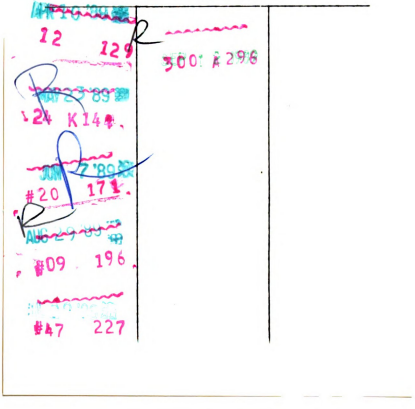

ISOLATION AND CHARACTERIZATION OF SPORULATION AMYLOGLUCOSIDASE-DEFICIENT STRAINS OF SACCHAROMYCES CEREVISIAE

By

Linda M. Smith

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Genetics Program

1985

Abstract

ISOLATION AND CHARACTERIZATION OF SPORULATION AMYLOGLUCOSIDASE-DEFICIENT STRAINS OF SACCHAROMYCES CEREVISIAE

By

Linda M. Smith

Diploid strains of the yeast Saccharomyces cerevisiae undergo meiosis and spore formation when incubated under the appropriate conditions. During sporulation they produce a sporulation-specific amyloglucosidase (SAG) that is responsible for the extensive glycogen degradation observed in sporulating cells. In this study, three SAG-deficient strains SL484, SL641 and SL382 were isolated and partially characterized. None of the mutants progressed through Meiosis I or sporulated at 34°C. Premeiotic DNA synthesis did not occur in SL484 or SL641. SL484 was temperature sensitive for SAG production and genetic analysis showed that the SL484 defect is the result of a mutation in a single gene. This gene is linked to the TRP5 gene on chromosome VII. SL641 was not a conditional mutant so was not amenable to standard genetic analysis. Instead, tetraploid strains heterozygous for the SL641 defect were constructed by fusing spheroplasts of SL641 and wild-type MATa/MATα diploids. Segregation analysis of the SAG-deficient phenotype indicated that the phenotype was the result of a defect in a single gene. One interesting finding of the fusion experiments was that many of the fusants were aneuploid. This suggests that karyogamy or

subsequent meiotic or mitotic segregation of chromosomes did not proceed normally. Complementation tests showed that SL641 and SL484 are probably not defective in SPO1, SPO3 or SPO7 genes. In addition, there was evidence that the SL484 and SL641 defects were not different alleles of the same gene.

To John, who has made my hopes and dreams seem possible again, and to my daughter Morgan, who is, by far, my most worthwhile and successful genetic experiment.

ACKNOWLEDGMENTS

Many individuals have worked in Dr. P. T. Magee's laboratory since I first joined the group. They include Dr. Don Primerano, Dr. Peter Alexander, Roger Partridge, B. B. Magee and others. I wish to thank all of them for their help, friendship and good wishes. I especially want to acknowledge Dr. P. T. Magee's continued support, and to thank Dr. Mary Clancy for her guidance and for being a role model; a responsibility I'm sure she did not relish. The many hours Donna Lehman spent at the spectrophotometer and fluorimeter are also gratefully acknowledged.

My committee members, Dr. Barry Chelm, Dr. Loren Snyder and Dr. Leonard Robbins, were always helpful and supportive. I would especially like to thank Dr. Robbins for the considerable time he spent helping me analyze the results of the protoplast fusion experiments.

Others have provided various forms of support. Roger Denome and Tim Adcock taught me how to use the departmental computer system; the Microbiology Women's Group reminded me that I was not alone in times of discouragement and doubt; and Estelle Hrabak encouraged me to make time to pick berries and make jam, for which my stomach and psyche are eternally grateful. Finally, I would like to thank Dr. Ellen Swanson, who I met my first day at Michigan State University and who became my good friend despite our first meeting.

TABLE OF CONTENTS

LIST OF TABLES

TEXT

LIST OF FIGURES

TEXT

APPENDIX

Figure | Page

INTRODUCTION AND LITERATURE REVIEW

Yeast as an experimental organism. The yeast Saccharomyces cerevisiae has long been an organism of interest for scientific investigation. Initially this was because of its economic importance in the production of beer and wine (103). More recently, S. cerevisiae has been used as a model system for developmental processes because of its relatively complex life cycle (for recent reviews see 41, 57, 86, 97 and 131). S. cerevisiae normally can grow mitotically in either the haploid or diploid state. Different mating types are conferred on haploid strains by two alleles, the a allele and the α allele, at the mating-type locus, MAT. Haploids of opposite mating type, when in proximity, undergo morphological and physiological changes, brought about by the action of the mating pheromones a-factor and α-factor, which lead to the fusion of a cells with α cells and formation of a diploid zygote that is heterozygous at the mating-type locus. The zygote can give rise to diploid cells which grow mitotically but are no longer able to mate. Instead, under the appropriate conditions, the diploid ceases mitotic cell division and enters the sporulation pathway which involves meiosis and ascospore formation. It is the process of sporulation that is the topic of this review and study.

In the last 10 years, a number of reviews have been written on sporulation (39-41, 46, 52, 53, 86). For a recent and thorough review of the physiology and genetics of sporulation, the reader is

particularly directed to Esposito and Klapholz (41). It is not the intention of this overview of sporulation to duplicate these earlier reviews. Rather, I will give a brief description of sporulation, its regulation and its genetic analyses. I will update and expand the discussion of certain processes in the sporulation pathway when relevant to this study.

Events in sporulation. A number of morphological and biochemical changes have been observed in yeast cells incubated in sporulation medium (41, 86). The exact response to incubation in sporulation medium depends on the cell type. When diploids heterozygous for the mating-type locus are shifted into sporulation medium from a growth medium, cell division arrests at G1. The cells then enter the meiotic pathway rather than initiating a new round of budding. During the course of sporulation, the chromosomes are replicated; recombination occurs and is followed by two meiotic divisions which yield four haploid nuclei. As in other ascomycetes, the nuclear membrane remains intact and the parental nucleus forms four protrusions or lobes which contain the haploid genomes. The lobes are eventually surrounded by a prospore wall which later is modified to form the mature spore wall (41). Concurrent with these morphological events are a variety of biochemical processes. These include DNA synthesis and RNA and protein synthesis and degradation (41, 62, 100, 138, 143), alterations in the synthesis or activity of particular proteins (18, 41, 91, 100), modification of histones (77), synthesis of storage molecules like lipids and carbohydrates (29, 41) and the eventual degradation of glycogen as mature ascospores appear (41).

When haploid cells or diploid cells homozygous for the mating-type locus are incubated in sporulation medium, the cells arrest at G1 but are unable to enter the meiotic pathway and so do not exhibit the morphological changes associated with sporulation (41, 86). However, many of the biochemical events seen in sporulating cells do occur in asporogenous cells. The sporulation-specific events, that is those occuring only in sporogenous cultures, are DNA synthesis (41, 100), RNA degradation (41), synthesis of neutral lipids after about 12 hours of incubation (41), synthesis of a spore-surface-specific antigen (18), and glycogen degradation (41). There are also reports of meiosis-specific increases in the activity of DNA polymerases I and II, two different deoxyribonucleases (100) and 1,3-β-glucanases (25, 60) as well as a loss of mitochrondrial circular RNAs (113). The events common to both sporulating and nonsporulating cells are thought to be a response to the starvation conditions that induce sporulation.

Control of sporulation. Normally, only diploid cells heterozygous at the mating-type locus that are starved for nitrogen and glucose but provided with a respirable carbon source (usually acetate) will sporulate. How these cellular characteristics and environmental conditions signal sporulation is poorly understood. Each will be considered below.

Glucose represses sporulation (41). Glucose repression of various catabolic pathways in yeast has been known and studied for some time. It is thought that the repression of sporulation is due to the repression of some of these pathways. In particular, enzymes of the TCA cycle (which are required for acetate utilization) and many enzymes involved in gluconeogenesis (which provide glucose for the synthesis of

storage carbohydrates) are repressed by glucose (41). Unfortunately, we do not understand the mechanism of glucose catabolite repression of the TCA and gluconeogenesis enzymes nor of any other catabolite repressible enzymes. There is evidence that repression occurs at the transcriptional level (7, 27, 41, 66, 151).

There has been a great deal of research into the role of cyclic AMP (cAMP) in catabolite repression (47, 83). The interest has been especially keen because yeasts are microbial eukaryotes. It is possible that cAMP may function in yeast, as in higher eukaryotes by activating protein kinases which in turn phosphorylate certain proteins modulating their activity (70). On the otherhand, cAMP may function as in bacteria where it combines with a receptor protein called CAP or CRP and the CAP-cAMP complex modulates the transcription of certain genes (74).

The role of chromatin structure in the regulation of glucose-repressible genes has also been examined. Sledziewski and Young (121) compared DNAase I digestion of two alcohol dehydrogenase genes, ADC1 and ADR2, from cells grown in the presence and absence of glucose. Increased sensitivity to DNAase I has been associated with actively transcribed genes. The increased sensitivity is thought to be the result of structural changes in the histone-DNA complex that make the DNA more accessible to DNAase I (50, 137). Using low concentrations of DNAase I, they found that in glucose-grown cells, the constitutively transcribed ADC1 gene was more sensitive to DNAase I digestion than the repressible ADR2 gene. When the cells were grown on ethanol, which derepresses the ADR2 gene, both genes were equally sensitive to DNAase I digestion.

