UNIVERSITY OF CINCINNATI

DATE: September 19,2003

I, Sarah Smith ,

hereby submit this as part of the requirements for the degree of:

Master of Science

in:

Biological Sciences

It is entitled:

Characterization of a High Amplification Rate Strain of *Saccharomyces cerevisiae*

Approved by:

Charlotte Paquin

CHARACTERIZATION OF A HIGH AMPLIFICATION RATE STRAIN OF *SACCHAROMYCES CEREVISIAE*

A thesis submitted to the

Division of Research and Advanced Studies
of the University of Cincinnati

in partial fulfillment of the
requirements for the degree of

MASTER OF SCIENCE

in the Department of Biological Sciences
of the College of Arts and Sciences

2003

by

Sarah Smith

B.S., Mount Union College, 2001

Committee Chair: Dr. Charlotte Paquin

ABSTRACT

Gene amplification is defined as the formation of extra functional copies of genes. This process is important because amplifications can provide drug resistance, are often found in cancer tumor cells and cell lines, and have been implicated in the evolution of new genes. I report here the characterization of B310, a yeast strain with a high rate of amplification isolated using the *ADH4:CUP1* amplification system in the model eukaryote, *Saccahromyces cerevisae*. B310 is especially interesting because a high proportion of its mutations are amplifications and it is incapable of sporulating.

The first set of experiments conducted in this study involved comparing the restriction maps of B310 amplifications to those of previously studied amplifications. In most cases, the B310 restriction maps were nearly identical to the control, suggesting that the B310 amplification structures are probably formed through a similar mechanism.

Next, in order to determine how the B310 amplifications changed over time, cultures were grown for two months and the structures present at the end of the growth periods were compared to those from the beginning. The structures were very dynamic and suggested that amplifications are sometimes integrated into specific chromosomal locations. In one strain, a linear amplification apparently integrated into the same chromosomal location in three independent experiments.

Finally, an experiment was conducted with the intent of identifying the mutation giving rise to the B310 phenotype through complementation of the sporulation deficiency with a yeast genomic library. Two sporulating B310 transformants were detected, and an amplification rate was calculated for a strain containing a deletion of *GPA2*, a candidate

gene identified from the plasmid in a sporulating transformant. Based upon control experiments, however, it was determined that the sporulating cells contained reversion mutations. While this experiment was unsuccessful in identifying the B310 mutation, it did result in the development of new ideas for solving this problem in the future.

ACKNOWLEGMENTS

First, I would like to thank Dr. Paquin for her guidance and support over the past two years. This project has been challenging to say the least, and she has always been the first to provide encouragement and offer new ideas. My lab mate, Madhuri Shirolkar, has been a good friend and an enjoyable person to work with. It has been very helpful to spend so much time with someone who can both discuss science and share a laugh at those moments when sanity is at stake! Julie Stacy has been very generous with her lab supplies and her expertise on certain protocols, while Dr. Keller is responsible for the photographs I was able to take of my genomic screen. My other two committee members, Dr. Grogan and Dr. DeBry, have offered ideas and constructive criticism during the planning and completion of my research.

I also want to acknowledge the friends and family who have helped me through graduate school. My husband, Carey, always believes in me and is my constant strength and motivation. My fellow graduate students have been a source of scientific feedback and have kept me balanced by providing much needed diversions from academia. My friends and family from home, despite not always understanding what I'm doing, have been incredible listeners and supporters.

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

LIST OF ABBREVIATIONS

A.....adenine

ADH.....alcohol dehydrogenase proteins

ATM.....ataxia-telangiectasia mutated protein

BCL-2.....B cell lymphoma/leukemia protein 2

BCR-ABL.....fusion of a breakpoint cluster region and the Abelson leukemia protein

BLAST.....Basic Local Alignment Search Tool

bp.....base pairs

C.....cytosine

°C.....degrees Celsius

C-ABL.....cellular Abelson leukemia protein

CHEF.....clamped homogenized electrophoretic field

C-KI-RAS.....cellular Kirsten rat sarcoma virus protein

CML..... chronic myeloid leukemia

C-MYC.....cellular myelocytomatosis protein

CUP1.....copper-inducible yeast metallothionein protein

DHFR.....dihydrofolate reductase protein

DNA.....deoxyribonucleic acid

ERCC6.....excision repair cross-complementing protein 6

FEN1.....flap endonuclease protein1

G.....guanine

GPA2.....G-protein 2 alpha subunit

HO.....homothallic switching protein

kb.....kilobase

KO.....knock out

LB.....liquid broth for growth of bacteria

MMS.....methyl methanesulfonate

MNNG .....*N*-methyl-*N*'-nitro-*N*-nitrosoguanidine

MRE11.....meiotic recombination protein 11

MSH2.....mutS protein homolog

MTX.....methotrexate

p53.....53 kilodalton protein

PALA.....N-(phosphonacetyl)-L-aspartic acid

PCR.....polymerase chain reaction

[^{32}P]dATP.....^{32}Phosphorus labeled deoxy-adenine tri-phosphate

RAD.....radiation sensitive mutants

rRNA.....ribosomal ribonucleic acid

SAE2.....sporulation in the absence of SPO eleven protein 2

SEPT9.....septin protein 9

SOD2.....superoxide dismutase protein 2

STI-571.....selective tyrosine kinase inhibitor protein 571

T.....thymine

TBE.....tris, boric acid, and ethylenediaminetetraacetic acid buffer

TFIIH.....subunit H of polymerase II transcription factor

tRNA.....transfer ribonucleic acid

Ty.....transposon yeast

UV.....ultraviolet

XPD.....xeroderma pigmentosum complementation group D protein

XRS2.....x-ray sensitive protein 2

YEPD.....yeast extract, peptone, and dextrose media for yeast

All gene names appear in italics, while protein names appear in regular font.

INTRODUCTION

Gene amplification, the formation of extra functional copies of genes, is an interesting phenomenon because of its occurrence in cancer cells and its potential for producing new genes. Some amplifications are regulated events, the classic examples being rRNA gene amplification in *Tetrahymena* and chorion gene amplification in *Drosophila* (Schimke 1984, Hamlin et al. 1984). However, this paper will focus on gene amplifications that are caused by mutations, examples of which have been found in organisms ranging from *E. coli* to humans. These amplifications are most frequently studied in cancer tumor cells or mammalian cell lines where the amplification of certain genes confers drug resistance. Amplification rates in these cells are often between 10^{-4} and 10^{-6} mutations per cell (Tlsty et al. 1989), while amplifications are undetectable in normal mammalian cells, occurring below a level of 10^{-9} (Tlsty et al. 1990, Wright et al. 1990). The fact that amplifications are found in cancer tumor cells but not in normal cells suggests that they are related to the genomic instability associated with cancer. It has also been suggested that amplifications are important in the evolution of new genes and gene families (Hamlin et al. 1984). Amplifications provide extra copies of genes that have the potential to mutate and encode new proteins.

Amplification Structures

Amplifications are defined as any structure that contains an additional copy of a gene. However, these structures can vary in size, shape, copy number, and genetic content. The two general types of amplifications are intrachromosomal and extrachromosomal amplifications. Intrachromosomal amplifications are sometimes called homogeneously staining regions because, in cases of large amplifications, two or

more chromosomal regions can sometimes be seen with similar banding patterns. However, intrachomosomal amplifications often have different banding patterns than the original copy of the amplified region (Hamlin et al. 1984). This may be due to a loss of normal regulation of chromatin structure. The amplified region of DNA is sometimes located adjacent to the original copy, but can also be found on an entirely different chromosome (Schimke 1984). Hamlin et al., 1984, reviews two explanations for the formation of intrachromosomal amplifications. First, it has been suggested that amplifications form next to the original copy and can recombine to change location or become extrachromosomal structures. Alternatively, amplifications may originate as extrachromosomal copies of genes and later become integrated into a chromosome through recombination.

Extrachromosomal amplifications can be linear or circular, very large or very small. Some extrachromosomal amplifications may contain centromeres and therefore segregate equally during mitosis. Many, however, do not contain centromeres and segregate at random. Because they may not segregate properly, extrachomosomal amplifications tend to be easily lost if there is no selection to ensure that they are maintained (Hamlin et al. 1984).

One of the difficulties with understanding amplifications is that the types of structures often vary between cell lines and culturing conditions (Hamlin et al. 1984). There are also a number of treatments that have been found to increase amplification rates and affect the types of amplifications observed. These include ultraviolet radiation, certain cytotoxic drugs, several carcinogens, and the transformation of cells with tumor promoter genes (Schimke 1984). This suggests that there may be several different

mechanisms through which amplifications can be formed. Most of the treatments mentioned above affect cells by hindering DNA synthesis, damaging DNA, or creating errors in replication, suggesting that these processes may be important in the formation of amplifications.

Mechanisms of Amplification

This paper will focus on the four models of gene amplification most commonly encountered in the literature. The first of these deals with disproportionate replication of certain genes. This model is based on the observation that, during eggshell formation, chorion genes in *Drosophila* are often replicated several times in a single cell cycle. This gives rise to a structure that resembles an onion skin due to multiple layers in the replication bubble (Hamlin et al. 1984). As reviewed in Schimke, 1984, the disproportionate replication model is supported by the fact that certain drugs that increase amplification rate, such as cyclohexamide and hydroxyurea, can stop replication temporarily. Presumably, when replication is reinitiated, certain regions of DNA may be replicated again while others will only have been replicated once. Duplicate copies may then connect to each other to form extrachromosomal amplifications, or recombine into chromosomes. However, there is thus far no experimental evidence to support the disproportionate replication model.

It has also been suggested that amplifications could develop as a result of unequal crossing-over between sister chromatids during mitosis or meiosis. Unequal crossing-over occurs when there are repetitive sequences on either side of a gene and recombination occurs between repeats upstream of the gene on one chromatid and

downstream from the gene on the other chromatid. This results in one daughter cell with two copies of the gene while the other daughter cell has none. This model is supported by the fact that high frequencies of homologous recombination have been observed between repetitive regions on chromosomes (Hamlin et al. 1984). There are also a number of examples of amplified genes that appear to be formed through unequal crossing over due to their tandem orientation, such as the genes for color vision (Nathans 1999) and the *CUP1* gene in yeast (Karin et al. 1994). However, it is difficult to explain with this model how some cells can have hundreds of copies of the same gene, often in inverted orientation and on different chromosomes than the original.

The third model, the intermolecular recombination model, involves the uptake of DNA from other sources, as reviewed in Schimke, 1984. This DNA most likely comes from the lysing of nearby dead cells and could persist in the new cell by recombining with the genomic DNA. Recombination between homologous regions in the genomic DNA and the foreign DNA can result in palindromic amplifications if the new copy recombines with the original copy in a head-to-head orientation. Butler et al., 2002, supported the intermolecular recombination model by showing recombination between two yeast plasmids transformed into the same cells. The two plasmids were identical except that they carried different selectable markers. Palindromic structures were obtained consisting of DNA from the first plasmid joined head-to-head with DNA from the second plasmid.

Lastly, the intrachromosomal recombination model depends upon the formation of double strand breaks in regions surrounding the DNA that is to be amplified. This model is based upon regulated amplification of rRNA genes in *Tetrahymena thermophila*

and the observed structures of yeast, protozoan, and mammalian amplifications isolated in the laboratory (as summarized in Butler et al. 1996). In *Tetrahymena*, double strand breaks are created on either side of an rRNA gene. On one side is a short sequence of inverted repeats. The inverted repeats on each strand join together at the break to form a hairpin structure and, after a round of replication, a palindromic amplification results (Butler et al. 1996). This palindrome has two copies of the original gene in inverted orientation with a novel joint region connecting them. The novel joint is simply the structure where two previously unassociated sequences come together after an amplification event. Many mammalian amplifications, including some associated with cancers, are palindromic with inverted repeats on either or both sides of the amplified region of DNA. It seems that this mechanism of amplification formation could easily be generalized to most cell types, considering that short inverted repeats, chromosome breaks, and homologous recombination are common features of most genomes.

The intrachromosomal recombination model is more easily testable than the other models and is supported by evidence involving fragile sites and experimentally induced chromosomal breaks. Fragile sites are simply regions in DNA, often A-T rich, where breaks tend to occur. Some are constitutive, some induced, and some are heritable (Yunis and Soreng 1984). Coquelle et al., 1997, treated cells with an agent that induces breaks only at fragile sites and found several intrachromosomal amplifications with fragile sites flanking them. Searches for known fragile sites in the Human Genome Data Bank have identified them as flanking at least 20 of the genes that are frequently found amplified in cancer cells (Coquelle et al. 1997, Yunis and Soreng 1984). If fragile sites are important in amplification formation, the fact that some are heritable could at least

begin to explain why certain cell lines tend to have different types and sizes of amplifications. Consumption of coffee and cigarette smoking are known to induce breaks at fragile sites, so it has been suggested that environmental factors can increase amplification rates through this mechanism (Coquelle et al. 1997).

Yeast strains with mutated *RAD27* have an increased frequency of breaks at fragile sites (Sutherland et al. 1998) and an increased amplification rate (Tseng 1998). RAD27 is a 5' to 3' flap endonuclease that removes the primer RNA from Okazaki fragments so that the fragments can be properly ligated together. *RAD27* mutants have an extremely high rate of gross chromosomal rearrangements, including translocations, deletions, inversions, and duplication (Chen and Kolodner 1999). These are the result of the chromosomal fragmentation associated with the persistence of RNA primers. *RAD27* mutants are unable to survive without a functional copy of *RAD52*, which is important in recombination, suggesting that recombination is essential for repairing *RAD27* defects (Debrauwere et al. 2001). It has been proposed that amplifications in *RAD27* mutants form through a double strand break at fragile sites or inverted repeats that lend themselves to palindrome formation (Gordenin et al. 1997). This mechanism would be consistent with the intrachromosomal recombination model. Humans do have a homolog of *RAD27*, called *FEN1*, and it appears to possess the same function as in yeast. In fact, transformation of a *RAD27* mutant strain with a copy of *FEN1* complements the *RAD27* mutation (Hansen et al. 2000).

To test the intramolecular recombination model, Butler et al., 1996, developed a *Saccharomyces cerevisiae* plasmid containing inverted repeats from *Tetrahymena*, a copy of the HO endonuclease, and a recognition site for the HO endonuclease near the inverted

repeats. They found that large palindromes resembling *Tetrahymena* palindromes are very efficiently formed by yeast. In a similar experiment with yeast, it was shown that mutations in the proteins that are normally involved in destroying hairpin structures result in higher rates of palindrome formation (Butler et al. 2002). These proteins include SAE2 and members of the MRE11/RAD50/XRS2 complex.

All of the models described above involve recombination to some extent. Several studies in yeast have demonstrated the importance of homologous recombination in amplification formation. Butler et al., 2002, measured the rates of palindrome formation with deletions of two genes involved in homologous recombination. Deletion of *RAD51*, which is involved in the formation of Holliday junctions, greatly reduces the rate of palindrome formation. Deletion of *RAD52* reduces palindrome formation to below the level of detection (Peterson et al. 2000, Butler et al. 2002). RAD52 possesses a variety of functions: single strand annealing, strand exchange, homologous pairing of strands, and break induced replication (Sung et al. 2000). There is a human homolog of RAD52 and it is also important in recombination, but not quite as critical as in yeast. It may be that there are other proteins with overlapping functions in mammals, or that mammalian RAD52 doesn't carry out so many functions as the yeast homolog.

A few studies have suggested that telomeres can also play a role in amplification formation. Patterson et al., 1999, found that proximity to telomeres can affect the structure of amplifications. The *SOD2* gene in fission yeast is normally found near a telomere and, when amplified, takes the form of a fairly small fragment attached to telomere sequences, probably on both ends. When the *SOD2* gene is deleted from its normal location and inserted at a location farther from telomeres, the amplifications are

found on large chromosomal fragments. Moore et al., 2000b, discovered telomere sequences at the novel joints of several palindromic amplifications in yeast. Mammalian chromosomes that are lacking telomeres are prone to developing amplifications, and cells with defects in telomere maintenance are prone to cancer (Desmaze et al. 2003). The connections between amplifications and cancer will be discussed below.

Despite the debate surrounding the mechanisms for amplification formation, it certainly seems possible that many, if not all, of the models described here can work under certain circumstances. Differences between cancer cells, cultured cells lines and yeast strains could effect how, at what point in the cell cycle, and at what locations in the genome amplifications are able to develop.

Drug Resistance through Gene Amplification

Amplifications of certain genes confer drug resistance both in laboratory and clinical settings. The amplifications used in research are generally identified in strains prone to amplification by treating cells with a selective agent, usually a cytotoxic drug, which will kill those cells not containing additional copies of a certain gene. Often, the amplified genes confer drug resistance by increasing the rate of efflux of the drug (Schimke 1984). Most laboratories have found that cultured cells treated with selective agents have high rates of amplification, usually between 10^{-3} and 10^{-6} (Schimke 1984).

To determine whether amplification occurs throughout the genome, Giulotto et al., 1987, identified cell lines that were resistant to two drugs often used for selection, PALA and MTX. These lines were found to also be resistant to three more drugs to which the cells had not previously been exposed. This suggests that amplification is not

simply occurring at the locus under selection in amplification-prone cells, but across the entire genome. In fact, as reviewed in Hamlin et al., 1984, amplifications have been found at nearly every locus that can be selected with a particular agent.

Several of the drugs used in the laboratory to select for amplifications are also used clinically. For example, MTX selects cells containing amplifications of the *DHFR* gene and is also used as a treatment for certain cancers. Amplified copies of *DHFR* have been found in leukemia patients who were treated with MTX (Wright et al. 1990).

The target gene of a tyrosine kinase inhibitor drug, STI-571, has been found to be amplified in some cases of drug resistance in chronic myeloid leukemia (CML) patients (Gorre et al. 2001). CML results from the creation of a fusion gene, *BCR-ABL*, due to chromosomal translocation. The BCR-ABL protein is a constitutive tyrosine kinase that can be blocked by STI-571. Amplifications of *BCR-ABL*, once created, increase in copy number during STI-571 treatment until the drug is no longer effective. If STI-571 use is discontinued, *BCR-ABL* amplifications rapidly decrease. This system shows that gene amplification can cause drug resistance in clinical cases and that the amplifications are maintained as long as the selective pressure is present. Understanding the relationship between gene amplification and drug resistance, in both clinical and laboratory settings, could provide clues as to how this problem might be combated.

