 24 hours of IPCD incubation at 30°C (**Figure 3-3**). Instead, accumulations of double-strand breaks were first seen after 48 hours of incubation in non-growth conditions at 30°C.

Accumulations of double-strand breaks in *blm5-1/blm5-1* cells were not observed at any time during incubations at 37°C (**Figure 3-3**).

2. Chromosomal degradation is dose dependent.

In yeast, the number of chromosomal double-strand breaks resulting from bleomycin treatment was determined to be a function of drug concentration, length of treatment incubation and the stage of growth at which treatment occurred (Moore et al. 1989). Many more breaks are generally seen in cells treated at stationary phase than at any other phase of growth. Observations made during these experiments agreed with previously reported data.

Since *BLM5/BLM5*, *BLM5/blm5-1* and *blm5-1/blm5-1* strains exhibited strikingly different levels of survival following treatment with bleomycin (**Figure 3–4**), experiments were conducted to determine the drug doses required to produce detectable accumulations of chromosomal double-strand breaks in each of the three strains. Significant levels of chromosomal degradation were not observed in the *BLM5/BLM5* strain after treatment with low doses of bleomycin (0 μg/ml – 10 μg/ml; data not shown). However, dose-dependent decreases in the intensities of chromosomal bands were observed after treatment with drug doses of 15 μg/ml, 30 μg/ml and 50 μg/ml (**Figure 3-5, A**). In spite of this dose-dependent chromosomal degradation, the chromosomes of the *BLM5/BLM5* diploid appeared to be unusually resistant and complete chromosomal degradation was never detected in the chromosomes of this strain following bleomycin treatment, even at drug doses as high as 50 μg/ml.

The chromosomes of the *BLM5/blm5-1* and *blm5-1/blm5-1* diploids displayed dramatic decreases in intensity as bleomycin treatment doses were increased from 0 µg/ml – 5 µg/ml (**Figure 3-5, B and C**). In addition, the amounts of the chromosomal degradation product increased and migrated further in the gel at higher drug doses. All chromosomal bands of both *BLM5/blm5-1* and *blm5-1/blm5-1* diploids appeared to be completely degraded after a bleomycin treatment dose of 5 µg/ml.

3. Repair of bleomycin-induced chromosomal double-strand breaks at 30°C and 37°C in *BLM5/BLM5*, *BLM5/blm5-1* and *blm5-1/blm5-1* diploids.

The repair of chromosomal double-strand breaks at 30°C and 37°C was examined in *BLM5/BLM5*, *BLM5/blm5-1* and *blm5-1/blm5-1* diploids to determine if the differences in bleomycin hypersensitivities in these strains could result from the accumulations of unrepaired double-strand breaks in chromosomes after bleomycin treatment. The *BLM5/BLM5*, *BLM5/blm5-1* and *blm5-1/blm5-1*diploids selected for this study (CM-1489, CM-1477 and CM-1492 respectively) displayed strikingly different levels of survival following drug treatment (**Figure 3–4**).

a. *BLM5/BLM5*.

The chromosomal bands of the *BLM5/BLM5* strain were always very strong, both after drug treatments with bleomycin doses ranging between 0 µg/ml and 30 µg/ml and during IPCD incubations at 30°C and 37°C (**Figure 3-6, 30 µg/ml**). Therefore, chromosomal degradation and regeneration could not be monitored in pulsed field gels at these doses. Repair processes were therefore followed after a bleomycin treatment dose of 50 μg/ml at both 30°C and 37°C (**Figure 3-6, 50 μg/ml**).

Chromosomal double-strand breaks were repaired at both temperatures in *BLM5/BLM5* cells. Analyses of pulsed-field gels indicated that before chromosomal bands were regenerated, chromosomes, already degraded by bleomycin treatment, underwent additional degradation during IPCDs at 30°C and 37°C. This observation was not surprising since the enlargement of double-strand breaks in DNA to double-strand gaps has been described in a model for recombination in double-strand break repair in yeast (Szostak et al. 1983). The additional chromosomal degradation was initiated earlier at 30°C (after 2 hours of IPCD) than at 37°C (after 4 hours), but chromosomal regeneration was initiated earlier at 37°C (regenerated bands have a greater intensity after 6 hours of IPCD) than at 30°C (**Figure 3-6, 50 μg/ml**). Despite the rapidity at which chromosomal bands reappeared at 37°C, stronger bands were regenerated after 48 hours of IPCD at 30°C than after IPCD of the same time at 37°C.

b. *BLM5/blm5-1.*

In contrast to the *BLM5/BLM5* strain, the chromosomal bands of the *BLM5/blm5-1* strain showed dramatic reductions in intensity after the very low bleomycin treatment dose of 0.2 μg/ml (**Figures 3–5 and 3–6 versus Figure 3–7**). Double-strand breaks incurred at this treatment dose were repaired at both 30°C and 37°C (**Figure 3-7, 0.2 μg/ml**). Pulsed-field gel analyses showed a pattern of chromosomal degradation and regeneration with time during IPCD incubations at both temperatures. The consistency of this pattern from experiment to experiment suggested that repair processes involved cycles of degradation and regeneration.

The reappearance of strong bands following 30 minutes of IPCD incubation at 30°C suggested that the first cycle of degradation and regeneration in the repair process was rapidly completed at this temperature (**Figure 3-7, 0.2 μg/ml**). A second cycle of repair began and was completed during the 30 minute to 24 hours of IPCD. A third cycle began during 24 hours to 48 hours of IPCD at 30°C. When IPCD temperatures were elevated to 37°C, chromosomes already fragmented by bleomycin treatment were further degraded during the first 2 hours of IPCD incubation (**Figure 3-7, 0.2 μg/ml**). Regenerated chromosomes first appeared after 4 hours of IPCD at 37°C and were still seen after 6 hours of IPCD at this temperature. A second repair cycle that began during the 6 hours to 48 hours of IPCD incubation at 37°C was not completed by 48 hours. Thus, unlike the *BLM5/BLM5* strain, repair processes in the *BLM5/blm5-1* strain seemed to occur at a faster rate at 30°C than at 37°C. In addition, regenerated chromosomal bands were always much stronger at 30°C.

In the *BLM5/blm5-1* strain, repair processes appeared to be limited by the number of breaks introduced into the chromosome. Thus, as bleomycin treatment doses were increased from 0.2 μg/ml to 1 μg/ml (**data not shown**) and 5 μg/ml (**Figure 3-7, 5 μg/ml**), chromosomal degradation was extensive and chromosomal regeneration was not detected in pulsed-field gels at either 30°C or 37°C.

c. *blm5-1/blm5-1.*

Like the *BLM5/blm5-1* strain, the chromosomes of the *blm5-1/blm5-1* strain were severely reduced in intensity after bleomycin treatment with a dose of only 0.2 µg/ml. More chromosomal double-strand breaks were introduced into *blm5-1/blm5-1* cells than in *BLM5/blm5-1* cells at this drug dose (**Figure 3–5**, **Figure 3–7 and Figure 3–8**). Like *BLM5/blm5-1* cells, breaks were repaired in *blm5-1/blm5-1* cells at both 30°C and 37°C (**Figure 3-8, 0.2 µg/ml**). The first cycle of chromosomal degradation and regeneration in *blm5-1/blm5-1* cells was completed during 0 to 4 hours of IPCD at both 30°C and 37°C. This suggested that, unlike *BLM5/BLM5* cells and *BLM5/blm5-1* cells, the rate at which repair cycles were initiated in *blm5-1/blm5-1* cells was the same at both temperatures. As in the *BLM5/BLM5* and *BLM5/blm5-1* strains, stronger bands were regenerated in the *blm5-1/blm5-1* strain after IPCD at 30°C than at 37°C (**Figure 3-8, 0.2 µg/ml**). These results strongly suggest that repair processes were more efficient at 30°C in the three strains.

A second repair cycle occurred in the *blm5-1/blm5-1* strain between 4 hours and 24 hours of IPCD at 30°C, and a third cycle appeared to have been initiated after 24 hours at this temperature. The completion of this third cycle was not detected within the experimental time frame. The second and third repair cycles in the *blm5-1/blm5-1* strain were not observed at 37°C. Indeed, the pattern of migration of DNAs in the gel, particularly in the lanes in the region of the arrow (**Figure 3–8**), suggested that repair processes failed in this strain after 4 hours of IPCD at the elevated temperature. Thus, although early repair functions in CM-1492 were initiated at 37°C, late repair functions appeared to be faulty.

The chromosomes in *blm5-1/blm5-1* cells were not repaired during IPCD incubations at either 30°C or 37°C following treatments with a bleomycin dose of 5 μg/ml (**Figure 3-8, 5 μg/ml**). Therefore, chromosomal regeneration was limited in the *blm5-1/blm5-1* strain as it was in the *BLM5/blm5-1* strain by the number of double-strand breaks introduced into the chromosomes.

4. Posttreatment survival at 30°C and 37°C.

The survival of each of the *BLM5/BLM5*, *BLM5/blm5-1* and *blm5-1/blm5-1* strains was examined in parallel with PFGE analyses. For each of the three strains, cycles of decreased survival and increased survival (**Figures 3-9, 3-10 and 3-11**) paralleled the chromosomal degradation and repair cycles observed in pulsed-field gels. That is, survival usually decreased when chromosomes were degraded and increased when chromosomes were regenerated.

a. *BLM5/BLM5*.

During IPCD incubations at both 30°C and 37°C, there was an initial decrease in the survival of the *BLM5/BLM5* strain CM-1489 after treatment with high bleomycin doses (15 μg/ml, 30 μg/ml and 50 μg/ml). This decrease in survival was more gradual at 30°C, where it lasted approximately 4 hours (**Figure 3-9, A**), than at 37°C, where it lasted 30 minutes (except for the 30-μg/ml treatment where notable changes in survival were not observed **Figure 3-9, B**). These decreases in survival were not observed following treatment with low drug doses (0.2 μg/ml, 1 μg/ml, 2.5 μg/ml and 5 μg/ml, **Figure 3–9, C and D**). Instead, survival increased dramatically within 30 minutes of IPCDs at both 30°C and 37°C.

Maximum survival in *BLM5/BLM5* cells usually occurred between 4 hours and 6 hours of IPCD at 30°C following treatment with low bleomycin doses (**Figure 3–9, C**) and after 48 hours following treatment with high drug doses (**Figure 3-9, A**). At 37°C however, cells treated with low and high drug doses showed maximum levels of survival after 2 hours (exceptions include cells treated with 2.5 μg/ml and 15 μg/ml where comparable levels of survival were seen after 2 hours and 24 hours of IPCD, **Figure 3–9, B and D**).

b. *BLM5/blm5-1.*

Dramatic increases in the survival of the *BLM5/blm5-1* strain were observed within 30 minutes of IPCD at 30°C following treatment with the relatively low bleomycin dose of 0.2 μg/ml (**Figure 3-10, A**). Such increases in cell survival were not observed after IPCDs of the same time period at 37°C (**Figure 3-10, B**). Unlike the *BLM5/BLM5* strain, maximum survival of the heterozygote occurred after 24 hours of IPCD at 30°C following treatment with 0.2 μg/ml and between 4 hours and 6 hours of IPCDs at 37°C. When bleomycin treatment doses were increased to 1 μg/ml and 5 μg/ml the survival of the heterozygote decreased dramatically (survival was approximately 4.9% and 1.6% respectively) and the strain showed poor recovery during IPCDs at both 30°C and 37°C (**Figure 3-10, A and B**).

c. *blm5-1/blm5-1.*

Maximum survival in the *blm5-1/blm5-1* strain after bleomycin treatment with 0.2 μg/ml occurred after 4 hours of IPCD at both 30°C and 37°C. The survival was considerably higher at 30°C than at 37°C in this strain. Survival fell after 4 hours of IPCD at each temperature, but increased again by 24 hours.

PART 4: Isolation and characterization of genes that complement the *blm5-1* mutation.

1. Selection of Ura$^+$ transformants with increased resistance to killing by bleomycin.

Two approaches were used to isolate genes with complementing activity to the *blm5-1* mutation. In the first approach, a *blm5-1* haploid was transformed with yeast genomic DNA carried in the YCp50 and YEp24 libraries. The mutant strain carried a selectable marker in the *URA3* gene. Both YEp24 and YCp50 plasmids carry the normal *URA3* gene of yeast. Therefore, after transformation, cells were allowed to recover at 30°C on SD media lacking uracil for the selection of plasmid-bearing transformants.

Approximately 2,500 Ura$^+$ transformants containing recombinant plasmids of YCp50 and 7,300 transformants containing recombinant plasmids of YEp24 were isolated. Each transformant was screened on solid SD media containing a range of bleomycin doses (0 μg/ml – 10 μg/ml) for resistance to killing by the drug. Eleven Ura$^+$ transformants with recombinant plasmids of YCp50 and twenty-two transformants with recombinant plasmids of YEp24 were selected with higher levels of resistance to killing by bleomycin than those displayed by *blm5-1* mutant haploids. A secondary screening on bleomycin-containing media was done to identify transformants with levels of drug resistance that were comparable with those of normal *BLM5* strains or were higher than those of *blm5-1* mutants transformed with either the YCp50 or YEp24 plasmid vectors. One transformant carrying a

YCp50 recombinant plasmid (pPM518y[1], **Figure 4-1**) and six transformants containing recombinant plasmids of YEp24 (**Figure 4-2; 1, 5, 6, 7, 20 and 21**) were selected based on their varying levels of resistance to killing by bleomycin. The transformant carrying the recombinant plasmid pPM518y displayed high levels of resistance to killing by the drug at both 30°C and 37°C. Transformants with recombinant plasmids of YEp24 were not screened at 37°C.

The second approach to isolate *blm5-1* complementing genes utilized the diminished ability of homozygous *blm5-1/blm5-1* diploids to grow at 37°C. Several mutant diploids were transformed with YEp24 library DNA. Unlike previous transformation experiments, cells were allowed to recover after transformation on solid selective media lacking uracil at 37°C instead of at 30°C. This strategy provided a double selection for the isolation of transformants that would be both Ura$^+$ and able to grow at 37°C.

There were significant differences in the numbers of Ura$^+$ transformants selected for each diploid. Of forty transformants selected on media without uracil, thirty-one were transformants of CM-1483, six were transformants of CM-1491 and three were transformants of CM-1493. The higher number of transformants selected from CM-1483 was attributed to the leaky nature of the temperature-sensitive phenotype of the CM-1483 diploid at 37°C. All forty Ura$^+$ transformants were re-screened at 37°C to ensure consistency in their ability to grow at the elevated temperature. In addition, the ability of transformants to survive bleomycin treatment

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[1] For the purpose of differentiation plasmids were always given the extension "y" if they were carried in or isolated from yeast transformants prior to their amplification in *E. coli*. This extension was dropped after the plasmid was amplified in *E. coli* and isolated from *E. coli* transformants.

at both 30°C and 37°C was compared with that of mutant strains transformed with the YEp24 vector.

Mutant diploids transformed with the YEp24 vector (**Figure 4-3, plates 1 and 2: K**) showed similar levels of growth inhibition at 37°C as untransformed mutants (**L and O**). Since diploids are more resistant to the lethal effects of bleomycin than haploid cells, it was not surprising that mutant diploids containing the YEp24 vector showed strong survival after treatment with a bleomycin dose of 1 µg/ml at 30°C (**plate 3**), while haploids carrying the empty YEp24 vector did not survive a similar treatment. The survival of mutant diploids carrying the YEp24 vector was significantly reduced in the presence of a drug dose of 3 µg/ml at 30°C and cells did not grow when the drug dose was increased to 5 µg/ml at the same temperature. While bleomycin sensitivity normally increased among YEp24-transformed *blm5-1* haploid cells when temperatures were elevated to 37°C, the growth of *blm5-1/blm5-1* cells was inhibited at 37°C in the absence of drug and thus their response to bleomycin treatment could not be analyzed.

Twenty-three of the thirty-one transformants of CM-1483 retained the ability to grow at 37°C. However, the level of survival among the twenty-three transformants ranged from very strong to poor at the elevated temperature. Eight of the twenty-three CM-1483 transformants showed varying degrees of survival after treatment with a bleomycin dose of 1 µg/ml at 37°C (**Figure 4–3; Plate 3 37°C, spot E and Plate 9 37°C, spots b, c, d, g, l, n, and p**). One of the eight Ura$^+$ transformants showed high survival at the bleomycin dose of 1 µg/ml at 37°C (**Plate**

9 37°C, spot n). When the drug dose was increased to 3 µg/ml, none of the eight transformants grew (**Plates 4 and 10, 37°C**).

Two transformants of CM-1493 (**H and I**) and one transformant of CM-1491 (J) showed consistency in their ability to grow at 37°C. However, there were differences in their ability to survive bleomycin treatment. The survival of transformant J following bleomycin treatment at 30°C was similar to that of mutant cells carrying the YEp24 vector and this transformant did not survive bleomycin treatment at 37°C. The two transformants, H and I, showed high levels of survival after bleomycin treatments at 30°C (**plates 4, 5 and 6**), even with drug doses as high as 10 µg/ml. One of these transformants (I) also displayed high levels of survival at 37°C after treatment with a bleomycin dose of 10 µg/ml. The transformant H was only capable of surviving a drug dose of 1 µg/ml at the same temperature. Five additional transformants of CM-1491 (**plates 1 and 2, spots M, and P through** S) and one additional transformant of CM-1493 (G) failed to maintain their ability to grow at 37°C, and these transformants also showed poor or no survival in the presence of a bleomycin dose of 3 µg/ml at 30°C.

In summary, Ura$^+$ transformants were selected both in haploid and diploid strategies. However, diploid transformants usually demonstrated higher levels of resistance to the lethal effects of bleomycin than haploid transformants. The remainder of this section describes in detail the characterization of both classes of transformants and the plasmids isolated from them.

2. Selection of Ura$^+$ transformants with plasmid-borne *blm5-1* complementing activity.

Cosegregation of plasmid markers with bleomycin resistance after mitotic plasmid loss from Ura$^+$ transformants would suggest the presence of a plasmid bearing *blm5-1* complementing activity in the transformed mutant. Mitotic plasmid loss experiments were conducted on the seven selected haploid transformants possessing high levels of complementing activity to the *blm5-1* mutation. Transformants were grown non-selectively in liquid YPAD for three days at 30°C, then plated onto solid YPAD to obtain isolated colonies. Colonies were screened for cosegregation of the uracil marker with the *blm5-1* complementing activity by replica plating onto SD media lacking uracil and onto SD containing bleomycin. These experiments demonstrated that the *blm5-1* complementing activity cosegregated with the plasmid markers of the YCp50 recombinant plasmid (pPM518y) and two of the six YEp24 recombinant plasmids (pPM5115y and pPM5116y isolated from Ura$^+$ transformants **5** and **6**, respectively; shown on **Figure 4-2**). Plasmid loss events were not observed among the remaining four haploid transformants (**Figure 4–2: spots 1, 7, 20 and 21**). Therefore, this method could not be used to determine if the *blm5-1* complementation was due to the presence of a plasmid in these transformants.

Southern hybridization studies were used to determine if each of the four haploid transformants (**Figure 4–2; spots 1, 7, 20 and 21**) contained a YEp24-based plasmid. Plasmid preparations, pPM5114y, pPM5117y, pPM5118y and pPM5119y, were isolated from the transformants (1, 7, 20 and 21 respectively) and separately hybridized to a ^{32}P-labelled *Pst*I – *Eco*RI fragment of the ampicillin gene. This gene is present on the YEp24 plasmid vector, but is not normally found in yeast cells. The empty YEp24 vector and λ DNA digested with *Hind*III were included in these experiments as positive and negative controls respectively. Each of the four-plasmid preparations hybridized strongly to the 751 bp fragment of the ampicillin gene (**Figure 4–4**), suggesting that the four transformants each carry a YEp24-based plasmid. Plasmid preparations were then amplified by transformation into *E. coli* and restriction analyses were conducted on the plasmids pPM5114, pPM5117 and pPM5118 that were isolated from *E. coli* transformants. All *E. coli* transformants were tetracycline sensitive. Since yeast chromosomal DNA was inserted at the *Bam*HI site within the tetracycline gene of the YEp24 plasmid in the *S. cerevisiae* library, it was expected that plasmids pPM5114, pPM5117 and pPM5118 each carried a chromosomal DNA insert fragment. However, analyses showed that pPM5114, pPM5117 and pPM5118 had restriction patterns that were similar to those of the empty YEp24 vector. In addition, when haploid and diploid cells were transformed with pPM5118 they remained hypersensitive to bleomycin treatments at both 30°C and at 37°C. These results suggested that the plasmids pPM5114, pPM5117 and pPM5118 had undergone a change that resulted in the loss of *blm5-1* complementing activity but did not reestablish a functional tetracycline gene.

3. Differential *blm5-1* complementing activity of recombinant plasmids.

DNA molecules may be altered in *E. coli* and in yeast. It is therefore important to verify that plasmids recovered from *E. coli* or yeast still complement the *blm5-1* mutation. For this purpose several haploid and diploid *blm5-1* mutant strains were transformed with recombinant plasmids recovered from *E. coli*. The *blm5-1* mutants were also transformed with recombinant plasmids recovered from each of the original yeast transformants and with the YEp24 vector. Bleomycin sensitivity of selected Ura$^+$ transformants was compared with that of normal diploids and the original transformants from which each plasmid was isolated.

a. Plasmids derived from haploid transformants.

We observed that after amplification in *E. coli*, the YCp50-based plasmid pPM518 no longer complemented the *blm5-1* mutation in haploid mutants. However, the YEp24-based plasmids, pPM5115y and pPM5116y isolated from yeast and pPM5115 and pPM5116 isolated from *E. coli*, still relieved the *blm5-1* mutation in mutant haploids (**Figure 4-5; F, I, H and K respectively**). The level of complementing activity observed among these plasmids was less than that observed in the original transformants (**G and J**) from which the plasmids were initially derived. Haploid cells transformed with any of the four plasmids grew in the presence of drug doses up to 1 µg/ml at 30°C, whereas haploid cells transformed with YEp24 did not grow at this drug dose. The original transformant (**G**) from which pPM5115y was isolated was less resistant to bleomycin treatment (capable of surviving doses up to 3 µg/ml) than the original transformant (**J**) from which pPM5116y (capable of surviving doses up to 5 µg/ml; data not shown) was isolated. The plasmids, pPM5115y and pPM5116y isolated from yeast, and pPM5115, and pPM5116 from *E. coli*, did not relieve the bleomycin hypersensitivity conveyed by the *blm5-1* mutation in the homozygous mutant diploids, CM-1483 and CM-1492, nor did they relieve the temperature-sensitive phenotype of these diploids.

b. Plasmids derived from diploid transformants.

Mutant *blm5-1/blm5-1* diploids with or without the YEp24 vector (**Figure 4-6, B and C respectively**) did not grow at 37°C. Each of the three original transformants (**D, I and L**) maintained an ability to grow at 37°C that was comparable to that of normal *BLM5/BLM5* diploids (**A**). The plasmids pPM5120y, pPM5121y and pPM5122y isolated from yeast transformants relieved the temperature-sensitive phenotype of homozygous mutant diploids (**F, H and K respectively**). After recovery from *E. coli*, the plasmids pPM5120, pPM5121 and pPM5122 still relieved the temperature sensitivity of mutant diploids (**E, G and J**).

The Ura$^+$ diploids transformed with the YEp24 vector showed reduced levels of survival after treatment with a bleomycin dose of 3 μg/ml at 30°C, and they did not survive a treatment dose of 5 μg/ml at the same temperature. The plasmids, pPM5120y, pPM5121y, pPM5122y, isolated from yeast relieved the bleomycin hypersensitivity conveyed by the *blm5-1* mutation in several homozygous diploids at both 30°C and 37°C (**Figure 4–6, plates 3 through 8**). These plasmids still relieved bleomycin hypersensitivity in homozygous mutant diploids at both 30°C and 37°C after they were amplified in *E. coli.*

There were significant differences in the level of the *blm5-1* complementing activity observed among the plasmids. These differences are summarized in **Table 4-1**. The plasmid pPM5120y generated the highest level of complementing activity among transformed mutant diploids. The complementing activity of this plasmid remained unchanged after amplification in *E. coli.* Transformed mutant diploids containing pPM5120y or pPM5120 showed strong growth even in the presence of bleomycin doses as high as 15 µg/ml at 30°C (**plates 7**) and 10 µg/ml at 37°C (**plates 6**).