A number of mutations affecting catabolite repression have been isolated. Their analysis indicates that there are both regulatory loci specific for a particular enzyme or pathway and regulatory loci that control a number of pathways (8, 32, 33, 47, 83, 84). Further complicating the story is the fact that the regulatory loci appear to be of two types, those that mediate repression and those that mediate derepression. Mutations in the former prevent glucose repression while mutations in the latter prevent derepression when glucose is removed from the growth medium. Whether one or more of the common control sequences or a regulatory sequence specific for sporulation acts to control sporulation is still unclear. Some mutations in common regulatory sequences have no affect on sporulation (11, 33, 85) whereas others, like the hex1^{r} (32), snf1-28 (8) and ccr3 (10) alleles sporulate poorly.

The presence of various nitrogen sources in the medium also represses sporulation. The mechanism of nitrogen repression is also poorly understood. Like glucose, ammonium salts and certain other nitrogenous compounds inhibit biochemical pathways that are known to function during sporulation. These pathways include the glyoxylate cycle, RNA and protein synthesis, and glycogen and protein degradative pathways (41). The effect on DNA synthesis is not clear at this time (41). The greatest effect on sporulation is brought about by ammonium ions, and it has been suggested that ammonia itself acts as the inhibitor rather than some metabolite generated from ammonia (14, 41). However, Cooper (14) has suggested that sporulation is triggered by nitrogen starvation due to a change in metabolite balance. That is, the concentration of certain metabolites relative to each other is the

signal for sporulation. The addition of nitrogen-containing compounds disrupts this balance and prevents sporulation. Supporting this argument is the existence of the cell-division-cycle mutants, cdc25, cdc35, and the sporulation-derepressed mutant, spd1, which are able to sporulate in a rich medium (14, 17). These will be discussed in more detail later. Additional support comes from a study by Freese, et al. (48). These authors found that cells partially starved for carbon, nitrogen or phosphate sporulated. They suggested that sporulation was triggered in response to a starvation-induced change in the balance of a metabolite or metabolites. It should be noted, however, that when glucose was present in a medium lacking nitrogen or phosphate, sporulation did not occur.

The final factor regulating sporulation is cell type as determined by the MAT gene. How the MAT alleles determine cell type has been intensively studied in the last several years and has led to the α1-α2 model (57). MATα contains two coding sequences, called α1 and α2, which are transcribed in opposite directions. The MATa allele also gives rise to two transcripts, however, only one, a1, has been shown to have MATa function. The α1 product is hypothesized to be a positive regulator of specific functions, whereas the α2 product is thought to be a negative regulator of a-specific functions. In a MATα haploid, the functions that make a cell α-type are turned on by α1 and the functions that make a cell a-type are turned off, so the cell produces α-factor and mates only with a-type cells. In MATa haploids, neither the α1 nor α2 products are synthesized, so α-specific genes are not expressed and a-specific genes are not repressed. These cells carry out a-specific functions, produce a-factor and mate only with α-type cells. The a1

product appears to function only in MATa/MATα diploids. In these cells, the α2 product represses the activity of a-specific genes and is also thought to interact in some manner with the a1 product to inhibit transcription of α1. Thus α-specific genes are not expressed. The cells produce neither a- nor α-factor and are unable to mate. However, when they are incubated in sporulation medium, sporulation-specific genes are expressed and the cells sporulate. There is evidence that sporulation-specific genes are negatively controlled by some other gene or genes, which in turn may be controlled by the a1 and α2 products. Candidates for negative regulators are the SCA, CSP and RME gene products. Mutations in these genes are known and will be discussed later.

As stated earlier, a MATa/MATα cell upon transfer from a growth medium to sporulation medium completes budding before it begins meiosis. The decision to enter the meiotic pathway is made in the G1 stage of the cell cycle. Two other developmental options are available to a cell at G1. These are initiation of a new round of cell division and initiation of the morphological changes which prepare the cell for conjugation. The control point for the initiation of cell division is called Start (97). A currently well-accepted model for the regulation of cell division proposes that the completion of Start is dependent on cell size (97, 116). How the cell monitors its size is unclear. The correlation between cell volume and the completion of Start is not precise and it has been suggested that the level of one or more macromolecules is monitored (97). Pringle and Hartwell (97) have proposed that subunits of the spindle pole body or proteins involved in the assembly of the spindle pole body are critical, and that the

completion of Start may actually be the completion of the doubling of the spindle pole body mass. Also implicated in the process of moving from G1 to S phase are cAMP via the action of a protein kinase (80, 132) and glycosylation of one or more proteins (68).

Start is also important to sporulation and conjugation. Cells defective in some of the Start genes are defective in mating (3, 97), karyogamy (31) and sporulation (17, 41). Furthermore, only cells arrested at Start are able to mate or sporulate (97). The only exception to this is that cells arrested at the cdc4 stage of the cell cycle, which is after Start, can sporulate directly (97). These observations are consistent with the hypothesis that Start involves the synthesis and assembly of spindle pole body subunits since the initial contact between two nuclei during karyogamy is at the spindle pole body (4) and an intact, functional spindle pole body is required for meiotic divisions (41). The exception, cdc4, also supports this hypothesis since cdc4 strains duplicate the spindle pole body but fail to separate the duplicates (97).

Cells are also arrested at G1 when incubated under nutrient-limited conditions (14). This is consistent with the model that a critical size must be reached before Start can occur. But the relationship between Start-gene products and environmental conditions is not limited to this. Mutations in certain Start genes respond differently to the presence or absence of glucose or nitrogenous compounds in the growth medium. At the restrictive temperature, cells with temperature-sensitive mutations in cdc28, cdc36, and cdc39 do not divide when incubated in glucose-containing media but do divide if the medium contains a non-fermentable carbon source such as pyruvate or acetate

(115). Cells with mutations in cdc25 and cdc35 exhibit a different phenotype. They fail to grow at the restrictive temperature, but sporulate at this temperature in nitrogen-rich media (41).

It seems likely then, that Start genes are important in sensing environmental conditions. How cell-type is integrated into this scheme is unclear. If the synthesis and assembly of the spindle pole body is an important Start event, then the MATa/MATα configuration must allow the cell to synthesize and assemble the spindle pole body subunits even though the cell is starved for nitrogen. Related to this are the results of experiments which suggest that spindle pole body development or separation is coincident with irreversible commitment to sporulation (41).

Isolation of mutations affecting sporulation. The traditional approach of geneticists to understanding biochemical or developmental pathways is to identify genes involved in the pathway. Classical geneticists have done this by isolating and characterizing mutations that affect the pathway. Then, based upon the phenotypes of the mutants, hypotheses regarding the function of the products of the wild-type alleles are made. The identification and characterization of mutations that affect sporulation requires the consideration of a number of problems and possibilities. These are:

1. How can one screen for recessive mutations in a diploid?
2. What will the phenotypes of sporulation mutants be?
3. Are the defects in genes necessary for and specific to sporulation?
4. If a mutant fails to sporulate or produces mostly inviable spores, how can the mutant be analyzed genetically?

In order to observe recessive mutations in genes that function only in diploids, procedures for isolating the defect in a homozygous state must be devised. Many sporulation mutations were isolated in haploids for the purpose of studying other cellular processes such as mitosis and DNA repair. They were later examined for their effect on sporulation by constructing homozygous diploids (41, 81). There have also been systematic searches for sporulation-specific defects. Two approaches have been used. Esposito and Esposito (38) mutagenized spores of a homothallic strain, then tested the surviving colonies for their ability to sporulate at elevated temperatures. A homothallic strain was used because individual cells are able to switch their mating type after having completed one cycle of cell division (57). Thus, a colony arising from a single spore briefly becomes a mixture of both a and α mating types which can intermate. Since all the cells in the developing colony are isogenic except for mating type, the resulting diploids are homozygous at all loci except mating type. Thus, any spore mutated in a sporulation gene will give rise to a diploid colony homozygous for that mutation and both recessive and dominant mutations will be observable. The second approach was taken by Roth (104, 105) and Roth and Fogel (106). They used a haploid strain that was a MATa/MATα, n+1 disome. Such strains, when incubated in sporulation medium, will undergo premeiotic DNA synthesis and recombination, but do not form normal, viable spores. By monitoring the occurance of intragenic recombination at elevated temperatures, these authors were able to isolate mutants defective in premeiotic DNA synthesis and recombination.

A number of different sporulation phenotypes are possible. Mutations that disrupt the regulation of sporulation should be

observable. For instance, diploid cells homozygous for the mating-type locus could sporulate, or cells could sporulate under conditions normally conducive to vegetative growth. Mutations could also cause a reduction in the number of asci produced or in the number of spores in each ascus. Finally the viability of the ascospores might be reduced. Any phenotype observed, might be the result of a mutation in a gene that functions only during sporulation and is, therefore, sporulation-specific or in a gene that functions in other cellular processes. Obviously, those mutants isolated initially for defects in mitosis or DNA repair fall into the latter, nonspecific class of genes. Sporulation-specific defects have usually been identified by requiring normal mitotic growth with a mutant phenotype evident only when the cells are incubated in sporulation medium (41, 104-106, 134).

Genetic analysis of mutants with reduced sporulation or reduced spore viability is extremely difficult, if not impossible, since the first step in the analysis is the construction of diploids heterozygous for the mutation. This problem is solved by using temperature-sensitive strains which can be sporulated at the permissive temperature, and the spores mated to a wild-type strain (41). The analysis of unconditional mutants requires nontraditional approaches. Tsuboi (134) isolated sporulation defects in a homothallic strain, then fused spheroplasts of the diploid mutants with spheroplasts of a wild-type diploid strain. The fused spheroplasts were incubated in sporulation medium. Nuclear fusion and cell-wall regeneration does not occur in sporulation medium. The wild-type nucleus in the fusant complemented the sporulation defect of the mutant nucleus and both nuclei divided meiotically and formed

spores. The spores, when incubated together in a germination medium, intermated to produce diploids heterozygous for the sporulation defect.