Amplifications in Cancer Cells

Amplifications are often found in tumor cells, including cultured cells and those taken directly from patients. It seems likely that these cancer cells, as well as immortalized cell lines, are prone to amplification because of other mutations in their

genomes. It has been observed that those cells that do develop amplifications tend to grow more slowly than normal cells, possibly due to defects in metabolism or cell division (Giulotto et al. 1987). They are often also more prone to other types of chromosomal rearrangements and point mutations (Bishop 1991).

A convincing line of evidence points to the loss of cell cycle controls as being an important step in making cells prone to amplification events. Mammalian cells with relaxed checkpoints, including most immortalized cell lines, have higher amplification rates than those with intact checkpoints (Sharma and Schimke 1994). In many cases, the loss of checkpoint controls in amplification-prone cells is probably due to loss of p53. p53 is a ubiquitin-conjugating enzyme known to function as a tumor suppressor. p53 defects are common in cancer cells and cell lines, while cells with normal p53 very rarely develop amplifications (Livingstone et al. 1992). p53 mutations are believed to increase amplification by decreasing cell cycle arrest and apoptosis.

The importance of apoptosis in the accumulation of amplifications has been demonstrated by several laboratories. Yin and Schimke, 1996, found that overexpression of the apoptosis regulator BCL-2 temporarily delays apoptosis and increases amplification rate. Montagna et al., 2003, showed that *SEPT9* is frequently amplified in mouse models of breast cancer and in human breast cancer cell lines. SEPT9, when overexpressed, downregulates apoptosis. Presumably, this decrease in apoptosis allows the errors that might give rise to amplifications to accumulate without fatal consequences. With apoptosis intact, cells with irreparable errors would be killed. Interestingly, BCL-2 has been found to affect apoptosis in cancers (Yin and Schimke 1996). This lack of

apoptosis may allow the genomic instability, including amplification events, that can eventually give rise to cancerous growth.

Mutations in the gene *ATM* cause ataxia telangiectasia, a disease associated with neurodegeneration, immunodefieciency, and predisposition to cancer. Most cell lines carrying mutations in *ATM* have unstable genomes, no cell cycle arrest, and high rates of gene amplification (Mondello et al. 2001). However, cells with mutations in only *ATM* do not have measurable amplification rates despite the fact that ATM is believed to be important in signaling cell cycle arrest through the regulation of p53. Therefore, Mondello et al., 2000, suggest that there might be another protein that overlaps ATM function with regard to p53. It is possible that ATM deficiency makes cells somehow prone to amplification and cancer, but that other mutations must accumulate before cell cycle controls are lost.

Checkpoint controls have also proven to be important in amplification formation in yeast. Patterson et al., 1999, found increased amplification rates in strains that had defects in the DNA damage checkpoint. The DNA damage checkpoint exists so that DNA damage can be detected and repaired if at all possible. Clearly, yeast are single-celled and therefore have no pathway for apoptosis. If an error is not detected, or is detected and can not be repaired, the cell will continue to live for as long as possible with the defect. This persistence of defects, although different from mammalian systems, is similar in that it increases the chances that amplifications will develop.

More links between amplifications and cancer can be found in the mismatch and excision repair systems. Individuals with defects in mismatch repair proteins are known to possess a predisposition to cancer, while cell lines containing certain mismatch repair

defects are known to have high amplification rates (Chen et al. 2001, Lin et al. 2001). In both prokaryotic and eukaryotic cells, functional mismatch repair systems suppress recombination occurring at sites of imperfect homology, including imperfect inverted repeats (Lin et al. 2001). Villemure et al., 2003, demonstrated that defects in the human mismatch repair protein hMSH2 reduced accurate homologous recombination at the sites of double strand breaks. Deletion of the yeast homolog *MSH2* increases recombination between non-identical sequences (Selva et al. 1995) and increases gene amplification (Peterson et al. 2000). It is also possible that defective mismatch repair proteins can cause pauses in replication when they can not effectively fix an error, and this could result in DNA breaks. Either of these consequences of mismatch repair defects could be consistent with high rates of amplification because, as described above, both recombination and replication appear to be important in amplification formation.

Nucleotide excision repair is the process of removing DNA fragments containing damaged bases, and is carried out through the interactions of at least 22 proteins (Friedberg 2001). TFIIH, a general transcription factor composed of six subunits, is important in excision repair because it contains helicases that unwind DNA as well as endonucleases that excise fragments containing damage. RAD3 in yeast, called XPD in humans, is one of the helicases. Defects in RAD3 increase the amplification rate in yeast (Peterson et al. 2000) while defects in ERCC6, another helicase involved in excision repair, increase amplification in Chinese hamster cell lines (Mondello et al 1995). Defects in nucleotide excision repair can lead to cancer predisposition, as in the case of Xeroderma pigmentosum. This is a disease characterized by an increased sensitivity to UV damage, and thus, an increased chance of developing cancer. XPD is one of the

genes that, when mutated, can lead to Xeroderma pigmentosum. Therefore, the accumulation of amplifications might be related to the cancer-prone phenotype of Xeroderma pigmentosum.

The amplifications found in cancer cells are simply additional copies of normal genes. In some cases, these amplifications may be caused by pre-existing defects that can give rise to cancer. However, it also seems possible that the amplifications themselves can be the cause of cancerous growth. As reviewed in Hamlin et al., 1984, several independent laboratories have identified amplifications of the oncogene *C-MYC* in leukemia patients and tumor cell lines. Some examples of *C-KI-RAS* and *C-ABL* oncogene amplification have also been observed. These oncogenes, then, are expressed at much higher levels than in normal cells. One can easily imagine how amplification of a gene that normally regulates cell growth could give rise to unregulated, pre-cancerous growth.

The degree of gene amplification in human cancer cells is sometimes used as an indicator of prognosis and tumor progression. Singh et al., 2002, showed that the percentage of cells containing amplifications of the 3q26.3 locus corresponds to the stage of progression of head and neck squamous cell carcinomas, ranging from 3% in normal cells to 56% in invasive cancers. These percentages also serve as indicators of prognosis, where individuals with lower levels of amplification have a better chance at long term survival. This data suggests that if researchers can find a way to decrease the level of gene amplification in cancer patients, it could greatly improve prognosis and slow the development of tumors.

Role of Amplification in Evolution

Although most amplification research revolves around cancer, evolutionary biologists regard amplification as an important source of new genes. Considering the complexity of gene regulation and the fact that there is no selection for non-coding sequences, building a gene from scratch would be a very time-consuming process. With amplified copies of pre-existing genes, however, regulatory and coding regions are often already intact and only a few mutations in the transcribed region could result in the expression of a new protein (Ohno 1970). In order for an amplified region to evolve into a new gene, it must be able to become a permanent fixture in the genome. Some amplifications do appear to be very stable, meaning that even without selection, they only gradually decline in copy number. Other amplifications are unstable and are quickly lost in the absence of selection. When grown long term under selective conditions, the stability and types of amplifications in mammalian cells often changes, as reviewed in Schmike et al., 1984. Early in the growth period, amplifications are usually extrachromosomal without centromeres and are unstable. Intrachromosomal structures are often found later in the growth period and these appear to be much more stable. It has been suggested that the extrachromosomal structures are being integrated into the genome over time. This integration could provide a selective advantage due to the fact that intrachromosomal amplifications are equally segregated during cell divisions and are not quickly lost if the selective pressure is temporarily removed.

Amplifications that are integrated into chromosomes may still be lost in some cases, but there is a great deal of evidence that some have become permanent parts of eukaryotic genomes. It has been reported that the human genome has at least 1520 gene

families consisting of genes with very high degrees of homology (Friedman et al. 2003). While the functions of most of these genes are not yet known, familiar examples include genes encoding tRNA, histones, and immunoglobulins (Hamlin et al. 1984). It's likely that there was only one original type of histone, for example, but that amplifications of that histone mutated over time and began encoding different proteins. Interestingly, the data presented by Friedman et al., 2003, suggests that intramolecular recombination can account for more of the recent amplifications than intermolecular recombination. There are also genes that exist in exact duplicates in higher eukaryotic genomes, including hexokinase, ferrodoxin, and alcohol dehydrogenase (Hamlin et al. 1984). An original copy was probably amplified and the new copies were maintained because they provided a selective advantage in the production of additional protein.

Again, amplification is undetectable in normal mammalian cells, but even a very low rate might be enough to occasionally generate a new gene. Also, considering that amplification appears to be common to most cell types, it may be a more significant source of new genes in organisms with higher amplification rates. The fact that some of the gene families found in humans are evolutionarily very old, like tRNAs for example, suggests that some advantageous amplifications have been maintained for millions of years.

Yeast as a Model system for Studying Gene Amplifications

Much of the research discussed thus far has been conducted with mammalian cells. Clearly, it would be ideal to learn everything about amplifications from mammals in order to best respond to cancers and drug resistance. However, there are problems

with mammalian systems that make the studying of gene amplification difficult. First, amplifications are undetectable in normal cells, and even in most cells that harbor just a few mutations. Generally, amplifications can only be identified in cells that already have many mutations, such as tumor cells or cell lines. Therefore, although it is possible to determine which genes are being amplified, it is difficult to attribute an amplification-prone phenotype to any single mutation. Secondly, most of the drugs used to select for amplifications in mammals have been shown to increase amplification rates themselves by inducing DNA damage (Yin and Schimke 1996).

Because of these problems with mammalian systems, some laboratories choose to study amplifications in yeast. Yeast have low but detectable levels of amplification in normal cells, about 10^{-10} mutations per cell per generation (Walton et al. 1986). This is probably because yeast, as single celled organisms, lack apoptosis. However, being eukaryotes, they do share several basic features with mammals, particularly in the systems that appear to be important in amplification. These include DNA replication, recombination, and repair. Yeast data with regard to amplification is very consistent with mammalian data, showing that cell cycle checkpoints, mismatch repair, excision repair, and recombination are very important (Patterson et al. 1999, Peterson et al. 2000, Butler et al. 2002). The structures of yeast amplifications also resemble those characterized from mammalian cells (Paquin et al. 1992, Dorsey et al. 1992, Dorsey et al. 1993, Butler et al. 1996, Butler et al. 2002).

ADH4:CUP1 System

In the Paquin lab, amplifications are identified using the *ADH4:CUP1* system (Dorsey et al. 1993). This system provides two levels of selection for strains carrying amplifications, allowing these strains to be more easily identified than using other selection protocols. First, *ADH1* is deleted from strains to be used in the screen. ADH1 is an alcohol dehydrogenase important in fermentation. There are three other known alcohol dehydrogenases in yeast, ADH2, ADH3, and ADH4. ADH2 is repressed in the presence of glucose, ADH4 is not normally expressed, and ADH3 is expressed only in mitochondria. This makes ADH1 critical for fermentation under normal circumstances. Without it, yeast switch to respiration when growing on glucose. However, strains can acquire the ability to ferment without ADH1 if ADH2 or ADH4 are expressed. This can happen through four different types of mutations, as summarized in Paquin et al., 1992. Ty elements can be inserted upstream from *ADH2* or *ADH4*, presumably disrupting normal regulation. Up promoter mutations or mutations in regulatory genes can also result in ADH2 or ADH4 expression. Lastly, amplifications of either *ADH2* or *ADH4* can allow expression of these genes. Therefore, fermentation in the absence of ADH1 can be used to find gene amplifications. In fact, one extra copy of *ADH4* is enough to make a cell capable of fermenting without ADH1, which is important for this system because it allows for detection of low copy number amplifications (Dorsey et al. 1992). In order to find *ADH1* knockout cells that can ferment, cells are grown on media containing antimycin A. Antimycin A blocks respiration so that cells subjected to antimycin A on glucose media will die if they can't ferment. As described above, antimycin A resistant cells don't always have amplifications, but this selection at least narrows the search for amplifications.

The antimycin A screen was used to identify several *ADH4* and *ADH2* amplifications (Walton et al. 1986, Paquin et al. 1992, Dorsey et al. 1992). However, amplifications of *ADH4* are present in only approximately 1% of antimycin A resistant mutants (Dorsey et al. 1992), requiring that many cells be studied before amplifications are found. A second level of selection was added by deleting the *CUP1* gene from its normal location on chromosome VIII and inserting it 490 bp downstream from the normal copy of *ADH4* on chromosome VII (Dorsey et al. 1993). CUP1 chelates copper and provides higher levels of copper resistance than normal when present in additional copies. Therefore, cells that are resistant to antimycin A can then be plated on copper media and those that can grow under both conditions are likely to have amplifications of the *ADH4:CUP1* construct.

Since selection for amplifications is fairly efficient, it is possible to create a mutant strain and quickly determine whether the mutation affects amplification rate. Alternatively, strains with high amplification rates can be isolated and the mutations identified. Using these strategies, the Paquin lab has discovered that mutations in *RAD3, RAD52, MSH2,* and *RAD27* affect amplification rate. The significance of each of these genes was discussed earlier in this paper.

The *ADH4:CUP1* system has also allowed several amplification structures to be observed and characterized. All three types of amplifications, intrachromosomal, extrachromosomal circles, and extrachromosomal linears have been identified. The intrachromosomal amplifications are generally found in low copy number, while circles and linear extrachromosomal structures are often present in high copy number (Dorsey et al. 1992). In most cases, the normal copy of *ADH4* is maintained on chromosome VII

despite amplification events, suggesting that a replication step is usually involved in the formation of these structures (Peterson et al. 1993).

Restriction maps have been generated for the novel joints of several of the *ADH4:CUP1* amplifications. Some amplifications have identical restriction maps, while others are different (Dorsey et al. 1992). This suggests that there may be sites where amplification events are frequently initiated, but that there are more than one of these sites near *ADH4*. There are several small inverted repeat sequences between *ADH4* and the centromere of chromosome VII within the region where novel joints were mapped (Peterson et al. 1992). These could be potential sites of recombination important in creating amplifications

The linear amplifications identified in the Paquin lab do not segregate equally during meiosis and therefore, must not possess centromeres (Walton et al. 1986). These linear amplifications, when denatured, decrease in size by fifty percent. This decrease indicates that single stranded linear amplifications are able to base pair with themselves, forming hairpin molecules half the length of the original amplification. The formation of hairpins is consistent with palindromic molecules. Restriction maps were made of these linear amplifications and the maps show a palindromic pattern of restriction sites. It was determined through hybridization experiments that there are telomeric sequences on the ends of these *ADH4* amplifications (Walton et al. 1986).

Four circular amplifications have been characterized in the Paquin lab. Each of these contains the centromere of chromosome VII. The same novel joint is found on every copy of the amplification in three of the strains, and the DNA between the *ADH4:CUP1* construct and the centromere appears to be on the amplification (Moore et

al. 2000a). In all four strains, a telomeric C-A repeat was found joined to a C-A rich region normally located upstream of *ADH4*. Therefore, it may be that the novel joints of these strains are formed by recombination between the C-A rich region and a telomere. These data, combined with the data concerning palindrome structure and inverted repeats in the region of novel joints, support the intrachromosomal replication model for formation of the amplifications found in the Paquin lab. This model was briefly described above, but has also been adapted from Butler et al., 1995, to fit the *ADH4:CUP1* system 1 (Figure 1). The intramolecular recombination model is consistent with the structures observed in *ADH4:CUP1* amplifications and can account for the formation of both circular and linear amplifications. There are two pathways that can be followed in this model. If the chromosome is broken at a location such that the amplified region will not contain a centromere, a hairpin structure can result and a palindrome will be formed during the next round of replication. This palindrome can remain linear or may in some cases circularize. If the chromosome is broken at a location such that the amplified region will contain a centromere, a similar hairpin and palindrome can form, but the palindrome will contain two centromeres. In this case, a breakage-fusion-bridge cycle can be initiated.