The *blm5-1* complementing activity generated by the plasmids pPM5121y and pPM5122y was not as high as that observed among diploid mutants transformed with pPM5120y or pPM5120. Mutant diploids transformed with pPM5121y or pPM5122y showed strong growth in the presence of a drug treatment dose of 5 µg/ml at 30°C (**plates 5**) but grew poorly at a treatment dose of 3 µg/ml at 37°C (**plates 4**). Following amplification, the plasmids pPM5121 and pPM5122 isolated from *E. coli* transformants conveyed similar levels of resistance to killing by bleomycin as pPM5121y and pPM5122y in transformed *blm5-1/blm5-1*diploids. The level of bleomycin resistance that was conveyed by pPM5120, pPM5121 and pPM5122 was always comparable with that observed in the original transformants from which each plasmid was initially derived. Thus, unlike plasmids isolated from haploid cells, plasmids isolated from diploid cells consistently maintained their *blm5-1* complementing activity.

4. Identification of the chromosomal origin of the inserts of pPM518, pPM5115 and pPM5116 by Southern hybridization.

Standard procedures were used to identify restriction sites and to generate restriction maps for the inserts of plasmids pPM518, pPM5115 and pPM5116. Restriction analyses were also used to determine that the relative sizes of the chromosomal inserts of pPM518, pPM5115 and pPM5116 were 16.8 kb, 6.6 kb and 5.8 kb respectively. Southern hybridization studies were then used to identify the chromosomal origin of each of the three-plasmid inserts. First, ^{32}P-labelled probes created from the plasmids pPM5115 and YEp24 were separately hybridized to a yeast chromoblot. The only chromosome not highlighted by YEp24 (**Figure 4–7, Lane A**) and highlighted by pPM5115 (**Lane B**) was chromosome I. In a second experiment a ^{32}P-labelled probe created from a 4.2 kb *Eco*RI – *Eco*RI fragment of the insert of pPM5115 (**Figure 4–8**) was hybridized to the yeast chromoblot. This fragment did not hybridize to any of the chromosomes of *S. cerevisiae* (**data not shown**). The result therefore suggested that the region bearing homologous sequences to chromosome I must be found within the remaining 2.4-kb region of the pPM5115 insert that includes a *Hind*III and a *Pst*I site (**Figure 4–8**).

^{32}P-labelled probes created from a 4 kb *Eco*RV – *Eco*RV fragment of the insert of pPM5116 and from a *Cla*I – *Hind*III fragment of the insert of pPM518 were also separately hybridized to yeast chromoblots. The insert fragment of pPM5116 hybridized strongly to chromosome I of *S. cerevisiae* (**Figure 4–7, Lane C**). However, the fragment of pPM518 highlighted chromosome XI of yeast (**data not shown**). Thus, the inserts of these plasmids were derived from different yeast chromosomes. Since pPM518 lost the ability to relieve bleomycin hypersensitivity in haploid strains after isolation from *E. coli*, the complementing activity of this plasmid was no longer pursued.

5. DNA sequence analyses of the chromosomal insert fragments of recombinant plasmids.

DNA sequence homology searches were used to identify or verify the chromosomal origin of insert fragments of recombinant plasmids, determine the size of the insert and identify genes and parts of genes present on the insert. Sequences on the right and left ends of each chromosomal insert were used to obtain 100% homology to sequences reported in the *Saccharomyces* Genome Databases at Stanford University (Stanford, CA) and at the National Center for Biotechnology Information (NCBI).

a. pPM5115.

The DNA sequence of the insert of pPM5115 had homology with sequences on several of the chromosomes of *S. cerevisiae*, including chromosome I. However, the amount of homology detected between the sequences of pPM5115 and any of the yeast chromosomes was never greater than 76%. When DNA homology searches were expanded to include yeast plasmid sequences, the search revealed that there was 100% homology between sequences on the left and right ends of the insert of pPM5115 and sequences on the 6.3-kb 2μ circle found in many yeast strains (Broach, 1981). In addition, the restriction map generated by standard procedures for the chromosomal insert of pPM5115 (**Figure 4–8**) contained common restriction sites with the computer-generated restriction map of the 2μ circle. Since the plasmid pPM5115 neither complemented the *blm5-1* mutation in homozygous diploid cells nor relieved the temperature sensitive phenotype of these cells, its complementing activity in haploid strains was not pursued.

b. pPM5116 and pPM5120.

The sequences of the inserts of pPM5116 and pPM5120 were 100% homologous to sequences on chromosome I of yeast. The DNA sequences on the left and right ends of the insert of pPM5116 defined a 5.8-kb region of chromosome I carrying the complete coding sequence of a single gene, *TFC3* (**Figure 4-9, A**).

The TFC3 gene, previously cloned and characterized (Lefebvre et al. 1992), encodes the large 138 kDa B-block binding subunit of transcription factor III C (TFCIII) of yeast. *TFC3* is a single copy gene that is essential for cell viability and is involved in tRNA and 5 sRNA synthesis. The pPM5116 plasmid insert also carried an 800-bp fragment of *CENI* on one side of the *TFC3* gene and a 131-bp fragment of the C-terminal end of the *VPS8* gene on the other side of the *TFC3* gene. The *VPS8* gene was also cloned and characterized (Robinson et al. 1988 and Chen et al. 1996), and was shown to be involved in vacuole biogenesis and protein sorting in yeast. A comparison between the restriction map generated by standard procedures for the insert of pPM5116 and a computer generated restriction map of the 5.8 kb region identified on chromosome I showed that the location of all restriction sites on the two maps were identical.

DNA sequences on the left and right ends of the insert of pPM5120 identified an 8.3-kb region of chromosome I that included 1.2-kb of overlapping sequences with the insert of pPM5116 (**Figure 4-9, B**). The complete coding sequences of two genes, the *VPS8* gene and a gene, *TEF5* (also called *EF-1β*, Hiraga et at 1993), which encodes the β-subunit of the elongation factor 1 (EF-1), were present on the pPM5120 chromosomal insert. Also present on the insert were 1,076 bp of the N-terminal end of the gene *SSA1* (Ingolia et al. 1982) that encodes a heat shock protein and 1,020 bp of the C-terminal end of *TFC3* (**Figure 4-9,** A).

c. pPM5121 and pPM5122.

The sequences on the left and right ends of the inserts of the plasmids pPM5121 and pPM5122 were 100 % homologous to chromosome IV of *S.*

cerevisiae (**Figure 4–10, 1A**). However, no overlapping sequences were found between the inserts of the two plasmids. The pPM5121 insert encompassed an 8.2-kb region of chromosome IV that carried the full coding sequence of the gene, *VPS3,* which was shown to be involved in vacuole biogenesis and protein sorting (Raymond et al. 1990). Four other open reading frames (ORFs) designated YDR491C, YDR492W, YDR493W and YDR494W were identified on the insert of pPM5121. The functions of these ORFs are unknown. The pPM5121 insert also carried a 1.1-kb fragment of the ORF YDR490C, which is believed to encode a probable protein kinase.

The insert of pPM5122 (**Figure 4–10, 2A**) contains the complete coding sequence for the gene, *PEP7* which encodes a protein that functions in vacuole biogenesis and protein sorting (Webb et al. 1997). The insert also includes the ORF, YDR324C, whose coding sequence bears similarity to the sequence of a G protein beta subunit, a 321-bp fragment of the ORF YDR322W and a 2.5-kb fragment of the ORF YDR325W. The functions of YDR322W and YDR325W are currently unknown.

6. Identification of genes that complement the *blm5-1* mutation on recombinant plasmids.

a. The *blm5-1* complementing activity of *TFC3.*

The plasmid pPM5116 carrying the *TFC3* gene conveyed weak complementing activity to the *blm5-1* mutation in haploid strains (**Table 4-1**). Since pPM5116 was a recombinant plasmid of YEp24, it was possible that the complementing activity associated with pPM5116 resulted from multiple copy suppression of the *blm5-1* mutation. To test this, the yeast strain yOL8α (kindly donated by Drs. Olivier Lefebvre and Andre Sentenac, CEA-Centre d'Etudes de Saclay, Gif-sur-Yvette Cedex, France), carrying a *tsv115* mutation in the *TFC3* gene (Lefebvre et al. 1994) was obtained and screened for comparable hypersensitivities to bleomycin treatment as the *blm5-1/blm5-1* mutant CM-1492. The bleomycin sensitivity of yOL8α was also compared with that of the normal *BLM5/BLM5* strain CM-1489, the *BLM5/blm5-1* strain CM-1477 and the *TFC3* strains YPH499 and YPH500. *In addition, yOL8α transformed either with the multiple copy plasmid* vector pFL44L or the plasmids pLR57 and pLR30 was screened for bleomycin hypersensitivity. The latter two plasmids (also donated by Dr. O. Lefebvre) were recombinant plasmids of pFL44L and carried the *TFC3* or the *BRF1* genes, respectively. The *BRF1* gene (also called *TDS4* and *PCF4*) encodes a 70-kDa subunit of the transcription factor TFIIIB and in high copy number the gene suppresses the *tsv115* mutation of *TFC3* (Lefebvre et al. 1994).

There were significant differences in the amount of growth shown by the *blm5-1/blm5-1* mutant and the *tsv115* mutant in the presence of bleomycin. Unlike the *blm5-1/blm5-1* mutant which did not grow in the presence of a bleomycin dose of 3 μg/ml (**Figure 4-11**), the *tsv115* mutant and both *TFC3* strains showed strong growth in the presence of drug doses as high as 20 μg/ml (**data not shown**). In fact, the bleomycin resistant phenotypes of the strains yOL8α, YPH499 and YPH500 were comparable with the *BLM5/BLM5* and *BLM5/blm5-1* strains. The *blm5-1/blm5-1* mutant was significantly more hypersensitive to the lethal effects of bleomycin than the *tsv115* mutant.

The *tsv115* strain yOL8α and the yOL8α strain carrying the empty pFL44L vector showed slightly less growth in the presence of a drug dose of 30 μg/ml than yOL8α bearing the plasmids pLR57 or pLR30 (**Figure 4-11**). This result suggests that multiple copies of the *TFC3* or the *BRF1* genes weakly enhanced the growth of the *tsv115* mutant in the presence of bleomycin. Thus, it was concluded that the weak *blm5-1* complementing activity associated with the plasmid pPM5116 in mutant haploids is likely the result of enhanced growth in the presence of multiple copy expression of the *TFC3* gene. Similar conclusions were drawn from an independent study conducted on *blm5-1* mutant haploids by Dr. O. Lefebvre.

b. The identity of the *blm5-1* complementing gene of pPM5120.

The pPM5120 plasmid was subcloned to identify the sequence involved in *blm5-1* complementing activity. The *SSA1* gene fragment was excised from pPM5120 following digestion with the restriction enzyme *Sal*I (**Figure 4-9**). Self-ligation of the larger fragment maintained the entire *TEF5* and *VPS8* genes in the smaller plasmid (pM5120-1) thus created (**Figure 4–9, B**). A second plasmid, pM5120-5, was similarly created by self-ligating the large fragment resulting from the digestion of pPM5120 with *Nhe*I. The *Nhe*I digestion removed most of the *VPS8* gene from pM5120-5, and left the *SSA1* gene fragment, the *TEF5* gene, and 750-bp of the N-terminal region of *VPS8*.

Three homozygous *blm5-1/blm5-1* diploids, CM-1489, CM-1492 and CM-1493, were transformed with the plasmids pM5120-1 and pM5120-5. Positive and negative controls were introduced by transforming mutant diploids with the original complementing plasmid pPM5120 and with the YEp24 vector. Following transformation, cells were allowed to recover either at 30°C or at 37°C in selective media lacking uracil. Several Ura^+ transformants carrying either pPM5120 or pM5120-1 were obtained at both 30°C and 37°C (**Table 4-2**). However, transformants carrying either the pM5120-5 or YEp24 plasmids were obtained only at 30°C. These results suggested that the sequences responsible for relieving the temperature-sensitive phenotype of *blm5-1/blm5-1* diploids were retained in pM5120-1 but were eliminated in pM5120-5.

The ability of selected transformants to survive both bleomycin treatment and the elevation of temperature to 37°C was compared with that of homozygous normal and *blm5-1/blm5-1* diploids and with mutant diploids transformed with YEp24. As expected, transformants carrying YEp24 showed the same level of hypersensitivity to bleomycin treatment as untransformed *blm5-1/blm5-1* diploids (**Figure 4-12**). Mutant cells and cells harboring the YEp24 plasmid were also incapable of growth at 37°C. Transformed mutants carrying either the original complementing plasmid pPM5120 or the subclone pM5120-1 maintained an ability to grow at 37°C. These transformants also showed similar levels of growth on solid bleomycin-containing media and their growth was comparable with that of *BLM5/BLM5* diploids at both 30°C and 37°C (**Figure 4-12**). These data suggested that either the *TEF5* or *VPS8* gene, the complete coding sequences for which are both present on the plasmids, was involved in rescuing *blm5-1* mutant phenotypes in mutant diploids. Transformants carrying pM5120-5 did not grow when temperatures were elevated to 37°C and they showed similar hypersensitivities to bleomycin treatment at 30°C as untransformed *blm5-1/blm5-1* diploids or mutant diploids bearing YEp24 (**Figure 4-**

12). The plasmid pM5120-5 carries the complete coding sequence for *TEF5* but only the N-terminal region of *VPS8*. These results suggested that the region involved in rescuing *blm5-1/blm5-1* cells was not present on pM5120-5, and that the *VPS8* gene was responsible for the *blm5-1* complementation observed in cells transformed with pPM5120 or pM5120-1.

c. Subcloning of pPM5121 and pPM5122.

Experiments are in progress to determine if the vacuole biosynthetic genes found on the plasmids pPM5121 and pPM5122 (*VPS3* and *PEP7*, respectively, **Figure 4-10**) contribute to the *blm5-1* complementing activity observed in transformed diploids. The plasmid pM5121-1 has been created by self-ligating the larger (11.3-kb) of the two fragments that resulted from the digestion of pPM5121 with *Bam*HI (**Figure 4-10, 1B**). The plasmid pM5121-1 carries the complete coding sequence of *VPS3* and 250-bp of the C-terminal of the ORF *YDR494W*. The plasmid pM5121-4 (**Figure 4-10, 1B**) is currently being constructed by ligating the 4.6-kb *Bam*HI - *Bam*HI fragment of pPM5121 into the *Bam*HI site of the YEp24 plasmid vector.

The plasmids pM5121-1, pM5121-4 and pBJ4272 (kindly provided by Dr. Elizabeth Jones, Carnegie Mellon University, Pittsburgh, PA) will be screened for their abilities to complement the *blm5-1* mutation in homozygous mutant diploids. The plasmid pBJ4272 was created by ligating the 3.9-kb *Sal*I – *Sal*I fragment carrying the *PEP7* gene and 1.6-kb of the N-terminal of the ORF *YDR324C* into the *Sal*I site of the YEp24 plasmid vector (**Figure 4-10, 2B**).

PART 5: Locating the *BLM5* gene of *S. cerevisiae.*

1. The 2 μ mapping method.

Mapping of genes to chromosomes in yeast is a difficult process that may be complicated by the number of chromosomes present in the organism and by the frequency of meiotic crossing over events. Mapping the *BLM5* gene was considered to prove even more difficult in the absence of a cloned gene fragment and because of the reduced viability of segregants of four spored asci from heterozygous diploids. A chromosomal mapping procedure developed by Wakem and co-workers (1986) was adapted and used to map the *BLM5* structural gene in an attempt to circumvent some of these difficulties. This procedure does not require a cloned gene fragment and would eliminate 15 of the 16 chromosomes of yeast, thus significantly reducing the number of crosses needed to identify the location of a single gene.

The mapping procedure of Wakem and co-workers utilizes the unique properties of the 2 micron (2μ) plasmid. This plasmid is an extrachromosomal DNA fragment that is a normal resident in wild-type yeast strains (called cir$^+$ strains). The 2μ plasmid possesses a *FLP* "flip" gene, whose product mediates site-specific recombination events between different 2μ plasmids that may be present in the same cell (Falco et al. 1983). Wakem and co-workers constructed plasmids carrying 2μ DNA sequences but excluded the *FLP* gene. The 2μ-plasmid construct also carried fragments of the YEp24 vector, the yeast *URA3* gene and segments of the bacterial cloning vector pBR322. Chromosomal DNA fragments taken from the centromeric region of each chromosome were also cloned into different 2μ-plasmid constructs. These researchers reasoned that if homologous recombination occurred between their plasmids and centromeric regions of homology, the plasmid would be stably maintained in the absence of a functional *FLP* gene within the chromosomes of cir^o yeast strains (strains lacking a resident 2µ plasmid). If these cir^o haploids (chromosome tester strains) were crossed to cir$^+$ haploids, the *FLP* function would be reestablished in the cir^o/ cir$^+$ diploids formed. Site-specific recombination may then occur between the 2µ chromosomal integrant and the incoming 2µ plasmid causing the integrated plasmid to "flip out" of its site on the chromosome. In so doing, regions of the chromosome around the site of plasmid integration are lost. If such recombination events occur, because the plasmid was integrated at the centromere of the chromosome donated by the cir^o strain, this chromosome is lost during subsequent mitotic division. Mutations carried on the remaining homologous chromosome of the cir$^+$ parent would then be revealed in the monosomic diploid. Standard meiotic mapping procedures could then be used to locate the mutation on the chromosome identified.

a. Frequency of the chromosomal loss events.

To test the rationale of the proposed method of Wakem and co-workers and the frequency of the chromosomal loss event, an attempt was first made to accurately assign certain genes to their known chromosomal locations. To do this, the cir$^+$ strains CM1069-40, CM1401-5C and CM1403-12D **(Table 1)** were crossed to the cir^o strain B-7180 **(Table 5-1)**. There were differences in the genetic backgrounds of each of the three-cir$^+$ strains, but each carried a mutation in the *ADE2* gene, which is located on chromosome 15. The strain B-7180 is the chromosome 15 tester strain, therefore, it was expected that chromosome 15 would be lost in any monosomic diploids that were formed from each of the three crosses.

Chromosomal loss events were easily detected with each of the three crosses (on SD media made selective against adenine auxotrophs) because such events were accompanied not only by a lack of growth of the monosomic diploid but also because all cells showing reversion to adenine auxotrophy turned red. This color change also enabled the detection of chromosomal loss events that occurred after cells were diluted and plated onto YPAD in preparation for velvet replica plating onto selective media. Such chromosomal loss events appeared as sectored colonies, where fractions of a colony turned red. The size of the sectors varied, depending upon the point during colony formation when the chromosomal loss event occurred.

The frequency of loss of chromosome 15, and consequently the loss of the *ADE2* gene, in each of the three cir$^+$ strains crossed to B-7180 was surprisingly low and remarkably different **(Table 5-2)**. Chromosome 15 was lost with the highest frequency from the diploid CM-1454 (6.08%) when B-7180 was crossed to CM1401-5C (a *blm1-1* mutant). The frequency of loss of chromosome 15 observed in the diploid CM-1456 (B-7180 crossed to the normal haploid CM1069-40) was 5.64%. In the diploid CM-1438, however, when the *blm5-1* mutant CM1403-12D was crossed to B-7180, the frequency of loss of chromosome 15 was only 0.7%. Thus, the frequency of loss of any one chromosome seemed to vary significantly in strains of different genetic backgrounds.

Crossing CM1401-5C to different cir^0 strains tested the frequency of loss of different chromosomes from cells with the same genetic background. CM1401-5C is auxotrophic for the isoleucine-valine gene (*ilv1-92*) which lies on chromosome 5, and for the tryptophan gene (*trp5-27*) which lies on chromosome 7. The chromosome 5 tester strain, B-7590, and the chromosome 7 tester strain, B-7173, were both crossed to CM1401-5C. Therefore, the expected chromosomes lost from monosomic diploids resulting from these crosses were chromosomes 5 and 7 respectively. There was no color change to aid in the detection of any chromosomal loss events that may have occurred in monosomic diploids formed from these crosses. It was therefore impossible to detect the sectoring observed for chromosomal loss events that occurred after the 25 generations of growth, when the cells were diluted and plated on YPAD medium in preparation for velvet replica plating. The frequencies at which chromosomes 5 and 7 were lost from the diploid strains created were very low **(Table 5-2)**. The frequency of loss of chromosome 5 from diploid CM-1444, (CM1401-5C crossed to B-7590) was 3.13%. The frequency of loss of chromosome 7 from the diploid CM-1446, when CM1401-5C was crossed to B-7173, was 1.23%. These frequencies were both lower than that observed for the loss of chromosome 15 in the diploids CM-1454 and CM-1456, and higher than the frequency of loss of chromosome 15 from the diploid CM-1438. That is, disparate frequencies of loss of each of the three chromosomes were discovered **(Table 5-2)**.

b. Identification of the yeast chromosome bearing *blm5-1* complementing activity.

The chromosomal assignment method used here would only work efficiently if the cir° haploid strains involved in each cross were more resistant to bleomycin treatment than the *blm5-1* test strain. The cir° strains were therefore screened on bleomycin-containing (0 μg/ml to 30 μg/ml) SD media for drug sensitivities that were comparable with the normal *BLM5* haploids CM1069-40 and CM1403-12A, but less than the *blm5-1* haploid CM403-12D used in the study. The growth of the normal haploids appeared unaffected on solid SD media containing 30 μg/ml bleomycin. However, the growth of the *blm5-1* haploid was severely reduced at a drug dose of 5 μg/ml and the mutant did not grow in the presence of drug doses of 10 μg/ml and higher, demonstrating the extreme sensitivity of the *blm5-1* mutant to killing by bleomycin. All cir° strains displayed consistently higher levels of growth in the presence of bleomycin than the *blm5-1* haploid. The strains B-7596, B7588 and B-7589 did not grow on SD media containing the highest bleomycin dose (30 μg/ml). All other cir° haploids were unaffected by the presence of this high drug dose.

To find the chromosome bearing complementing activity to the *blm5-1* mutation, CM1403-12D was crossed to each of the 16 cir° tester strains **(Table 5-3)**. Diploids were isolated and grown on non-selective YPAD media as previously described to allow chromosomal loss events to occur. After chromosomal loss, velvet replica plating onto SD media containing 50 μg/ml of bleomycin, identified monosomic diploids that were hypersensitive to bleomycin. This high drug concentration was used to increase the selection pressure and improve the likelihood that only bleomycin-sensitive monosomic diploids would be selected.

It was expected that monosomic diploids that were hypersensitive to bleomycin would result from only one of the 16 cir$^+$/cir° diploid strains. However, 13 of the 16 cir^+/cir^o diploid strains showed low frequencies of chromosomal loss which produced monosomic diploids that were sensitive to bleomycin **(Table 5–4).** Each of the monosomic diploids isolated was therefore rescreened over a range of bleomycin concentrations (0 µg/ml to 30 µg/ml). All monosomic diploids screened in this manner showed high levels of growth in the presence of the highest bleomycin concentration (30 µg/ml) except the monosomic diploids resulting from the cross between the *blm5-1* mutant, CM1403-12D and the chromosome 16 tester strain B-7598. Monosomic diploids resulting from this cross displayed hypersensitivities to bleomycin that were within the same range as the *blm5-1* mutant segregants CM1403-12C and CM1403-12D **(Figure 5-1).** This result suggested that the *BLM5* structural gene was located on the yeast chromosome XVI.

2. Meiotic mapping and tetrad analysis.

The four-spored product (tetrad) of meiotic division in *S. cerevisiae* represents a single meiotic event. When released, the segregation patterns of heterozygous markers among the four haploid segregants can be used to identify the chromosomal location of mutations. Additionally, genetic analyses of crossover events between heterozygous markers in each tetrad are useful in determining the order and distance between genes on the same chromosome and their positions relative to the centromere. In a heterozygous cross between two genes, A and B, if no crossover events occurred only parental ditypes (PD: example AB, AB, ab, ab) will be observed within each of the tetrads examined. If one crossover event has occurred, then the tetrads formed are all tetratypes (T: AB, Ab, aB, ab). Multiple crossover events will result in the formation of three kinds of tetrads. These include non-parental ditypes (NPD: Ab, Ab, aB, aB), as well as PD and T tetrads.