Using the schemes described above, a large number of sporulation-defective strains have been isolated. They will be discussed below.

Mutations affecting the regulation of sporulation. A number of genes have been identified which function in the initiation of either mitotic or meiotic development in MATa/MATα cells. The cell-division-cycle genes, CDC28, CDC25, CDC35 and the amino acid biosynthesis gene, TRA3 are required for the initiation of a new mitotic cycle (41). TRA3 is also required for entry into the meiotic pathway since mutations in TRA3 block sporulation. There is conflicting evidence on the role of CDC28 in sporulation. Dawes and Calvert (17) observed nearly wild-type levels of sporulation at 30°C and 34°C for two different cdc28 alleles. However, earlier studies of cdc28 strains showed greatly reduced sporulation at either 25°C or 34°C (41). As discussed earlier, cdc25 and cdc35 mutants are capable of sporulating at both permissive and nonpermissive temperatures when incubated in sporulation medium. However, they will also sporulate at the nonpermissive temperature for growth in a medium containing acetate and a good nitrogen source. It has been proposed that the normal function of the CDC25 and CDC35 genes is to make a choice between meiosis and mitosis based on nutritional signals (41).

Other mutations that behave abnormally in media containing nitrogen or a variety of carbon sources have been isolated. The spd1 mutant is able to sporulate in a nitrogen-containing medium, and is unable to grow on a number of nonfermentable carbon sources (17, 41). The spd1 locus

has been mapped and is located next to the SUP3 gene (17). Interestingly, homozygotes for the ocher suppressing allele of the SUP3 gene exhibit decreased sporulation unless they are shifted from logarithmic growth in a rich glucose-containing medium. Normally, cells sporulate very poorly when shifted to sporulation medium under such conditions (41). Recently, two more SPD loci, SPD3 and SPD4, were identified (17). None of the SPD loci are linked to CDC25, CDC35, or CDC28 (17). Using a spd1 strain, Calvert and Dawes (6) isolated three new sporulation (spo) mutants they felt were defective in the initiation of sporulation. These mutations were pleiotropic. When grown to stationary phase in a rich glucose-containing medium, the spo mutants formed large aberrantly shaped cells that had a pseudomycelial appearance. When incubated further, the cells rapidly lost viability. The cells were also able to reduce triphenyltetrazolium chloride, an indication of respiratory activity, even when grown on glucose. Glucose normally represses respiratory enzymes. Protein differences were also observed.

Entian and Frolich (32) recently described a new HEX1 allele called hex1^{r}. The HEX1 locus, also known as HXK2, codes for PII, one of the two isoenzymes of hexokinase. The original HEX1 mutants exhibited reduced hexokinase activity and failed to show glucose repression of invertase, maltase, malate dehydrogenase and a number of respiratory enzymes (33). Entian and Frolich (32) hypothesized that the PII isoenzyme is bifunctional, having a catylytic site for hexose phosphorylation and a regulatory site for triggering carbon catabolite repression of a number of catabolic pathways. This hypothesis predicts that mutants altered in the regulatory site, but not the catalytic site, could be isolated. The

hex1^{r} allele appears to be such a mutant. In hex1^{r}/hex1 diploids, hexokinase PII activity was normal, but there was no repression by glucose of maltase, invertase or malate dehydrogenase. In addition, these strains were unable to sporulate, unlike the hex1 homozygotes which sporulate normally.

As mentioned earlier, cAMP and protein kinase are hypothesized to have a role in the Start event of the cell cycle (80, 132). A temperature-sensitive mutant defective in the structural gene for adenylate cyclase, cyr1, has been tested for its ability to sporulate. It sporulated poorly, and most asci formed contained only one or two spores. However, this strain could sporulate in both a rich acetate-containing medium and a rich glucose-containing medium (81). A mutant with a temperature-sensitive defect in CYR3, the structural gene for the regulatory subunit of cAMP-dependent protein kinase was also examined. It had decreased sporulation at the restrictive temperature but, like the cyr1 mutant, could sporulate in a rich acetate-containing medium (81). Finally, bcy1 mutants were tested. bcy1 mutations suppress the need for exogenous cAMP for growth in cyr1 and cyr3 strains by causing a deficiency in the regulatory subunit of cAMP-dependent protein kinase and an increase in cAMP-independent protein kinase activity (80). bcy1 homozygotes failed to sporulate (81). Based on these results, Matsumoto, et al. (81) suggested that the initiation of meiosis requires a decrease in cAMP production as well as the inactivation of cAMP-dependent protein kinase.

Mutations that affect mating-type control of sporulation have been described. Originally, four different mutants, rme1, sca, csp1 (41) and SAD (61) were isolated. The SAD locus has been determined to be an

extra copy of a information located in a site distinct from MAT and the silent mating-type information loci HMR and HML (65). The biochemical nature of the SCA, CSP1 and RME1 genes is unknown, but RME1 is hypothesized to code for a negative regulator of sporulation-specific genes that is itself regulated by the action of the MATa and MATα products (41, 99).

Mutations that reduce sporulation or spore viability. Mutants defective in premeiotic DNA synthesis, spindle pole body duplication and separation, spindle formation, synaptonemal complex formation, meiotic recombination, chromosome segregation, spore wall formation, or enclosure of haploid nuclei in spore walls have been isolated (5, 16, 41). These include the spo strains isolated by Esposito and Esposito (38), most cell-division cycle (cdc) mutants (41), a number of radiation-sensitive mutants (41), and others (5, 16, 41). Tsuboi (134) has begun to characterize a number of new sporulation-specific defects which fall into four different phenotypic classes based on premeiotic DNA synthesis and meiotic nuclear division. Allelic relationships between Tsuboi's mutants and the other spo strains have not been determined.

Mutations that result in the formation of two-spored asci have also been described. One mutant, called hfd1-1 forms four haploid nuclei, but fails to incorporate two of the nuclei into spores (89). It is thought that this strain is defective in the morphogenesis of the outer plaques and prospore wall membranes at two of the spindle poles, two steps in ascospore formation. Two temperature-sensitive cell-cycle mutants, cdc5 and cdc14 will, at intermediate temperatures, produce two diploid spores per ascus. These mutants successfully complete the first

meiotic division but do not complete meiosis II (112). In the case of cdc5, unusually short meiosis II spindles are formed. After the chromosomes segregate to opposite poles, both spindle poles are encapsulated by a single spore wall (112). Strains that produce two-spored asci due to the sucessful completion of Meiosis II but not Meiosis I are also known and have been characterized (41, 78, 79).

The availability of a large number of sporulation-defective strains has made it possible to try to order the defects along the sporulation pathway. This has been done by examining the phenotypes of single mutants and double mutants, and by reciprocal shift experiments (41, 86, 97). Although all of the relationships have not been ascertained, it is apparent that sporulation is a branched pathway, with certain genes functioning independently of others. The pathways eventually converge resulting in mature ascospores. One problem with the attempt to relate various gene products both spatially and temporally is that the precise enzymatic or biochemical function of only a very few of the genes is known (41).

Molecular attempts to identify sporulation-specific genes. Recently, Clancy, et al. (12) differentially screened a Lambda Charon 28 library of yeast genomic DNA using two complementary DNA (cDNA) probes; one complementary to poly(A)+ RNA isolated from a MATa/MATα diploid incubated in sporulation medium, and the other complementary to poly(A)+ RNA isolated from a MATα/MATα diploid also incubated in sporulation medium. DNA from Lambda plaques that hybridized to the a/α probe, but not the α/α probe were considered to be sporulation-specific clones. Fifteen different sporulation-specific genes were thought to be represented by the 46 clones they identified. It has been estimated by

classical genetic approaches that about 50 sporulation-specific genes exist in S. cerevisiae (41). Four of the clones were chosen for further analysis. Transcripts complementary to the four clones appeared after about seven hours of incubation in sporulation medium. Furthermore, three of the clones hybridized to two distinct transcripts. The nature of the two transcripts was not determined.

Using a similar approach, Percival-Smith and Segall (94), isolated 38 clones from a pBR322 yeast genomic DNA library, that hybridized preferentially to an a/α cDNA probe. Comparisons of restriction endonuclease digestions of the clones and RNA blot analysis of RNAs isolated from a/α, a/a, and α/α strains incubated in either a growth medium or in sporulation medium suggested that 14 different sequences had been cloned. Final proof that the genes isolated by these two groups are indeed sporulation-specific requires the construction of mutations in the genes and the observation of a corresponding mutant phenotype. This has not been reported.

Identification of sporulation-specific proteins. Although both molecular and classical genetic studies have shown that sporulation-specific genes exist, the identification of sporulation-specific proteins has not been so successful. Several investigators have compared one-dimensional and two-dimensional gels of labelled proteins from sporulating MATa/MATα cells and asporogenous MATa/MATa or MATα/MATα cells, but few reported the synthesis of new proteins specific to sporulating cells (41, 95, 144). Recently, changes in translatable mRNA during sporulation have been examined (71, 138). Weir-Thompson and Dawes (138) isolated RNA from sporulating diploids and asporogenous strains and used it to program an in vitro translation

system. They observed sporulation-specific increases and decreases in the concentrations of translatable mRNA species as well as the synthesis of four new sporulation-specific products from the mRNA. Most of the differences observed between the sporulating and asporogenous cultures occured after 6-8 hours of incubation in sporulation medium. This coincides with the time of commitment to sporulation. Kurtz and Lindquist (71) observed the coordinate induction of a set of sporulation-specific mRNAs encoding eight different proteins. These RNAs appeared after six hours of incubation in sporulation medium. The synthesis of RNAs for these eight proteins is similar to the synthesis of RNAs encoding the four proteins described by Weir-Thompson and Dawes (138). The observations by Clancy et. al. (12) of the appearance of transcripts complementary to their sporulation-specific clones is in good agreement with both of these studies.