The breakage-fusion-bridge cycle was first described by McClintock and is reviewed in McClintock, 1984. This cycle was later adapted as a model for gene amplification (Stark 1993), and has been applied to the *ADH4:CUP1* system (Figure 2). In this model, two chromosomes or chromosomal segments containing centromeres fuse to form a dicentric chromosome. As described above, a dicentric chromosome could also result from palindrome formation. These centromeres pull apart during cell division,

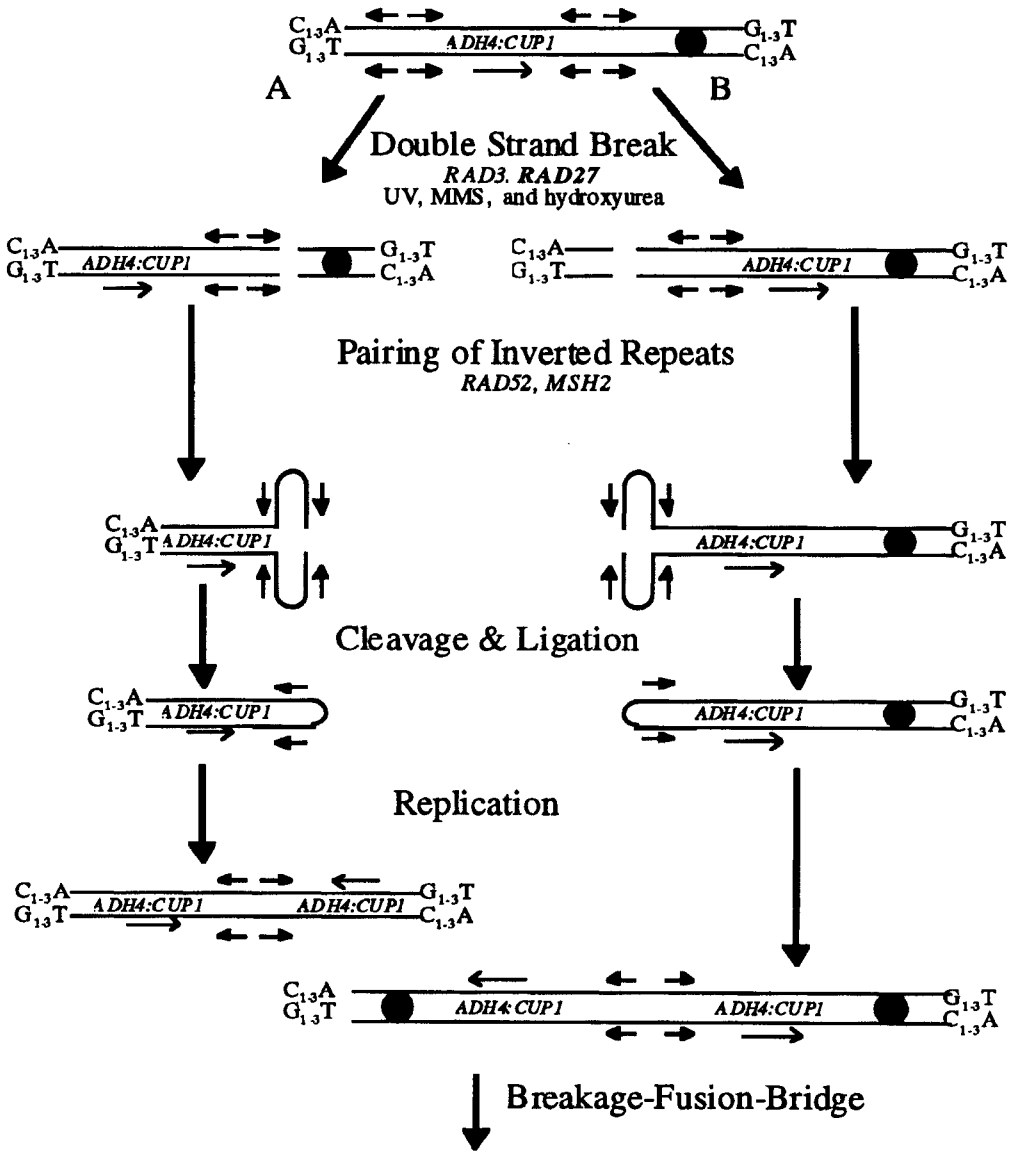

Figure 1: Intrachromosomal recombination model of gene amplification, adapted to fit the *ADH4:CUP1* system from Butler et al., 1995. $G_{1\text{-}3}T/C_{1\text{-}3}A$ sequences represent telomeres and filled in circles represent centromeres. Open arrows show the direction of *ADH4:CUP1* transcription. Filled arrows show the orientation of *ADH4:CUP1* constructs at the novel joints.

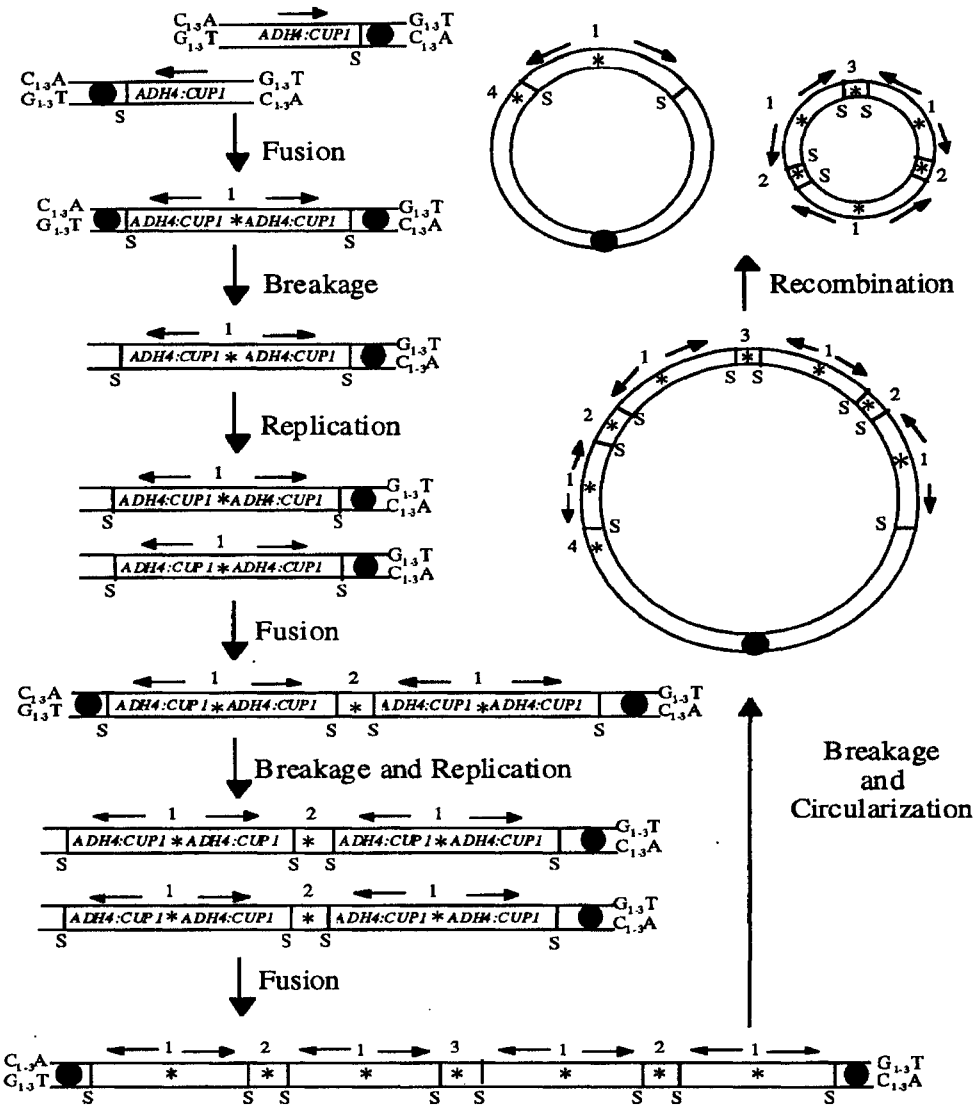

Figure 2: The breakage-fusion-bridge model of gene amplification adapted from Stark, 1993, to account for the *ADH4:CUP1* system. Telomeres are represented by $G_{1-3}T/C_{1-3}A$ sequences. Filled circles represent centromeres and S represents *Sfi*I restriction sites adjacent to *ADH4*. Arrows indicate the direction of transcription of *ADH4:CUP1*. Asterisks represent novel joints, which are numbered in the order in which they are created.

breaking the chromosome. The broken chromosomes replicate before the next cell division and fuse to one another, creating the bridge, because of the instability associated with having broken ends. The same process will repeat, increasing the copy number of the amplified region during each cycle. The cycle can end when a broken chromosome circularizes with only one centromere or if telomeres are added to the broken end to form a more stable linear molecule with only one centromere. These structures could of course be various sizes depending upon the amount of DNA amplified, the number of cycles completed before stabilization, and the location of the break where the molecule either circularized or had telomeres added to the ends.

Characterization of a High Amplification Rate Strain of Yeast

The strains and amplification structures studied in the Paquin lab have helped to elucidate the importance of certain genes and pathways important in amplification formation. Structural data have supported the intramolecular recombination model, a model also consistent with much of the mammalian data. However, because the *ADH4:CUP1* system is so effective in isolating strains with high amplification rates, strains and amplifications have been isolated that have not yet been characterized. One such strain was named B310. This strain was created by MNNG mutagenesis of a wild-type strain with the *ADH4:CUP1* background and has an amplification rate of approximately 10^{-8} (Peterson et al. 2000). About 99% of B310 mutants that can grow on antimycin A and copper media contain amplifications, which is a much higher percentage than in any other strain studied in the Paquin lab (Peterson et al. 2000). This suggests that B310 is specific for the formation of amplification mutations. Unlike some other

high amplification rate strains, B310 apparently has no defects in DNA damage repair, since it is not sensitive to MMS, hydrogen peroxide, UV radiation, or gamma radiation. However, B310 is incapable of sporulating. Because it is unique, it may be that B310 is the result of a mutation that has not been previously identified as being related to amplification. It is also possible that the mechanism of amplification formation in B310 is different from that of the other strains studied in the Paquin lab.

The work described in this thesis involves the characterization of B310, with three major research goals. First, restriction maps were created for the novel joints of several B310 amplifications. These were compared to the restriction maps already generated for a circular amplification strain, A4C-EA1. A4C-EA1 is known to possess amplification structures consisting of two copies of *ADH4:CUP1*, one with a restriction pattern identical to the normal copy of the construct and one with a unique pattern (Moore et al. 2000b). Examples of circular, linear, and intrachromosomal B310 amplifications were studied. Most of the restriction patterns of the B310 strains resemble those already mapped, so it seems likely that the B310 amplifications are formed through a similar mechanism and have similar structures.

The second objective was to determine how B310 amplifications change over time. Members of the Paquin lab have observed in the past that the types of *ADH4:CUP1* amplifications (intrachromosomal, linear, or circular) sometimes change within a given strain during long term growth. To further explore these changes, B310 amplification strains beginning with all three types of structures were grown in liquid culture over long periods of time. The types of structures present at the end of the growth periods were compared to the structures at the beginning. In the case of a particularly

interesting change in structure, weekly intermediates throughout a growth period were examined and the genetic content of the amplifications was studied in more detail. The changes in structures observed may serve as clues as to how amplification structures evolve over time. For example, it appears that some extrachromosomal amplifications became integrated into chromosomes, which serves as evidence that even the less stable amplifications have the potential to become permanent fixtures in the genome.

The third research objective was to determine which gene is mutated in the B310 strain. A genomic screen based upon complementation of the sporulation deficiency was used (see Rose and Broach, 1991, for a discussion of cloning by complementation). Since DNA breaks and recombination are important processes in sporulation, it seems likely that the mutation giving rise to the high amplification rate is the same mutation that gives rise to the sporulation deficiency. Genomic clones with restored sporulation were sequenced and a candidate gene, *GPA2*, was chosen for further study. A *GPA2* deletion strain was created in the *adh1Δ* background, and the effects of the deletion on amplification rate were measured.

MATERIALS AND METHODS

Strains and Media

The *Saccharomyces cerevisiae* strains used in this study are shown in Table 1. 411B, 411B-2N and A4C2 are wild type strains with the *ADH4:CUP1* background. A4C-EA1 contains circular amplifications and was derived from a parent strain nearly identical to A4C2 (Moore et al. 2000). The B310 amplification strains were isolated from the haploid B310 parent strain. The GPA2Δ and its parent strain (KO parent) were purchased from Open Biosystems. These strains were created through PCR-based gene disruption (described in Johnston et al. 2002) with *Kan Tn 903*, a gene that confers resistance to the antibiotic G418. *E. coli* strain DH5α was used for transformations.

Yeast strains were maintained on antimycin A plates (Dorsey et al. 1993), YEPD plates, or YEPD liquid culture (Ciriacy 1979). In yeast transformations, successful transformants were detected on minimal media lacking leucine (Sherman 2002)). Sporulation was conducted on SPO media, as described in Kassir and Simchen, 1991. G418 media (Johnston et al. 2002) was used for identifying *GPA2Δ* strains. *E. coli* was maintained on LB or LB-amplicillin (Maniatis et al. 1982), and 2XL media was used for *E. coli* transformations. All yeast cultures were grown at 30°C and *E.coli* was grown at 37°C.

DNA Preparation and Southern Blots

Yeast genomic DNA was prepared from overnight cultures in YEP by the phenol/chloroform method of Denis and Young, 1983. DNA was digested with restriction enzymes as recommended by New England Biolabs, except that the amount of enzyme was doubled. This DNA was electrophoresed in TBE buffer at 10 volts

Table 1: *Saccharomyces cerevisiae* strains used in this study.

Strains	Genotypes	Sources
411B	MATa, *adh1Δ-1, ADH4::CUP1, ADH2, ADH3, cup1Δ*	Peterson et al. 2000
411B-2N	MATa/α, *adh1Δ-1/adh1Δ-1, ADH4::CUP1/ADH4::CUP1, ADH2/ADH2, ADH3/ ADH3, cup1Δ/cup1Δ*	Peterson et al. 2000
A4C2	*MATa/α, adh1Δ-1/adh1Δ-1, ADH4::CUP1/ADH4::CUP1, ADH2/ADH2,ADH3/ ADH3, cup1Δ/cup1Δ*	Dorsey et al. 1993
A4C-EA1	*MATa/α, adh1Δ-1/adh1Δ-1, ADH4::CUP1/ADH4::CUP1, ADH2/ADH2, ADH3/ ADH3,cup1Δ/cup1Δ, ADH4::CUP1-EA1*(extrachrachromosomal amplification-1)	Moore et al. 2000
B310	*MATa, adh1Δ-1, ADH4::CUP1, ADH2, ADH3, cup1Δ*	Peterson et al. 2000
B310-2N	*MATa/α, adh1Δ-1/adh1Δ-1, ADH4::CUP1/ADH4::CUP1, ADH2/ADH2, ADH3/ ADH3,cup1Δ/cup1Δ, leu2Δ/leu2Δ ura3Δ/ura3Δ*	this study
B310-5-1-738C	*MATa, adh1Δ-1, ADH4::CUP1, ADH2, ADH3, cup1Δ, ADH4::CUP1*(circular amplification)	Peterson et al. 2000
B310 -5-2-777C	*MATa, adh1Δ-1, ADH4::CUP1, ADH2, ADH3, cup1Δ, ADH4::CUP1*(circular amplification)	Peterson et al. 2000
B310-5-3-831LP	*MATa, adh1Δ-1, ADH4::CUP1, ADH2, ADH3, cup1Δ, ADH4::CUP1*(linear palindrome)	Peterson et al. 2000
B310-5-4-865 LP	*MATa, adh1Δ-1, ADH4::CUP1, ADH2, ADH3, cup1Δ, ADH4::CUP1*(linear palindrome)	Peterson et al. 2000
B310-5-6-964CI	*MATa, adh1Δ-1, ADH4::CUP1, ADH2, ADH3, cup1Δ, ADH4::CUP1*(circular amplification)	Peterson et al. 2000
B310-6-6-1243CI	*MATa, adh1Δ-1, ADH4::CUP1, ADH2, ADH3, cup1Δ, ADH4::CUP1*(circular and intrachromosomal amplifications)	Peterson et al. 2000
GPA2Δ	*MATα, ADH1, ADH2, ADH3, ADH4, CUP1, gpa2Δ, ura3Δ*	Winzeler et al. 1999
KO parent	*MATα, ADH1, ADH2, ADH3, ADH4, CUP1, ura3Δ*	Winzeler et al. 1999

overnight on 0.7% agarose gels. The gels were then stained in a bath of ethidium bromide in TBE buffer and viewed under UV light.

Intact chromosomal DNA was extracted while embedded in low melt agarose, as described in Carle and Olson, 1985. Intact DNA was electrophoresed on CHEF (clamped homogenized electrophoretic field) pulsed-field gels (Chu et al. 1986). The gels were then stained and viewed as above.

Southern blots were prepared as described in Klessig and Berry, 1983. Probes were gel purified from plasmids and labeled with [^{32}P]dATP by random priming (Sambrook et al. 1989). The *ADH4:CUP1* probe was isolated from the p7a plasmid (Dorsey et al. 1993) and the IKM-2, *RAD54*, *CEN7*, and *RAD2* probes were isolated from plasmids described in Moore, 1997. Regions where the probes hybridized to the blots were visualized using a Packard Cyclone phosphoimager and OptiQuant software.

Long Term Growth of B310 Strains

Amplification and control strains were grown in 25 ml YEP+glucose. Approximately 10^8 cells from each culture were transferred to fresh media twice a week. 0.5 ml samples from the cultures of each strain were frozen in 0.5 ml 50% glycerol once a week. These frozen strains are listed in Appendix B. The cultures were continued for one to two months. This was repeated for three independent long term growth experiments. For the initial blot and the 5-4-865LP intermediates, intact DNA was prepared from liquid cultures derived from single colonies. For the blots representing the end of each long term growth experiment, intact DNA was prepared directly from the 25 ml cultures.

Sporulation Screen

The *leu-* B310 diploid strain was constructed by mating two *leu-* B310 haploids. A *LEU2* genomic library was transformed into this diploid using a high-efficiency yeast transformation protocol (Gietz and Woods 2002), and transformants were detected on *leu-* minimal media. Transformed colonies were tested for sporulation using the assay described in Esposito et al., 1991. In this assay, cells are grown on filters, transferred to sporulation media, and then the cell walls are broken open and treated with ammonium hydroxide. Under long wavelength UV light, a fluorescent membrane protein can be seen in sporulating cells.

Plasmid DNA Preparation and Sequencing

Plasmid DNA was extracted from yeast cells using a QIAGEN Plasmid Midi Kit. The cells were treated with yeast lytic enzyme, and then the directions for the Midi Kit were followed as described in the manual. The plasmids were transformed into *E. coli* using the heat shock method described in Maniatis et al., 1982, then extracted from *E. coli* with the QIAprep Spin Miniprep Kit, following the directions for >10 kb plasmids. Restriction digests of plasmid DNA were performed as described for yeast genomic DNA, except that the gels were run at 95 volts for approximately 1.5 hours.

Sequencing reactions were carried out on an ABI PRISM® 3700 DNA Analyzer, located at the University of Cincinnati Children's Hospital. Sequences were analyzed using the BLAST program on the *Saccharomyces* Genome Database website (http://www.yeastgenome.org).

Rate of Mutation to Antimycin A

Ten independent cultures of each transformant (SPO1, SPO1-2, B310-2N with p366) were grown in *leu-* minimal media for one week. Each culture was plated on an antimycin A plate, and dilutions from each culture were plated on YEPD. For each of the three transformants, the number of antimycin A plates containing growth was counted. The average number of cells plated on antimycin A was determined based upon the amount of growth on YEPD. The rates of mutation to antimycin A were calculated by the P_0 method in Lea and Coulson, 1949. This method is described in Appendix A.