The ratio of PD: NPD: T tetrads can be used to determine if two genes are linked to each other on the same chromosome or if they are linked to the centromere (Spencer and Spencer, 1996, and Sherman and Wakem, 1991). When two genes are tightly linked on the same chromosome, the ratio of PD: NPD tetrads formed is >1: <1, and T tetrads are not produced. A PD: NPD: T ratio of 1: 1: 4 is indicative of the independent or random assortment of genes that either lie on different chromosomes or are widely separated on the same chromosome. Genes on different chromosomes where at least one is linked to the centromere have a PD: NPD: T ratio of 1: 1: <4. The number of crossover events per chromatid can also be used to calculate the map distance (measured in units called centiMorgans [cM]) between two genes using the formula:

$$\mathrm{cM} = 100/2\ ([\mathrm{T} + 6\ \mathrm{NPD}]/[\mathrm{PD} + \mathrm{NPD} + \mathrm{T}])$$

(Spencer and Spencer, 1996, and Sherman and Wakem, 1991). The accuracy of this formula decreases as map distances increase beyond 35 cM. Map distances greater than 35 cM up to approximately 75 cM can be corrected using the formula:

$$\mathrm{cM}_{(\mathrm{corrected})} = \frac{(80.7)(\mathrm{cM}) - (0.883)(\mathrm{cM})^2}{83.3 - \mathrm{cM}}$$

(Sherman and Wakem, 1991). Additional crosses are required if map distances greater than 75 cM are obtained.

To determine the map position of the *blm5-1* mutation on chromosome XVI, several *blm5-1* mutant haploids were crossed to strains bearing mutations in the *rad1* and *aro7* genes (**Table 2**). These mutations map to positions on opposite sides of the centromere of chromosome XVI of *S. cerevisiae*, and the *rad1* gene is linked to the centromere (Sherman and Fink, 1991 and Genbank accession No. M15435). The *blm5-1* mutation was complemented in heterozygous diploids formed by separate crosses with strains bearing either the *rad1* or *aro7* mutations. This suggested that the *blm5-1* mutation was not the same as the *rad1* or *aro7* mutations.

Strains bearing mutations in the *RAD1* gene are unable to repair UV-induced DNA damage and are therefore sensitive to UV exposure. Mutations in the *ARO7* gene convey auxotropies to the aromatic amino acids. Following sporulation of heterozygous diploids and dissection of tetrads, segregants were screened for their hypersensitivities to the lethal effects of bleomycin and UV and tested for their ability to grow in the absence of aromatic amino acids. Analyses of sixteen tetrads resulting from heterozygous diploids carrying single copies of the *blm5-1* and *rad1* mutations showed a PD: NPD: T ratio of 4: 3: 9. This ratio was similar to that expected for the segregation of widely separated or unlinked genes where one gene is centromere-linked. A map distance of 84.4 cM was calculated for the *blm5-1* and *rad1* mutations, but this did not identify the chromosomal location of the *blm5-1* mutation relative to the centromere of chromosome XVI. Nine tetrads analyzed from heterozygous diploids with single copies of the *blm5-1* and *aro7* mutations gave a PD: NPD: T ratio of 1: 0: 8. This ratio suggested that the *blm5-1* and the *aro7* mutations were some distance apart. The map distance calculated between the two mutations was 47.41 cM.

The map distances calculated between the *blm5-1* mutation and the *rad1* or *aro7* mutations respectively, suggested that if the *blm5-1* mutation is on chromosome XVI it lies within a region of approximately 20.47-cM from the *ARO7* gene. To verify the chromosome XVI location and identify a single locus for the *blm5-1* mutation, a *blm5-1* haploid, CM1403-12D, was crossed to a strain, GR67-6, bearing a mutation in the *CDC67* gene (**Table 2**). The *cdc67* mutation was selected for this cross not only because of its relative proximity to the *aro7* mutation on chromosome XVI, but also because it was positioned within the 20.47-cM region presumed to carry the *BLM5* gene. It was therefore expected that the map distance calculated between the *blm5-1* and *cdc67* mutations would be very small. Strains carrying the *cdc67* mutation are cell division cycle mutants, and unlike *blm5-1* haploids, they do not grow when temperatures are elevated to 37°C. Heterozygous diploids carrying one copy each of the *blm5-1* and *cdc67* mutations were sporulated, tetrads dissected and segregants were screened for bleomycin hypersensitivities and their abilities to grow at 37°C. Of 17 tetrads analyzed in this manner, the ratio of PD: NPD: T tetrads obtained was 0: 4: 13. The excess number of NPD and T tetrads was unexpected and suggested that the *blm5-1* and *cdc67* mutations were in fact unlinked or widely separated. The map distance calculated between the two mutations was 108.8 cM. Thus, in spite of the evidence obtained using the 2μ mapping procedure of Wakem and co-workers, this cross clearly demonstrated that the *blm5-1* mutation was not located on chromosome XVI of yeast.

DISCUSSION

1. Bleomycin cytotoxicity is not related to increased drug accumulations in *blm5-1* cells.

Several methods have been described which may control bleomycin cytotoxicity. One method described by Jani and co-workers (1992a and 1992b) showed that reductions in cellular toxicity were usually accompanied by decreased accumulations of the drug within the cell. We therefore examined the accumulations of [S-*methyl*-^{3}H]bleomycin in *blm5-1* mutant cells. This was done by comparing the amount of radioactive bleomycin associated with normal and mutant cells during drug treatment with the amount of radioactive material released from treated cells over time. Since *blm5-1* mutants displayed elevated sensitivities to the lethal effects of bleomycin, we expected to see high accumulations of the drug in mutant cells during treatment. Surprisingly, *blm5-1* mutants were shown to associate extremely low quantities of the drug in comparison to normal cells. It was possible to draw two conclusions from these results. First, the drug's entry into the cell may be partially inhibited, or second, the drug enters the cell and is rapidly expelled. Comparisons between the timed release of radioactive material from normal and *blm5-1* cells after treatment with [S-*methyl*-^{3}H]bleomycin showed the fraction of the associated radioactivity initially released from normal cells was significantly greater than that released from mutant cells. However, there was little difference in the final fraction of associated radioactivity released from normal and *blm5-1* cells. Thus, if bleomycin molecules were rapidly expelled after entry into *blm5-1* mutant cells, it is likely that some intracellular modifications of the drug would not have occurred (exception might include enzymatic conversion of the drug). Similarly, bleomycin molecules that did not enter the cell would have remained unchanged. Differentiation between each of these molecules would have been difficult under the conditions used in this study. Nevertheless, the lack of association of high levels of bleomycin molecules coupled with the final release of comparable fractions of radioactive material from normal and *blm5-1* cells led to the conclusion that the hypersensitivity of *blm5-1* mutants to bleomycin treatment must result from something other than increases in intracellular drug concentrations.

2. Double-strand breaks in chromosomes are repaired as synchronized global events in the cell.

Bleomycin cytotoxicity is also reduced in different cell types with increased levels of DNA repair (Begleiter et al. 1992). The ability of *BLM5/BLM5*, *BLM5/blm5-1* and *blm5-1/blm5-1* strains to repair double-strand breaks in DNA was therefore examined. The use of PFGE made it possible to analyze the induction and repair of double-strand breaks through variations in the patterns of chromosomal degradation and regeneration over time. Double-strand breaks were introduced into the chromosomes of untreated *BLM5/BLM5* and *BLM5/blm5-1* strains during IPCD incubations at 30°C and 37°C. The induction of such spontaneous breaks may be the result of the age of the cells, extended incubations in non-growth media or part of the normal repair processes after aberrations in the DNA are recognized by the cell. If the accumulation of spontaneous breaks was due to the age of the cell or to extended incubations, then the number of breaks should increase with time. Instead, maximum accumulations were observed within the first 30 minutes of IPCD incubations in the *BLM5/BLM5* and *BLM5/blm5-1* strains. Additionally, with the exception of the *BLM5/BLM5* strain where breaks accumulated only within the first 30 minutes of IPCD incubations at 30°C, breaks generally accumulated and disappeared periodically throughout the 48 hours of IPCD incubation at 30°C and 37°C. These observations suggested that the accumulation of double-strand breaks may be part of the DNA repair process, perhaps serving as a trigger to turn on repair processes in the cell once a threshold accumulation of spontaneous double-strand breaks is reached. Since cells accumulated more breaks at 30°C rather than at 37°C, the threshold may be higher at 30°C.

Double-strand breaks introduced spontaneously into the chromosomes of the *BLM5/BLM5* and *BLM5/blm5-1* strains appeared to be repaired at both 30°C and 37°C. The accumulation of breaks in the chromosomes also suggests that individual breaks may not be recognized and repaired as independent events within the cell. Instead there may be periods of global monitoring and recognition of DNA damage followed by the synchronized global processing and repair of the damage.

Spontaneous chromosomal double-strand breaks did not accumulate to levels that could be detected in pulsed-field gels in the *blm5-1/blm5-1* strain except after 48 hours at 30°C. Three possibilities may explain this lack of detectable accumulations of double-strand breaks in the *blm5-1/blm5-1* strain. First the system responsible for monitoring the DNA and/or recognizing aberrations may be defective in this strain. Second, it took longer for DNA aberrations to be recognized in the *blm5-1/blm5-1* strain than in either the *BLM5/BLM5* or *BLM5/blm5-1* strains so repair processes in the *blm5-1/blm5-1* strain were turned on later than in the other two strains. Third, aberrations in the DNA were recognized and repaired too quickly to be detected under the experimental conditions used.

3. Differences in the susceptibilities of *BLM5/BLM5*, *BLM5/blm5-1* and *blm5-1/blm5-1* strains to bleomycin reflect differences in their abilities to repair double-strand breaks at 30°C and 37°C.

The *BLM5/BLM5*, *BLM5/blm5-1* and *blm5-1/blm5-1* strains displayed different susceptibilities to the lethal effects of bleomycin. After treatment with low bleomycin doses (0.2 μg/ml) it was not possible to detect chromosomal double-strand breaks in the *BLM5/BLM5* strain, and the *BLM5/blm5-1* strain showed fewer breaks than the *blm5-1/blm5*-1 strain. There are two possibilities that may account for this observation. First, of the three strains, the *BLM5/BLM5* strain was the least susceptible to bleomycin-induced chromosomal double-strand breaks. Second, the *BLM5/BLM5* strain was significantly more proficient in the repair of double-strand breaks then either the *BLM5/blm5-1* or the *blm5-1/blm5-1* strains, and these breaks must be repaired rapidly, perhaps during the 30-minute treatment period, thus making them undetectable in pulsed-field gels.

Cleavage of chromosomes by low bleomycin treatment doses occurs preferentially at actively transcribed sites in the DNA within the linker region between two nucleosomes, and is limited by the drug's accessibility to the G-C base pairs it recognizes (Kuo, 1981 and Mir et al. 1996). It is unlikely that either of the *BLM5/BLM5*, *BLM5/blm5-1* and *blm5-1/blm5-1* strains tested here possess significantly more recognizable G-C base pairs or actively transcribed regions than the other because of the genetic relationship among the three strains. In addition, although the number of chromosomal double-strand breaks was shown to increase in each of the strains tested as bleomycin doses were increased, attempts made to introduce equivalent numbers of detectable breaks in each strain by varying drug treatment doses were unsuccessful because *BLM5/BLM5* cells were much more tolerant of DNA damage than *BLM5/blm5-1* and *blm5-1/blm5-1* cells. It is likely that the *BLM5/BLM5* cells are more efficient in the repair of bleomycin-induced double-strand breaks in chromosomes than either the *BLM5/blm5-1* or the *blm5-1/bml5-1* strains and that this repair begins during drug treatment.

4. Global repair of bleomycin-induced double-strand breaks requires cycles of chromosomal degradation and regeneration.

The migration patterns of chromosomal bands and degraded chromosomal DNA in several pulsed-field gels suggested that the global repair of DNA double-strand breaks induced by bleomycin occurred in a series of steps or cycles involving the sequential degradation and regeneration of chromosomal bands. This was unexpected because repair was considered complete when whole chromosomes were regenerated. The probability that all damage was not removed from chromosomal DNA during the first round of chromosomal degradation and regeneration was considered. If this were true, subsequent rounds of degradation would be required to remove the remaining damage or the cell's ability to replicate its DNA would be limited. Additionally, each successive round of chromosomal degradation and regeneration would be expected to result in progressively higher levels of cell survival. It was noted, however, that double-strand breaks induced by the cell during subsequent rounds of chromosomal degradation seemed more extensive than expected for the removal of residual amounts of unrepaired damage. Furthermore, studies conducted on the same cell populations used to follow repair processes showed that cell survival mirrored the cyclic nature of chromosomal degradation and regeneration, decreasing when chromosomes were degraded and increasing when they were regenerated. Thus, changes in cell survival were considered to directly reflect repair processes in the cell even when slight changes in the intensities of chromosomal bands could not be detected in pulsed-field gels. These observations suggested that DNA replication was not inhibited in surviving cells after chromosomes were regenerated and mitotic division occurred normally. Therefore it seemed unlikely that damage was still present in regenerated chromosomes and the purpose for continued rounds of chromosomal degradation and regeneration after bleomycin-induced damage was repaired remains a mystery.

5. The rate of double-strand break repair influenced bleomycin sensitivities in *BLM5/BLM5*, *BLM5/blm5-1* and *blm5-1/blm5-1* strains.

Bleomycin hypersensitivity and cell survival are usually linked with the accumulation of double-strand breaks in the cell (Umezawa et al. 1966, and 1972; Povirk, 1983; Mir et al. 1996). Experiments conducted here agreed with these findings, but also suggested that the rate at which breaks were repaired influenced the accumulations of double-strand breaks and hence cell survival after bleomycin treatment. The *BLM5/BLM5*, *BLM5/blm5-1* and *blm5-1/blm5-1* strains used in this study all demonstrated some proficiency in the global repair of bleomycin-induced chromosomal double-strand breaks. However, there were notable differences in the amount of time required by the each strain to regenerate whole chromosomes. It seemed unlikely that significantly different numbers of breaks were introduced into each strain as a function of bleomycin treatment, and rather that there were variations in the rate at which double-strand breaks were repaired in the three strains.

High levels of chromosomal degradation coupled with low levels of cell survival were never detected in *BLM5/BLM5* cells following treatments with low bleomycin doses. However, severely degraded chromosomes and low cell survival were observed in the *BLM5/blm5-1* and *blm5-1/blm5-1* strains after treatment with similar drug doses. Additionally, regeneration of chromosomal bands accompanied by increased cell survival occurred earlier in the *BLM5/blm5-1* than in the *blm5-1/blm5-1* strain. Thus, it appeared that of the three strains, the *BLM5/BLM5* strain had the fastest rate of repair followed by the *BLM5/blm5-1* strain, and the *blm5-1/blm5-1* strain exhibited the slowest rate of repair. The rapid repair rate of the *BLM5/BLM5* strain may have prevented high accumulations of double-strand breaks after bleomycin treatment whereas breaks accumulated in the *BLM5/blm5-1* and *blm5-1/blm5-1* strains where repair appeared to proceed at slower rates. The *BLM5/BLM5* strain was therefore capable of surviving treatments with significantly higher drug doses than the *BLM5/blm5-1* and *blm5-1/blm5-1* strains.

Despite the rapidity of repair in *BLM5/BLM5* cells, double-strand breaks accumulated in all three strains as drug dose was increased and the time required by each strain to regenerate whole chromosomes also increased. When chromosomal damage became excessive, repair processes were ineffective so that chromosomal regeneration did not occur and cell survival decreased. Thus, there appeared to be a distinct relationship among bleomycin hypersensitivities, the accumulation of double-strand breaks and the rate at which these breaks were repaired, and cell survival in the *BLM5/BLM5*, *BLM5/blm5-1* and *blm5-1/blm5-1* strains.

The observations made here led to the theory that the presence of a single copy of the *blm5-1* mutation may be responsible for the reduction in the rate of repair in the *BLM5/blm5-1* strain. Similarly, repair rates may have been further reduced in the *blm5-1/blm5-1* strain because of the presence of two copies of the *blm5-1* mutation. This theory can only be confirmed after several more strains have been screened for repair. Nevertheless, it appeared that the ability of cells to survive bleomycin treatment might not depend only on their ability to repair bleomycin-induced double-strand breaks. The rate at which repair processes are induced may also play a major role in the survival of the cell following bleomycin treatment.

6. The repair of double-strand breaks was affected by elevated temperature in the *BLM5/BLM5*, *BLM5/blm5-1* and *blm5-1/blm5-1* strains.

Chromosomal bands degraded by bleomycin treatment were regenerated at both 30°C and 37°C in the *BLM5/BLM5*, *BLM5/blm5-1* and *blm5-1/blm5-1* strains. However, repair processes appeared to be more efficient at 30°C since regenerated chromosomal bands in the three strains were always stronger at 30°C than at 37°C. In addition to its effect on the efficiency of repair in the *BLM5/BLM5*, *BLM5/blm5-1* and *blm5-1/blm5-1* strains, the elevation of temperature during repair produced different effects on the repair process in each strain. Chromosomes were regenerated earlier at 37°C in the *BLM5/BLM5* strain than at 30°C. This suggested that elevating the temperature to 37°C further increased the rapid rate of repair already noted in the normal strain. Unlike the *BLM5/BLM5* strain, there was a significant reduction in the repair rate in the *BLM5/blm5-1* strain when the temperature was elevated to 37°C. This observation contributed to the theory that repair rates might be reduced when one copy of the *blm5-1* mutation is present as it is in the *BLM5/blm5-1* strain.

The rate of double-strand break repair appeared to be unaffected by the elevation of temperature in the *blm5-1/blm5-1* strain and whole chromosomes were first regenerated at the same time at both 30°C and 37°C. However, the cycles of chromosomal degradation and regeneration associated with repair in the *BLM5/BLM5* and the *BLM5/blm5-1* strains were not observed in the *blm5-1/blm5-1* strain at 37°C. Whole chromosomes were regenerated once at 37°C, then they were continually degraded. Repair processes, therefore, appeared to be inactivated with time by the elevation in temperature to 37°C. Thus, while one copy of the *blm5-1* mutation reduces repair rates in cells, two copies further reduces the rate of repair (at 30°C) and inhibits late repair processes (at 37°C). These conclusions can be verified after repair processes in other strains have been studied.

7. The vacuole biosynthetic gene, *VPS8*, relieved bleomycin hypersensitivity in *blm5-1/blm5-1* strains of yeast.

A gene, *VPS8*, known to function at the junction of the endocytic and vacuole biosynthetic pathways of *S. cerevisiae* (Jones et al. 1997), was shown to complement the *blm5-1* mutation in homozygous mutant diploids. Plasmids bearing two other vacuole biosynthetic genes, *VPS3* and *PEP7* respectively, were also shown to confer *blm5-1* complementing activity. With the evidence that *blm5-1* mutants were hypersensitive to the lethal effects of ionizing radiation and bleomycin, are defective in meiosis, and have impaired repair rates in mitotic cells, this result was unexpected.

The yeast vacuole is similar to the lysosome of mammalian cells and influences many diverse cellular functions, including the modification of proteins manufactured in the endoplasmic reticulum (ER) and the detoxification of drugs (Matile and Wiemken, 1967; Matile, 1978; Wiemken, 1980). Molecules from the extracellular environment or from the ER are trafficked to and from the vacuole in membrane-bound vesicles via the endosome. The products of the *VPS8*, *VPS3* and *PEP7* genes function with other gene products to mediate the fusion of these vesicles onto the endosome. Thus the isolation of the *blm5-1* complementing gene, *VPS8*, and *blm5-1* complementing plasmids carrying the *VPS3* and *PEP7* genes suggested a possible function for the *BLM5* structural gene in vesicular docking and fusion onto the endosome. It also offers a surprising and previously unreported link between vacuolar metabolism and the initiation of repair of chromosomal double-strand breaks. It could be speculated that a protein or proteins functioning in the recognition of double-strand breaks and/or initiation of repair processes must first be modified in the vacuole before it becomes functionally active. Defects in the vacuolar delivery system may therefore result in inadequate amounts of such functionally active repair-recognition/initiation proteins in *blm5-1/ blm5-1* cells hence the apparent delay in the initiation of double-strand break repair in these cells.

It was demonstrated that *blm5-1* mutants accumulate very small quantities of bleomycin during treatment. However, very few molecules of bleomycin are required to cause extensive damage in DNA (Povirk et al. 1989). If bleomycin molecules are inactivated in the vacuole, defects in the vacuolar delivery system could result in inadequate amounts of these molecules becoming inactive. The extreme hypersensitivity of *blm5-1* mutants to the lethal effects of bleomycin could then result from such inadequate inactivation of bleomycin molecules coupled with the delay in the initiation of double-strand break repair in these cells.

Nevertheless, it is possible that the *BLM5* gene might be the *VPS8*, *VPS3* or *PEP7* gene. Alternatively, expression of these vacuole biosynthetic genes could suppress the mutant effect of *blm5-1* during growth at 37°C or in the presence of bleomycin. Studies must be completed to identify if any of the three genes is the same as the *BLM5* structural gene.

8. The *blm5-1* complementing activity of chromosome XVI.

The yeast chromosome XVI was found to carry *blm5-1* complementing activity using the 2μ chromosomal mapping procedure of Wakem and coworkers. Yet standard meiotic mapping procedures revealed that the *BLM5* structural gene was not located on chromosome XVI. Two factors were considered to contribute to these conflicting results. First, the frequency of chromosomal loss events was generally low, and the numbers of monosomic diploids created was highly variable and appeared to be dependent upon which of the sixteen yeast chromosomes was lost in cir^{+}/cir^{o} diploids. For example, chromosome XVI was lost with the highest frequency of any of the other yeast chromosomes, whereas chromosomes I and IV which bear the *VPS8* gene and the *VPS3* and *PEP7* genes, respectively, displayed very low chromosomal loss frequencies (ranging bet. 0% – 0.64%). Thus, the numbers of monosomic diploids available for screening was perhaps insufficient to produce conclusive results for some chromosomal markers.

The low frequency of chromosomal loss events in this mapping procedure has been observed by other researchers (Dr. Rodney Rothstein, Columbia University, N.Y., N.Y. personal communication), and has been attributed to the possible insertion of the 2μ sequences outside of the centromeric region of some chromosomes in marker strains. *FLP*-mediated recombination events occurring between such chromosomally located 2μ sequences and the incoming 2μ sequences in cir$^+$/cir^o diploids may still cause chromosomal breakage. However, of the resulting fragments, one would retain an intact centromere so the fragment may be propagated in the progeny of the aneuploid and genes present on the fragment would be expressed, whereas the genes present on the chromosomal fragment lacking centromeric sequences would be lost.

The second factor believed to contribute to the *blm5-1* complementing activity associated with chromosome XVI is the presence of a gene or genes with *blm5-1* suppressor activity on this chromosome. The identification of *VPS8* as the gene involved in *blm5-1* complementing activity, coupled with the isolation of *blm5-1* complementing plasmids bearing the *VPS3* and *PEP7* genes, led to the theory that chromosome XVI probably carried another gene or genes that functioned with *VPS8*, *VPS3* and *PEP7* in the vacuole biosynthetic pathway. Analyses of the genetic and physical maps of yeast chromosomes recorded in the *S. cerevisiae* database at Stanford revealed that chromosome XVI did indeed carry a gene, *VPS28*

(Jones et al. 1997), which functioned in mediating the docking and fusion of vesicles onto the yeast endosome.

9. Phenotypic comparisons between vacuolar and *blm5-1* mutants.

Like *vps* and *pep* mutants (Jones et al. 1997), *blm5-1* mutants displayed a reduced growth rate during the logarithmic phase of growth and reductions in their ability to sporulate. The *vps* and *pep* mutants were isolated on the basis of their inability to transport the precursors of the vacuolar hydrolases, carboxypeptidase Y (CPY), proteinase A (PrA) and proteinase B (PrB) into the vacuole for modification (Rothman et al. 1989 and Webb et al. 1997). These hydrolase precursors were instead secreted through the plasma membrane in *vps* and *pep* mutants. The current work led to the speculation that the *blm5-1* mutants might secrete bleomycin since *blm5-1* cells associated less drug than *BLM5* cells. It is possible that like vacuolar mutants, the *blm5-1* mutants may also secrete CPY, PrA and PrB, but this has not yet been tested. Thus, although we have not yet determined if the *BLM5* structural gene is the same as *VPS3*, *VPS8*, *PEP7* or another as yet unidentified gene, the existing data suggested the possibility that the *blm5-1* mutant may be a vacuolar mutant. This led to the speculation that bleomycin molecules entering the cell via the endocytic pathway may eventually be inactivated in the vacuole of yeast.