Other investigators have focused on proteins with a known function. Dawes, et al. (18) reported that an antigen, specific to the spore surface and probably proteinaceous, was synthesized in a soluble form several hours before the appearance of the spore surface. The amount of soluble antigen decreased during the course of sporulation, presumably as the spore surface was assembled. In 1979, del Rey, et al. (24) reported that the activities of both exo- and endo-1,3-β-glucanases changed during mitotic and meiotic cycles. The change in exo-1,3-β-glucanase activity was due to the synthesis of a new sporulation-specific enzyme (25) which was purified, characterized biochemically and found to be different from the vegetatively produced exo-1,3-β-glucanase (26). More recently, Hien and Fleet (59) isolated and characterized six 1,3-β-glucanases, two of which were exoglucanases,

the other four having endoglucanase activity. One of the endoglucanases was active almost exclusively during sporulation, and was considered to be sporulation-specific (60). Hien and Fleet (60) found no evidence of the sporulation-specific exoglucanase activity reported by del Rey, et al. (25).

Another carbohydrate-degrading enzyme has been reported to be sporulation-specific. Colonna and Magee (13) described an enzyme that appeared only in sporulating MATa/MATα cells after about 8-10 hours of incubation in sporulation medium. Its appearance coincided with the onset of glycogen degradation, which is specific to MATa/MATα cells, and with the first appearance of mature spores. Glycogen is a highly branched chain of glucose moieties joined by α-1,4 linkages with the branch points being α-1,6 linkages. Using a partially purified enzyme preparation, Colonna and Magee (13) observed both 1,4- and 1,6-glucosidase activity. These two activities were partially resolved by Sephadex G-150 chromatography. Glucose is the only product released by the enzyme. They were able to rule out the possibility that the glycogen degrading activity was the result of the combined action of glycogen phosphorylase plus phosphatase, or of amylase plus maltase.

This glycogen-degrading enzyme, later called sporulation amyloglucosidase (SAG), has also been characterized developmentally (see Appendix). Several mutants defective at various stages in sporulation were examined for the production of SAG. It was found that some event or events in the pachytene stage of meiosis must be successfully completed for SAG to appear. This suggested that completion of recombination might be the critical event controlling SAG synthesis. To test this possibility, a diploid strain homozygous for the mutation,

rad52-1, was sporulated and SAG assayed. The rad52-1 allele was isolated for its sensitivity to radiation. It is defective in DNA repair and fails to complete recombination (56). The rad52-1 homozygous diploid had wild type levels of SAG, thus, though recombination events may be critical, the successful completion of recombination is not.

Purpose of this study. The purpose of this study was to attempt to isolate and characterize mutant strains of S. cerevisiae that do not produce SAG. This was desirable for several reasons. First, if the mutants exhibited a sporulation-specific phenotype, this would be further proof that SAG was indeed a sporulation-specific protein. Second, in order to understand a complex developmental process such as sporulation, it is important not only to identify genes involved in the process, but to ascertain their exact biochemical nature and function. Despite considerable effort in characterizing the many genes implicated in sporulation, the functions of only a very few genes have been elucidated. Thus, with respect to understanding the interplay of various enzymes, structural proteins and other molecules that results in the formation of spores, the analysis of both sporulation-specific and sporulation-required genes has left many questions unanswered. By isolating mutations in a gene whose function is known, more progress might be made. Third, a procedure that screens for SAG-deficient strains would also detect mutations in genes that function prior to the critical event(s) that trigger SAG appearance. Fourth, the isolation of defects in the structural gene for SAG would possibly enable one to isolate the gene by functional complementation so that it might be studied at the molecular level.

In this study I describe the screening procedure used to isolate SAG-deficient strains and their partial physiological and genetic characterization. Both temperature sensitive and unconditional, nonsporulating mutants were examined. The former were characterized genetically by standard procedures. The latter, were manipulated using the parasexual technique of spheroplast fusion to construct strains of desired genotypes.

MATERIALS AND METHODS

Yeast strains. The strains of Saccharomyces cerevisiae used in these experiments and their genotypes are given in Table 1.

Media. The media given below were used either in liquid form or in a solid form made by the addition of 20 g of Bactoagar per liter of medium. When necessary, the media were supplemented with adenine, arginine, histidine, leucine, lysine, methionine, tryptophan or uracil, each at a final concentration of 40 mg per liter.

Normally, cells were grown and maintained in a rich medium, YEPD (43). In a number of experiments, a minimal (MIN) medium (43) or various supplemented forms of MIN were used. For regeneration of spheroplasts and fusants, solid MIN and YEPD media containing 1 M sorbitol were used. Respiratory-competence was tested using YPGlycerol (114). BBMB medium has been described elsewhere (125).

For some sporulation experiments, the presporulation (PSP) medium of Roth and Halvorson (107) was used. In other experiments, YEPacetate [10 g yeast extract, 10 g peptone, 10 g potassium acetate and 100 ml 0.1 M phthalate buffer (pH 5.2) brought to one liter with distilled water] was used to adapt cells to growth on acetate. Cells were sporulated in SPM (13).

Sporulation of cells in liquid culture. Cells were grown at 22, 30, or 34°C depending on the experiment, in either PSP or YEPacetate to a cell density of 1×10^7 to 3×10^7 cells per ml. The cells were

Table 1. Genotypes of Saccharomyces cerevisiae.

Strain	Genotype	Source
SCMS7-1	a/α (HO leu2-3 leu2-112 his4 Ade-)	This laboratory
SL382	same as SCMS7-1 except SAG-	This study
SL484	same as SCMS7-1 except Ade+/Ade- and SAG-	This study
SL641	same as SMCS7-1 except SAG-	This study
M12a	a ilv5 trp2	This laboratory
M12α	α ilv3 his1	This laboratory
XMB4-12b	a sst1 Killer+ arg9 ilv3 ura1	I. Herskowitz
RC757	α sst2-1 met1 his6 can1 cyh2	I. Herskowitz
W66-8A	a/α (HO ade2-1 leu1 trp5-2 ura3-1 met4-1 lys2-1)	R. Rothstein
74-1A	a/α (HO arg4-1 spo1-1)	R. E. Esposito
89-1D	a/α (HO arg4-1 met4 spo3-1)	R. E. Esposito
C52-4B	a/α (HO arg4-1 spo7-1)	R. E. Esposito
A364a	a adel ade2 ura1 his7 lys2 tyr1 gal1	This laboratory

harvested by centrifugation, washed two times in sterile distilled water and resuspended in SPM at a concentration of approximately 2 x $10^7$to 3 x 10^7 cells per ml. The cells were incubated with shaking at the indicated temperature.

Sporulation of cells on solid medium. Patches of cells grown overnight on YEPD were replicated onto PSP and incubated 24-48 hours. The patches were then transferred using toothpicks to SPM, and incubated for three days. All incubations were done at either 30 or 34°C.

Preparation of cell extracts. Washed cells were suspended at a cell density of 1 x 10^9 to 3 x 10^9 cells per ml in 0.1 M sodium citrate buffer (pH 6.2), hereafter called citrate buffer, that contained 0.3 mg per ml of the protease inhibitor phenylmethylsulfonylflouride (PMSF) that had been predissolved in 90% ethanol (96) and the protease inhibitor aprotinin (Sigma Chemical Co.) at a concentration of 200 KU per ml (49). Cells were broken by blending in a vortex with glass beads (0.45 mm diameter; B. Braun Melsugen AG, Germany) by a method similar to that of Kraig and Haber (69). The cells were vortexed for 15 seconds then cooled on ice for 15 seconds until they had been vortexed a total of 3-5 minutes. Breakage was always greater than 90%. The broken cell suspension was immediately centrifuged at 12,000 x g for 20 minutes and then at 45,000 rpm for 2 hours in a Beckman Type 65 rotor. The supernatant was dialyzed for 16-24 hours at 4°C against two changes of citrate buffer. The extract was generally assayed immediately after dialysis for SAG.

SAG and protein assays. Qualitative and quantitative assays for SAG were done using a coupled assay system which has been described previously (13, Appendix). For qualitative analyses, a whole cell assay

was employed. In these assays, 0.2 ml of citrate buffer containing 0.33 mM p-chloromercuribenzoate (PCMB) was added to cells permeabilized by air drying on Whatman 3MM filter paper (88). Duplicate filters were made in each experiment. To one filter, 0.4 ml of a 1% solution of glycogen in citrate buffer was added. To the second filter, 0.4 ml of citrate buffer was added. The final volume of the reaction mixture for both filters was 0.6 ml. The filters were incubated at 30°C or 34°C for 18-24 hours and then 1.2 ml of glucose oxidase reagent was added. After 30-60 minutes of incubation at 30°C, 0.8 ml of concentrated HCl was added and the reaction mix vortexed immediately. The duplicate filters were compared, and SAG activity was indicated by formation of greater color by the cells incubated with glycogen.