Amplification Rates

GPA2Δ was mated with 411B and sporulated on sporulation media. The asci were broken open with glusulase and the spores separated by sonication (Sherman 2002). Spores were grown into colonies on YEPD media. Approximately 400 colonies were replica plated onto antimycin A and G418 media. One of the antimycin A sensitive/G418 resistant strains (an ADH1Δ/GPA2Δ strain) was plated on YEPD and allowed to grow for one week. Sixty of the resulting colonies were plated on 60 antimycin A plates. After one week, intact DNA was prepared from colonies on each of the antimycin A plates that contained growth. Amplifications were identified on CHEF gels and an estimate of amplification rate was calculated by the P_0 method in Lea and Coulson, 1949. The same procedure was followed for creating an *ADH1* deletion in the parent strain of GPA2Δ and determining its amplification rate.

RESULTS

B310 is an interesting strain because of its high amplification rate, specificity for amplification mutations, insensitivity to DNA damaging agents, and inability to sporulate. Since B310 is unique, it was hypothesized that its amplifications might be formed through a different mechanism than the other high amplification rate strains studied in the Paquin lab. To test that hypothesis, the structures of B310 amplifications were compared to those of strains that had previously been characterized. B310 amplification strains were also used in a long term growth study in order to investigate how the structures changed over time. B310 was created through mutagenesis, so the mutation giving rise to the B310 phenotype was unknown. Identification of this mutation was attempted through complementation of the sporulation deficiency with a genomic library.

Restriction Digests of B310 Novel Joints

To determine whether B310 amplification structures are unique, restriction digests of their novel joints were compared to those of previously studied strains. DNA from B310 and control strains (411B, A4C2, A4C-EA1) was digested using three individual restriction digests and two double digests. The blots of these digests were probed with the *ADH4:CUP1* construct, which was isolated through an *Eco*RI/*Sal*I digest of p7a (Figure 3). The B310 restriction fragments that hybridized to the probe were compared to those of A4C-EA1 and the previously published restriction maps of p7a (Figure 3), A4C and A4C-EA1 (Figure 4). Most of the B310 restriction patterns are very similar to those of A4C-EA1. The *Xba*1 and *Eco*RI/*Xba*1 digests most clearly show that there are amplified bands corresponding to both the normal copy of *ADH4:CUP1* and the unique

copy (Figures 5 and 6). For example, an Xba1 digest of the normal *ADH4:CUP1* construct would be expected to produce 2.4 and 1.3 kb fragments (Figure 3). However, the *Xba*I site upstream from ADH4 is in a different position in the A4C-EA1 novel joint (Figure 4). This gives rise to a 3.2 kb fragment. All but one of the B310 amplification strains share this 3.2 kb band with A4C-EA1, and all but the intrachromosomal amplifications possess the normal 2.4 and 1.3 kb bands (Figure 5). The *Eco*RI, *Eco*RI/*Hind*III, and *Hind*III digests are consistent with the B310 structures being similar to A4C-EA1, but they are not as conclusive as the *Xba*1 and *Eco*RI/*Xba*1 digests and therefore are not shown in this paper. Both of the restriction digests below are labeled with an arrow pointing to the band that differs between the normal *ADH4:CUP1* construct (present in A4C) and the restriction map of A4C-EA1 novel joints. Despite unique bands in some of the B310 strains, these A4C-EA1 bands are shared by most of the strains studied. This suggests that the B310 and A4C-EA1 amplifications have similar novel joints.

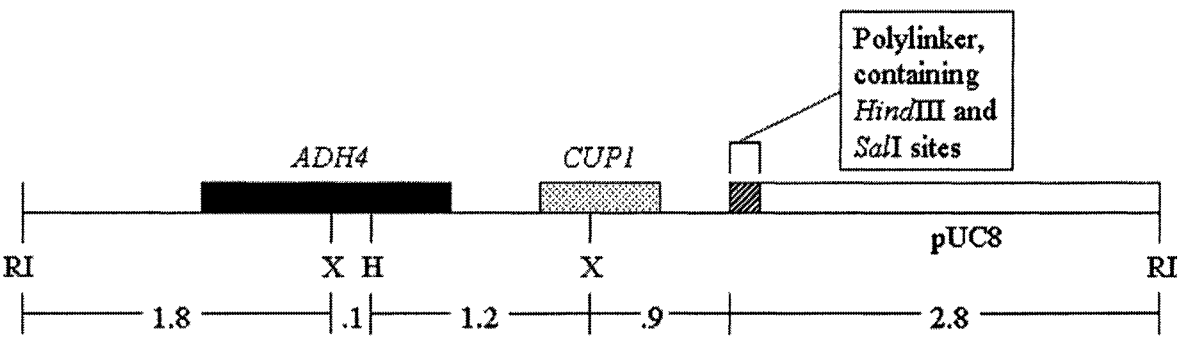

Figure 3: Restriction map of p7a, the plasmid containing the *ADH4:CUP1* construct. RI=*Eco*RI, X=*Xba*1, H=*Hind*III. The distance between the *Xba*I and *Hind*III sites within *ADH4* is actually only 26 bp.

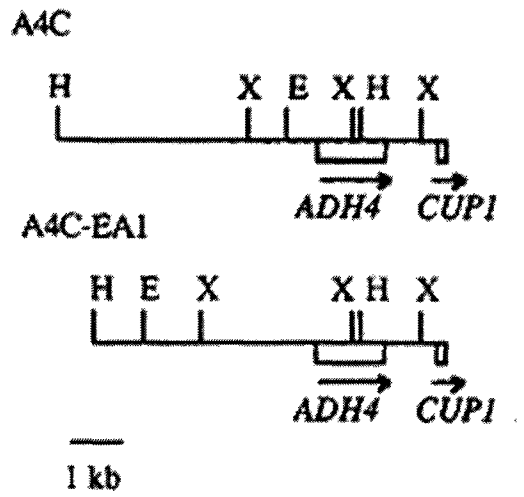

Figure 4: Restriction maps of novel joints of A4C and A4C-EA1 (Moore et al. 2000).

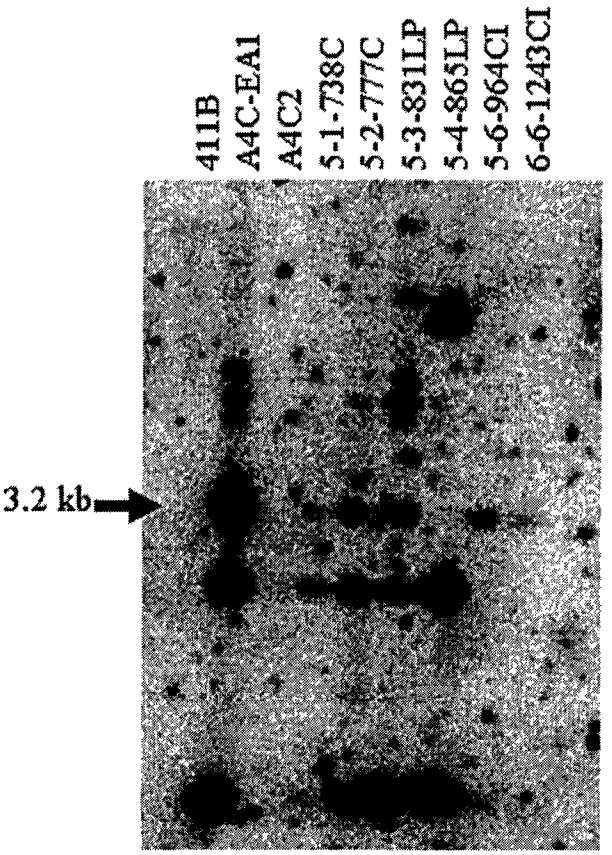

Figure 5: Southern blot of *Xba*I restriction digest of B310 and control strains.

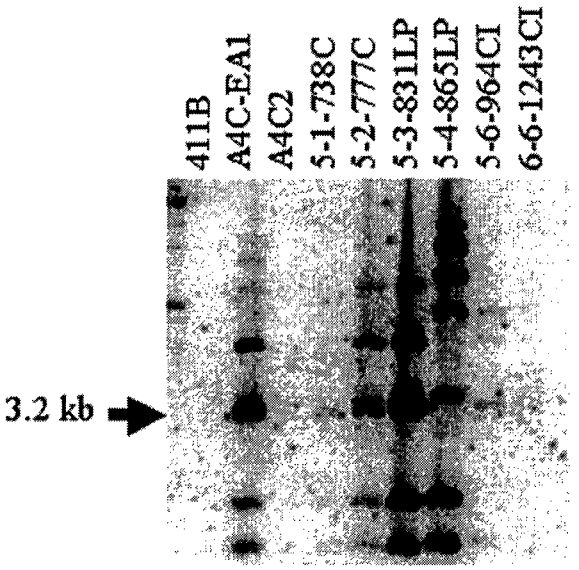

Figure 6: Southern blot of *Eco*RI/*Xba*I restriction digest of B310 and control strains.

Long Term Growth of B310 Strains

In order to determine how B310 amplification structures change over time, B310 and control strains were grown in liquid culture for one to two months and the beginning and ending structures were compared. The amplification structures can be seen on Southern blots of CHEF gels probed with the *ADH4:CUP1* construct. Figure 7 shows an ethidium bromide-stained gel next to its Southern blot. Circular amplifications migrate independently of size and tend to be found near the top of the blot. Linear amplifications tend to be smaller than any of the yeast chromosomes and exist in high copy number, so they appear as dark bands near the bottom of the blot. Intrachromosomal amplifications can be on any of the chromosomes, so they appear toward the center of the blot, between the circles and linear palindromes. The normal copy of *ADH4* on chromosome VII appears as a lighter chromosomal band in all of the strains used. In some cases, DNA

will remain in the wells and hybridize to the probe, but this should not be confused with amplification structures. The locations of the wells are marked on each of the figures.

The initial B310 amplification strains were characterized by Peterson et al., 2000, and were believed to consist of two strains containing circular amplifications, two containing linear palindromes, and two containing both circular and intrachromosomal amplifications. However, in this study, it was observed that 5-6-964CI contained only a circle and no intrachromosomal amplification (Figure 7).

The B310 and control strains were grown long term for three independent experiments. CHEF blots were prepared from each of these strains at the end of each growth period. These final amplification structures were compared to the initial structures shown in Figure 7. The CHEF blot from the end of the first growth period is shown in 8. Early in the period the B310 parent strain developed a flocculating mutation, so this strain is not represented in the final blot. 5-1-738C lost its circular amplification, while 5-2-777C gained at least two new circles. 5-3-831LP and 5-4-865LP have new intrachromosomal amplifications. The blot from the end of the second growth period is shown in Figure 9. The B310 parent strains developed a circular amplification, while 5-1-838C lost its circle. 5-3-831LP developed an intrachromosomal band and 5-4-865LP developed two intrachromosomal bands. The 5-6-964CI circle is more intense, and 5-6-964CI and 6-6-1243CI both have new linear palindromes. The blot from the third long term growth period is shown in Figure 10. A4C-EA1 and 5-1-738C appear to have lost circles, while 5-3-831LP lost its linear palindromes. 5-6-964CI has a new circle, A4C-EA1 and 5-3-831LP gained intrachromosomal amplifications, and 5-4-865LP gained two intrachromosomal amplifications. These changes are circled on the blots.

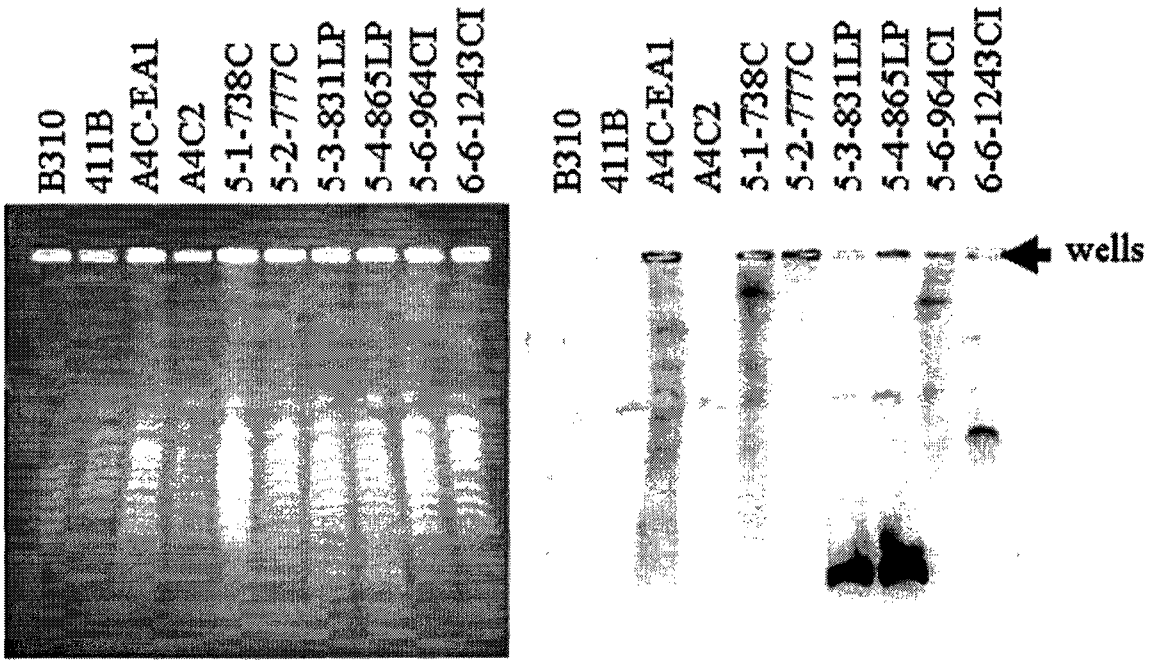

Figure 7: Ethidium bromide-stained CHEF gel (left) and its Southern blot (right) showing amplifications in B310 and control strains before long term growth.

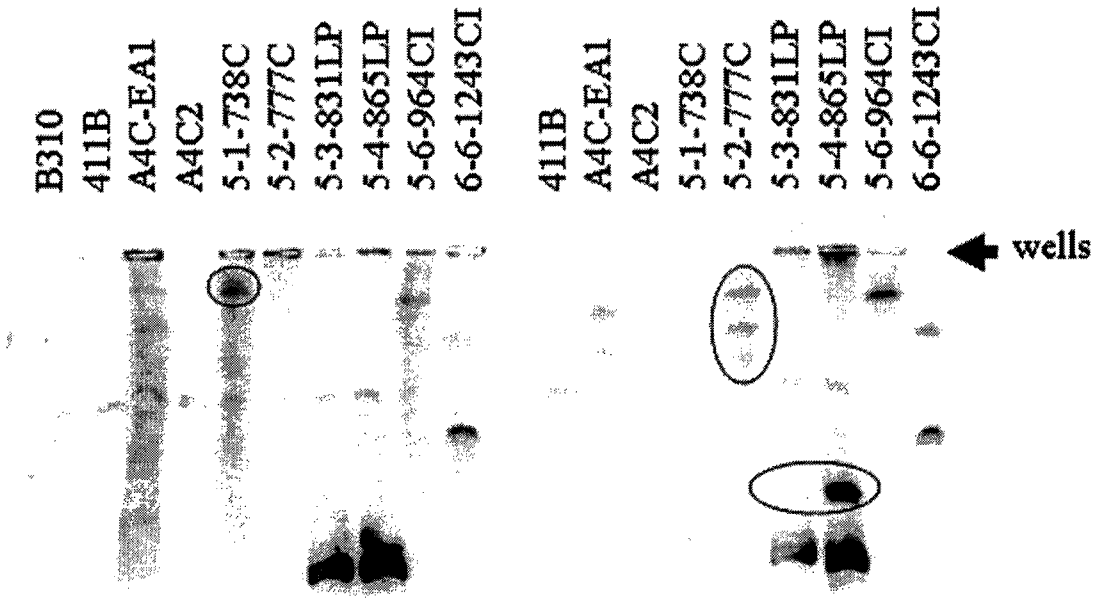

Figure 8: Southern blots of CHEF gels showing initial amplifications (left) and amplifications after first long term growth period (right). Bands that were lost are circled on the left and bands that were gained are circled on the right.

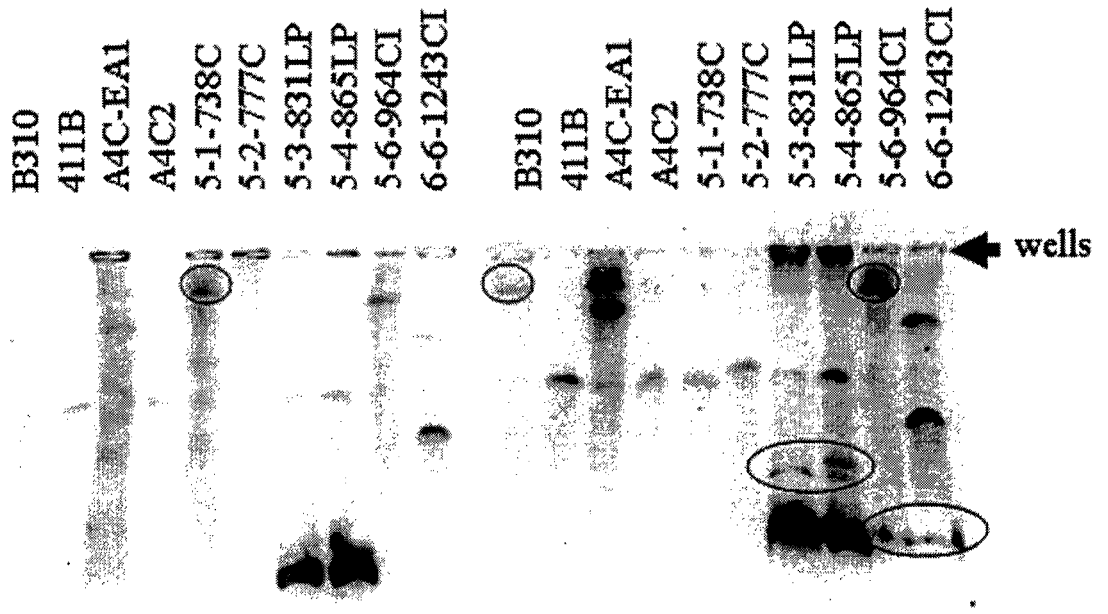

Figure 9: Southern blots of CHEF gels showing initial amplifications (left) and amplifications after second long term growth period (right).