A model was therefore developed to predict the function of the Blm5p as a participant mediating the docking and/or fusion of bleomycin containing vesicles onto the yeast endosome and describe the movement of bleomycin through the cell (**Figure 2**). This model suggests that bleomycin molecules enter the endocytic pathway after binding to receptors on the plasma membrane (Pron et al. 1993 and

1994). The isolation of the *blm5-1* complementing gene, *VPS8*, and *blm5-1* complementing plasmids carrying the *VPS3* and *PEP7* genes allowed the speculation that the drug enters the cell via membrane bound vesicles and is conveyed into the vacuole via the endosome. This laboratory has shown that bleomycin is modified within the cell (manuscript in preparation). Further, it is speculated that vesicle bound bleomycin molecules that do not enter the vacuole in *blm5-1* mutants are returned to the plasma membrane and exocytosed in a manner that is similar to the secretion of CPY, PrA and PrB from vacuolar mutants.

Table 1. List of haploid yeast strains and bacterial strains used in this study.

Strains	Genotype
Yeast strains	
CM1069-40	*MATα ade2-40 trp5-12 ilv1-92 cyc1-45*
CM1234-105	*MATa*
CM1403-12A	*MATa ade2-1* or *ade2-40*
CM1403-12B	*MATa ade2-1* or *ade2-40 trp1-1* or *trp5-12 his3-11,15 ilv1-92*
CM1403-12C	*MATα ade2-1* or *ade2-40 trp1-1* or *trp5-12 his3-11,15 ilv1-92 ura3-1 blm5-1*
CM1403-12D	*MATα ade2-1* or *ade2-40 trp5-12 leu2-3,112 ura3-1 blm5-1*
CM1477-1B	*MATα ade2-1* or *ade2-40 his3-11,15 leu2-3,112 blm5-1*
CM1477-1D	*MATα ade2-1* or *ade2-40 trp1-1* or *trp5-12 leu2-3,112 ura3-1*
CM1477-3C	*MATα ade2-1* or *ade2-40 trp1-1* or *trp5-12 his3-11,15 leu2-3,112*
CM1477-4A	*MATa ade2-1* or *ade2-40 trp1-1* or *trp5-12 leu2-3,112 blm5-1*
CM1477-4B	*MATa ade2-1* or *ade2-40 trp1-1* or *trp5-12 his3-11,15 ura3-1 blm5-1*
CM1477-12B	*MATα ade2-1* or *ade2-40 trp1-1* or *trp5-12 ilv1-92 leu2-3,112 blm5-1*
CM1477-12D	*MATa ade2-1* or *ade2-40 trp1-1* or *trp5-12 ura3-1 ilv1-92*
CM1483-7A	*MATa ade2-1* or *ade2-40 trp1-1* or *trp5-12 his3-11,15 ura3-1 blm5-1*
CM1483-7B	*MATa ade2-1* or *ade2-40 trp1-1* or *trp5-12 his3-11,15 ura3-1 blm5-1*
CM1483-7C	*MATα ade2-1* or *ade2-40 trp1-1* or *trp5-12 leu2-3,112 ura3-1 blm5-1*
CM1483-7D	*MATα ade2-1* or *ade2-40 trp1-1* or *trp5-12 leu2-3,112 ura3-1 blm5-1*
A236-57B	*MATa his3 leu2-3 lys11 met4 trp1 can1 suc2 mal3 aro7*
GR67-6	*MATa ade6 his6 leu1 ura1 cdc67*
2110-222	*MATa ade2-1 lys2 his3 ura1 rad1*
X5194-23C	*MATa ade1 arg4 his2 leu1 lys1 trp1 ura3 rad52-1 aro7*

Table 1. Continued

Strains	Genotype
***E. coli* strains**[a]	
HB101	F^- *mcr*B *mrr hsd*S20 (r_B^-, m_B^-) *rec*A13 *sup*E44 *ara*14 *gal*K2 *lac*Y1 *pro*A2 rpsL20(Sm^r) *xy*15λ^- *leu mt*l1
SURE	*e14*$^-$(*mcr*A) *Δ*(*mcr*CB-*hsd*SMR-*mrr*)171 *sup*E44 *thi*$^-$1 *gyr*A96 *end*A1 *rel*A1 *lac rec*B *rec*J *sbc*C *umu*C::*Tn*5 (*kan*^{r}) *uvr*C (F', proAB, lacρZΔM15, *Tn*10 [*tet*^{r}])

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[a] Maximum efficiency competent *E. coli* cells were purchased when required for use in bacterial transformation procedures. HB101 cells were obtained from Life Technologies Inc. and SURE cells were obtained from Strategene Ltd.

Table 2. List of diploid yeast strains used in this study.

Strains	Parents[a]		
	MATa		*MATα*
CM-1293	CM1234-105	x	CM1069-40
CM-1471	A236-57B	x	CM1403-12D
CM-1477	CM1403-12B	x	CM1403-12D
CM-1483	CM1477-4B	x	CM1403-12D
CM-1487	CM1477-4A	x	CM1477-1D
CM-1488	CM1477-12D	x	CM1477-1B
CM-1489	CM1403-12B	x	CM1477-3C
CM-1490	CM1403-12B	x	CM1477-1D
CM-1491	CM1477-4B	x	CM1483-7D
CM-1492	CM1483-7B	x	CM1483-7C
CM-1493	CM1483-7A	x	CM1483-7D
CM-1505	A236-57B	x	CM1483-7B
CM-1506	X5194-23C	x	CM1483-7B;
CM-1508	A236-57B	x	CM1477-12B
CM-1515	2110-222	x	CM1403-12D
CM-1520	GR67-6	x	CM1403-12D

---

[a] Genotypes of parental strains are shown on TABLE 1.

Table 1-1. Sporulative abilities of *BLM5/BLM5*, *BLM5/blm5-1* and *blm5-1/blm5-1* strains at 30°C and 37°C.

Strains	No. of spores in asci	Total cells counted 30°C	Total cells counted 37°C	% sporulation[a] 30°C	% sporulation[a] 37°C
BLM5/BLM5					
CM-1293	four	69 (3.94%)	33 (2.23%)	37.3	36.4
	three	188 (10.73%)	163 (11.36%)		
	two	397 (22.66%)	333 (23.21%)		
	unsporulated[b]	1098 (62.65%)	907 (63.20%)		
CM-1489	four	120 (8.81%)	4 (0.36%)	16.3	14.0
	three	44 (3.80%)	55 (4.74%)		
	two	43 (3.71%)	92 (7.93%)		
	unsporulated	969 (83.68%)	1008 (86.97%)		
BLM5/blm5-1					
CM-1477	four	92 (6.76%)	0 (0%)	15.4	0
	three	28 (2.06%)	0 (0%)		
	two	90 (6.61%)	0 (0%)		
	unsporulated	1151 (84.57%)	1990 (100%)		
CM-1487	four	11 (0.95%)	0 (0%)	27.7	0
	three	36 (3.24%)	0 (0%)		
	two	266 (23.47%)	0 (0%)		
	unsporulated	819 (72.33%)	1329 (100%)		
CM-1490	four	74 (6.19%)	0 (0%)	29.2	0
	three	134 (11.15%)	0 (0%)		
	two	143 (11.86%)	0 (0%)		
	unsporulated	852 (70.80%)	1050 (100%)		
blm5-1/blm5-1					
CM-1483	four	1 (0.10%)	0 (0%)	1.3	0
	three	5 (0.43%)	0 (0%)		
	two	9 (0.81%)	0 (0%)		
	unsporulated	1117 (97.38%)	1082 (100%)		
CM-1491	four	0 (0%)	0 (0%)	0.2	0
	three	2 (0.16%)	0 (0%)		
	two	1 (0.08%)	0 (0%)		
	unsporulated	1246 (99.76%)	1270 (100%)		
CM-1492	four	3 (0.29%)	0 (0%)	0.7	0
	three	3 (0.29%)	0 (0%)		
	two	1 (0.10%)	0 (0%)		
	unsporulated	1025 (99.32%)	1293 (100%)		
CM-1493	four	2 (0.16%)	0 (0%)	1.0	0
	three	3 (0.24%)	0 (0%)		
	two	7 (0.55%)	0 (0%)		
	unsporulated	1255 (99.05%)	1210 (100%)		

---

[a] Asci with two, three or four spores were included in the fraction of the population that was sporulated.

[b] Unsporulated cells and aborted asci were not distinguished and were not included in the calculation.

Table 1-2. Viability of spores from *BLM5/BLM5*, *BLM5/blm5-1* and *blm5-1/blm5-1* strains.

Strains	No. Tetrads Dissected	Survival of Segregants (% of total tetrads)[a]				
		4	3	2	1	0
BLM5/BLM5 (CM-1293)	13	61.5	23.1	15.5	0	0
BLM5/blm5-1 (CM-1477)	17	11.8	17.7	17.6	47.1	5.9
blm5-1/blm5-1 (CM-1483)	23	9.1	31.8	18.2	40.9	0

Cells were sporulated at 30°C and, after dissection, spores were grown for 7 days at 30°C on solid YPAD.

[a] Only four-spored asci were dissected. Numbers are percent of tetrads with four segregants, three segregants, two segregants, one segregant or no segregants surviving.

Table 2-1. Efflux of radioactivity from *BLM5* and *blm5-1* cells treated with [S-*methyl*-^{3}H]bleomycin A$_2$.

Posttreatment incubation time (Hours)	*BLM5* cells actual efflux (nCi)	*BLM5* cells % of associated bleomycin[a]	*blm5-1* cells actual efflux (nCi)	*blm5-1* cells % of associated bleomycin[a]
1	5.6	10.8	0.1	3.4
2	8.1	15.5	0.2	5.5
3	8.4	16.0	0.5	12.9
14.5	15.4	29.6	0.7	20.2
22	14.0	26.9	0.9	25.1
27	23.5	45.1	1.5	42.2
37	30.7	58.9	2.1	58.8

---

[a] The total amount of radioactivity associated with *BLM5* (CM1403-12A) and *blm5-1* (CM1403-12D) strains following treatment with 33.5-μM [S-*methyl*-^{3}H]bleomycin A$_2$ was determined to be 52.2 nCi and 3.6 nCi respectively. The radioactivity associated with material released during posttreatment incubation was calculated as a percent of the total radioactivity associated with each strain.

Table 2-2. Cytotoxicity of radioactive efflux from *BLM5* and *blm5-1* strains.

Strains	Radioactivity[a] (nCi)	% Survival[b]
BLM5	9.2	11.8
BLM5	6.2	12.8
blm5-1	0.6	45.2
blm5-1	0.7	36.0

---

[a] The radioactivity contained in each treatment was determined by scintillation counts.

[b] A364A yeast cells were treated with 200 μl aliquots of the radioactive efflux from *BLM5* and *blm5-1* strains.

Table 4-1. Summary of the *blm5-1* complementing activity and genes present on plasmids used in this study.

Plasmid [a]	Insert	*blm5-1* [b] Complementation	Yeast Genes or Gene Fragments	Vector
pPM518	not identified	-	not identified	YCp50
pPM5114	none	-	none	YEp24
pPM5115	2μ plasmid	-	none	YEp24
pPM5116	5.8-kb genomic library	+	*VPS8*, *TFC3*	YEp24
pPM5117	none	-	none	YEp24
pPM5118	none	-	none	YEp24
pPM5119y [a]	not identified	++	not identified	YEp24
pPM5120	8.3-kb genomic library	+++++	*SSA1*, *TEF5*, *VPS8*, *TFC3*	YEp24
pM5120-1	6.7-kb *Sal*I fragment of pPM5120	+++++	*TEF5*, *VPS8*	YEp24
pM5120-5	4.4-kb *Nhe*I fragment of pPM5120	-	*SSA1*, *TEF5*	YEp24
pPM5121	8.2-kb genomic library	+++	*VPS3*	YEp24
pPM5122	6.4-kb genomic library	+++	*PEP7*	YEp24
pFL44L [a]	none	-	none	pFL44L
pLR30 [a]	*BRF1*	+	*BRF1*	pFL44L
pLR57 [a]	*TFC3*	+	*TFC3*	pFL44L

---

[a] Plasmids listed here (except for pPM5119y, pFL44L, pLR30 and pLR57) were isolated from Ura$^+$ transformants and amplified in *E.coli*. The *blm5-1* complementing activitiy of pPM5119y was not pursued after selection in yeast. Plasmids pFL44L, pLR30 and pLR57 were kindly donated by Dr. O. Lefebvre.

[b] The degree of *blm5-1* complementation is indicated by the number of "+" symbols if the plasmid relieved bleomycin hypersensitivity in mutant cells, or by a "-" symbol if the plasmid did not relieve bleomycin hypersensitivity.

Table 4–2. Selection of pPM5120 subclone bearing the temperature-resistant gene.

Plasmid	Ura$^+$ Transformants Selected[a]		
	30°C	37°C	Total
pPM5120	50	35	85
pM5120-1	63	44	107
pM5120-5	45	0	45
YEp24	475	0	475

[a] Transformed *blm5-1/blm5-1* cells were plated on selective media lacking uracil and allowed to recover at 30°C or 37°C.

Table 5-1. List of *cir*^{0} chromosome tester strains.

Strain	Genotype	Chromosome tester[a]
B-7588	*MATa cir*^{0} *ura3-52 leu2-3,112 trp1-289 met2*	Chromosome 1
B-7170	*MATa cir*^{0} *ura3-52 leu2-3,112 trp1-289 met2 his3-1 cyhR*	Chromosome 2
B-7171	*MATa cir*^{0} *ura3-52 leu2-3,112 trp1-289 met2 his3-1 cyhR*	Chromosome 3
B-7589	*MATa cir*^{0} *ura3-52 leu2-3,112 trp1-289 met2 his3-1 cyhR*	Chromosome 4
B-7590	*MATa cir*^{0} *ura3-52 leu2-3,112 trp1-289 met2 his3-1 cyhR*	Chromosome 5
B-7591	*MATa cir*^{0} *ura3-52 leu2-3,112 trp1-289 met2 his3-1*	Chromosome 6
B-7173	*MATa cir*^{0} *ura3-52 leu2-3,112 trp1-289 met2 his3-1 cyhR*	Chromosome 7
B-7174	*MATa cir*^{0} *ura3-52 leu2-3,112 trp1-289 met2 his3-1 cyhR*	Chromosome 8
B-7175	*MATa cir*^{0} *ura3-52 leu2-3,112 trp1-289 met2 his3-1 cyhR*	Chromosome 9
B-7593	*MATa cir*^{0} *ura3-52 leu2-3,112 trp1-289 met2 his3-1*	Chromosome 10
B-7178	*MATa cir*^{0} *ura3-52 leu2-3,112 trp1-289 met2 his3-1 cyhR*	Chromosome 11
B-7595	*MATa cir*^{0} *ura3-52 leu2-3,112 trp1-289 met2 his3-1 cyhR*	Chromosome 12
B-7255	*MATa cir*^{0} *ura3-52 leu2-3,112 trp1-289 met2 his3-1 cyhR*	Chromosome 13
B-7596	*MATa cir*^{0} *ura3-52 leu2-3,112 trp1-289 met2 his3-1 cyhR*	Chromosome 14
B-7180	*MATa cir*^{0} *ura3-52 leu2-3,112 trp1-289 met2 his3-1 cyhR*	Chromosome 15
B-7598	*MATa cir*^{0} *ura3-52 leu2-3,112 trp1-289 met2 cyhR*	Chromosome 16

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[a] Dr. Paul Wakem (The laboratory of Dr. Fred Sherman, at The University of Rochester Medical School, Dept. of Microbiology, Rochester, N.Y.) kindly donated Chromosome tester strains used in this study.

Table 5-2. Tests for the loss of different chromosomes.

Diploid	Gene lost[a]	Total No. of Viable cells	No. of colonies unable to grow in the absence of nutritional markers	Frequency of chromosomal loss[b] (%)
CM-1438	*ADE2*	1556	11	0.7
CM-1454	*ADE2*	658	40	6.1
CM-1456	*ADE2*	1188	67	5.6
CM-1444	*ILV1*	734	23	3.1
CM-1446	*TRP5*	567	7	1.2

[a] Chromosomes 15, 5 or 7 were lost in strains that reverted to adenine, isoleucine/valine and tryptophan auxotrophy respectively.

[b] The frequency of chromosomal loss was determined as the percent of viable cells that became auxotropic for the test nutritional marker.

Table 5-3. List of *cir*$^+$/*cir*^{0} diploids created in this study.

Diploid	Parents[a]		Diploid Genotype	Chromosome Lost[b]
	MATa	*MATα*		
CM-1456	B-7180	CM1069-40	wild type	15
CM-1444	B-7590	CM1401-5C	wild type	5
CM-1446	B-7173	CM1401-5C	wild type	7
CM-1454	B-7180	CM1401-5C	wild type	15
CM-1424	B-7588	CM1403-12D	*trp1-1* or *trp1-289 leu2-3,112*	1
CM-1425	B-7170	CM1403-12D	*trp1-1* or *trp1-289 leu2-3,112*	2
CM-1426	B-7171	CM1403-12D	*trp1-1* or *trp1-289 leu2-3,112*	3
CM-1427	B-7589	CM1403-12D	*trp1-1* or *trp1-289 leu2-3,112*	4
CM-1428	B-7590	CM1403-12D	*trp1-1* or *trp1-289 leu2-3,112*	5
CM-1429	B-7591	CM1403-12D	*trp1-1* or *trp1-289 leu2-3,112*	6
CM-1430	B-7173	CM1403-12D	*trp1-1* or *trp1-289 leu2-3,112*	7
CM-1431	B-7174	CM1403-12D	*trp1-1* or *trp1-289 leu2-3,112*	8
CM-1432	B-7175	CM1403-12D	*trp1-1* or *trp1-289 leu2-3,112*	9
CM-1433	B-7593	CM1403-12D	*trp1-1* or *trp1-289 leu2-3,112*	10
CM-1434	B-7178	CM1403-12D	*trp1-1* or *trp1-289 leu2-3,112*	11
CM-1435	B-7595	CM1403-12D	*trp1-1* or *trp1-289 leu2-3,112*	12
CM-1436	B-7255	CM1403-12D	*trp1-1* or *trp1-289 leu2-3,112*	13
CM-1437	B-7596	CM1403-12D	*trp1-1* or *trp1-289 leu2-3,112*	14
CM-1438	B-7180	CM1403-12D	*trp1-1* or *trp1-289 leu2-3,112*	15
CM-1439	B-7598	CM1403-12D	*trp1-1* or *trp1-289 leu2-3,112*	16

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[a] Genotypes of parents are shown on TABLE 1 and TABLE 5-1.

[b] Chromosome or chromosome fragment lost in monosomic diploids resulting from cir$^+$/cir^0 crosses.

Table 5-4. Loss of bleomycin resistance in cir^{+}/cir^{0} diploids.

Diploid	No. of viable cells (0 µg/ml bleomycin)	Colonies lost (50 µg/ml bleomycin)	Chromosomal loss [a] (%)	Adjusted frequency [b] (%)
CM-1424	1299	0	0	0
CM-1425	2318	0	0	0
CM-1426	2808	19	0.7	0
CM-1427	1552	10	0.6	0
CM-1428	1093	7	0.6	0
CM-1429	1266	4	0.3	0
CM-1430	1863	1	0.1	0
CM-1431	1179	1	0.1	0
CM-1432	1253	0	0	0
CM-1433	1343	11	0.8	0
CM-1434	854	3	0.4	0
CM-1435	783	10	1.3	0
CM-1436	791	21	2.7	0
CM-1437	1523	6	0.4	0
CM-1438	556	11	0.7	0
CM-1439	1176	42	3.6	2.6

---

[a] Chromosomal loss was calculated as the percent of viable cells unable to grow in the presence of 50 µg/ml bleomycin. The chromosome lost in each monosomic diploid is shown on Table 5-3.

[b] The frequency of the chromosomal loss event was adjusted on the basis of a secondary screening for bleomycin sensitivity. The bleomycin sensitivity of monosomic diploids was compared with that of normal bleomycin resistant strains CM1403-12A, CM1403-12B and each of the cir^{0} haploid parents, as well as with bleomycin sensitive strains CM1403-12C and CM1403-12D.

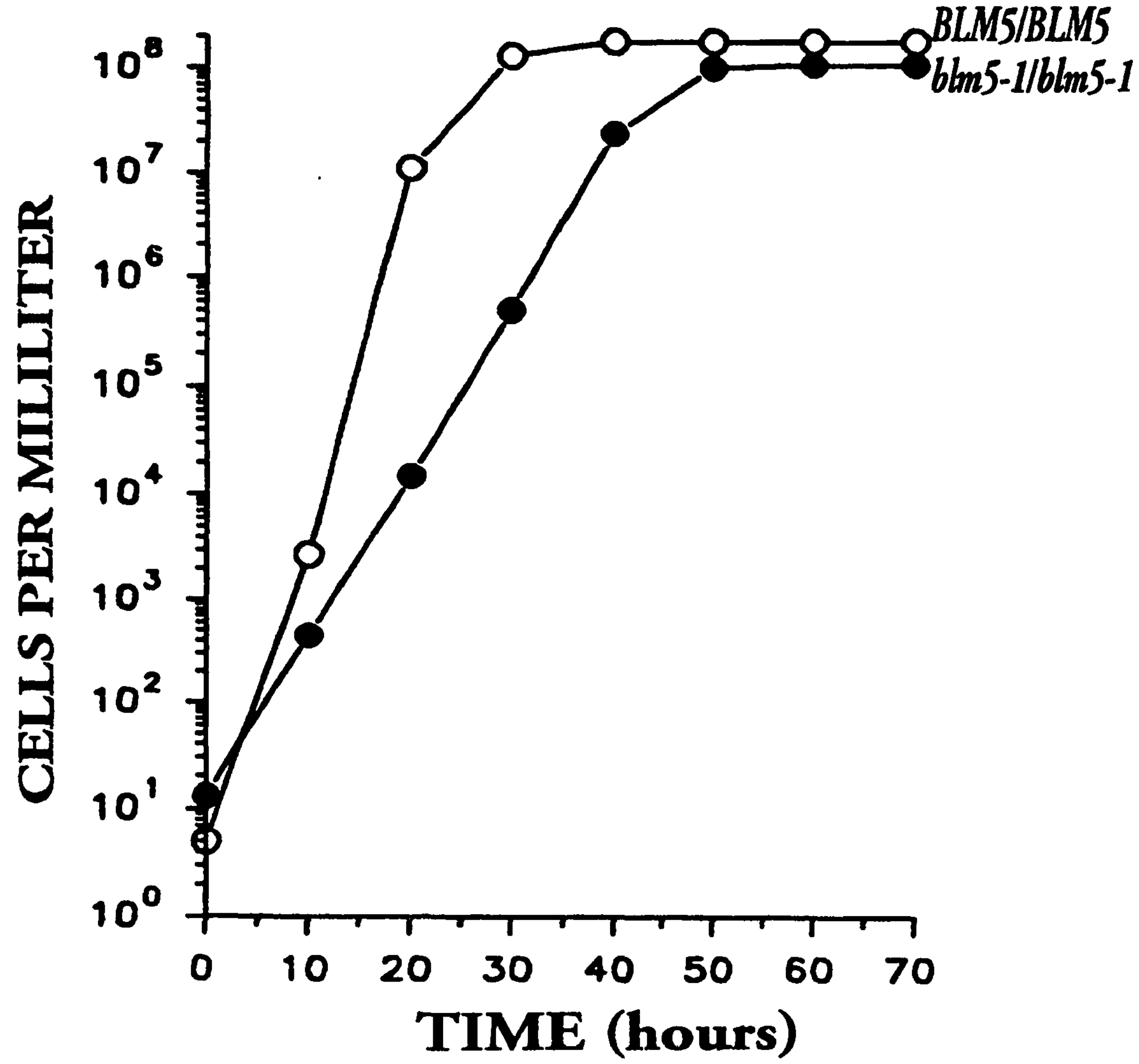


Figure 1-1. Comparisons of growth curves of (*BLM5/BLM5*) and (*blm5-1/blm5-1*) strains. Logarithmic growth of strains grown in sterile complete liquid media (YPAD) was determined by hemocytometer counts of cells/ml with time.