For quantitative determinations, 0.05-0.1 ml of a cell extract was incubated at 22, 30, or 34°C in an assay mixture containing citrate buffer, 0.66% glycogen and 0.33 mM PCMB. At various times, 0.6 ml samples were removed and boiled for 10 minutes. The samples were centrifuged and the amount of glucose present in the supernatant was determined using glucose oxidase reagent (13). D-glucose was used as a standard. Specific activity was expressed in milliunits per milligram of protein, where one unit is defined as 1 μmole of glucose released per minute. Protein was determined by the method of Lowry, etal (73).

Isolation of spores for mutagenesis. A 300 ml culture was sporulated and the sporulating cells harvested by centrifugation. The cells were resuspended in 10 ml of 24 mM phosphate buffer (pH 7.0) containing 20 μl of 2-mercaptoethanol and 15 mg Zymolyase 60,000 (Kirin Brewery, Japan). The digestion mixture was incubated at 30°C with gentle shaking for one hour. The spores were harvested by

centrifugation and washed three times with sterile distilled water. To disrupt aggregates of spores, the spore suspension was sonicated for three 30 second bursts, then washed two times in a sterile 1% solution of Tween 80 in distilled water. The spores were finally washed two times in sterile distilled water and plated onto YEPD at a concentration of 1000-2000 cells per plate.

Ultraviolet irradiation and filter paper screen for SAG. The homothallic strain SCMS7-1 was sporulated and the spores harvested, isolated and spread onto YEPD plates. The spores were mutagenized by ultraviolet (UV) irradiation for 60 seconds. This length of irradiation had been shown earlier to kill about 90% of the spores. The irradiated plates were placed immediately in the dark and incubated at 34°C. All subsequent incubations were also done at 34°C.

The surviving ascospore colonies that were to be screened for SAG activity were patched to YEPD, incubated overnight, then replicated twice to 3MM Whatman filter paper that had been premoistened with PSP and then placed onto plates of PSP containing histidine, leucine and adenine. After two days of incubation, the ascospore colonies growing on the filters were shifted to SPM by simply transferring the filter to leucine supplemented SPM plates. The colonies were incubated on SPM for three days, then the filters were removed and air dried for 4-5 hours at room temperature. The individual colonies were separated and assayed using the whole cell assay for SAG.

Tests for mating ability and pheromone production. Strains were tested for their ability to mate by cross-replication with the haploid tester strains M12a and M14α on MIN plates. The tester strains harbored nutritional defects that complemented defects in the strains to be

tested. A positive mating response was indicated by growth in the area of overlap between the strain being tested and the tester strain.

The ability to produce mating pheromones was determined using the procedure of Sprague and Herskowitz (125). Patches of cells to be tested were replicated onto BBMB plates spread with either XMB4-12b or RC757. Production of pheromones was evidenced by a halo caused by inhibition of growth of the lawn of the tester strain.

Nuclear staining. Progress through the two nuclear divisions of meiosis was monitored by the use of the fluorescent stain 4,6-diamidino-2-phenylindole (DAPI) (142). Fluorescence of the cells was observed with a Zeiss epifluorescence phase-constrast microscope and the different cell types were counted. At least 300 cells were counted from each sample.

Measurement of DNA synthesis. At various times, one ml samples of cells incubated in SPM were collected in triplicate then frozen and stored until assayed. DNA content was measured using the fluorescent compound 3,5-diaminobenzoic acid (DABA) (58). Calf thymus DNA was used as a standard.

Isolation of spores for genetic analyses. Cells harvested from five ml of a sporulating culture were washed once in sterile distilled water and resuspended in five ml sterile distilled water containing 20 µl of 2-mercaptoethanol. The cells were incubated for 30 minutes at room temperature, then washed three times in sterile distilled water to which was added 0.25 ml of glusulase which digests cell and ascus walls. The digestion mix was swirled gently at room temperature until the tetrads could be dissected easily by micromanipulation. This generally took 20-30 minutes.

Procedure for fusion of spheroplasts. A modification of the procedure used by Kakar and Magee (63) was used. A 10 ml culture of cells was grown overnight in YEPD at 30°C then diluted with 15 ml fresh YEPD and incubated for 1.5-3 hours to allow the cells to reenter log phase growth. Generally, about 1 x 10^8 to 3 x 10^8 cells were harvested which provided enough spheroplasts for a single fusion experiment. The harvested cells were washed once in distilled water, once in sorbitol-phosphate [1 M sorbitol, 0.1 M potassium phosphate (pH 7.5)] and then resuspended in sorbitol-phosphate containing 5 mg Zymolyase 5,000 (Kirin Brewery, Japan) and 12.5 µl 2-mercaptoethanol. The cells were incubated at room temperature in the spheroplasting mix until greater than 90% of the cells were converted to spheroplasts. This was determined by checking the degree of lysis in either 5% sodium dodecyl sulfate or distilled water. The spheroplasts were washed five times by centrifugation for 10 minutes at half-speed in a desk-top centrifuge (approximately 700 x g). All subsequent centrifugations were done in this manner. Spheroplasts to be fused were mixed in a 1:1 ratio. Generally, between 1 x 10^8 and 5 x 10^8 spheroplasts of each strain were used. The mixed spheroplasts were incubated for 20 minutes at room temperature then centrifuged and resuspended in five ml of fusion mix [30% (wt/vol) polyethylene glycol 4,000, 10 mM $CaCl_2$, and 10 mM Tris (pH 7.5)]. The spheroplasts were incubated in the fusion mix for one hour at room temperature and then stored overnight at 4°C. Prior to being plated, the spheroplasts were centrifuged, resuspended and incubated for 20 minutes at room temperature in five ml SOS, a recovery broth (114). They were then centrifuged, resuspended in one ml of sorbitol-phosphate and either 0.01 or 0.1 ml of spheroplasts was mixed with five ml molten

MIN-sorbitol agar medium held at 50°C, then poured immediately onto MIN-sorbitol plates. The plates were incubated at 30°C for 4-10 days. Prototrophic colonies were streaked onto MIN plates and in some cases transferred to YEPD plates where they were stored at 4°C until needed.

Segregation analysis of mutants. Strains heterozygous for the SAG defect were constructed by either spore-to-spore matings, spore-to-cell matings, or spheroplast fusions. The heterozygotes were sporulated and the tetrads dissected by micromanipulation. Spore viabilities were determined and for most experiments only complete tetrads were analyzed further. Nutritional requirements were determined by replicating from YEPD master plates to various MIN-nutrient drop plates. The SAG phenotype and the ability to sporulate were determined in most experiments by incubating 10 ml cultures at either 30 or 34°C in SPM after pregrowth in YEPacetate. Cultures were examined microscopically to ascertain the percent sporulation and SAG was assayed using the whole cell assay by spotting 50 µl of cell suspension on 3MM Whatman filter paper circles (2.3 cm) and air drying for 2-3 hours. In some experiments, only the percent sporulation was determined after cells were sporulated on solid SPM.

Complementation tests. Strains heterozygous for two different sporulation defects were constructed by either spore-to-spore matings or spheroplast fusions. These strains were sporulated in 10 ml cultures at 25, 30 or 34°C. The ability to sporulate and the percent sporulation was determined. SAG was assayed using the whole cell assay after spotting cells onto 3MM Whatman filter paper circles and air drying as described above.

ISOLATION AND CHARACTERIZATION OF STRAINS DEFICIENT IN SAG ACTIVITY

Mutagenesis. Both recessive and dominant mutations in genes that function only in diploids, such as sporulation-specific genes, can be isolated by mutagenizing the haploid ascospores of a homothallic strain (38). Through the action of the homothallism allele, HO, and usually within two to three generations, the mating type of some cells, but not others, is switched. Cells of opposite mating type then mate and give rise to diploid cells that are homozygous for all loci except the mating-type locus. Thus, any mutation generated in the original haploid ascospore will be present in a homozygous condition in the resulting diploid colony.

In this study, the homothallic strain SCMS7-1 was used. A culture of SCMS7-1 was sporulated, ascospores isolated and spread onto YEPD plates at a density of 1000-2000 spores per plate. The spores were mutagenized by UV irradiation. Three rounds of mutagenesis were performed. For two of the rounds, ascospores from the same culture were used. In one round the spores were used immediately after harvest and in the second round after storage for several days at 4°C. For all three rounds of mutagenesis, the number of viable spores per ml was determined. A total of 2,528 colonies arose from spores that survived UV irradiation (Table 2) and, in agreement with earlier experiments, 93% of the spores plated were killed.

Table 2. Results of mutagenesis and filter paper screen for SAG-deficient strains.

No. of colonies	Description
2,528	Survived UV irradiation
2,168	Respiratory competent and able to grow on MIN plus histidine, leucine and adenine
2,023	Screened
428	failed to grow on PSP
1,299	exhibited SAG activity
296	did not exhibit SAG activity

The surviving ascospore colonies were prescreened in two ways. First, the ability of the survivors to grow on MIN medium that contained histidine, leucine, and adenine was tested, to eliminate nutritional defects other than those present in the parent strain. Colonies that failed to grow were not considered further. Second, the colonies were tested for their ablility to grow on YPGlycerol, a medium that is commonly used to determine respiration competence (114), since cells must be able to respire in order to sporulate. Those colonies that failed to grow were not considered further. A total of 2,168 colonies respired and grew on MIN medium containing histidine, leucine, and adenine (Table 2).

Filter paper screen for SAG activity. The protocol used to screen for SAG activity is shown in Figure 1. Generally, about 40 colonies were patched on a single plate and about 200 colonies were tested in each round of screening. Included in each round were both positive and negative controls. Duplicates of each colony were sporulated on 3MM Whatman filter paper then air dried and assayed for SAG. For each ascospore colony tested, one of the duplicates was assayed in a reaction mixture containing glycogen, the substrate for SAG, and the other was assayed in a reaction mixture lacking glycogen. A visual comparison was made between the two reactions. If the duplicate assayed with glycogen produced more color than the duplicate assayed without glycogen, the colony was scored as positive for SAG. If the duplicates had an equal amount of color, the colony was scored as negative for SAG.