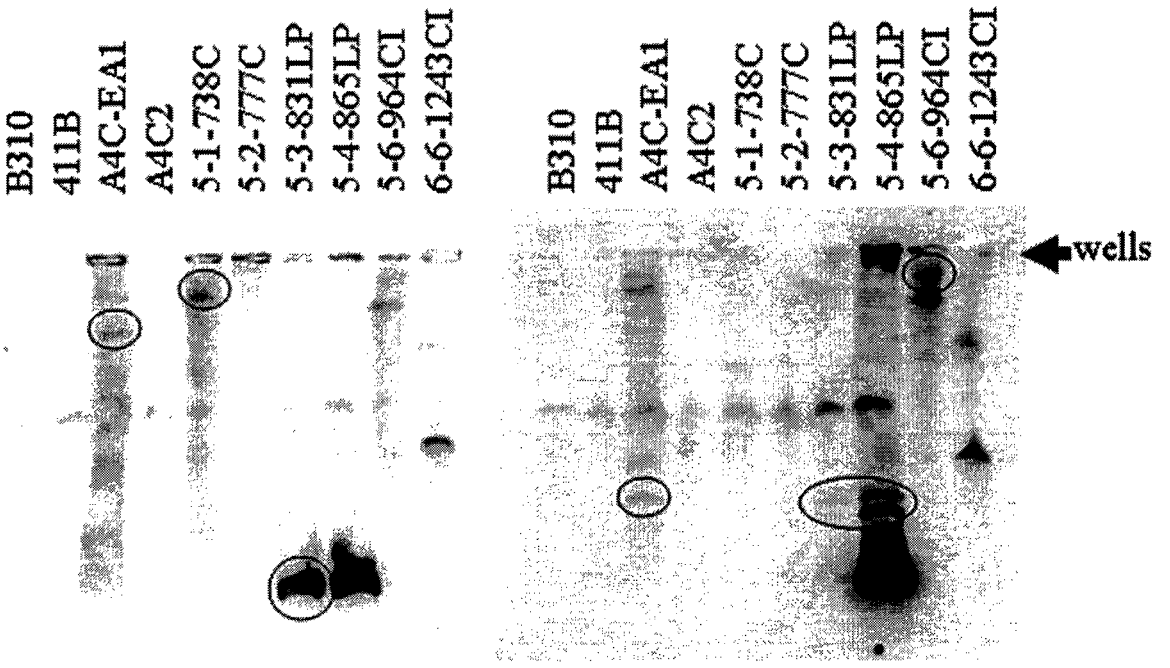

Figure 10: Southern blots of CHEF gels showing initial amplifications (left) and amplifications after third long term growth period (right).

5-4-865LP developed intrachromosomal amplifications in all three long term growth experiments, and was therefore chosen as an especially interesting strain for further study. A CHEF gel was prepared from weekly frozen cultures of this strain from the first long term growth period. Figure 11 shows that the intrachromosomal amplification appeared approximately four weeks into the growth period and, from that point on, increased in copy number relative to the linear amplifications.

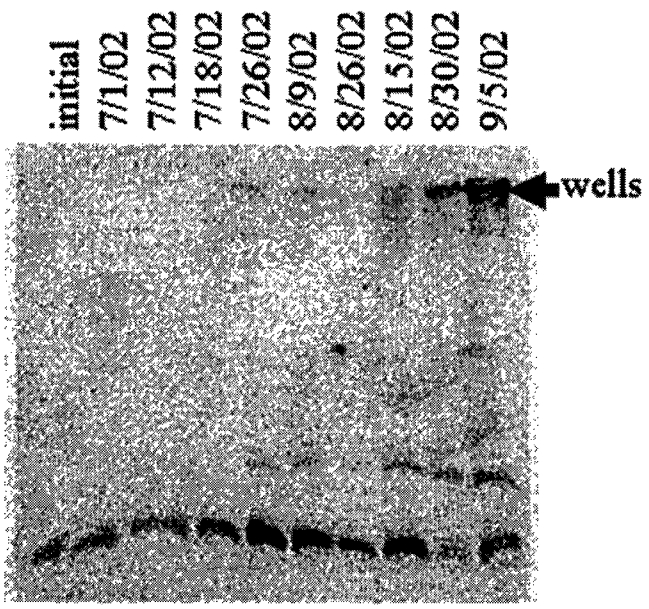

Figure 11: Southern blot of CHEF gel, showing weekly changes in 5-4-865LP linear and intrachromosomal amplifications during the first long term growth period.

In order to determine approximately how much of chromosome VII was amplified in the 5-4-865LP linear and intrachromosomal amplifications, blots of CHEF gels were probed with four different markers. The locations of the markers on chromosome VII are shown in Figure 12. A4C-EA1 was used as a control because the circular amplifications in this strain are known to contain most of chromosome VII, including all four of these

markers (Moore et al. 2000a). Although the A4C-EA1 amplification bands are faint, probably due to partial degradation of the DNA, the circles are present on all four blots (Figure 13). Samples of 5-4-865LP were prepared from the initial strain, the week that the intrachromosomal amplifications first appeared, and the final week of the growth period. The IKM-2 probe hybridized to the linear amplifications, but not to the intrachromosomal structures, while none of the other probes hybridized to any of the 5-4-865LP structures (Figure 13). This indicates that only a small part of the left end of chromosome VII is present on the 5-4-865LP amplifications and that the intrachromosomal structures are different than the linear structures. Considering this and the changes identified in all of these experiments, amplification structures appear to be highly variable and dynamic structures.

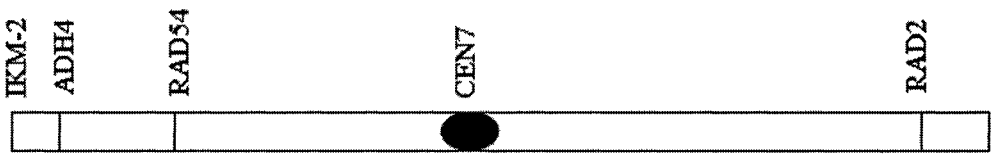

Figure 12: Map of chromosome VII, showing the locations of the probes used to characterize the structures of 5-4-865LP amplifications.

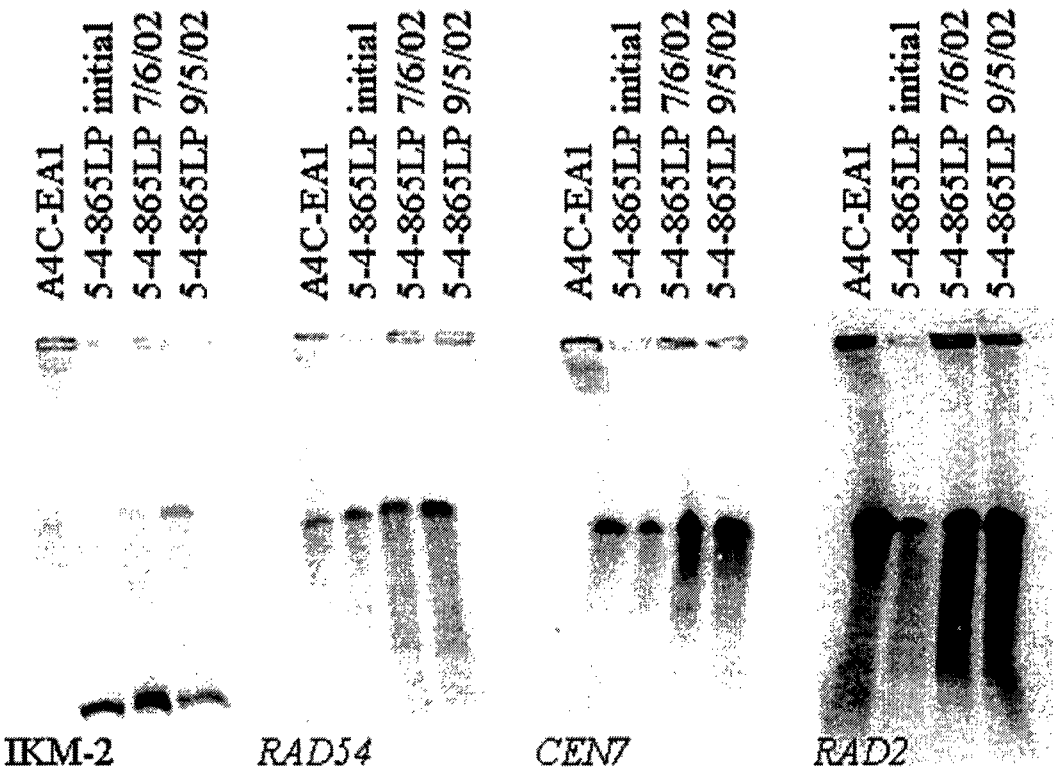

Figure 13: Blots of CHEF gels showing hybridization of chromosome VII probes to A4C-EA1 and 5-4-865LP strains.

Identification of the B310 Mutation

Since the B310 phenotype is unique, one of the objectives of this study was to determine what gene is mutated in this strain. In order to narrow the search for candidate genes, a yeast genomic library was used to screen for complementation of the B310 sporulation deficiency. It was assumed that the B310 mutation was recessive because it had been observed in the past that a B310 haploid mated with a wild type haploid is capable of sporulation. The library was produced by inserting fragments of the yeast genome into a *Bam*HI site on the plasmid p366 (Figure 14). Complementation was detected using a sporulation assay based upon the presence of a fluorescent membrane protein in sporulating cells. Approximately 4000 transformants should be screened to

have a 95% chance of finding an insert that complements the B310 mutation. However, due to problems with the screen in its early stages, this number was doubled. Two B310-2N transformants were found that were able to sporulate and these were named SPO1 and SPO2. Figure 15 shows the filter from the sporulation assay that contained SPO1.

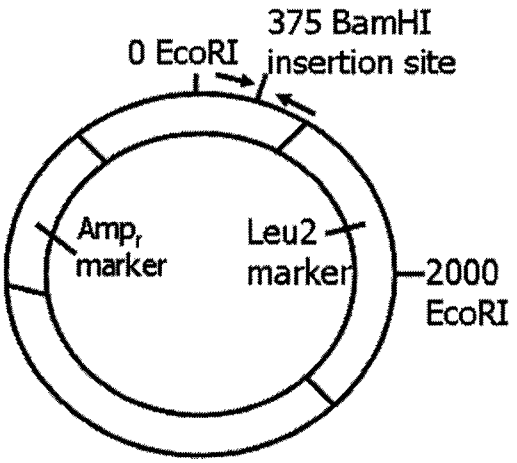

Figure 14: 8.4 kb p366 plasmid used to construct genomic library. The arrows on either side of the *Bam*HI insertion site represent clockwise and counter-clockwise primers.

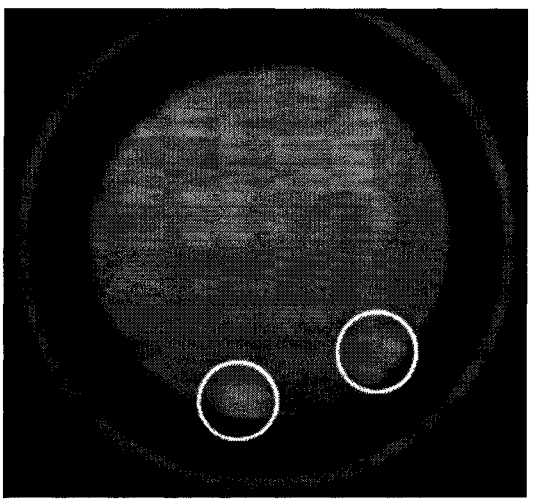

Figure 15: Sporulation assay showing two fluorescent sporulating colonies. The positive control (411B-2N) is at the bottom of the filter, with the SPO1 colony to its right.

Plasmid DNA was extracted from SPO1 and SPO2 and digested with *Eco*RI to check for the presence of yeast genomic inserts. A plasmid without an insert would be expected to have 6.4 kb and 2.0 kb *Eco*RI restriction fragments, while a plasmid containing an insert would be expected to have the 6.4 kb fragment but not the 2.0 kb fragment (Figure 14). This is because the *Bam*HI site into which the genomic DNA was inserted falls within the 2.0 kb fragment. An insert should result in the presence of one or more *Eco*RI fragments in addition to the 6.4 kb fragment, depending upon the number of *Eco*RI sites within the insert. As shown in Figure 16, SPO1 clearly has an insert, but the SPO2 digest is inconclusive. There is a faint 2.0 kb band which suggests that there is no insert, but there is also a band larger than the 6.4 kb band, suggesting that there might be an insert.

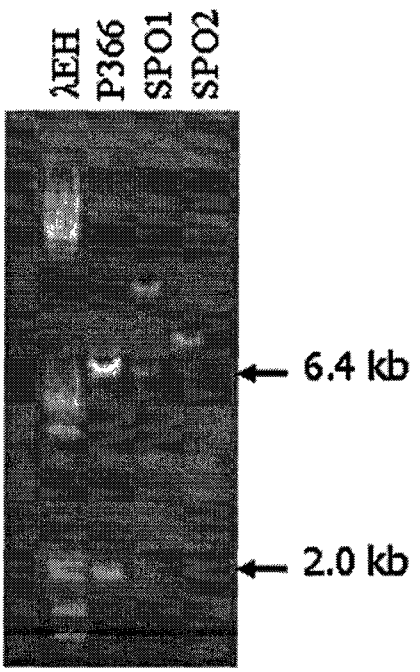

Figure 16: *Eco*RI digest of p366 and plasmids isolated from the screen for complementation of the B310 sporulation deficiency. λEH=lambda standard digested with *Eco*RI and *Hind*III.

To determine which genes are on the SPO1 insert and whether there is a SPO2 insert, the two plasmids were sequenced. A clockwise primer and a counterclockwise primer, each based upon the sequences surrounding the plasmid's *Bam*HI insertion site, were used to sequence the inserts from either end (Figure 14). No sequences were obtained with the counterclockwise primer. The sequences obtained with the clockwise primer were analyzed using BLAST on the *Saccharomyces* Genome Database website (http://www.yeastgenome.org). The clockwise sequence for the SPO2 plasmid contained the plasmid's *LEU2* marker, so it was determined that SPO2 probably contained no insert. The clockwise sequence for the SPO1 plasmid contained part of chromosome V (Figure 17).

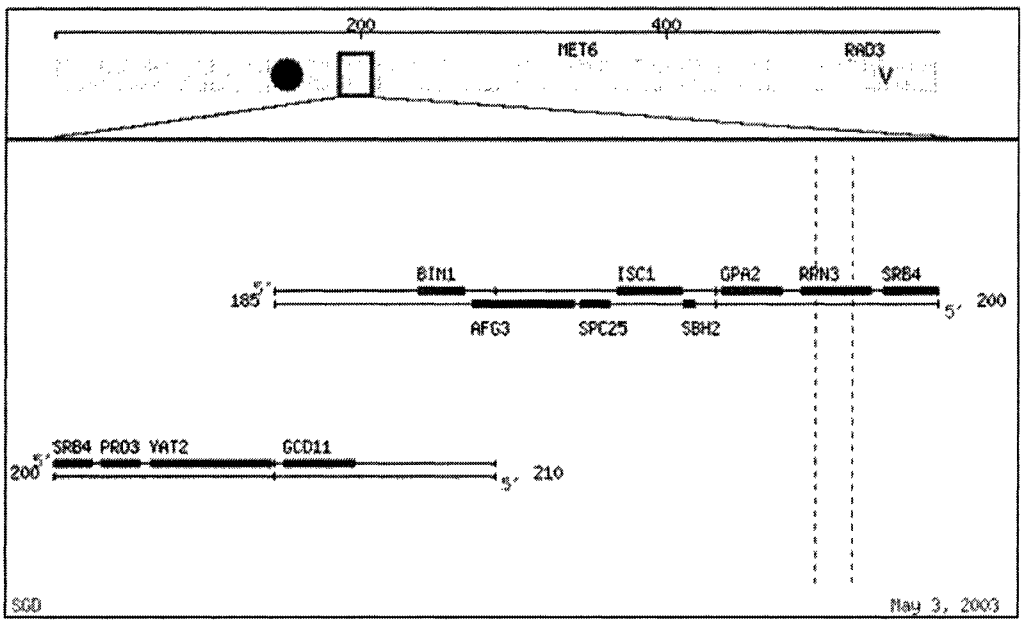

Figure 17: Map of chromosome V, highlighting the 25 kb surrounding the SPO1 sequence obtained with the clockwise primer (http://www.yeastgenome.org). The hollow box to the right of the centromere at the top represents the 25 kb region shown on the bottom part of the figure. The sequence obtained from SPO1 lies between the dotted vertical lines.

Based upon the *Eco*RI restriction digest (Figure 16), the SPO1 insert was determined to be approximately 10 kb long. However, because sequence information was only obtained from one of the primers, it was unclear whether the SPO1 insert contained the 10 kb region upstream or downstream from the sequence. To solve this problem, the *Eco*RI restriction map posted on the *Saccharomyces* Genome Database (http://www.yeastgenome.org) was consulted. There are *Eco*RI restriction sites at 184025 and 199478 kb on chromosome V. The sequence obtained from the clockwise primer spanned the 197720-198022 kb region of chromosome V. There appear to be no EcoRI sites in the SPO1 insert, since only one new restriction fragment is present on Figure 16. This is consistent with the region upstream from the clockwise sequence because there are no restriction sites between 184025 and 199478 kb. There should have been an EcoRI restriction fragment of at least 1.8 kb if the insert had contained the region downstream from the clockwise sequence (197720-199478).