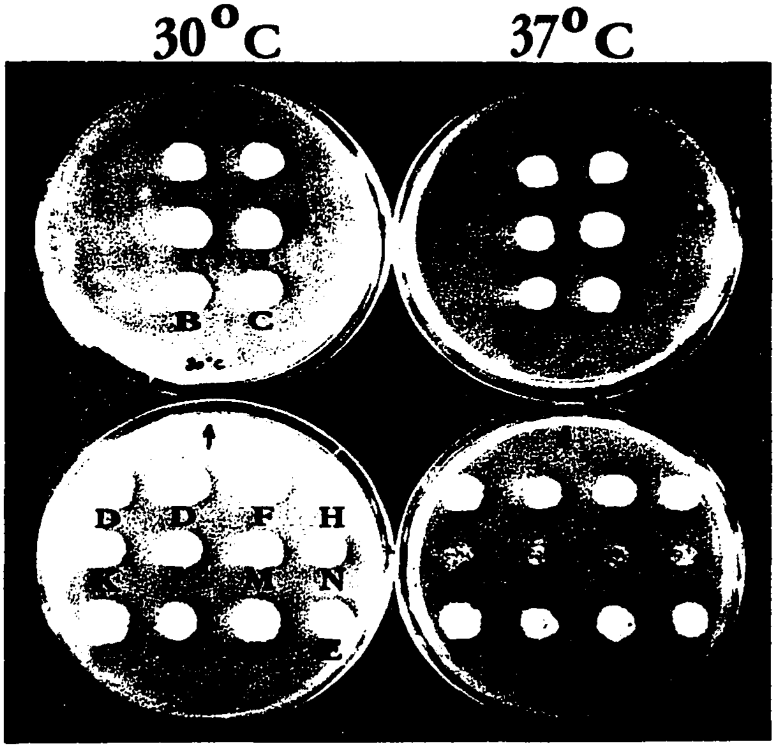


Figure 1-2. Mitotic growth at 30°C and 37°C.
Normal and mutant haploid and diploid cells were patch-plated onto solid YPAD plates, then incubated for 3 days at 30°C and 37°C. Haploid strains (top and bottom rows on each plate) were mated to give diploid strains (middle row on each plate). All haploid cells grew at 30°C and 37°C. The *BLM5*/*BLM5* and *BLM5*/*blm5-1* diploids also grew at 30°C and 37°C. The *blm5-1*/*blm5-1* diploids grew at 30°C but not at 37°C.

KEY: List of strains.

HAPLOIDS

***BLM5* (Normal)**
A: CM1403-12B
B: CM1477-3C

***blm5-1* (Mutant)**
C: CM1403-12D
D: CM1477-4B
E: CM1483-7D
F: CM1483-7B
G: CM1483-7C
H: CM1483-7A

DIPLOIDS

***BLM5*/*BLM5* (Normal)**
I: CM-1489

***BLM5*/*blm5-1* (Heterozygote)**
J: CM-1477

***blm5-1*/*blm5-1* (Mutant)**
K: CM-1483
L: CM-1491
M: CM-1492
N: CM-1493

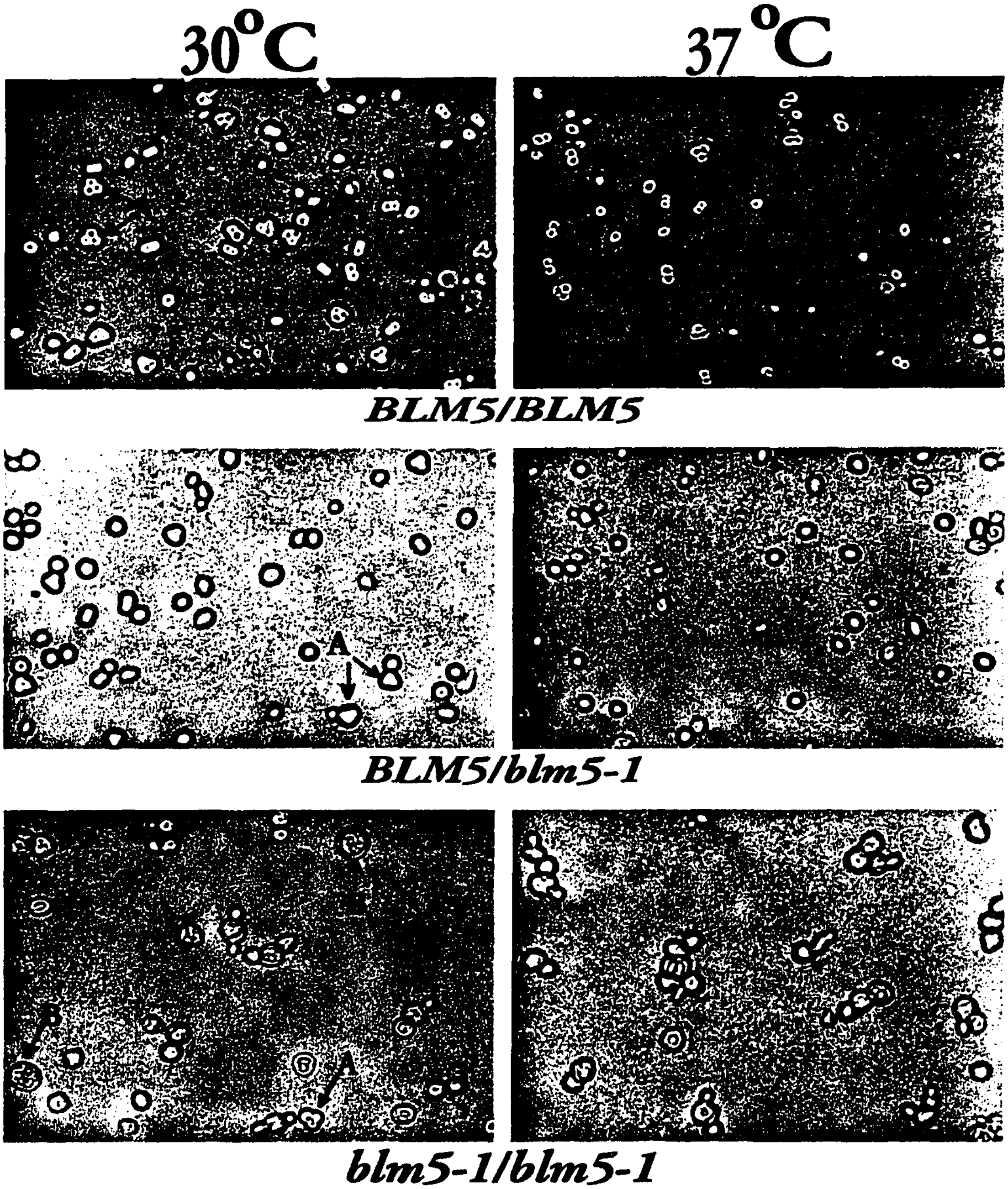


Figure 1-3. Light micrographs of *BLM5/BLM5*, *BLM5/blm5-1* and *blm5-1/blm5-1* populations sporulated at 30°C and 37°C. Sporulated cells (A) were present in *BLM5/BLM5* populations sporulated at 30°C and 37°C. Sporulated cells were also present in *BLM5/blm5-1* populations sporulated at 30°C, but were not present in *BLM5/blm5-1* populations sporulated at 37°C. Very few sporulated cells were found in *blm5-1/blm5-1* populations sporulated at 30°C, but enlarged cells (B) were present in these cell populations. Sporulated cells were not found in *blm5-1/blm5-1* populations sporulated at 37°C.

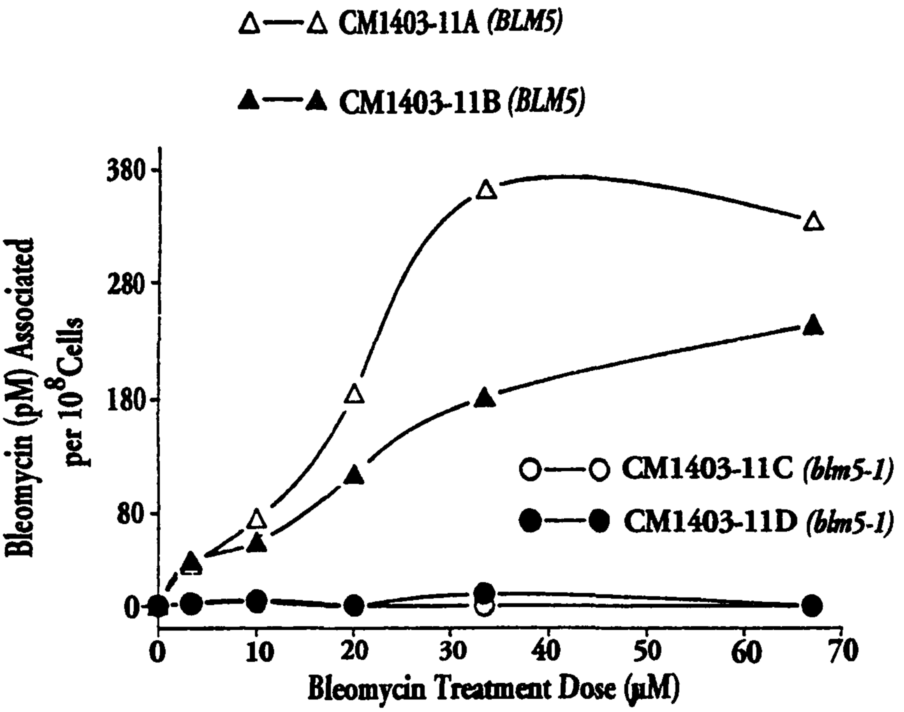


Figure 2-1. Association of [S-*methyl*-^{3}H]bleomycin A_2 in normal *(BLM5)* and *blm5-1* mutant cells. The amount of bleomycin associated with treated cells was determined as the amount of radioactivity in counts per minute (cpm) associated with the pelleted cells.

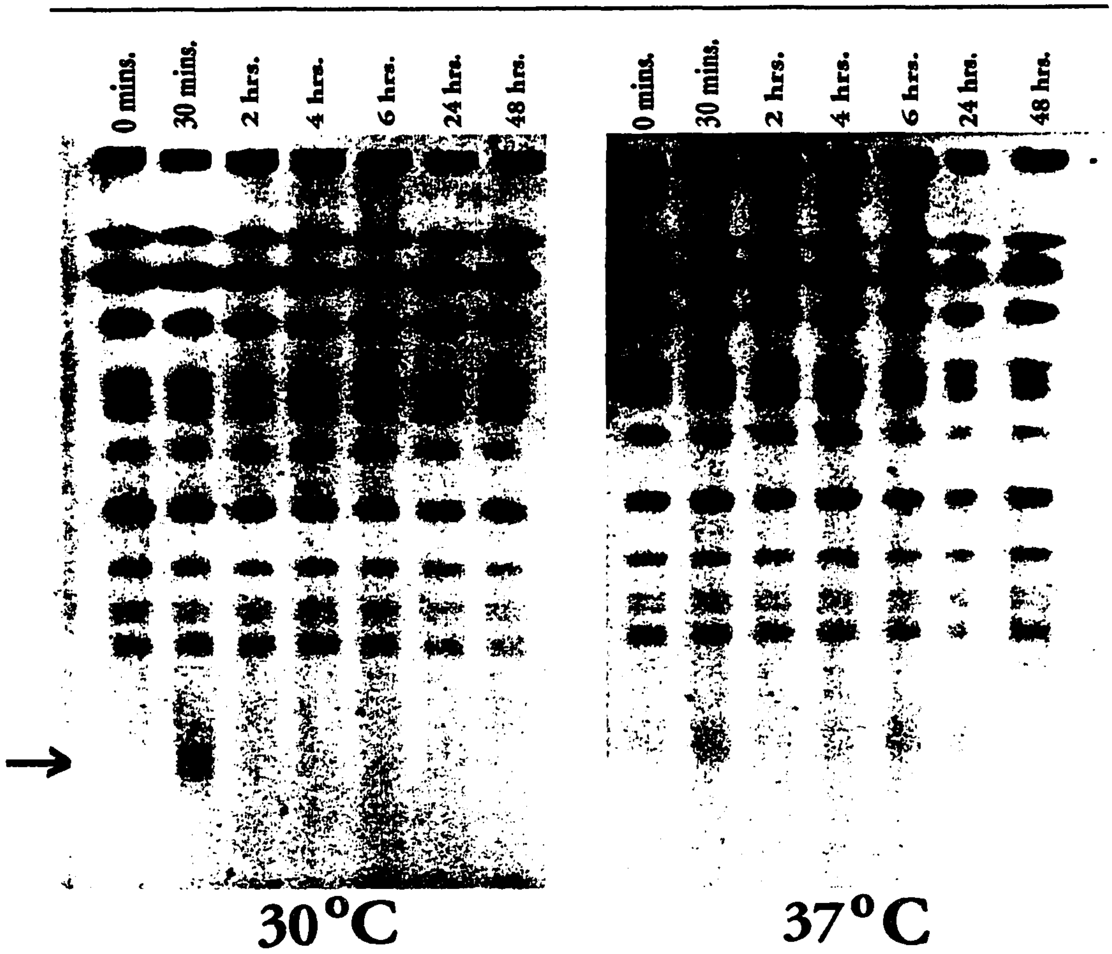


Figure 3-1. Induction and repair of spontaneous chromosomal double-strand breaks at 30°C and 37°C in stationary phase *BLM5/BLM5* (CM-1489) cells. Cells were prepared and treated as described in Materials and Methods. Chromosomes released from cells following IPCD from t = 0 mins. to t = 48 hrs. were separated by PFGE. Broken chromosomes accumulate as smears at the bottom of pulsed-field gels (indicated by the arrow). When breaks are repaired, the smear disappears.

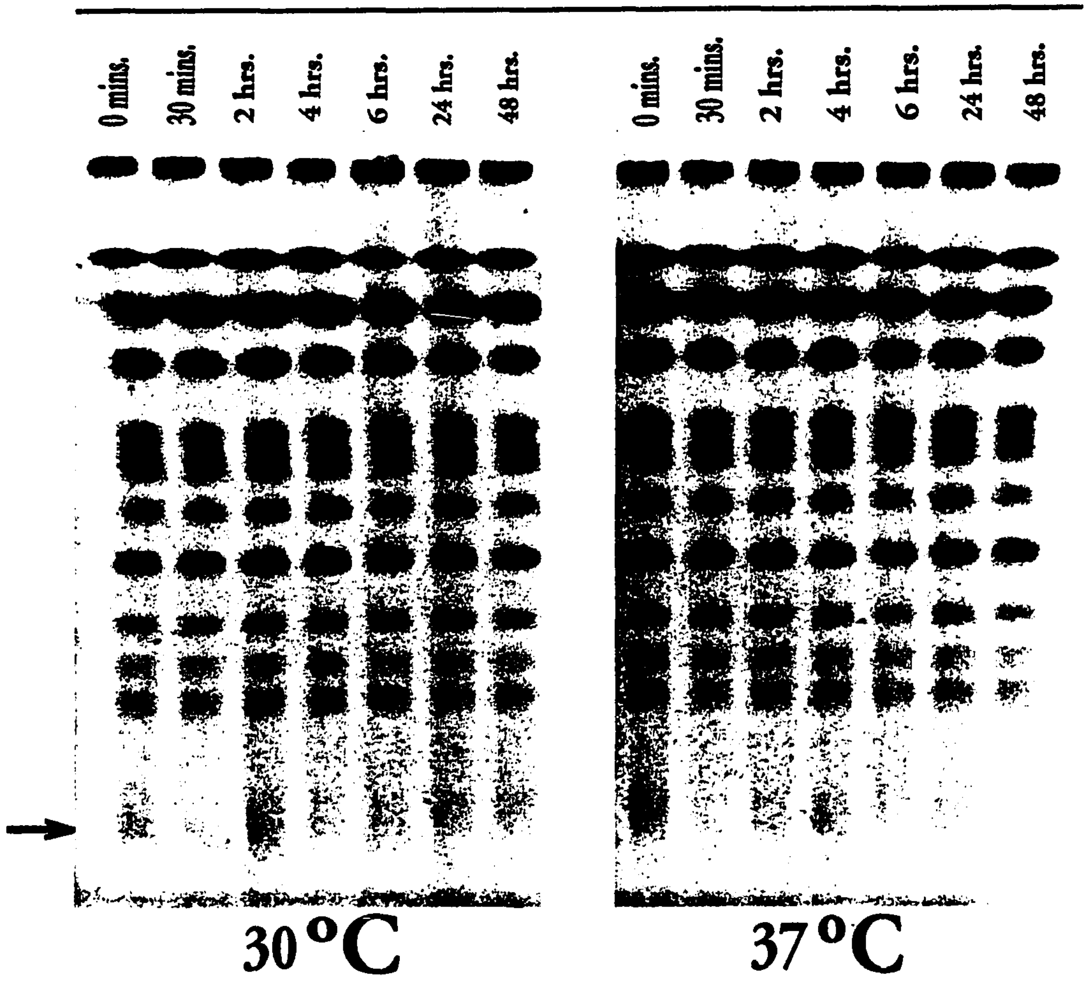


Figure 3-2. Induction and repair of spontaneous chromosomal double-strand breaks at 30°C and 37°C in stationary phase *BLM5/blm5-1* (CM-1477) cells. Cells were prepared as described in Materials and Methods. Broken chromosomes (indicated by the arrow) detected prior to IPCD incubations, were repaired within 30 mins. at 30°C and 37°C. Double-strand breaks (broken chromosomes) accumulated again after 2 hrs. at 30°C and 4 hrs. at 37°C.

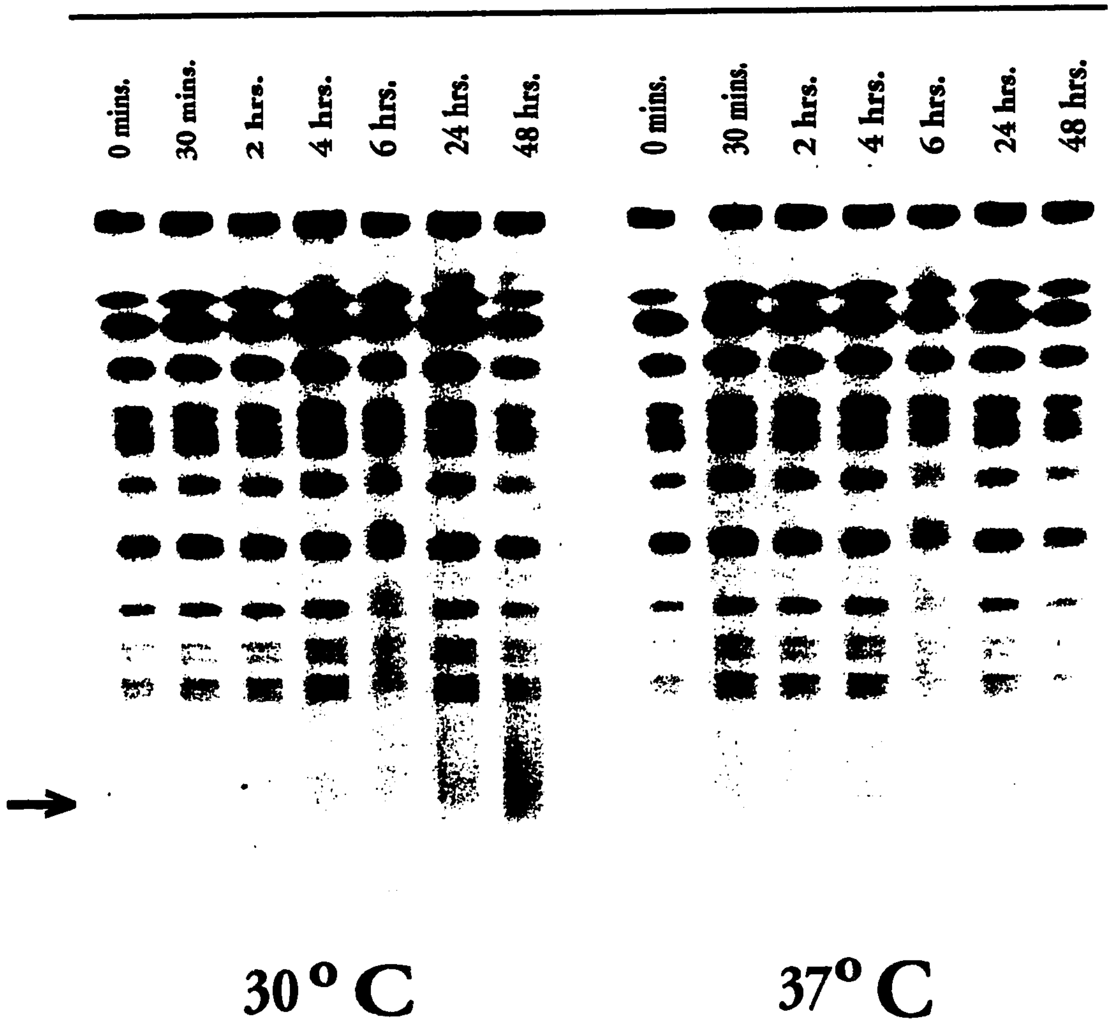


Figure 3-3. Induction and repair of spontaneous chromosomal double-strand breaks at 30°C and 37°C in stationary phase *blm5-1/blm5-1* (CM-1492) cells. Cells were prepared and treated as described in Materials and Methods. Accumulations of broken chromosomes (indicated by the arrow) were detected after 48 hrs. of IPCD at 30°C. Double-strand breaks did not accumulate significantly during IPCD at 37°C.

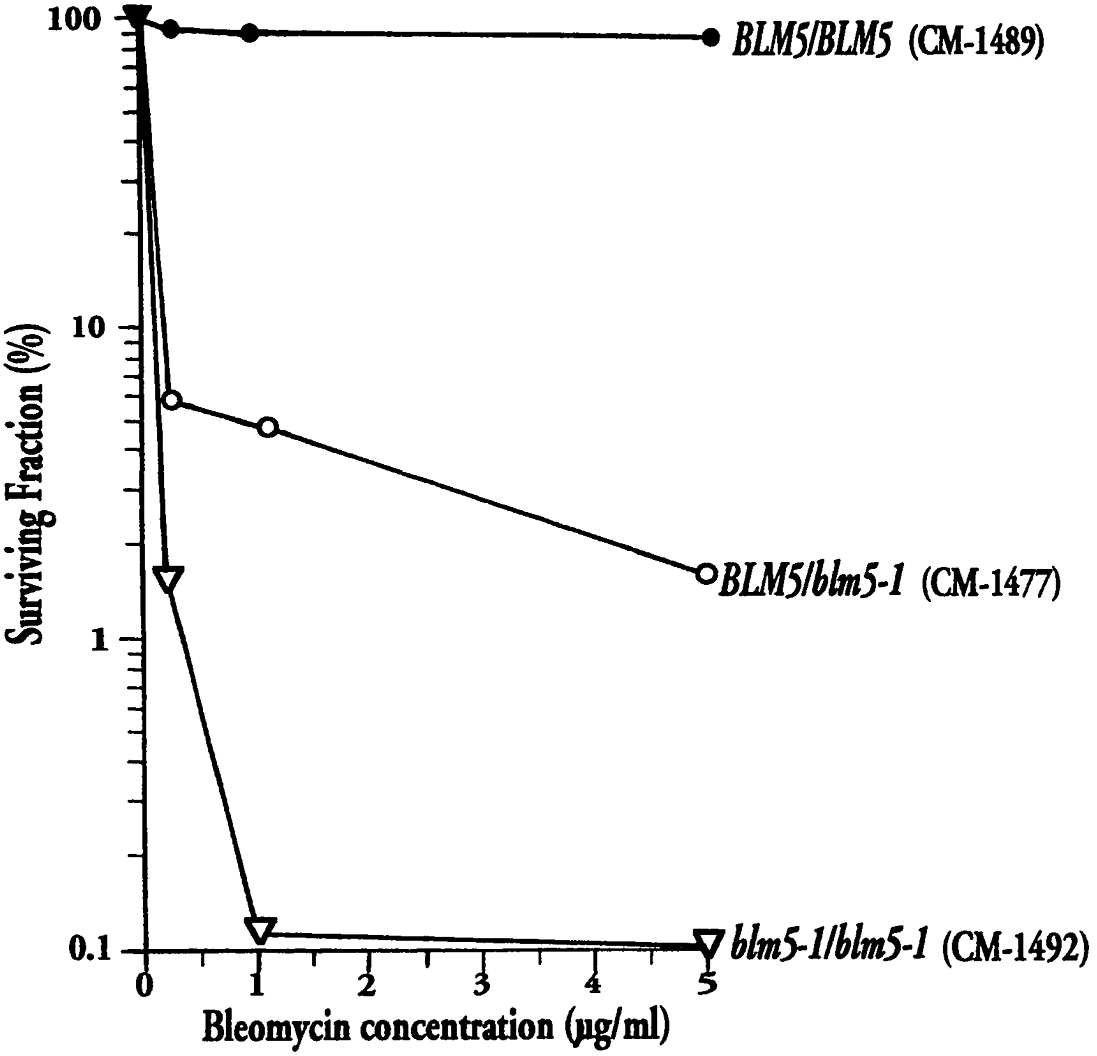


Figure 3-4. Survival of *BLM5/BLM5*, *BLM5/blm5-1* and *blm5-1/blm5-1* strains after bleomycin treatment. The surviving fraction represents the average number of colony forming units (cfu) after 3 to 5 days of incubation at 30°C. Cells were treated in sterile deionized water for 30 mins at 4°C, then plated for growth on solid YPAD media. Comparable results were obtained in multiple experiments.