As shown in Table 2, 428 colonies failed to grow on PSP even though they were able to respire. Each PSP-negative colony was tested at least twice. The reason for their failure to grow was not determined. Of the

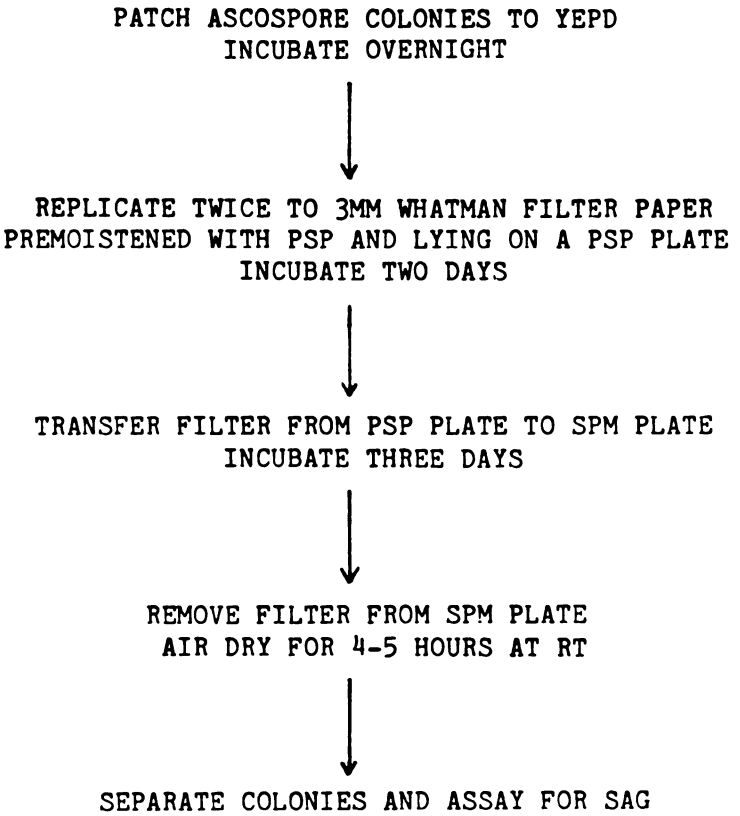

Figure 1. Filter paper screen for SAG-deficient strains. All incubations were done at 34°C. RT is room temperature.

remaining PSP-positive colonies, 296 were found to lack SAG and were considered to be potential SAG mutants.

Retest for SAG activity in liquid cultures. Small, 5-10 ml, liquid cultures were used to retest 141 potential mutants for SAG activity. After 48-72 hours of incubation in SPM at 34°C, samples of the cultures were spotted onto Whatman filter paper, air-dried and assayed for SAG activity using the whole cell assay. Twenty-one colonies were deficient for SAG and were characterized further.

Mutations that can confer a SAG-deficient phenotype. The screening procedure used in this study asked only whether or not the cells produced SAG. Mutations in the SAG structural gene would be deficient for SAG; however a number of other mutations could also cause a SAG-deficient phenotype (Table 3). Some of these other mutations were of interest: for instance, mutations in regulatory sequences for SAG, and mutations in genes that function prior to the critical event or events in pachytene that must be completed for SAG to appear. Unfortunately, SAG would not be produced by cells with defects in genes that function in the mating process, mating-type determination, and mating-type switching since such cells remain haploid and therefore are unable to sporulate. As seen in Table 3, most of these other mutations would produce mating pheromones and in some cases be able to mate. However, defects in MATα1 and defects in pheromone production would have the same phenotype as the sporulation mutations with respect to mating and pheromone production. The major difference between these two defects in mating functions and the sporulation defects is that MATα1 and pheromone production mutants are haploid. Thus to distinguish sporulation mutants from the other mutations shown in Table 3, the

Table 3. Mutations that can confer a SAG-minus phenotype.

Defect in	Sporulates	Mates	Produces Pheromones	Ploidy
SAG structural gene	?	-	-	2N
SAG regulatory loci	?	-	-	2N
Early sporulation functions	-	-	-	2N
HO	-	+	+	1N
MATα1	-	-	-	1N
MATα2	-	-	+	1N
MATa1	-	+	+	2N
Pheromone production	-	-	-	1N
Pheromone sensitivity	-	-	+	1N
Other mating functions	-	-	+	1N

colonies must be tested for their ability to mate and produce pheromones. The ploidy of any nonmating colonies that were deficient in SAG and pheromone production could then be determined either biochemically or genetically.

Of the 21 SAG-minus colonies identified after retesting for SAG activity in liquid cultures, two, SL484 and SL641, were nonmaters and did not produce either mating pheromone. These strains were characterized further. A third colony, SL382, also failed to mate or produce pheromones; however, because of problems later encountered with this strain it was not characterized to the same extent as SL484 and SL641. The ploidy of these strains will be considered later. A fourth SAG-deficient isolate, SL572, will be described later (see Preliminary Experiments with Mutant Strain SL572).

Specific activity of SAG in mutant strains. SL484, SL382, SL641 and their parent were incubated at 22, 30, or 34°C in SPM. At the indicated times, samples of cells were harvested and used to determine the specific activity of SAG. The data for a number of experiments are summarized in Table 4 and the mean specific activity has been used for comparison. Most of the extracts were assayed at all three temperatures. No significant difference was found between these different assay temperatures and only the results obtained when the extracts were assayed at 30C are presented.

When incubated in SPM at 34 and 22°C, SL484 had, at the most, one-fifth the specific activity of the parent strain, SCMS7-1. However, at 30°C, and after 72 hours in SPM, SL484 exhibited wild type levels of SAG. The only difference between SL484 and SCMS7-1 at 30°C was that SCMS7-1 attained full activity by 24 hours and changed little

Table 4. Specific activity of SAG in mutant strains and their parent.[a]

Strain	Specific activity at following time in sporulation medium (h):												
	0				24			48			72		
	$\bar{X}$[b]	SD[c]	n[d]		$\bar{X}$	SD	n	$\bar{X}$	SD	n	$\bar{X}$	SD	n
SL484	0.170	0.158	5	34°C	1.13	1.32	5	2.52	2.21	5	8.34	8.35	5
				30°C	3.22	2.46	3	19.9	4.46	3	35.1	12.0	3
				22°C	0.345	0.443	2	0.560	0.481	2	0.817	0.229	2
SL641	0.138	0.119	3	34°C	1.06	0.474	3	1.10	1.03	3	1.99	0.278	3
				30°C	0.157	--	1	1.04	--	1	1.81	--	1
				22°C	0.531	--	1	0.267	--	1	0.312	--	1
SL382	0.403	--	1	34°C	3.02	2.34	2	5.18	--	1	6.18	--	1
				30°C	1.75	--	1	7.65	--	1	10.6	--	1
SCMS7-1	0.415	0.583	4	34°C	48.6	23.7	4	47.8	22.2	4	46.2	25.2	4
				30°C	28.0	18.8	4	28.6	5.94	2	27.4	7.07	2
				22°C	54.8	--	1	88.2	--	1	111	--	1

a. Cells were incubated in SPM at the temperature indicated after pregrowth in PSP.
b. Unless otherwise noted, $\bar{X}$ is the mean specific activity of two or more experiments.
c. SD is standard deviation.
d. n is number of determinations.

thereafter. SL484, on the otherhand, did not reach maximal activity until after approximately 48 hours of incubation. Thus SL484 is temperature sensitive for SAG production, with 30°C being the permissive temperature.

SL641 exhibited approximately the same activity at all temperatures and at all times. Its specific activity varied from 1/15 to 1/200 of wild type activity. SL382, showed somewhat higher levels of SAG, varying from 1/3 to 1/30 of the wild type activity. Neither SL641 nor SL382 was temperature sensitive for SAG production.

Sporulation and meiotic behavior of the mutants. The ability of the mutant strains to sporulate is shown in Table 5. The results presented are the average of at least two determinations. As can be seen, neither SL382 nor SL641 sporulated at the three temperatures tested. SL484 sporulated very poorly at 34 and 22°C, but, paralleling the SAG activity, it sporulated at nearly wild-type levels at 30°C.

The progression of the mutants along the meiotic pathway was also examined. Cells were incubated in SPM at 34°C and sampled at the times indicated. The nuclei of the cells were stained with the fluorescent stain DAPI which binds DNA and renders the nucleus visible when the cells are observed by fluorescence microscopy. The results of one experiment are shown in Table 6. For all three mutants, most cells remained mononucleate. For SL382 and SL641, the small percentage of cells that did progress through both nuclear divisions should be regarded with caution. There are artifacts of the DAPI staining procedure that are difficult to distinguish from meiotic nuclei. Thus, these results are best interpreted as an indication that none of the mutants progressed through the first meiotic division.

Table 5. Ability of mutants and their parent to sporulate.[a]

Strain		Percent sporulation at following time in SPM (h): 24	48	72
SL484	34°C	0.3	1.3	2.3
	30°C	3.9	21.5	38.7
	22°C	0[b]	0.6	1.3
SL641	34°C	0	0	0
	30°C	0	0	0
	22°C	0	0	0
SL382	34°C	0	0[b]	0[b]
	30°C	0	0[b]	0[b]
SCMS7-1	34°C	35.1	47.1	46.8
	30°C	38.2	67.9	69.4
	22°C	44.4[b]	72.0[b]	73.6[b]

a. Cells were incubated in SPM at the indicated temperature after pregrowth in PSP. Unless otherwise noted, the values presented are the average of at least two determinations.
b. The results of a single experiment.