Once it was determined that the SPO1 plasmid contained approximately 10 kb upstream from the clockwise sequence, the functions of the genes that fall within that region were checked for logical candidates. These genes are shown on Figure 17. *GPA2* is the only one of these genes known to be involved in sporulation, so it was chosen as a candidate gene for further study. To make certain that *GPA2* was on the SPO1 insert, the plasmid was sequenced using a *GPA2* primer. A sequence from *GPA2* was obtained, confirming its presence on the SPO1 plasmid.

In order to ensure that the SPO1 insert was responsible for rescuing sporulation in B310, the plasmid was again transformed into B310-2N. This second SPO1 clone was called SPO1-2. p366, without an insert, was also transformed into B310-2N as a control.

These three transformants, along with 411B-2N as a positive control, were plated on sporulation media. The cells were checked for sporulation under a light microscope during three separate trials and the sporulation screen was used twice. 411B-2N and SPO1 sporulated during 4 of the 5 trials. SPO1-2 and B310-2N-p366 never sporulated.

These same three transformants (SPO1, SPO1-2, and B310-2N-p366) were also used in an assay to estimate the rate of mutation to antimycin A. Since amplification is one of the ways through which *ADH1Δ* strains can become resistant to antimycin A, rate of mutation to antimycin A should roughly correlate to amplification rate. It was expected that, if the SPO1 plasmid was complementing the mutation in B310, SPO1 and SPO1-2 should have lower rates of mutation to antimycin A than B310-2N-p366. Ten independent cultures from each transformant were plated on antimycin A, and the number of plates containing growth after a week was used to calculate mutation rates using the method of Lea and Coulson, 1949. As shown in Table 2, these three strains are not significantly different in their rates of mutation to antimycin A.

The effect of *GPA2* deletion on amplification rate was determined by creating a *gpa2Δ/adh1Δ* strain from GPA2Δ and 411B. Sixty colonies from this strain were plated on separate antimycin A plates. After one week, CHEF gels were prepared from colonies on each of the antimycin A plates that contained growth. Blots of these gels revealed that five plates contained amplifications (Figures 18-20). The estimated amplification rate for the *gpa2Δ/adh1Δ* strain is $5.1\pm4.6\times10^{-9}$ mutations per cell per generation (Table 2). As a control, an *adh1Δ* background was also mated into the parent strain of GPA2Δ. The CHEF blots for the parent strain revealed that nine plates contained amplifications (Figures 21-22), giving an estimated amplification rate of $1.3\pm0.83\times10^{-8}$ (Table 2). This

amplification rate for the parent strain is not significantly different from that of GPA2Δ. Based upon these results, it appears that the SPO1 plasmid is not responsible for complementation of the B310 phenotype, and that *GPA2* does not have an effect on amplification rate.

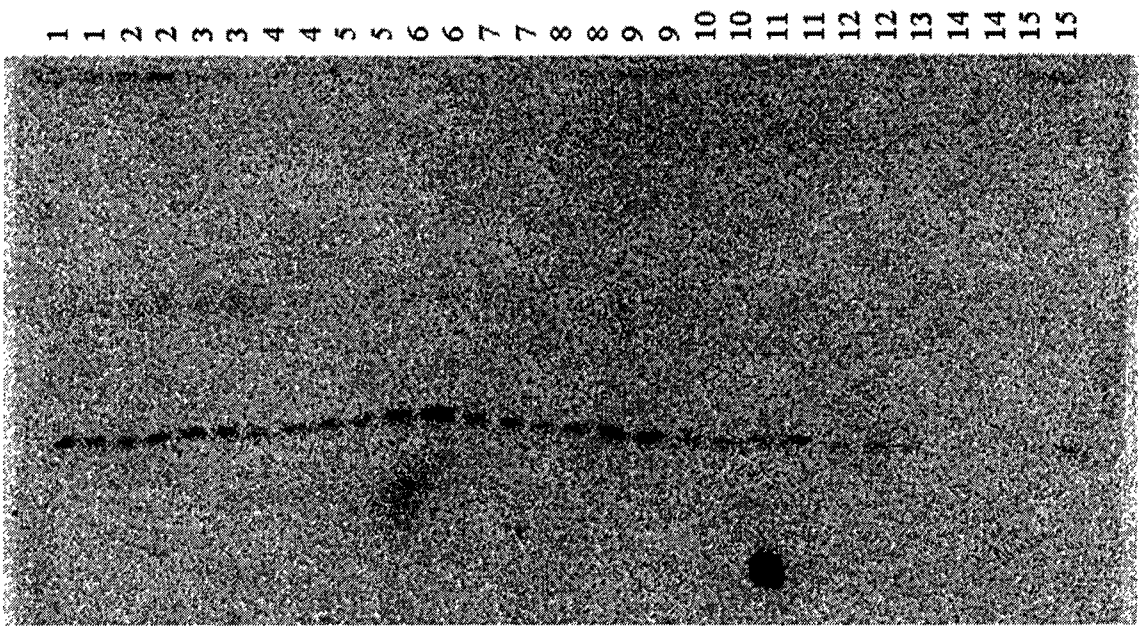

Figure 18: Southern blot of first CHEF gel prepared from colonies isolated on antimycin A plates in the GPA2Δ amplification rate. The wells are labeled with the plate number from which the colonies were chosen.

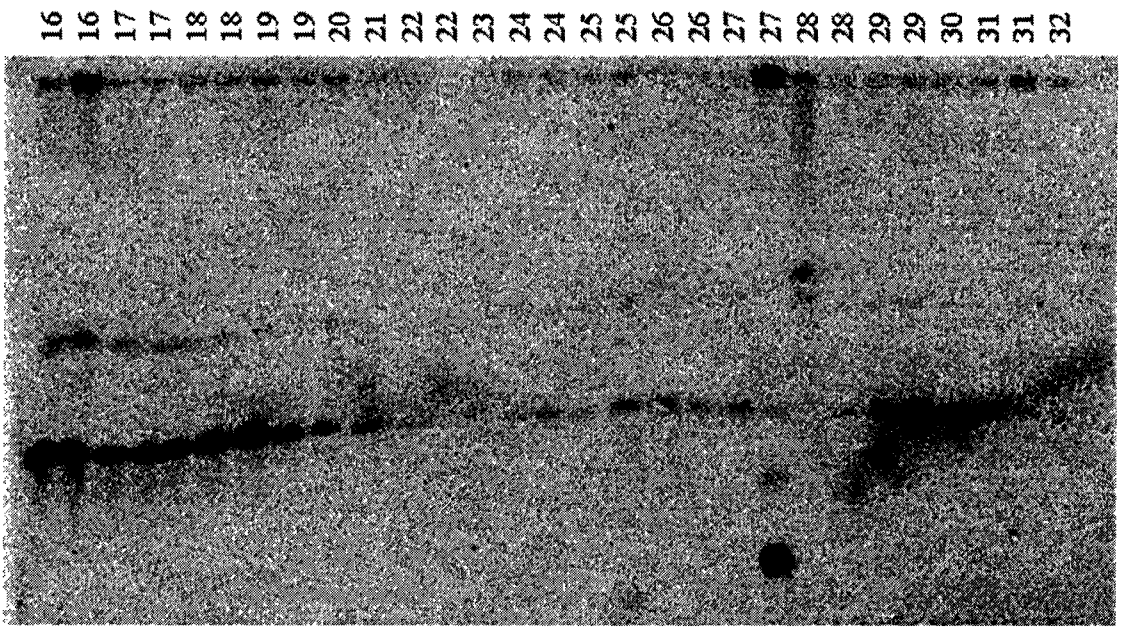

Figure 19: Southern blot of second CHEF gel prepared from colonies isolated on antimycin A plates in the GPA2Δ amplification rate.

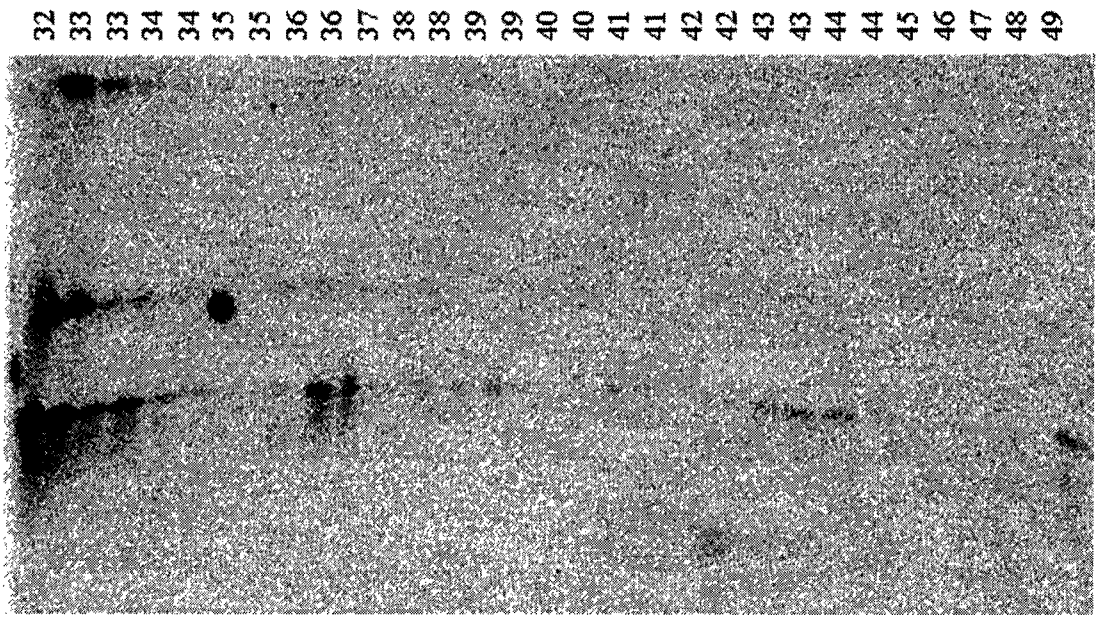

Figure 20: Southern blot of third CHEF gel prepared from colonies isolated on antimycin A plates in the GPA2Δ amplification rate.

Table 2: Rates of mutation to antimycin A and amplification of *ADH4*. Rates and 95% confidence intervals were calculated as described in Lea and Coulson 1949. These calculations are explained in Appendix A.

Strain	Number of cells/culture	Number of plates with antimycin A-resistant colonies/ total number of plates	Estimate of mutation rate to antimycin A	Percentage of antimycin A-resistant colonies with *ADH4* amplifications	Number of cultures containing amplifications/ total number of cultures	Estimate of amplification rate
B310 with p366	1.2×10^{7}	8/10	$1.3\pm1.1\times10^{-7}$	—	—	—
SPO1	1.8×10^{7}	5/10	$3.9\pm3.5\times10^{-8}$	—	—	—
SPO1-2	1.4×10^{7}	9/10	$1.6\pm1.4\times10^{-7}$	—	—	—
gpa2Δ/adh1Δ	1.7×10^{7}	49/60	$1.0\pm0.32\times10^{-7}$	6% (5/87)	5/60	$5.1\pm4.6\times10^{-9}$
KO parent/*adh1Δ*	1.3×10^{7}	39/60	$8.1\pm2.7\times10^{-8}$	21% (12/56)	9/60	$1.3\pm0.83\times10^{-8}$

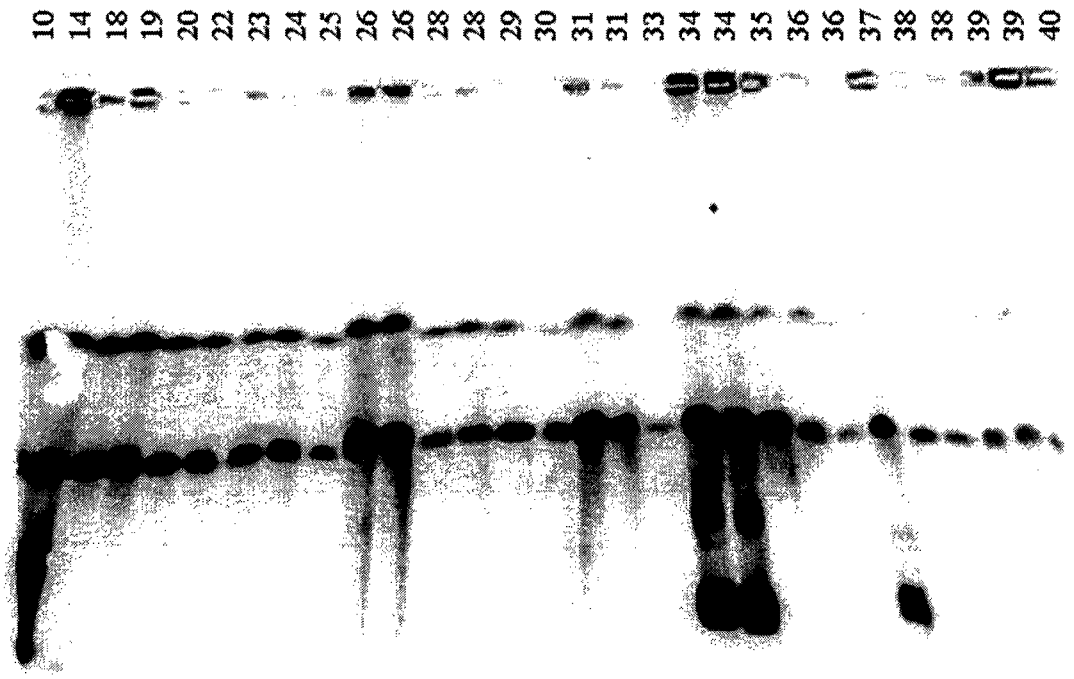

Figure 21: Southern blot of first CHEF gel prepared from colonies isolated on antimycin A plates in the KO parent strain amplification rate.

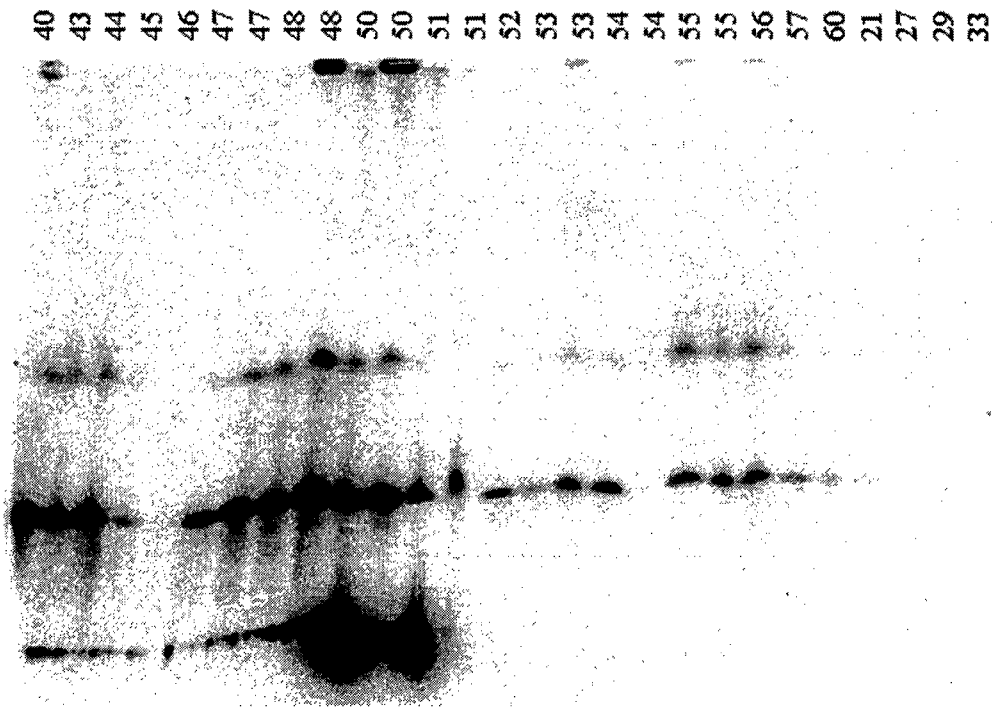

Figure 22: Southern blot of second CHEF gel prepared from colonies isolated on antimycin A plates in the KO parent strain amplification rate.

DISCUSSION

B310 Novel Joints

The restriction fragments observed when B310 novel joints are probed with the *ADH4:CUP1* construct are similar to those described in Moore et al., 2000b. This indicates that the B310 amplification structures are comparable to those that have been previously characterized. Therefore, they are probably formed by a similar mechanism. This suggests that the mechanism of amplification formation is likely to be the same between yeast strains, even when the mutations causing the high amplification rates are different.

The A4C amplifications described in Moore et al., 2000b, consist of a novel joint region flanked by inverted repeats. Further study of these structures revealed that they were circles containing centromeres, as well as the DNA that normally lies between *ADH4* and the centromere (Moore et al. 2000a). These data are consistent with the intrachromosomal recombination model of gene amplification (Butler et al. 1996), as well as the breakage-fusion-bridge model of resolving dicentric molecules (Stark 1993). The restriction fragments produced from A4C-EA1 amplifications reflect the change in restriction sites that occurs when a novel joint is present between two inverted copies of *ADH4:CUP1*. Interestingly, the same fragment sizes that reflect this change in restriction sites in the circular A4C-EA1 molecules are also present in both of the B310 circles, one of the linears, and both of the intrachromosomal amplifications. The bands corresponding to amplification of a normal copy of *ADH4:CUP1* are present in both circles, both linears, but not in the intrachromosomals. Although the B310 amplifications have not been tested for the presence of centromeres, all circular amplifications previously studied in the Paquin lab contained centromeres (Moore et al. 2000a) while

linears lacked centromeres (Walton et al. 1986, Dorsey et al. 1992). Therefore, the breakage-fusion-bridge model is thus far only consistent for the formation of circular *ADH4:CUP1* amplifications. However, the similarity in restriction patterns suggests that all three of the known types of amplifications may be formed through a similar mechanism, quite possibly through intramolecular recombination. Some of the differences observed between strains may be explainable by processing of the structures after the initial amplification event, for example during integration into chromosomes.