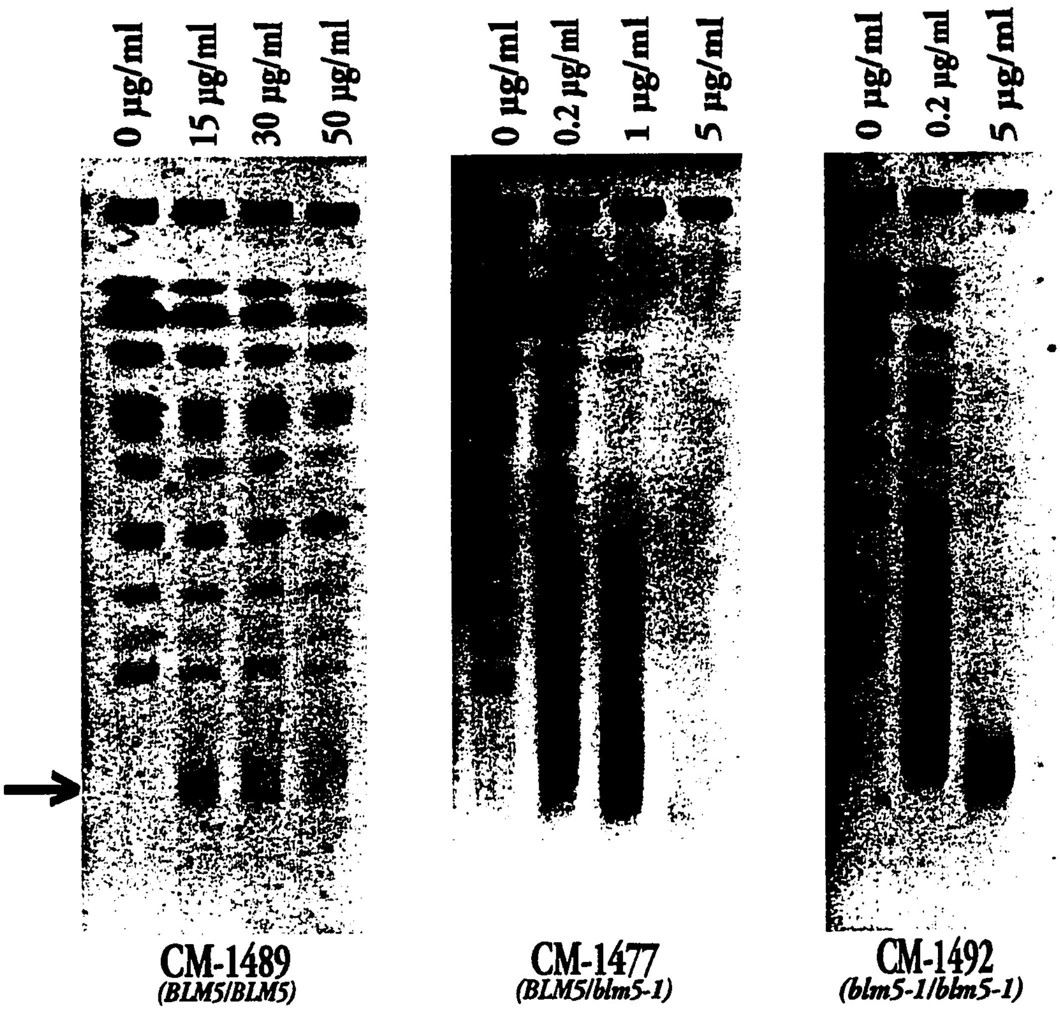


Figure 3-5. Dose dependency of bleomycin-induced chromosomal degradation. Stationary phase *BLM5/BLM5* (CM-1489), *BLM5/blm5-1* (CM-1477) and *blm5-1/blm5-1* (CM-1492) cells were treated with increasing concentrations of bleomycin (0 µg/ml to 50 µg/ml) at 4°C as described in Materials and Methods. Chromosomes were separated by PFGE. As bleomycin concentrations were increased, the chromosomal bands of all three strains decreased in size and broken chromosomes accumulated as a smear at the bottom of the gel (indicated by the arrow).

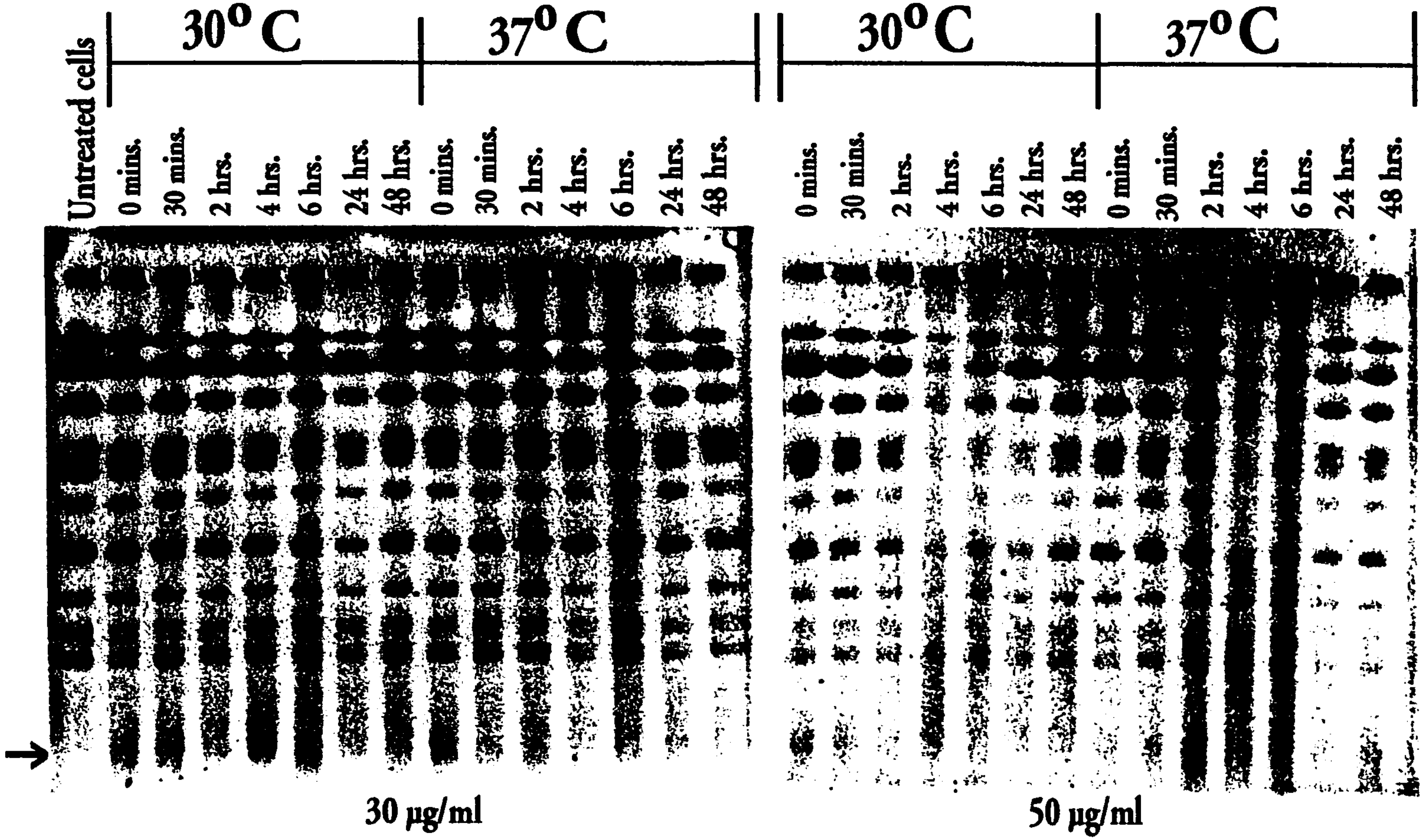


Figure 3-6. Repair of bleomycin-induced chromosomal double-strand breaks at 30°C and 37°C in *BLM5/BLM5* (CM-1489) cells. Cells were treated with 30 µg/ml or 50 µg/ml of bleomycin at 4°C as described in Materials and Methods. Following IPCD incubations (from t=0 mins. to t=48 hrs.) at 30°C and 37°C. Bleomycin-induced double-strand breaks were accompanied by a reduction in the sizes of chromosomal bands and accumulations of broken chromosomes as smears at the bottom of the gel (indicated by the arrow). Repair of chromosomal double-strand breaks was determined by comparisons between the chromosomal bands of untreated and bleomycin-treated (No IPCD) cells and the bands of treated cells following IPCD incubations at 30°C and 37°C.

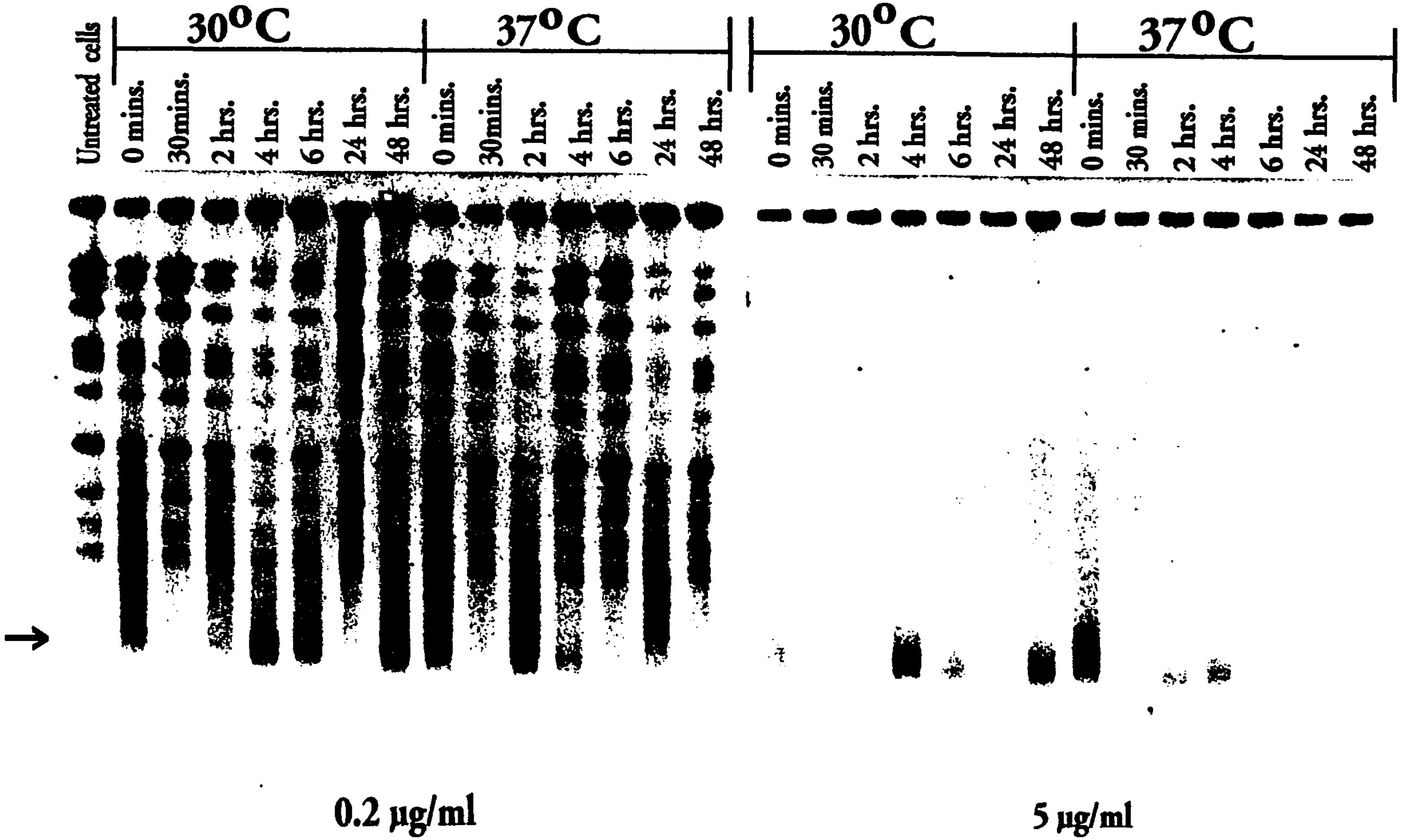


Figure 3-7. Repair of bleomycin-induced chromosomal double-strand breaks at 30°C and 37°C in *BLM5/blm5-1* (CM-1477) cells. Cells were treated with 0.2 µg/ml or 5 µg/ml of bleomycin at 4°C as described in Materials and Methods. Chromosomes were separated by PFGE and double-strand break repair following IPCD incubations (from t=0 mins. to t=48 hrs.) at 30°C and 37°C was determined as described in Figure 3-6.

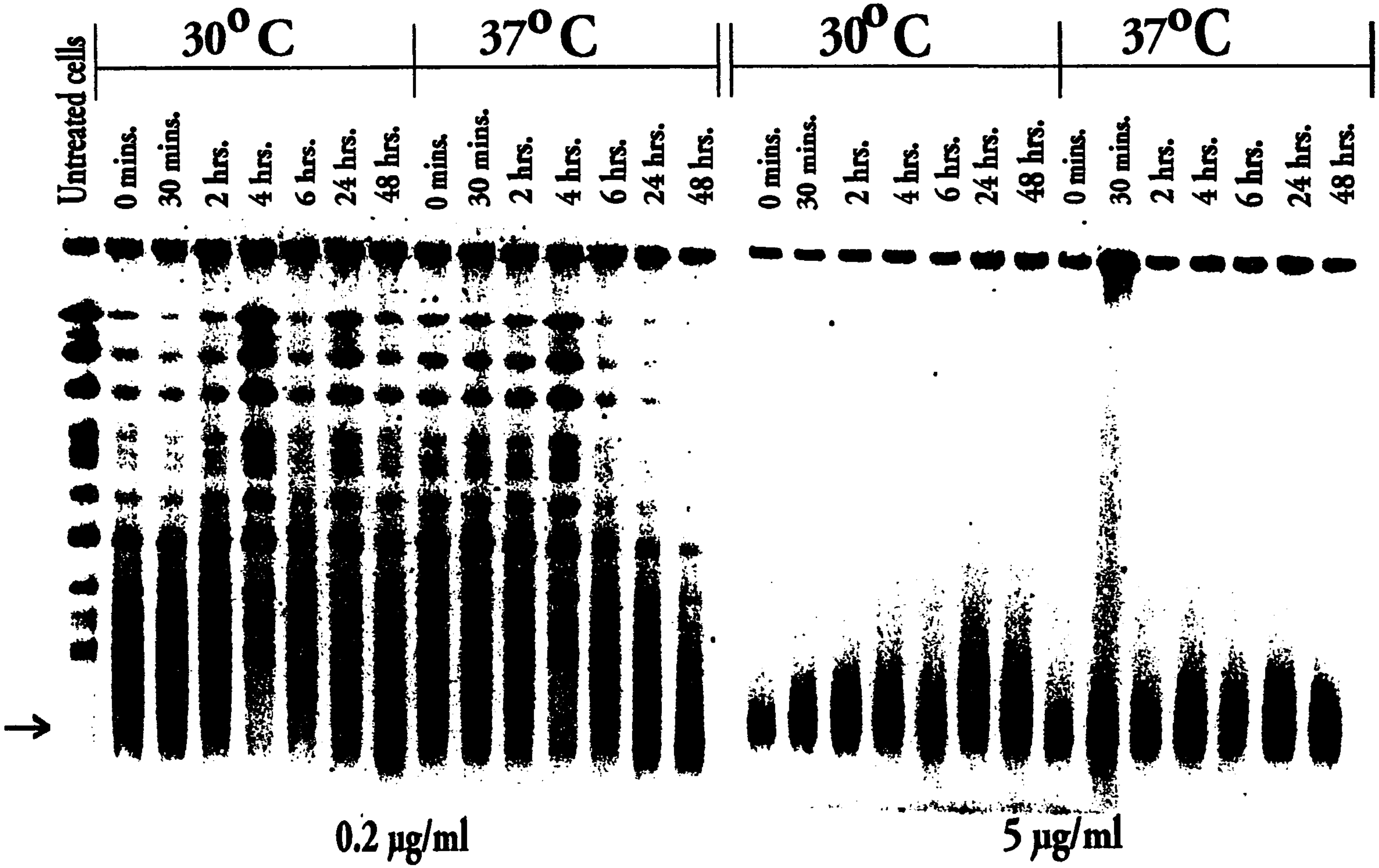


Figure 3-8. Repair of bleomycin- induced chromosomal double- strand breaks at 30°C and 37°C in *blm5-1/blm5-1* (CM-1492) cells. Cells were treated with 0.2 µg/ml or 5 µg/ml of bleomycin at 4°C as described in Materials and Methods. Chromosomes were separated by PFGE and double-strand break repair following IPCD incubations (from t=0 mins. to t=48 hrs.) at 30°C and 37°C was determined as described in Figure 3-6.

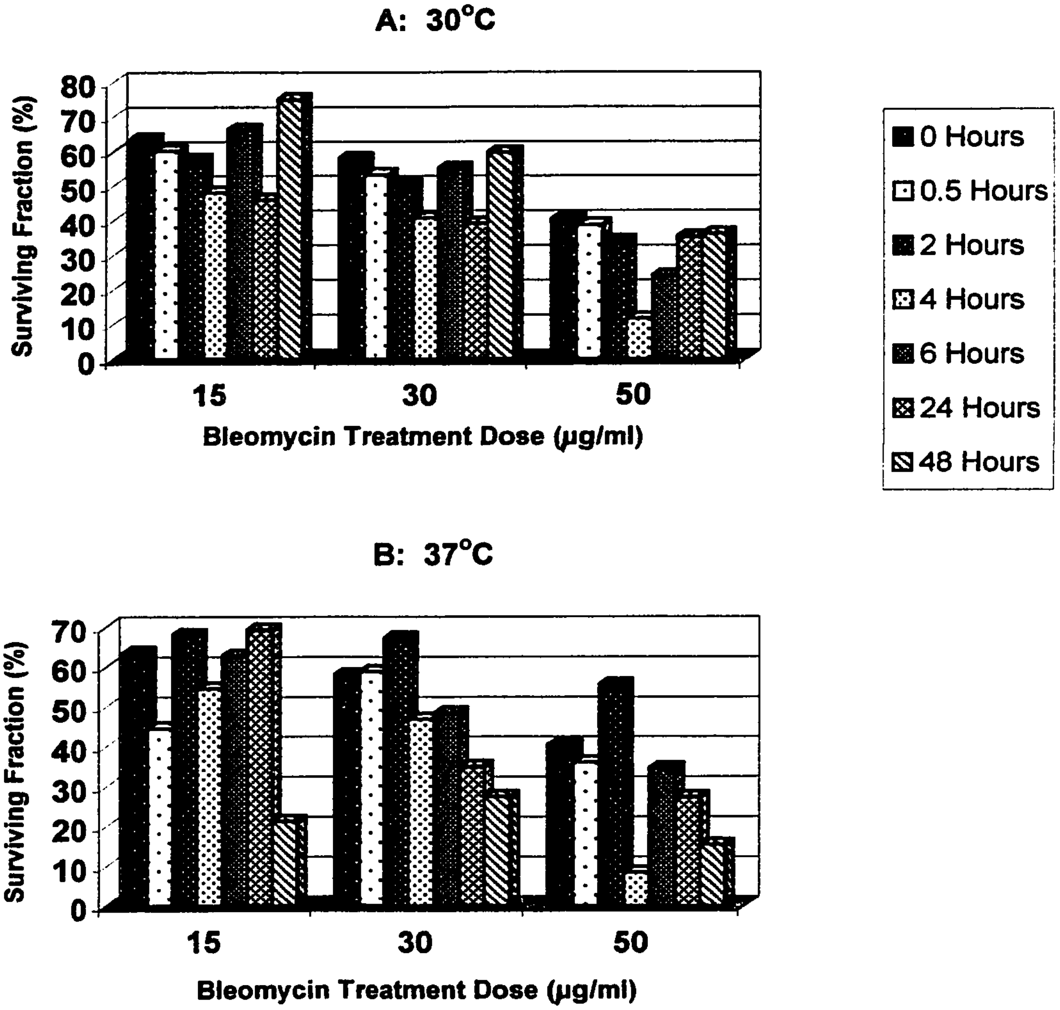


Figure 3 – 9. Survival of *BLM5/BLM5* (CM-1489) cells after IPCD at 30°C and 37°C. Survival was determined by plating for growth on solid medium after IPCD incubations from t = 0 mins. to t = 48 hrs. Surviving fraction represents the number of colony forming units on YPAD media after 3 to 5 days incubation at 30°C. A and B: High doses. C and D: Low doses.

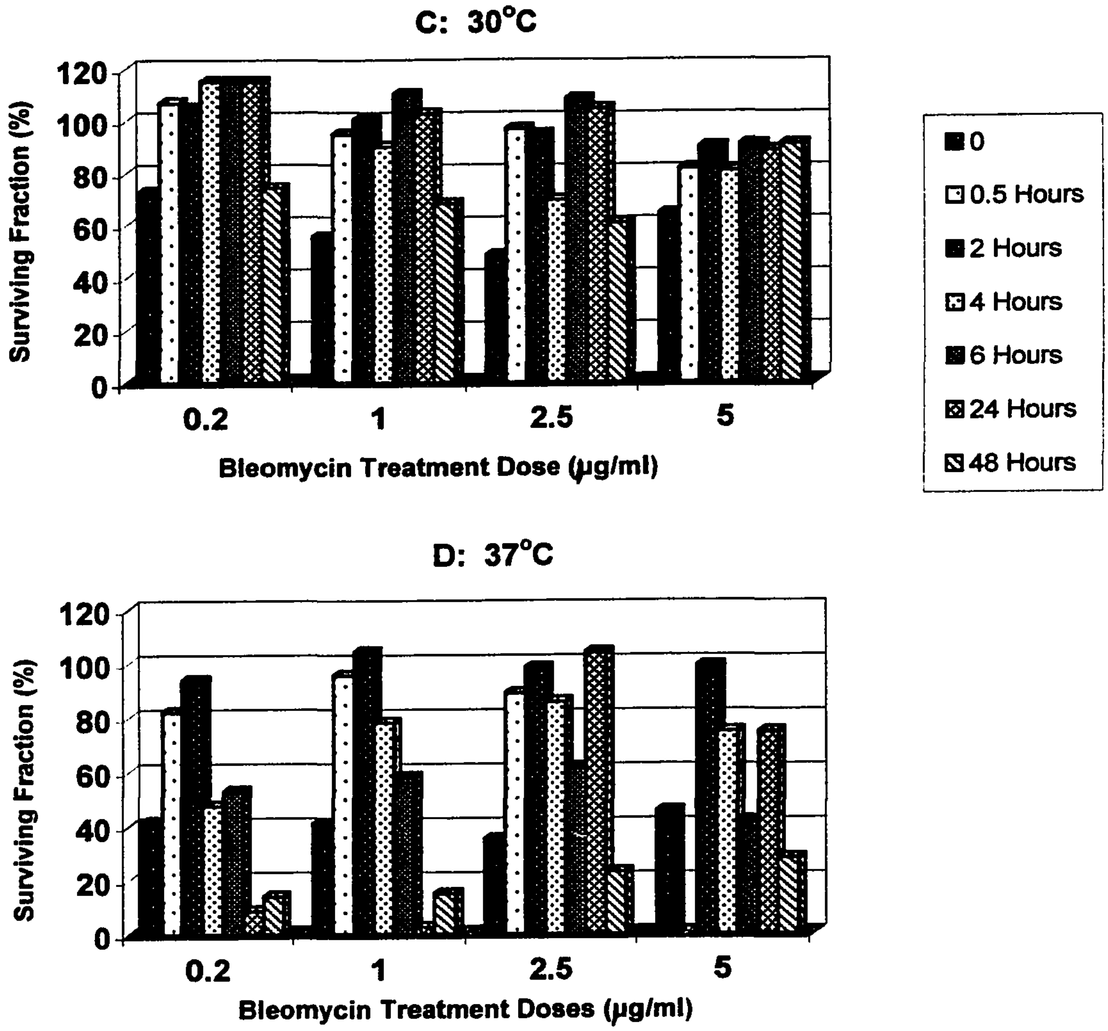


Figure 3 – 9 (continued).