Table 6. Progress of the mutants through meiosis.[a]

Strain	Percent mono-, bi-, tetranucleate cells, and asci at following incubation times in SPM (h):											
	24				48				72			
	Mono	Bi	Tetra	Asci	Mono	Bi	Tetra	Asci	Mono	Bi	Tetra	Asci
SL484	99.0	1.0	0	0	99.3	0.7	0	0	100	0	0	0
SL641	99.0	0.3	0.7	0	97.3	2.0	0.7	0	89.7	7.9	2.3	0
SL382	94.0	2.6	3.3	0	--ND[b]--				--ND--			
SCMS7-1	21.0	20.3	25.2	33.4	33.9	4.6	4.3	57.2	20.6	6.0	12.3	61.1

a. Cells were incubated in SPM at 34°C after pregrowth in PSP.
b. ND is not determined.

Premeiotic DNA synthesis. The occurence of premeiotic DNA synthesis in SL484 and SL641 was determined. Cells incubated in SPM at 34°C were sampled after various times of incubation, and the DNA content of the cells measured using the fluorescent compound DABA. The results are shown in Figure 2. The parent strain began synthesizing DNA almost immediately, and completed the synthesis within six hours of incubation. During that time, the DNA content was almost doubled. The failure to completely double the DNA content reflects the fraction of cells that failed to enter the meiotic pathway. In contrast, neither SL484 or SL641 synthesized DNA. The DNA content of the strains at zero time was 0.301, 0.446, and 0.286 μg of DNA per 1 x 10^7 cells for SL484, SL641, and SCMS7-1, respectively. These values are within 72-112% of the values reported for diploid S. cerevisiae strains, using the same and other methods of measurement (139, 72, 75).

Ploidy of the mutants. Based on the DNA determinations discussed above, SL484 and SL641 are diploids. The viabilities of spores produced by the fusion products of the mutants and the diploid strain W66-8A (see Genetic Analysis of Mutant Strain SL641) also support this conclusion. If the mutants were haploid, fusion with W66-8A would have created triploid strains. Triploid yeast strains sporulate but generally produce less than 15% viable spores (92). If the mutants were diploid, then the fusion products would be tetraploid. Tetraploids exhibit good spore viability. Both SL484 and SL641 derived fusants exhibited greater than 50% spore viability. Only 22% of the spores produced by the SL382 derived fusant were viable. Because of this low spore viability, and because of the later failure of this fusant to sporulate, SL382 was not characterized further.

Figure 2. Premeiotic DNA synthesis in mutant strains and their parent. Cells were pregrown in PSP then incubated at 34°C in SPM. At the indicated times one ml samples of cells were removed and frozen until assayed for DNA content. Premeiotic DNA synthesis in SCMS7-1 (●—●), SL484 (○—○) and SL641 (■-■).

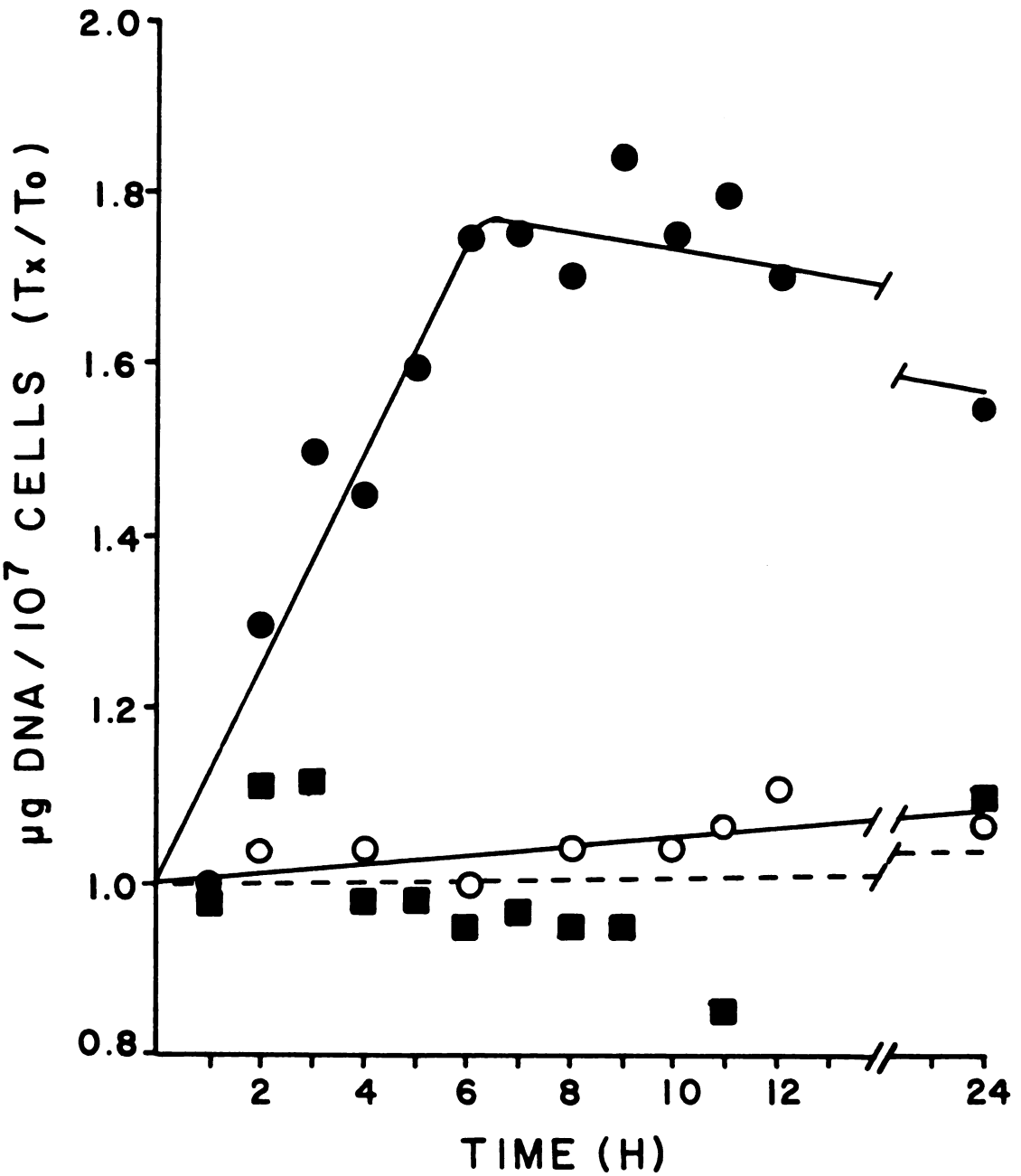

Figure 2.

GENETIC ANALYSIS OF MUTANT STRAIN SL484

Segregation of the SL484 defect. A conditional sporulation mutation can be studied genetically using standard genetic procedures. Strains heterozygous for the sporulation defect are constructed by mating spores of the mutant strain with spores of a wild-type homothallic strain. The diploid progeny of such a mating are heterozygous at all loci except the HO gene and when sporulated will produce ascospores of various genotypes but all containing the HO allele. Because of the presence of the HO allele, each haploid spore will diploidize making it possible to test each ascospore colony for its ability to sporulate (Figure 3).

Strains heterozygous for the SL484 defect were constructed by spore-to-spore matings of SL484 and the wild type strain W66-8A. Two diploid progeny were examined. Forty-eight tetrads were dissected from one of the diploid progeny but only 23 complete tetrads survived. Only one complete tetrad of the five tetrads isolated from the second diploid survived. The ascospore colonies derived from these 24 tetrads were characterized nutritionally by replicating from YEPD master plates to various MIN-nutrient drop plates. The colonies were also examined for their ability to sporulate after incubation on SPM plates for three days at 34°C. SAG activity was not assayed. The patterns of segregation of all phenotypes are shown in Table 7. Five tetrads which showed aberrant segregation for two or more genes were not included in the analysis since they were probably false tetrads. In diploids, a single gene