There are a number of experiments that could be performed to expand upon the data collected in this study. It would be interesting to know which of the B310 amplifications actually contain centromeres. As mentioned above, this would allow for further hypothesizing on the importance of the breakage-fusion-bridge mechanism in the formation of the circular and linear structures. PCR primers have been developed for the A4C amplifications which have allowed the size of the novel joint regions as well as their sequences to be determined (Moore et al. 2000b). Problems with PCR have prevented the use of these primers in this study, but if PCR could be optimized in the future, the primers could be used to quickly characterize the novel joints of many amplification structures. Clearly there are several more experiments that could be conducted with the B310 structures, but the data collected in this study at least suggest that future experiments will show similarities between amplification structures and mechanisms even in strains with different mutations.

Long Term Growth of B310 Strains

Long term growth experiments conducted with the B310 and control strains show that the amplification structures in these strains are very dynamic. Some amplifications were lost over time, some maintained, and new ones were formed. It appears that in the high amplification rate strains, amplifications are being formed and lost constantly, despite the fact that they are growing under conditions that select for *ADH4* amplifications. The glucose in the media during the long term growth periods does not prevent aerobic respiration for those cells lacking *ADH1*, but it does provide a selective advantage to those that have amplified *ADH4* and can ferment the glucose. Therefore, *ADH4:CUP1* amplifications that are lost even in the presence of glucose must be quite unstable. The structures that were lost were mostly circles, although one strain lost its linear palindromes in one of the experiments. The original intrachromosomal amplification was never lost, but several new intrachromosomal amplifications were formed. This is consistent with observations of mammalian amplifications, where the extrachromosomal structures are unstable and the intrachromosomal structures are more stable and tend to appear later in growth periods (Schmike et al. 1984).

One particularly interesting observation made from the long term growth experiments was that 5-4-865LP gained new intrachromosomal amplifications in the same location in all three independent experiments. In two of the experiments, a second intrachromosomal band was formed on another chromosome that migrates near to the first one. In the second growth period, 5-3-831LP clearly gained an intrachromosomal band at a similar location on the blot to the 5-4-865LP bands. Although the bands are very faint, it looks as though 5-3-831LP may have that same intrachromosomal band in both of the other experiments as well. The new intrachromosomal amplification in A4C-

EA1 is also at a similar location on the blot. These data suggest that there might be something about the structures of the *ADH4:CUP1* amplifications that allows for their incorporation into particular chromosomal locations, probably through homologous recombination.

The blot prepared from the weekly frozen cultures of 5-4-865LP showed that the intrachromosomal amplification formed four weeks into the growth period and increased in copy number relative to the linear palindromes for the remainder of the experiment. This suggests that this location of chromosomal integration is an active site for recombination, and that the intrachromosomal structure is fairly stable once it is formed.

The 5-4-865LP amplifications contain only the left end of chromosome VII, extending at most up to the beginning of *RAD54*. This means that these amplifications do not contain centromeres and were, therefore, probably not formed through the breakage-fusion-bridge mechanism. The linear amplifications contain the sequence that hybridizes to IKM-2, but the intrachromosomal amplifications do not. This suggests that the intrachromosomal amplifications were either formed independently of the linears or that, upon integration into the chromosome, part of the linear amplifications were lost. The latter might be possible if recombination occurred at a site on the linear amplifications that resulted in the loss of the extreme left end of chromosome VII. In the future, it would be interesting to probe these blots with more markers that lie between *ADH4* and *RAD54* in order to determine exactly how large the amplifications are and how similar the linears are to the intrachromosomal amplifications.

At this point, it is assumed that the bands migrating toward the middle of the gels are intrachromosomal bands, but it has not been proven. If the strains containing these

bands are mated with another haploid strain, sporulated, and the tetrads dissected, it would be possible to trace the segregation of these amplifications. If they segregate as would be expected from a chromosome, then they are definitely intrachromosomal. If they segregate unequally, then they are extrachromosomal.

The observations made above suggest that the extrachromosomal amplifications are unstable and that the intrachromosomal amplifications are more stable. However, the experiments started with only one intrachromosomal amplification strain and the strains were grown under selective pressure for maintaining the amplifications. The strains could be grown in glycerol, a carbon source that does not preferentially select cells that can ferment. If extrachromosomal amplifications are quickly lost and intrachromosomal amplifications are maintained, then it would be reasonable to conclude that the intrachromosomal structures are indeed more stable. This would be very consistent with the hypothesis that amplifications can become permanent fixtures in a genome, giving rise to the potential for mutation into new genes (Ohno 1970).

Microarrays would be very useful tools for analyzing amplification structures, as well as determining how many genes are amplified on a genome-wide scale. Microarrays have been developed which contain all known open reading frames in the yeast genome. If these were probed with the B310 *ADH4:CUP1* amplifications, all the open reading frames contained on the amplifications would hybridize to the probe, allowing for quick determination of the genetic content of the amplification structures. Alternatively, the total DNA from a yeast strain could be used as the probe and those genes that are amplified should show much stronger hybridization than those genes that are present in only one copy. This would allow all the genes that are amplified to be identified, rather

than using just *ADH4* and *CUP1* as indicators of amplification. DNA extracted from the strains frozen during this study could then be used to demonstrate genome-wide changes over time. The observations made and the amplification strains isolated in these long term growth experiments can serve as a starting point for a variety of experiments concerning the dynamic nature of gene amplifications and their potential for being integrated into the genome.

Identification of the B310 Mutation

Identification of the B310 mutation through complementation of its sporulation deficiency was unsuccessful. The sporulation assay allowed for the identification of sporulating cells, but it appears that the transformants that regained the ability to sporulate did so through reversion mutations and not through complementation. The SPO1 plasmid did not restore sporulation when reinserted into B310, indicating that it was not responsible for rescuing sporulation. Neither the SPO1 strain nor SPO1-2 had any significant effect on rate of mutation to antimycin A, suggesting that the plasmid does not restore amplification to its wild type level. However, ignoring the 95% confidence interval, the rate of mutation to antimycin A is lower for SPO1 than for B310-2N-p366 and SPO1-2. It may be that SPO1 is a revertant with a lower amplification rate, but that the sample sizes were too small for this to be determined with any certainty. The second sporulating transformant, SPO2, is also likely to be a revertant of the B310 mutation since its plasmid probably does not contain an insert.

GPA2 was chosen as a candidate gene for complementing the B310 sporulation deficiency because it was present on the SPO1 plasmid and it is known to be important in

sporulation (Donzeau and Bandlow 1999). GPA2 is the alpha subunit of a heterotrimeric G-protein important in regulating growth in response to glucose. Although this does not provide an obvious link to amplification, this G-protein interacts with so many other proteins that it seemed possible that a mutation could somehow affect amplification. The *GPA2* deletion strain studied during this project, however, does not have a higher amplification rate than its parent strain. This is not surprising since it is now clear that the SPO1 plasmid did not actually rescue sporulation. What is surprising is the fact that both the *GPA2* deletion strain and its parent strain had higher amplification rates than wild-type strains previously studied in the Paquin lab (see Peterson et al. 2000). It seems as if there are probably mutations in the parent strain that increase its amplification rate. If this parent strain or any knock-outs derived from it are to be used in further amplification-related experiments, the mutations in the parent strain must be identified.

The fluorescence-based sporulation screen used in this study is reliable in that spores always fluoresce and vegetative cells do not. However, because there are several steps in the screen and the cells are transferred to new plates on two occasions, the filters often become contaminated and the cells become very old. Contamination and age are both factors that have been observed in this study to decrease sporulation efficiency. On many of the filters, even the positive control did not sporulate. This suggests that several cells that might have been capable of sporulating might have been missed by this screen. Therefore, it may be worth trying a different sporulation assay that allows a larger number of transformants to be screened in a shorter period of time. For example, there is a test that subjects cells to ether vapor, killing vegetative cells while spores can survive (Rockmill et al. 1991). Cells could be transferred directly to sporulation plates, cutting at

least a week out of the screen. However, in addition to the obvious problems in dealing with ether fumes, this method is not 100% effective in killing vegetative cells. It would probably take a great deal of time to optimize the screen in a way that minimizes false positives. It is also a possibility that the B310 mutation simply can not be complemented. It seemed as if complementation was an option because a B310 haploid mated with 411B was capable of sporulating. However, the amounts and types of proteins present in a B310 diploid transformed with a plasmid are different than those in a B310/411B diploid.

Another possible approach to identifying the B310 mutation is to choose candidate genes based upon the B310 phenotype. These candidate genes could then be sequenced in B310 and the sequences searched for mutations. Amplification rates of mutant strains of the candidate genes could be determined as was done in this study. Candidate genes could be identified by searching those genes known to be required for sporulation for functions that are also consistent with high amplification rates. Using this technique, *MRE11* was identified as a good candidate. MRE11 has endonuclease and exonuclease functions and has been implicated in DNA repair, meiotic double strand break induction and processing, regulation of replication, checkpoint signaling, and telomere maintenance (D'Amours and Jackson 2002). MRE11 is part of the MRE11/RAD50/XRS2 complex, and some of its functions are linked to its interactions with these other two proteins. One of the nuclease functions of MRE11 is to resolve hairpin structures (D'Amours and Jackson 2002, Lobachev et al. 2002). Since hairpins are thought to be intermediates in the intramolecular recombination model of gene amplification (Butler et al. 1996), this function may link MRE11 to high amplification rates. In fact, at least one laboratory has found high amplification rates in MRE11

mutants and they attribute this to a lack of hairpin processing (Lobachev et al. 2002). MRE11 is particularly consistent with the B310 phenotype because MRE11 separation-of-function mutants have been found that are deficient in some of the MRE11 functions but normal with respect to others (Chamankhah et al. 2000). Therefore, it might be possible to have a sporulation-deficient mutant, like B310, that is still capable of DNA repair. The MRE11 separation-of-function mutants mentioned above result from point mutations that most likely affect only one motif of the MRE11 protein while the rest of the protein remains intact. The fact that B310 reversion mutations were found in the sporulation screen suggests that the B310 mutation is also a point mutation. More severe mutations would be far less likely to revert. Although this study was unsuccessful in identifying the B310 mutation, it would be worth continuing this effort by sequencing *MRE11* and other candidate genes or experimenting with a different sporulation screen.

Conclusion

This study has been successful in showing that the amplification structures formed in B310 are similar to those that had been previously characterized in the Paquin lab. This indicates that, although the B310 phenotype is very different from strains that have been studied thus far, the mechanism of gene amplification may be the same. The second goal of this research was to determine how the B310 amplifications change over time, and three independent experiments have shown that they are very dynamic. It seems as though *ADH4:CUP1* amplifications are frequently integrated into chromosomes at particular locations, which suggests an important role for homologous recombination in chromosomal integration. It also appears that intrachromosomal structures are fairly

stable, which is consistent with hypotheses that amplifications can become permanent genomic fixtures. The screen for complementation of the sporulation deficiency was unsuccessful in determining the gene that is mutated in B310, but it did provide clues as to what approaches should be taken in the future.

BIBLIOGRAPHY

Bishop JM. 1991. Molecular themes in oncogenesis. Cell 64: 235-248.

Butler DK, Gillespie D, Steele B. 2002. Formation of large palindromic DNA by homologous recombination of short inverted repeat sequences in Saccharomyces cerevisiae. Genetics 161: 1065-1075.

Butler DK, Yasuda LE, Yao MC. 1995. An intramolecular recombination mechanism for the formation of the rRNA gene palindrome of Tetrahymena thermophila. Mol. Cel. Biol.15: 7117-7126.

Butler DK, Yasuda LE, Yao MC. 1996. Induction of large DNA palindrome formation in yeast: implications for gene amplification and genome stability in eukaryotes. Cell 87: 1115-1122.

Carle GF, Olson MV. 1985. An electrophoretic karyotype for yeast. PNAS 82: 3756-3760.

Chamankhah M, Fontanie T, Xiao W. 2000. The *Saccharomyces cerevisiae mre11(ts)* allele confers a separation of DNA repair and telomere maintenance functions. Genetics 155: 569-576.

Chen C, Kolodner RD. 1999. Gross chromosomal rearrangements in *Saccharomyces cerevisiae* replication and recombination defective mutants. Nature Genetics 23: 81-85.

Chen S, Bigner SH, Modrich P. 2001. High rate of CAD gene amplification in human cells deficient in MLH1 or MSH6. PNAS 98(24): 13802-13807.

Chu G, Vollrath D, Davis RW. 1986. Separation of large DNA molecules by contour

clamped homogenous electric fields. Science 234: 1582-1585.

Ciriacy M. 1979. Isolation and characterization of further *cis*- and *trans*-acting regulatory elements involved in the synthesis of glucose repressible alcohol dehydrogenase (ADHII) in *Saccharomyces cerevisiae*. Mol. Gen. Genet. 176: 427-431.

Coquelle A, Pipiras E, Toledo F, Buttin G, Debatisse M. 1997. Expression of fragile sites triggers intrachromosomal mammalian gene amplification and sets boundaries to early amplicons. Cell 89: 215-225.

D'Amours D, Jackson SP. 2002. The Mre11 complex: at the crossroads of DNA repair and checkpoint signaling. Nature Reviews 3: 317-327.

Debrauwere H, Loeillet S, Lin W, Lopes J, Nicolas A. 2001. Links between replication and recombination in *Saccharomyces cerevisiae*: a hypersensitive requirement for homologous recombination in the absence of Rad27 activity. PNAS 98(15): 8263-8269.

Denis CL, Young ET. 1983. Isolation and characterization of the positive regulatory gene *ADR1* from *Saccharomyces cerevisiae*. Mol. Cell. Biol. 3: 360-370.

Desmaze C, Soria JC, Freulet-Marriere MA, Mathieu N, Sabatier L. 2003. Telomere-driven genomic instability in cancer cells. Cancer Lett. 194(2): 173-182.

Dolinski K, Balakrishnan R, Christie KR, Costanzo MC, Dwight S S, Engel SR, Fisk DG, Hirschman JE, Hong EL, Issel-Tarver L, Sethuraman A, Theesfeld CL, Binkley G, Lane C, Schroeder M, Dong S, Weng S, Andrada R, Botstein D, Cherry JM. "Saccharomyces Genome Database." `http://www.yeastgenome.org/` (May 3, 2003).

Donzeau M, Bandlow W. 1999. The yeast trimeric gu;anine nucleotide-binding protein α

subunit, Gpa2p, controls the meiosis-specific kinase Ime2p activity in response to nutrients. Mol. Cell. Biol.19(9): 6110-6119.

Dorsey M, Peterson C, Bray K, Paquin CE. 1992. Spontaneous amplification of the ADH4 gene in *Saccharomyces cerevisiae*. Genetics 132: 943-950.

Dorsey MJ, Hoeh P, Paquin CE. 1993. Phenotypic identification of amplifications of the ADH4 and CUP1 genes of *Saccharomyces cerevisiae*. Curr. Genet. 23: 392-396.

Esposito RE, Dresser M, Breitenback M. 1991. Identifying sporulation genes, visualizing synaptomemal complexes, and large-scale spore and spore wall purification. Methods in Enzymology 194: 110-131.

Friedberg, EC. 2001. How nucleotide excision repair protects against cancer. Nature Reviews Cancer 1: 22-33.

Friedman R, Hughes AL. 2003. The temporal distribution of gene duplication events in a set of highly conserved human gene families. Molecular Biology and Evolution 20(1): 154-161.

Gietz RD and Woods A. 2002. Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. Methods in Enzymology 350: 87-96.

Giulotto E, Knights C, Stark R. 1987. Hampster cells with increased rates of DNA amplification, a new phenotype. Cell 48: 837-845.

Gordenin DA, Kunkel TA, Resnick MA. 1997. Repeat expansion-all in a flap? Nature Genetics 16: 116-118.

Gorre ME, Mohammed M, Ellwood K, Hsu N, Paquette R, Rao PN, Sawyers CL. 2001. Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. Science 293: 876-880.

Hamlin JL, Milbrandt JD, Heintz NH, Azizkan JC. 1984. DNA sequence amplification in mammalian cells. International Review of Cytology 90: 31-82.

Hansen RJ, Friedberg EC, Reagan MS. 2000. Sensitivity of a *S. cerevisiae RAD27* deletion mutant to DNA-damaging agents and in vivo complementation by the human *FEN-1* gene. Mutation Research/DNA Repair 461(3): 243-248.

Johnston M, Riles L, Hegemann JH. 2002. Gene disruption. Methods in Enzymology 350: 290-315.

Karin M, Najarian R, Haslinger A, Valenzuela P, Welch J, Fogel S. 1984. Primary structure and transcription of an amplified genetic locus: The *CUP1* locus of yeast. PNAS 81: 337-341.

Kassir Y, Simchen G. 1991. Monitoring meiosis and sporulation in *Saccharomyces cerevisiae*. Methods in Enzymology 194: 94-110.

Klessig DL, Berry JO. 1983. Improved filter hybridization method for detection of single copy sequences in large eukaryotic genomes. Plant Mol. Biol. Rep. 1: 12-18.

Lea DE, Coulson CA. 1949. The distribution of the numbers of mutants in bacterial populations. J. Genet. 49: 264-285.

Lin CT, Lyu YL, Xiao H, Lin WH, Whang-Peng J. 2001. Suppression of gene amplification and chromosomal DNA integration by the DNA mismatch repair system. Nucleic Acids Research 29(16): 3304-3310.

Livingstone LR, White A, Sprouse J, Livanos E, Jacks T, Tlsty TD. 1992. Altered cell cycle arrest and gene amplification potential accompany loss of wild-type p53. Cell 70: 923-935.

Lobachev KS, Gordenin DA, Resnick MA. 2002. The Mre11 complex is required for

repair of hairpin-capped double-strand breaks and prevention of chromosome rearrangements. Cell 108: 183-193.

Maniatis T, Fritsch EF, Sambrook J. 1982. Molecular Cloning; A Laboratory Manual. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

McClintock B. 1984. The significance of responses of the genome to challenge. Science 226: 792-801.