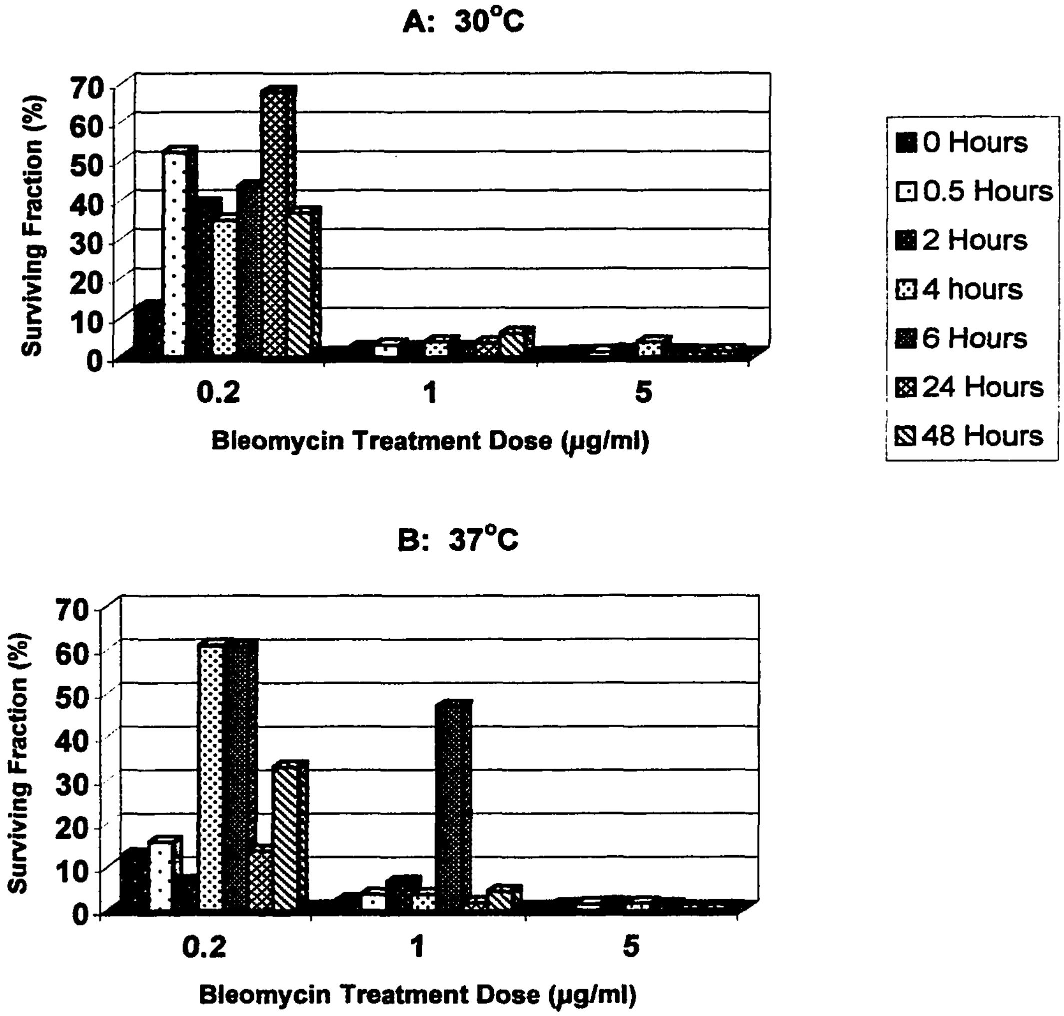


Figure 3 – 10. Survival of *BLM5/blm5-1* (CM-1477) cells after IPCD at 30°C and 37°C. Survival was determined by plating for growth on solid YPAD after IPCD incubations from t = 0 minutes to t = 48 hours. Surviving fraction represents the number of colony forming units on YPAD media after 3 to 5 days incubation at 30°C.

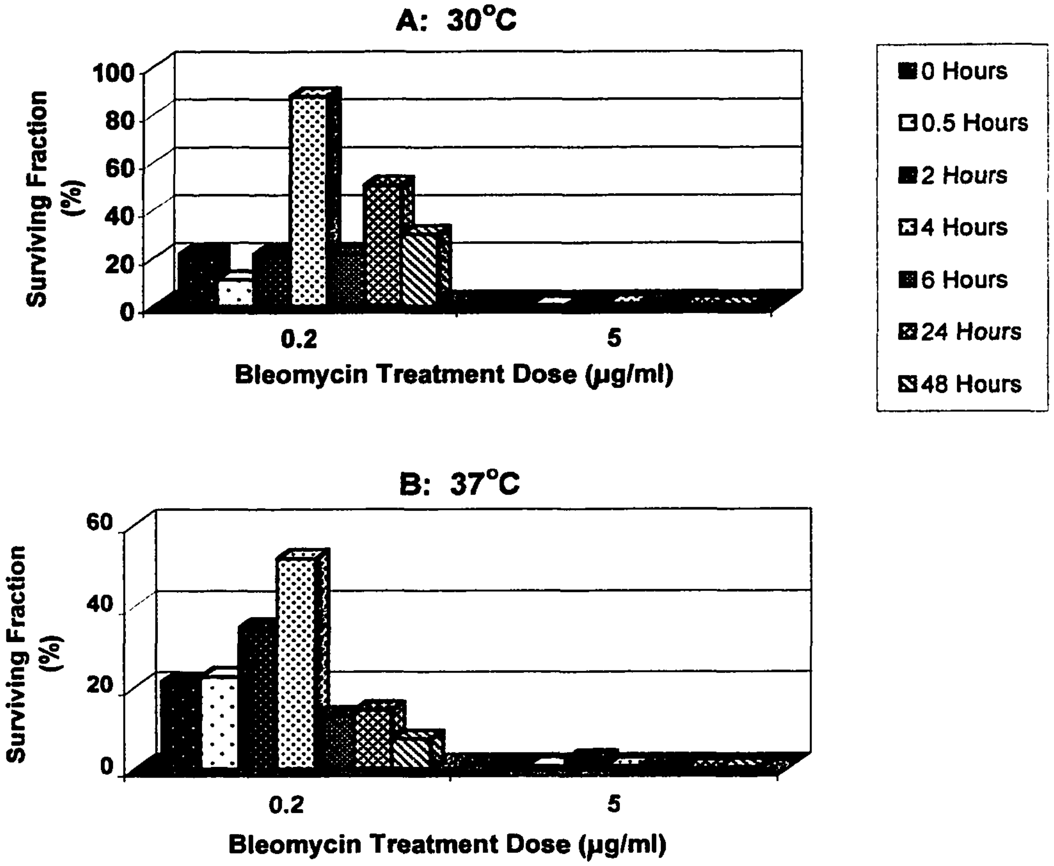


Figure 3 – 11. Survival of *blm5-1/blm5-1* (CM-1492) cells after IPCD at 30°C and 37°C. Survival was determined by plating for growth on solid YPAD after IPCD incubations from t = 0 mins. to t = 48 hrs. Surviving fraction represents the number of colony forming units on YPAD media after 3 to 5 days incubation at 30°C.

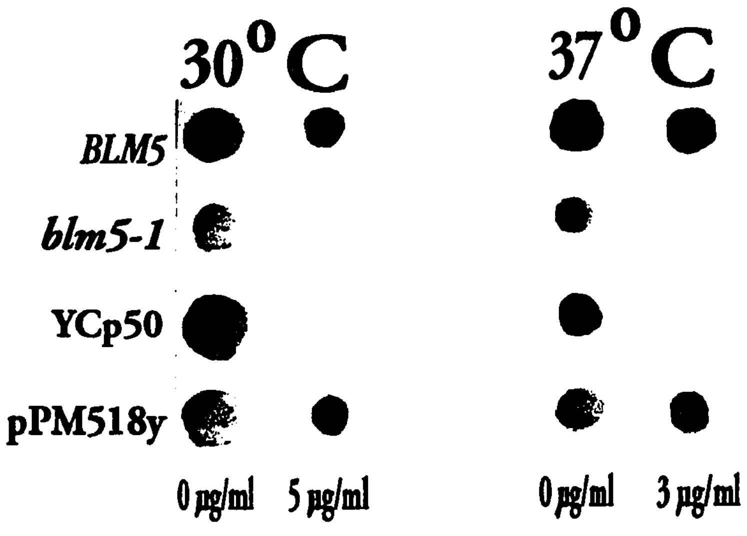


Figure 4-1. Selection of *blm5-1* complementing plasmid pPM518y. Strains were replica plated onto solid YPAD (0 µg/ml) and solid bleomycin containing SD media (3 µg/ml and 5 µg/ml). A Ura$^+$ haploid transformant bearing the YCp50-based plasmid pPM518y was selected with levels of resistance to killing by bleomycin at 30° C and 37° C that were comparable with normal *BLM5* haploid strains and greater than transformed *blm5-1* mutant strain, CM1403-12D or the mutant haploid bearing the empty YCp50 vector.

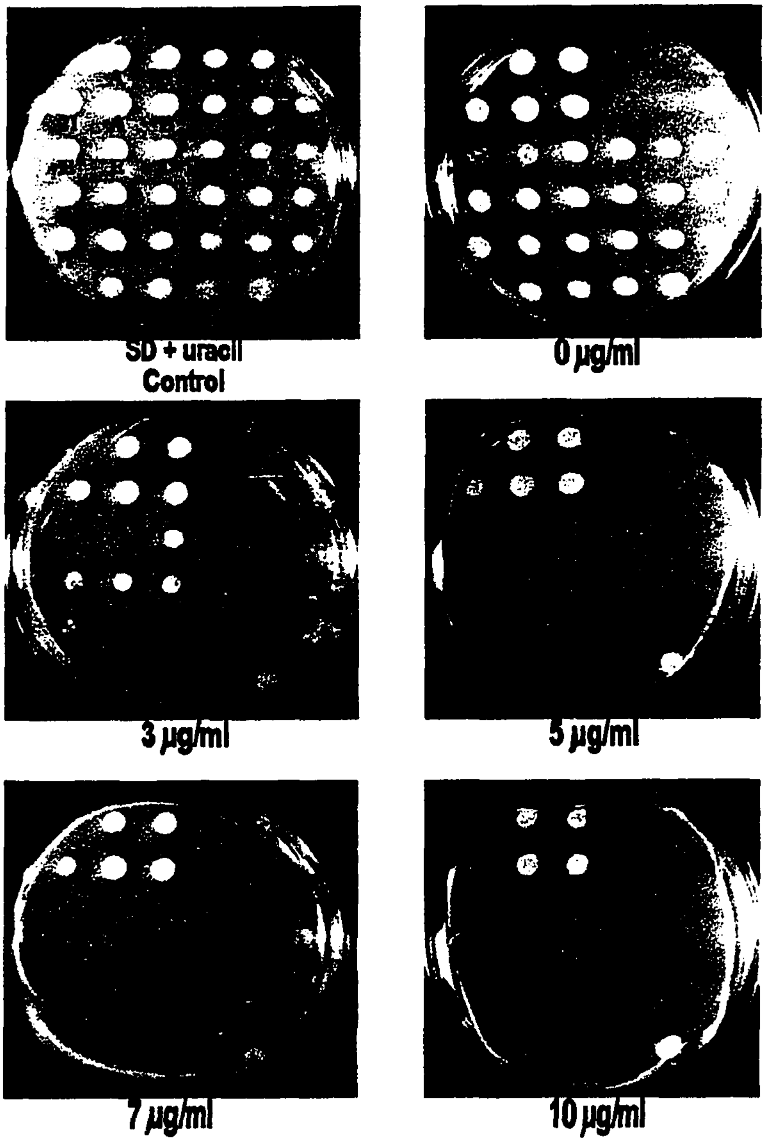


Figure 4-2. Selection of bleomycin resistant haploid transformants. Transformants were replica plated on bleomycin-containing SD media lacking uracil (0 µg/ml to 10 µg/ml). Control plate: A and B are *BLM5* (normal) haploids, C and D are untransformed uracil-requiring *blm5-1* cells and E is a mutant cell transformed with YEp24 vector. 1 to 22 represent different Ura^+ transformants bearing S288C genomic DNA in the YEp24 vector. The six transformants selected with varying resistances to bleomycin could be ordered based on their level of resistance to bleomycin (from high to low) as follows: 20 > 21 > 6 > 1 > 7 >5.

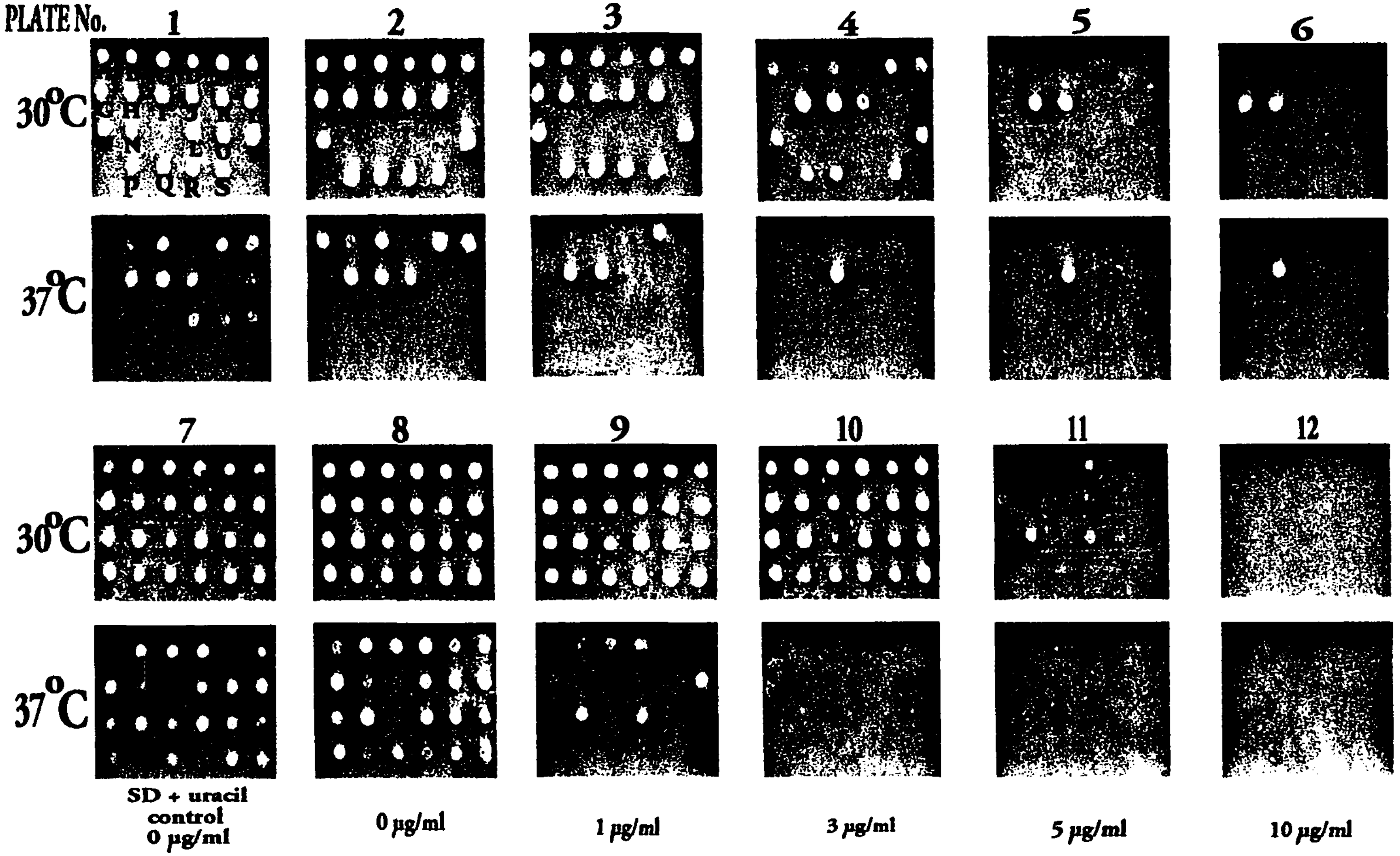


Figure 4-3. Selection of bleomycin resistant diploid transformants. Three uracil requiring *blm5-1/blm5-1* strains were transformed with S288C genomic DNA in the YEp24 vector. Ura$^+$ transformants were screened on bleomycin-containing (0 μg/ml to 10 μg/ml) SD media lacking uracil. Two masters (1 and 7) were replica plated onto drug plates (2 through 6 and 8 through 12 respectively). Each spot represents a single transformant as follows: L and O are *blm5-1/blm5-1* diploids (CM-1491 and CM-1483 respectively). K is a Ura$^+$ transformant bearing the empty YEp24 vector. A through F, N, and a through x are Ura$^+$ transformants of CM-1483 bearing S288C library in YEp24. G through I are Ura$^+$ transformants of CM-1493 and J, M, and P through S are Ura transformants of CM-1491. These transformants also carry S288C library in YEp24.

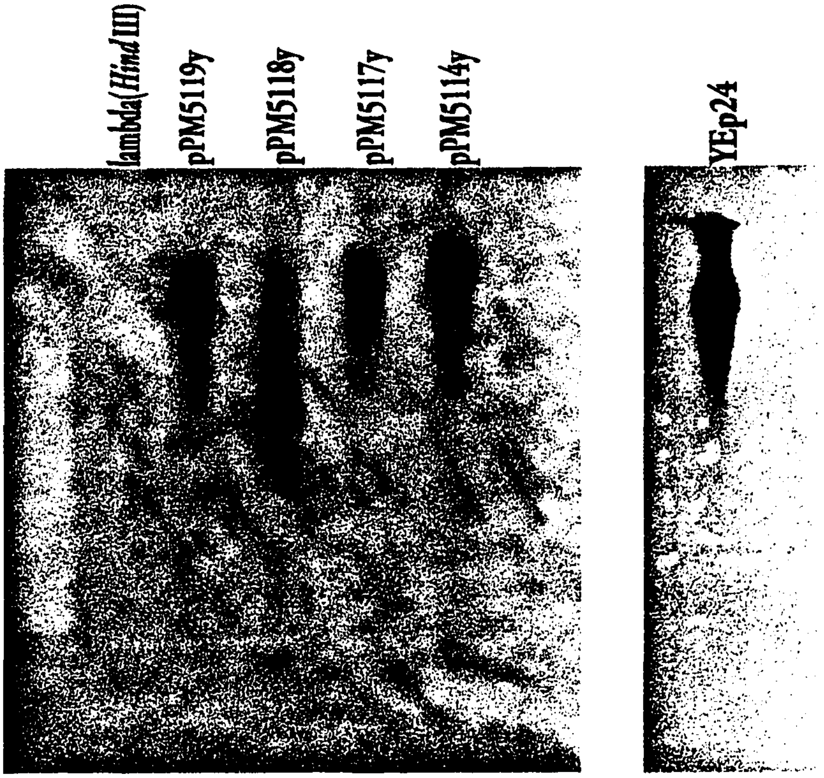
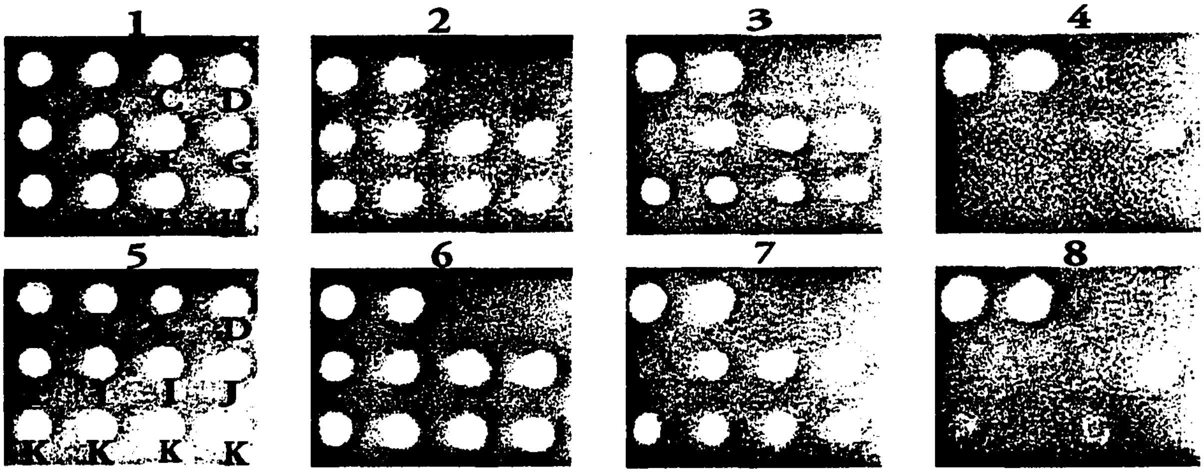


Figure 4-4. Identification of plasmid-containing preparations from yeast. Plasmid preparations, pPM5114y, pPM5117y, pPM5118y and pPM5119y, were isolated from Ura$^+$ transformants, run in a 1% agarose gel, transferred to a nitrocellulose filter and hybridized with a ^{32}P-labeled fragment of the ampicillin gene (Materials and Methods). The YEp24 vector and lambda DNA cut with *Hind*III were used as positive and negative controls respectively.



SD + uracil
control
0 µg/ml

0 µg/ml

1 µg/ml

3 µg/ml

Figure 4-5. The *blm5-1* complementing activity of plasmids in transformed haploids. Plates 1 and 5 were replica-plated onto bleomycin-containing SD media lacking uracil (plates 2 through 4 and 6 through 8 respectively). Strains and transformants are listed below.

KEY

A: CM1403-12A *(BLM5)*
B: CM1403-12B *(BLM5)*
C: CM1403-12C *(blm5-1)*
D: CM1403-12D *(blm5-1)*
E: CM1403-12D(YEp24)
F: CM1403-12D(pPM5115y)
G: Transformant #5 (Figure 4-2)
H: CM1403-12D(pPM5115)
I: CM1403-12D(pPM5116y)
J: Transformant #6 (Figure 4-2)
K: CM1403-12D(pPM5116)

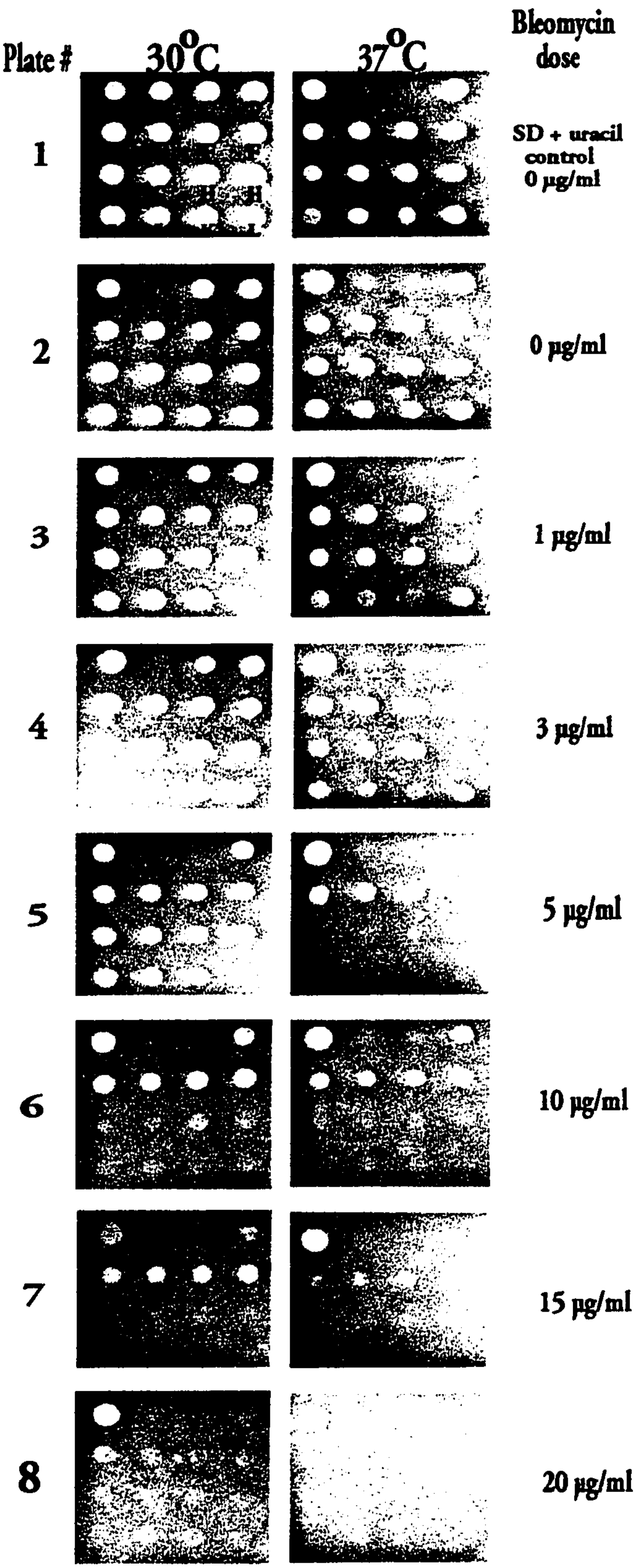


Figure 4-6. The *blm5-1/blm5-1* complementing activity of plasmids in transformed diploids. Strains (listed below) were replica-plated onto SD media containing bleomycin (0 µg/ml to 20 µg/ml) but lacking uracil. Plates were incubated for 3 days at 30°C or 37°C.

KEY

A: CM-1489 *(BLM5/BLM5)*
B: CM-1483 *(blm5-1/blm5-1)*
C: CM-1483(YEp24)
D: CM-1493(pPM5120) Transformant I, Figure 4-3.
E: CM-1483(pPM5120)
F: CM-1483(pPM5120y)
G: CM-1483(pPM5121)
H: CM-1483(pPM5121y)
I: CM-1493(pPM5121) Transformant H, Figure 4-3.
J: CM-1483(pPM5122)
K: CM-1483(pPM5122y)
L: CM-1483(pPM5121) Transformant n, Figure 4-3.

Figure 4-7. Chromosomal location of insert fragments of pPM5115 and pPM5116. Yeast chromoblots carrying the 16 chromosomes of *S. cerevisiae* were hybridized to P-labelled probes created from the YEp24 (Lane A) and the pPM5115 (Lane B) plasmids or from an *Eco*RV-*Eco*RV fragment of pPM5116 (Lane C).

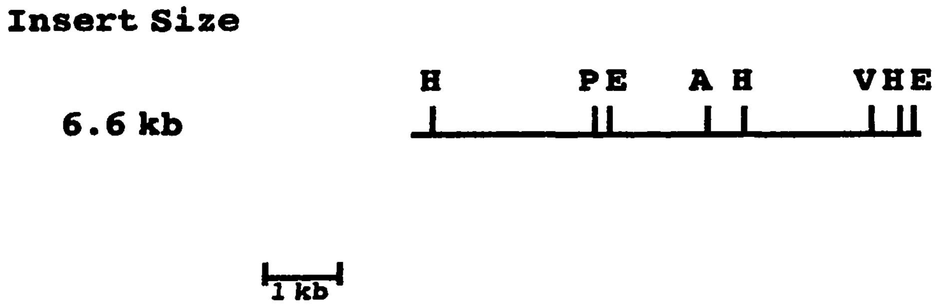


Figure 4 - 8. Restriction map of the chromosomal insert of pPM5115. A, *ApaI*; C, *ClaI*; E, *EcoRI*; H, *HindIII*; N, *NheI*; P, *PstI*; V, *EcoRV*.

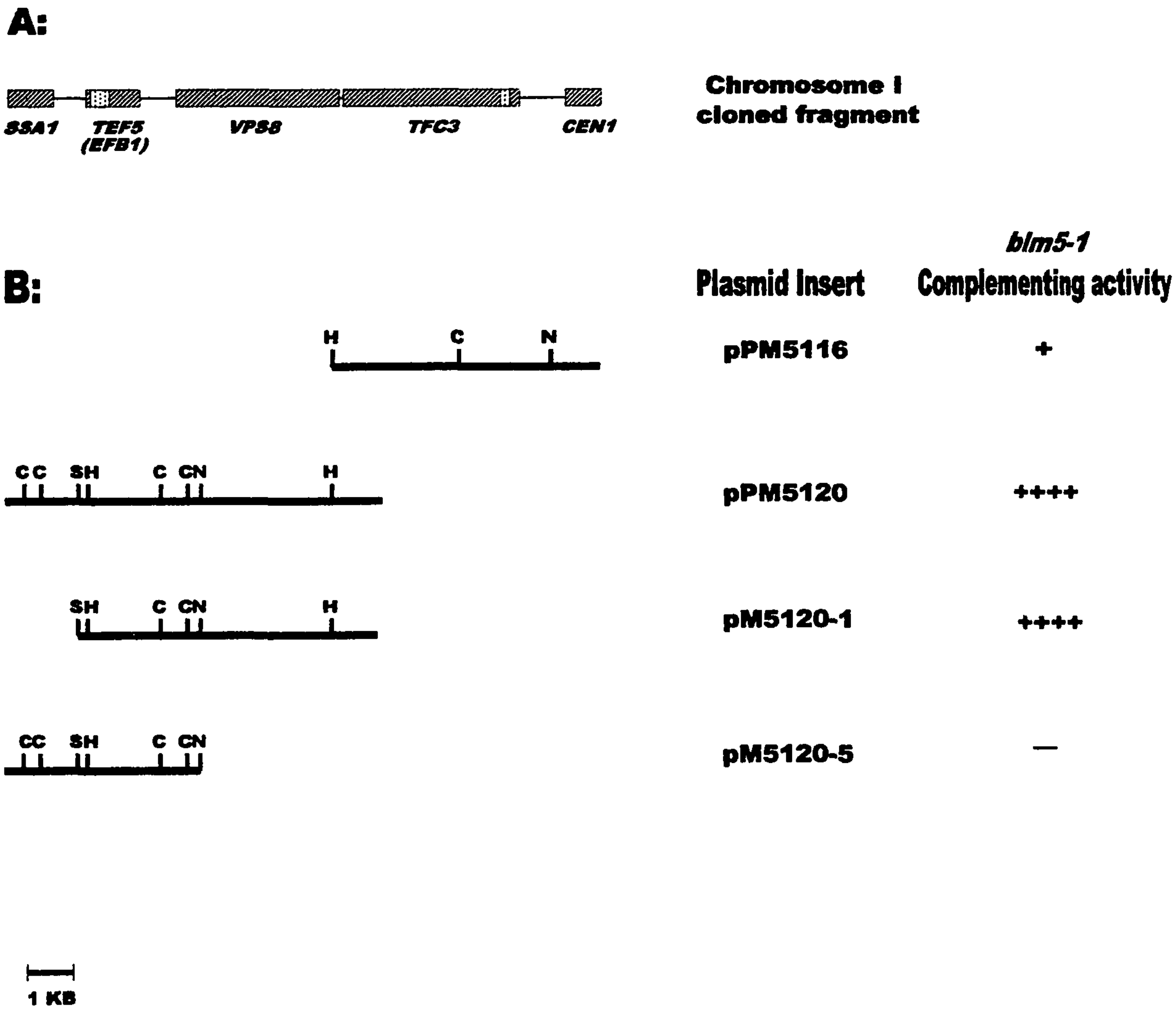
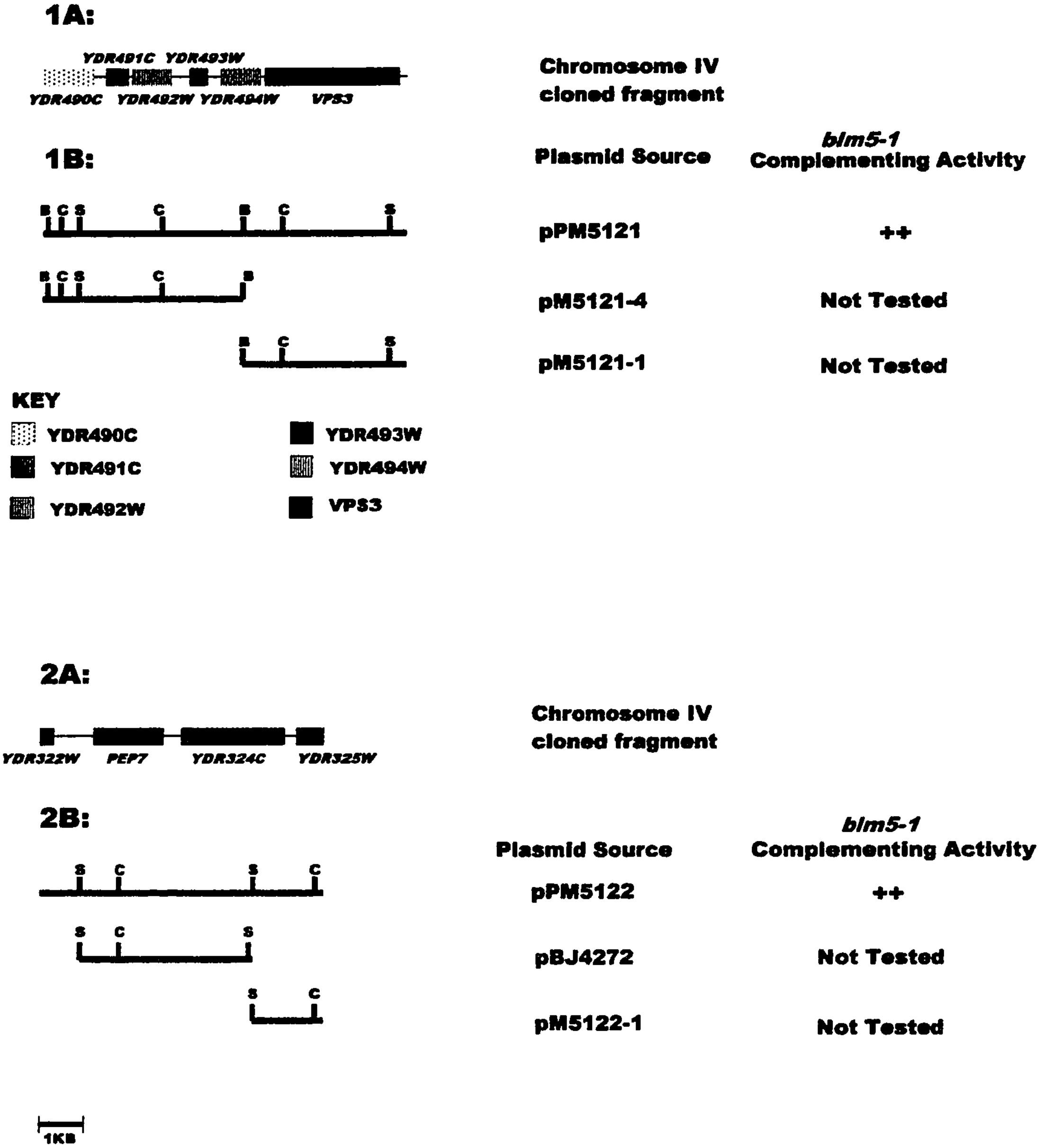


**Figure 4 - 9. Cloned fragment and restriction maps of *blm5-1* complementing region of Chromosome I. A: Cloned fragment of Chromosome I with *blm5-1* complementing activity. Genes included on the fragment are shown.
B: Restriction maps of chromosome I plasmid inserts. Cloned genes included on each insert, regions of overlap between inserts and the relative *blm5-1* complementing activity are shown (+ = complements: - = does not complement). C: *ClaI*;: H: *HaeII*; S: *SalI*; N: *NheI*.**



Plasmid Source	*blm5-1* Complementing Activity
pPM5121	++
pM5121-4	Not Tested
pM5121-1	Not Tested

Plasmid Source	*blm5-1* Complementing Activity
pPM5122	++
pBJ4272	Not Tested
pM5122-1	Not Tested

Figure 4 - 10. Cloned fragments and restriction maps of *blm5-1* complimenting regions of Chromosome IV. 1A and 2A: Genes included on each fragment are shown. 1B and 2B: Restriction maps of Chromosome IV plasmid inserts. Cloned genes included on each insert, regions of overlap between inserts and the complementing activity associated with each insert are shown.
++ = relieves the hypersesitivity to bleomycin conveyed by the *blm5-1* mutation.
B: *Bam*HI; C: *Cla*I; S: *Sal*I.

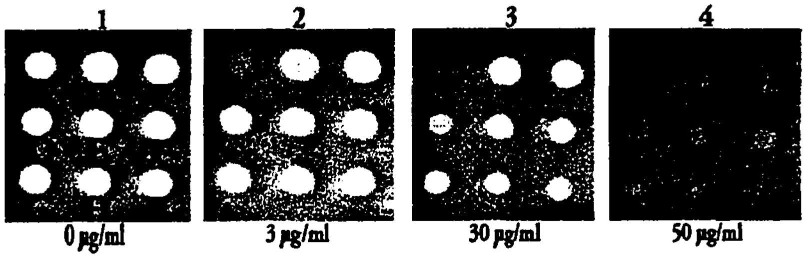


Figure 4-11. Bleomycin hypersensitivities of transcription factor genes *TFC3* and *BRF1* Strains (listed below) were replica-plated onto bleomycin-containing (0 µg/ml to 50 µg/ml) SD media. Plates were incubated at 30°C for 3 days.

KEY

SPOT	STRAIN
A	CM-1492 *(blm5-1/blm5-1)*
B	CM-1477 *(BLM5/blm5-1)*
C	CM-1489 *(BLM5/BLM5)*
D	yOL8 x *(tsv115)*
E	YPH499 *(TFC3)*
F	YPH500 *(TFC3)*
G	yOL8 x (pLR57-*TFC3)*
H	yOL8 x (pLR30-*BRF1)*
I	yOL8 x (pFL44)

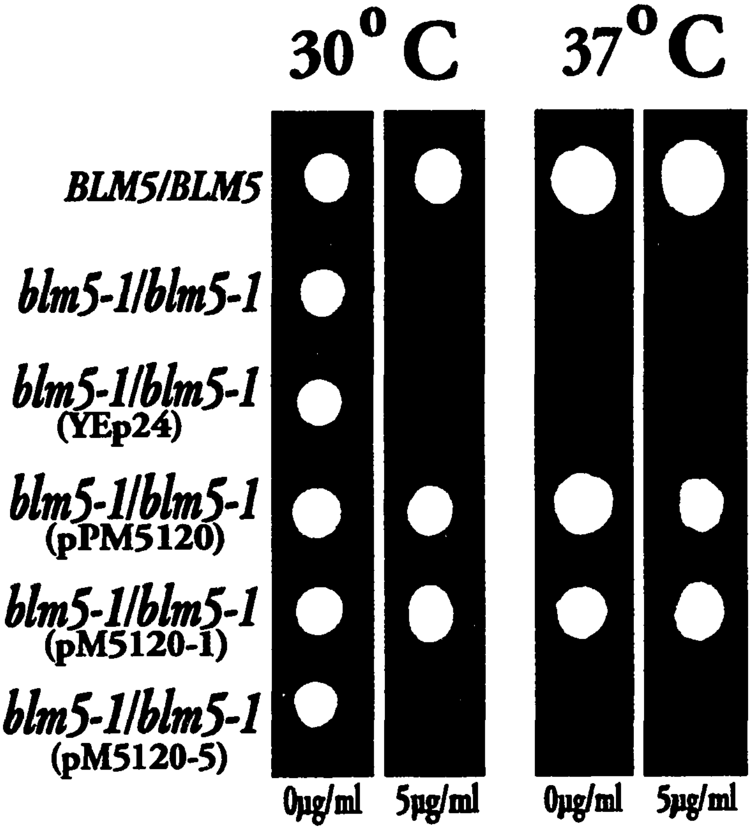


Figure 4-12. Identification of *blm5-1* complementing fragment of pPM5120. Normal (*BLM5/BLM5*), mutant (*blm5-1/blm5-1*) and mutant diploids carrying either the YEp24, pPM5120, pM5120-1 or pM5120-5 plasmids were replica plated onto bleomycin-containing SD media. Plates were incubated for 3 days at 30°C or 37°C.

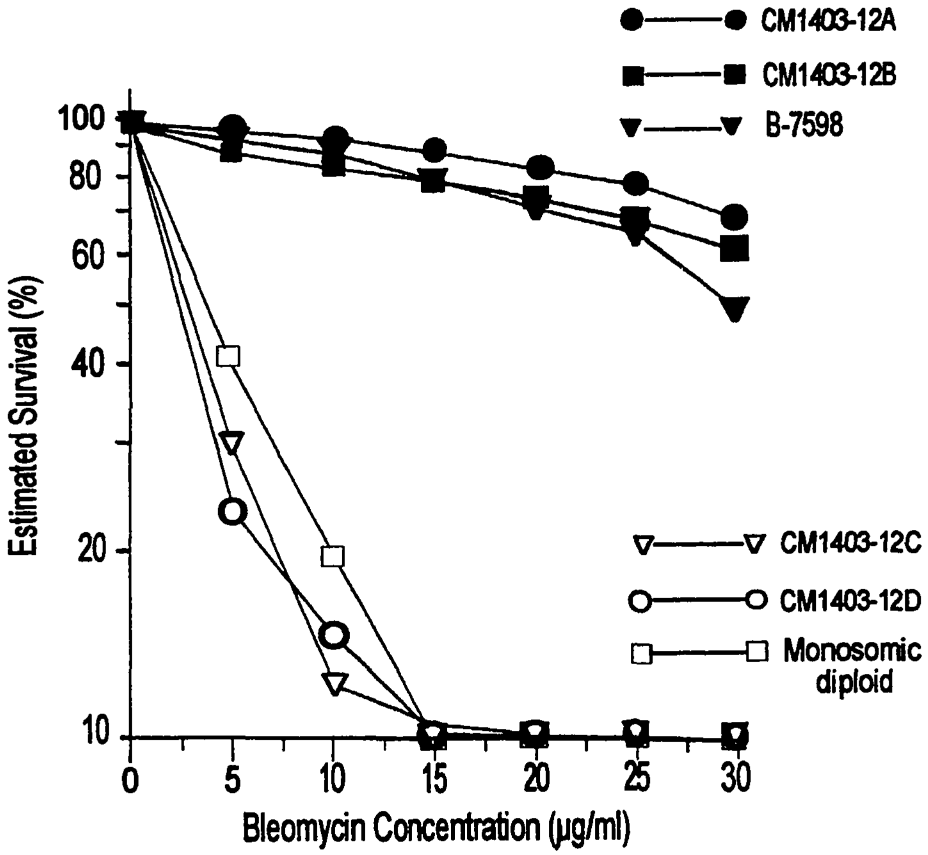


Figure 5-1. Comparative survival of the chromosome XVI monosomic diploid. Strains were grown on solid YPAD plates containing bleomycin for 4 days at 30°C. Survival was estimated by allowing the growth of each strain on YPAD without drug be equivalent to 100% survival and the absence of growth be equivalent to 0% survival.

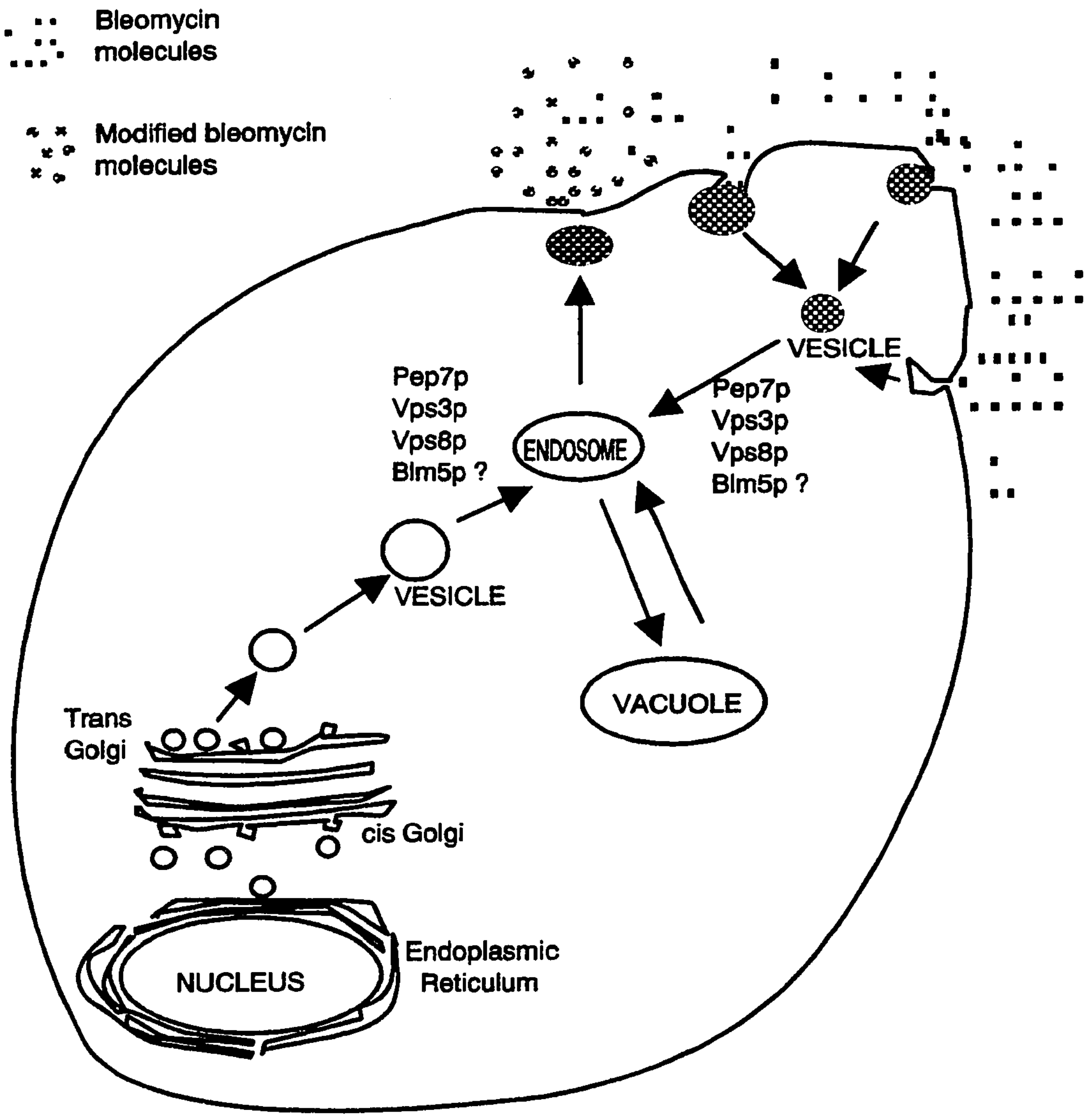


Figure 2. Proposed function of Blm5p in the cell.

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