Mondello C, Riboni R, Rady M, Giulotto E, Nuzzo F. 1995. Gene amplification in Chinese hamster DNA repair deficient mutants. Mutat. Res. 346: 61-67.

Mondello C, Faravelli M, Pipitone L, Rollier A, Leonardo AD, Giulotto E. 2001. Gene amplification in fibroblasts from ataxia telangiectasia (AT) patients and in X-ray hypersensitive AT-like Chinese hamster mutants. Carcinogenesis 22(1): 141-145.

Montagna C, Lyu MS. Hunter K, Lukes L, Lowther W, Reppert T, Hissong B, Weaver Z, Ried T. 2003. The septin 9 (MSF) gene is amplified and overexpressed in mouse mammary gland adenocarcinomas and human breast cancer cell lines. Cancer Res. 63(9): 2179-2187.

Moore IK. 1997. Characterization of extrachromosomal, circular amplification structures in *Saccharomyces cerevisiae*. Doctoral thesis: University of Cincinnati.

Moore IK, Martin MP, Dorsey MJ, Paquin CE. 2000a. Formation of circular amplifications in *Saccharomyces cerevisiae* by a breakage-fusion-bridge mechanism. Environmental and Molecular Mutagenesis 36: 113-120.

Moore IK, Martin MP, Paquin CE. 2000b. Telomere sequences at the novel joints of four independent amplifications in *Saccharomyces cerevisiae*. Environmental and Molecular Mutagenesis 36: 105-112.

Nathans J. 1999. The evolution and physiology of human color vision: insights from molecular genetic studies of visual pigments. Neuron 24: 299-312.

Ohno S. 1970. Evolution by gene duplication. Springer-Verlag, Berlin-Heidelberg-New York.Patterson TE, Albrecht EB, Nurse P, Sazer S, Stark GR. 1999. Effects of genome position and the DNA damage checkpoint on the structure and frequency of sod2 gene amplification in fission yeast. Molecular Biology of the Cell 10: 2199-2208.

Patterson TE, Albrecht EB, Nurse P, Sazer S, Stark GR. 1999. Effects of genome position and the DNA damage checkpoint on the structure and frequency of *sod2* gene amplification in fission yeast. Molecular Biology of the Cell 10: 2199-2208.

Paquin CE, Dorsey M, Crable S, Sprinkel K, Sondej M, Williamson VM. 1992. A spontaneous chromosomal amplification of the ADH2 gene in *Saccharomyces cerevisiae*. Genetics 130; 263-271.

Peterson C, Kordich J, Milligan L, Bodor E, Siner A, Nagy K, Paquin CE. 2000. Mutations in RAD3, MSH2, and RAD52 affect the rate of gene amplification in the yeast *Saccharomyces cerevisiae.* Environmental and Molecular Mutagenesis 36: 325-334.

Rockmill B, Lambie EJ, Roeder GS. 1991. Spore enrichment. Methods in Enzymology 194: 146-149.

Rose MD, Broach JR. 1991. Cloning genes by complementation in yeast. Methods in Enzymology 194: 195-230.

Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Schimke RT. 1984. Gene amplilfication in cultured animal cells. Cell 37: 705-713.

Selva E, New L, crouse GF. 1995. Mismatch correction act as a barrier to homeologous recombination in *Saccharomyces cerevisiae*. Genetics 139: 1175-1188.

Sharma RC, Schimke RT. 1994. The propensity for gene amplification: a comparison of protocols, cell lines, and selection agents. Mutat. Res. 304(2): 243-260.

Sherman F. 2002. Getting started with yeast. Methods in Enzymology 350: 3-41.

Singh B, Stoffel A, Gogineni S, Poluri A, Pfister DG, Shaha AR, Pathak A, Bosl G, Cordon-Cardo C, Shah JP, Rao PH. 2002. Amplification of the 3q26.3 locus is associated with progression to invasive cancer and is a negative prognostic factor in head and neck squamous cell carcinomas. Am. J. Pathol. 161(2): 365-371.

Stark GR. 1993. Regulation and mechanisms of mammalian gene amplification. Adv. Can. Res. 61: 87-113.

Sung P, Trujillo KM, VanKomen S. 2000. Recombination factors of Saccharomyces cerevisiae. Mutation Research 451: 257-275.

Sutherland GR, Baker E, Richards RI. 1998. Fragile sites still breaking. Trends in Genetics 14(12): 501-506.

Tlsty TD. 1990. Normal diploid human and rodent cells lack a detectable frequency of gene amplification. PNAS 87(8): 3132-3136.

Tlsty TD, Margolin BH, Lum K. 1989. Differences in the rates of gene amplification in nontumorigenic and tumorigenic cell lines as measured by Luria-Delbruck fluctuation analysis. PNAS 86: 9441-9445.

Tseng E. 1998. Characterization of a high amplification rate (HAR) strain of *Saccharomyces cerevisiae*. Masters thesis: University of Cincinnati.

Villemur JF, Abaji C, Cousineau I, Belamaaza A. 2003. MSH2-deficient human cells exhibit a defect in the accurate termination of homology-directed repair of DNA double strand breaks. Cancer Res. 63(12): 3334-3339.

Walton JD, Paquin CE, Kaneko K, Williamson VM. 1986. Resistance to antimycin A in yeast by amplification of ADH4 on a linear, 42 kb palindromic plasmid. Cell 46: 857-863.

Winzeler EA, Shoemaker DD, Astromoff A, Liang H, Anderson K, Andre B, Bangham R, Benito R, Boeke JD, Bussey H, Chu AM, Connelly C, Davis K, Dietrich F, Sow SW, Bakkoury ME, Foury F, Friend SH, Gentalen E, Giaever G, Hegemann JH, Jones T, Laub M, Liao H, Liebundguth N, Lockhart DJ, Lucau-Danila A, Lussier M, M'Rabet N, Menard P, Mittmann M, Pai C, Rebischung C, Revuelta JL, Riles L, Roberts CJ, Ross-MacDonald P, Scherens B, Snyder M, Sookhai-Mahadeo S, Storms RK, Veronneau S, Voet M, Volckaert G, Wark TR, Wysocki R, Yen GS, Yu K, Zimmermann K, Philippsen P, Johnston M, Davis RW. 1999. Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis. Science 285: 901-906.

Wright JA, Smith HS, Watt FM, Hancock MC, Hudson LD, Stark GR. 1990. DNA amplification is rare in normal human cells. PNAS 87: 1791-1795.

Yin DX, Schimke, RT. 1996. Inhibition of apoptosis by overexpressing Bcl-2 enhances gene amplification by a mechanism independent of aphidicolin pretreatment. PNAS 93: 3394-3398.

Yunis JJ, Soreng AL. 1984. Constitutive fragile sites and cancer. Science 226: 1199-1204.

APPENDIX A

Calculation of Mutation and Amplification Rates

The P_0 method of Lea and Coulson, 1949, was designed to describe the Poisson distribution of mutants in bacterial populations. The equation relates the logarithmic growth rate of bacterial cells to the emergence of mutants in the population. In this study, the P_0 method is used to calculate mutation rates to antimycin A and rates of amplification of the *ADH4:CUP1* construct in yeast. P_0 represents the fraction of cultures without mutations. Using the amplification rate calculation for the KO parent strain as an example (Table 3), P_0 equals 0.83 (51/60). To calculate mutation rate (m), the following formula is used:

$$P_0=e^{-m}$$

To solve for m, the natural log is taken from both sides of the equation, as shown below:

$$\ln(P_0)=\ln(e^{-m})$$

$$\ln(0.83)=\ln(e^{-m})$$

$$m=0.16$$

To calculate the number of amplifications per cell, m is divided by the average number of cells per culture:

$$m/\#\text{cells per culture} = 0.16/1.3\times10^{7} = 1.3\times10^{-8}$$

95% confidence intervals were also determined as described in Lea and Coulson, 1949. First, standard error is calculated by the following formula, where N represents the total number of cultures:

$$\sigma_m=[(e^m-1)/N]^{1/2}$$

$$\sigma_m=[(e^{0.16}-1)/60]^{1/2}$$

$$\sigma_m=0.054$$

The standard error is divided by the average number of cells per culture and multiplied by 2 to obtain the 95% confidence intervals:

$$2(\sigma_m/\#\text{ cells per culture}) = 2(.054/1.3\times10^7) = 8.3\times10^{-9}$$

Using this confidence interval, the amplification rate of the KO parent strain is $1.3\pm0.83\times10^{-8}$ amplifications per cell.

APPENDIX B

Frozen Stocks from Long Term Growth Experiments

Sarah's Box:

Location	Strain	Date frozen	Location	Strain	Date frozen
A1	p366- *E.coli*		D8	A4C2	7/26/02
A2	YEP24-*E.coli*		D9	5-1-738C	7/26/02
A3	pYA4-2-*E.coli*		D10	5-2-777C	7/26/02
A4	p7a- *E.coli*		E1	5-3-831LP	7/26/02
A5	B310	7/1/02	E2	5-4-865LP	7/26/02
A6	411B	7/1/02	E3	5-6-964CI	7/26/02
A7	A4C-EA1	7/1/02	E4	6-6-1243CI	7/26/02
A8	A4C2	7/1/02	E5	B310	8/9/02
A9	5-1-738C	7/1/02	E6	411B	8/9/02
A10	5-2-777C	7/1/02	E7	A4C-EA1	8/9/02
B1	5-3-831LP	7/1/02	E8	A4C2	8/9/02
B2	5-4-865LP	7/1/02	E9	5-1-738C	8/9/02
B3	5-6-964CI	7/1/02	E10	5-2-777C	8/9/02
B4	6-6-1243CI	7/1/02	F1	5-3-831LP	8/9/02
B5	B310	7/12/02	F2	5-4-865LP	8/9/02
B6	411B	7/12/02	F3	5-6-964CI	8/9/02
B7	A4C-EA1	7/12/02	F4	6-6-1243CI	8/9/02
B8	A4C2	7/12/02	F5	B310*	8/15/02
B9	5-1-738C	7/12/02	F6	411B	8/15/02
B10	5-2-777C	7/12/02	F7	A4C-EA1	8/15/02
C1	5-3-831LP	7/12/02	F8	A4C2	8/15/02
C2	5-4-865LP	7/12/02	F9	5-1-738C	8/15/02
C3	5-6-964CI	7/12/02	F10	5-2-777C	8/15/02
C4	6-6-1243CI	7/12/02	G1	5-3-831LP	8/15/02
C5	B310	7/18/02	G2	5-4-865LP	8/15/02
C6	411B	7/18/02	G3	5-6-964CI	8/15/02
C7	A4C-EA1	7/18/02	G4	6-6-1243CI	8/15/02
C8	A4C2	7/18/02	G5	B310	8/26/02
C9	5-1-738C	7/18/02	G6	411B	8/26/02
C10	5-2-777C	7/18/02	G7	A4C-EA1	8/26/02
D1	5-3-831LP	7/18/02	G8	A4C2	8/26/02
D2	5-4-865LP	7/18/02	G9	5-1-738C	8/26/02
D3	5-6-964CI	7/18/02	G10	5-2-777C	8/26/02
D4	6-6-1243CI	7/18/02	H1	5-3-831LP	8/26/02
D5	B310	7/26/02	H2	5-4-865LP	8/26/02
D6	411B	7/26/02	H3	5-6-964CI	8/26/02
D7	A4C-EA1	7/26/02	H4	6-6-1243CI	8/26/02
H5	B310	8/30/02	I5	B310	9/5/02

H6	411B	8/30/02	I6	411B	9/5/02
H7	A4C-EA1	8/30/02	I7	A4C-EA1	9/5/02
H8	A4C2	8/30/02	I8	A4C2	9/5/02
H9	5-1-738C	8/30/02	I9	5-1-738C	9/5/02
H10	5-2-777C	8/30/02	I10	5-2-777C	9/5/02
I1	5-3-831LP	8/30/02	J1	5-3-831LP	9/5/02
I2	5-4-865LP	8/30/02	J2	5-4-865LP	9/5/02
I3	5-6-964CI	8/30/02	J3	5-6-964CI	9/5/02
I4	6-6-1243CI	8/30/02	J4	6-6-1243CI	9/5/02

*B310 developed its flocculating mutation by 8/9/02 and the original culture was discarded. A new B310 culture was begun on 8/12/02. All B310 frozen cultures in this box from this point on are descendants of the new culture.

Sarah's Box 2:

Location	Strain	Date frozen	Location	Strain	Date frozen
1	B310	9/23/02	40	6-6-1243CI	10/15/02
2	411B	9/23/02	41	B310	10/22/02
3	A4C-EA1	9/23/02	42	411B	10/22/02
4	A4C2	9/23/02	43	A4C-EA1	10/22/02
5	5-1-738C	9/23/02	44	A4C2	10/22/02
6	5-2-777C	9/23/02	45	5-1-738C	10/22/02
7	5-3-831LP	9/23/02	46	5-2-777C	10/22/02
8	5-4-865LP	9/23/02	47	5-3-831LP	10/22/02
9	5-6-964CI	9/23/02	48	5-4-865LP	10/22/02
10	6-6-1243CI	9/23/02	49	5-6-964CI	10/22/02
11	B310	10/1/02	50	6-6-1243CI	10/22/02
12	411B	10/1/02	51	B310	10/29/02
13	A4C-EA1	10/1/02	52	411B	10/29/02
14	A4C2	10/1/02	53	A4C-EA1	10/29/02
15	5-1-738C	10/1/02	54	A4C2	10/29/02
16	5-2-777C	10/1/02	55	5-1-738C	10/29/02
17	5-3-831LP	10/1/02	56	5-2-777C	10/29/02
18	5-4-865LP	10/1/02	57	5-3-831LP	10/29/02
19	5-6-964CI	10/1/02	58	5-4-865LP	10/29/02
20	6-6-1243CI	10/1/02	59	5-6-964CI	10/29/02
21	B310	10/8/02	60	6-6-1243CI	10/29/02
22	411B	10/8/02	61	B310	11/15/02
23	A4C-EA1	10/8/02	62	411B	11/15/02
24	A4C2	10/8/02	63	A4C-EA1	11/15/02
25	5-1-738C	10/8/02	64	A4C2	11/15/02
26	5-2-777C	10/8/02	65	5-1-738C	11/15/02
27	5-3-831LP	10/8/02	66	5-3-831LP*	11/15/02
28	5-4-865LP	10/8/02	67	5-4-865LP	11/15/02
29	5-6-964CI	10/8/02	68	5-6-964CI	11/15/02
30	6-6-1243CI	10/8/02	69	6-6-1243CI	11/15/02
31	B310	10/15/02	70	B310	11/13/02
32	411B	10/15/02	71	411B	11/13/02
33	A4C-EA1	10/15/02	72	A4C-EA1	11/13/02
34	A4C2	10/15/02	73	A4C2	11/13/02
35	5-1-738C	10/15/02	74	5-1-738C	11/13/02
36	5-2-777C	10/15/02	75	5-3-831LP	11/13/02
37	5-3-831LP	10/15/02	76	5-4-865LP	11/13/02
38	5-4-865LP	10/15/02	77	5-6-964CI	11/13/02
39	5-6-964CI	10/15/02	78	6-6-1243CI	11/13/02

*The 5-2-777C culture stopped growing by 11/5/02. When gels were prepared representing the end of the second long term growth period, the frozen strains from 10/29/02 were used.

Sarah's Box 3:

Location	Strain	Date frozen	Location	Strain	Date frozen
1	B310	12/10/02	40	6-6-1243CI	1/6/13
2	411B	12/10/02	41	B310	1/13/03
3	A4C-EA1	12/10/02	42	411B	1/13/03
4	A4C2	12/10/02	43	A4C-EA1	1/13/03
5	5-1-738C	12/10/02	44	A4C2	1/13/03
6	5-2-777C	12/10/02	45	5-1-738C	1/13/03
7	5-3-831LP	12/10/02	46	5-2-777C	1/13/03
8	5-4-865LP	12/10/02	47	5-3-831LP	1/13/03
9	5-6-964CI	12/10/02	48	5-4-865LP	1/13/03
10	6-6-1243CI	12/10/02	49	5-6-964CI	1/13/03
11	B310	12/23/02	50	6-6-1243CI	1/13/03
12	411B	12/23/02	51	B310	1/22/03
13	A4C-EA1	12/23/02	52	411B	1/22/03
14	A4C2	12/23/02	53	A4C-EA1	1/22/03
15	5-1-738C	12/23/02	54	A4C2	1/22/03
16	5-2-777C	12/23/02	55	5-1-738C	1/22/03
17	5-3-831LP	12/23/02	56	5-2-777C	1/22/03
18	5-4-865LP	12/23/02	57	5-3-831LP	1/22/03
19	5-6-964CI	12/23/02	58	5-4-865LP	1/22/03
20	6-6-1243CI	12/23/02	59	5-6-964CI	1/22/03
21	B310	12/30/02	60	6-6-1243CI	1/22/03
22	411B	12/30/02	61	B310	1/28/03
23	A4C-EA1	12/30/02	62	411B	1/28/03
24	A4C2	12/30/02	63	A4C-EA1	1/28/03
25	5-1-738C	12/30/02	64	A4C2	1/28/03
26	5-2-777C	12/30/02	65	5-1-738C	1/28/03
27	5-3-831LP	12/30/02	66	5-2-777C	1/28/03
28	5-4-865LP	12/30/02	67	5-3-831LP	1/28/03
29	5-6-964CI	12/30/02	68	5-4-865LP	1/28/03
30	6-6-1243CI	12/30/02	69	5-6-964CI	1/28/03
31	B310	1/6/13	70	6-6-1243CI	1/28/03
32	411B	1/6/13	71	KO parent	
33	A4C-EA1	1/6/13	72	SPO1-yeast	
34	A4C2	1/6/13	73	SPO2-yeast	
35	5-1-738C	1/6/13	74	SPO3-yeast	
36	5-2-777C	1/6/13	75	SPO1-*E.coli*	
37	5-3-831LP	1/6/13	76	SPO2-*E.coli*	
38	5-4-865LP	1/6/13	77	SPO3-*E.coli*	
39	5-6-964CI	1/6/13			