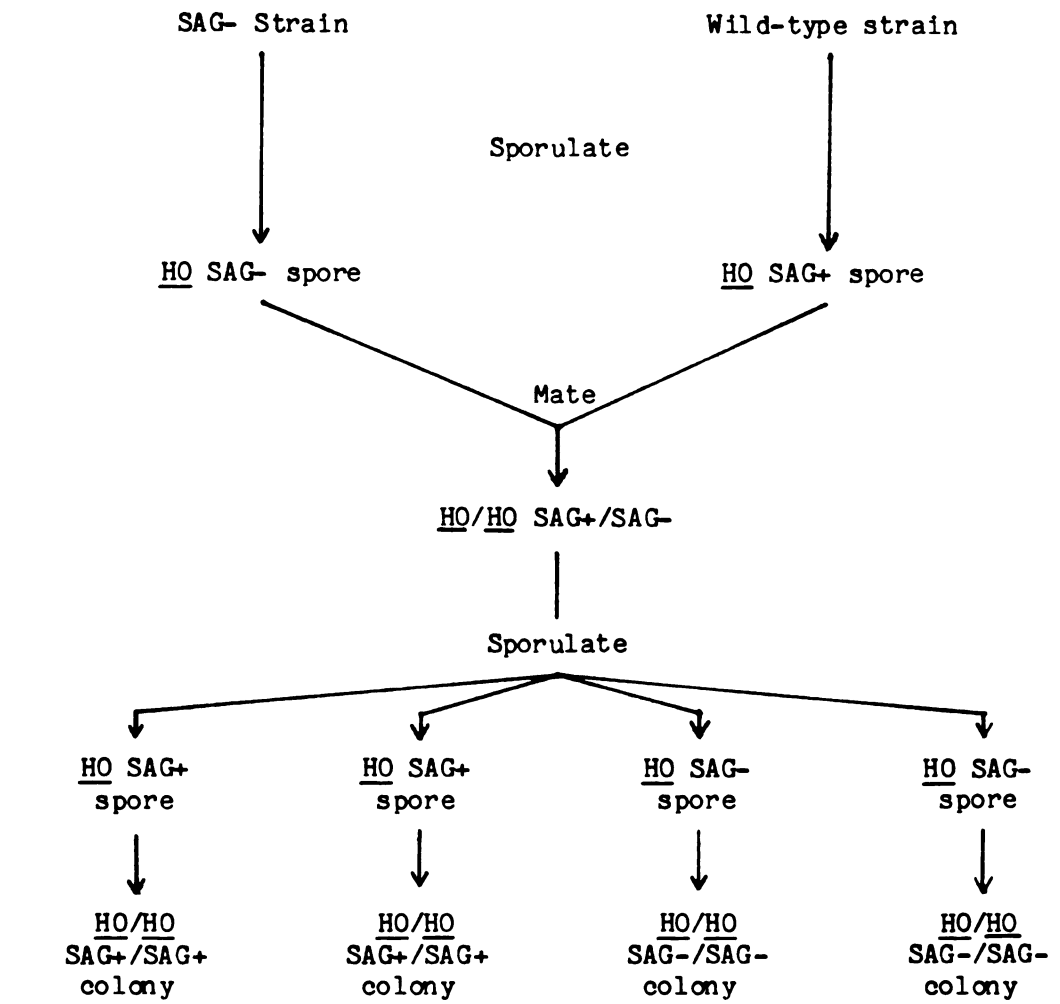

Figure 3. Segregation of the SAG defect in heterozygous strains.

Table 7. Segregation of phenotypes in ascospore colonies derived from SL484 x W66-8A diploids.

Phenotype	Number of tetrads segregating:				
	4+ : 0-	3+ : 1-	2+ : 2-	1+ : 3-	0+ : 4-
Ability to sporulate [a]	0	1	17	1	0
Ability to grow on media lacking: [b]					
histidine	0	2	17	0	0
leucine	0	0	9	4	6
adenine	0	0	1	13	5
uracil	0	0	19	0	0
methionine	0	0	19	0	0
lysine	1	0	18	0	0
tryptophan	0	0	19	0	0

a. Ascospore colonies were patched to YEPD plates, incubated overnight at 30°C then replicated to PSP plates and incubated for about 24 hours at 30°C. The colonies were then transferred using toothpicks to SPM plates and incubated three days at 34°C. Each colony was examined microscopically to determine if it contained asci.

b. Ascospore colonies were patched to YEPD plates, incubated overnight at 30°C then replicated to minimal plates lacking the indicated nutrient.

segregrates in a 2+ : 2- fashion. With the exception of the requirements for leucine and adenine, where two genes are segregating for each phenotype, all other phenotypes, including the ability to sporulate segregated 2+ : 2-. These results indicate that the SAG deficiency in SL484 is the result of a defect in a single gene. The aberrant tetrads included in the analysis were probably the result of gene conversion. The frequency of gene conversions for each phenotype is within the range reported for a number of different loci in S. cerevisiae (44).

Preliminary linkage analysis. A formal mapping procedure was not carried out with SL484. However, the presence of several markers in the SL484 x W66-8A diploids made it feasible to determine if the SL484 mutation was linked to any of these other markers. An analysis was only done with the single gene defects, since the genotype of the adenine and leucine requiring colonies was not known. The numbers of parental ditype, nonparental ditype and tetratype asci are shown in Table 8. Only tetrads showing normal 2+ : 2- segregation for each gene were considered. For the HIS4, URA3, MET4 and LYS2 genes, the ratio of parental ditypes to nonparental ditypes was approximately 1:1 indicating that these genes are not linked to the SL484 defect. However, with the TRP5 gene, most of the tetrads were parental ditypes suggesting that the two genes are linked and 8.8 map units apart on chromosome VII.

Complementation tests. Three strains, C52-4B, 74-1A and 89-1D, which are defective in premeiotic DNA synthesis (spo7), the first meiotic division (spo1), and spore formation (spo3), respectively, were available in this laboratory for complementation tests. Trans-heterozygotes of spo7 and SL484, and spo1 and SL484 were

Table 8. Linkage analysis of SL484 defect.

	PD[a]	NPD[b]	TT[c]	Map distance[d]
HIS4 - SAG	4	3	8	unlinked
URA3 - SAG	7	2	8	unlinked
MET4 - SAG	0	4	13	unlinked
LYS2 - SAG	3	1	12	unlinked
TRP5 - SAG	14	0	3	8.8 m.u.

a. PD is parental ditype ascus.
b. NPD is nonparental ditype ascus.
c. TT is tetratype ascus.
d. Map distance is expressed as the recombination frequency times 100.

constructed by spore-to-spore matings. A tetraploid heterozygous for both the spo1 and the SL484 defects was constructed by fusing spheroplasts of the two strains (see Genetic Analysis of Mutant Strain SL641). Ten ml cultures of the heterozygotes were incubated at 34 and 30°C in SPM and examined for SAG activity using the whole cell assay for SAG. In earlier experiments, it had been shown that only spo7 mutants failed to exhibit SAG activity at 34°C. Thus even though spo1 and spo3 mutants failed to sporulate, they progressed far enough along the sporulation pathway to produce SAG. Thus, in addition to SAG, the trans-heterozygotes needed to be checked for their ability to sporulate. The results are shown in Table 9. At 34°C, the nonpermissive temperature, all three heterozygotes sporulated. Thus the SL484 defect complemented the sporulation defect of all three SPO mutants. In addition, the SL484/spo7 heterozygote exhibited SAG activity at the nonpermissive temperature. These results indicate that the SL484 mutation is probably not in the SPO1, SPO3 or SPO7 genes.

In addition to the complementation tests described above, a complementation test between SL484 and SL641 was attempted. It will be described in the next chapter.

Table 9. Complementation tests: SL484 vs spo1, spo3, and spo7 strains.[a]

Cross	Sporulation defect	% Sporulation	SAG Activity
SL484 x spo7	DNA synthesis	35.1	+
SL484 x spo1[b]	Meiosis I	17.0	+[c]
SL484 x spo3	Spore formation	50.5	+

a. Unless otherwise noted, a culture of cells growing in PSP was shifted into SPM and half of the culture was incubated at 34°C and the other half was incubated at the permissive temperature. After 48 hours of incubation the cultures were examined for sporulation and SAG was assayed using the whole cell assay described in Materials and Methods. All cultures sporulated and produced SAG at the permissive temperature.

b. Cells were pregrown in YEPacetate then shifted to SPM and incubated as above.

c. Results after 24 hours of incubation.

GENETIC ANALYSIS OF MUTANT STRAIN SL641

The use of spheroplast fusion as a genetic tool. Since SL641 was unable to sporulate, traditional genetic analysis was not possible. In recent years a number of parasexual techniques have been developed to study yeasts and other organisms that do not have a sexual cycle. One of these techniques is spheroplast fusion. It seemed reasonable that this technique might be used with SL641 to construct strains of appropriate genotypes which could then be studied genetically. Spheroplast fusions were done for two reasons: first, to create tetraploid strains heterozygous for the SAG defect so that the segregation of the mutant allele could be studied and secondly, to create trans-heterozygotes for complementation tests. In all of the fusion experiments, strains with different nutritional requirements were fused and the prototrophic fusants selected by plating onto MIN-sorbitol medium. The procedure used to fuse spheroplasts was similar to that used by Kakar and Magee (63) in this laboratory with Candida albicans. They reported very efficient regeneration (>90%) in all their experiments. However, with the Saccharomyces strains used in this study, regeneration was much worse, generally 0.1% or less. Regeneration frequencies of 50-70% have been reported for S. cerevisiae spheroplasts (93, 127). The reason for the poor regeneration observed in these experiments was not determined. Despite the low frequency of regeneration, the desired fusants, with one exception, were isolated.

The stability of the fusants varied and will be discussed after the results of the genetic analyses are reported.

Segregation of nutritional markers. A tetraploid yeast strain was constructed by fusing spheroplasts of SL641 and W66-8A, and the segregation of alleles at several loci was examined. For comparison, fusants of SL484 and SCMS7-1 with W66-8A were also constructed and analyzed.

If a tetraploid of the genotype +/+/-/- for a single locus is sporulated, three types of tetrads are possible, 4+ : 0- (Type I), 2+ : 2- (Type II) and 3+ : 1- (Type III) (102) (Figure 4). The frequencies of the three ascus types are dependent on the distance between the gene and its centromere. Because of the complexity of the segregation of alleles in tetraploids, only the nutritional phenotypes conferred by a single gene were examined. The genes studied were, MET4 located on chromosome XIV, TRP5 located on chromosome VII, LYS2 located on chromosome II, HIS4 located on chromosome III, and URA3 located on chromosome V. All five genes have been mapped to their centromeres using diploid strains. None have been mapped in tetraploids. Although map distances derived from tetraploids are generally in good agreement with those derived from diploids, it was not certain that the tetraploid fusants would behave the same as tetraploid conjugants. Therefore, the maximum likelihood method of parameter estimation (67) was used to estimate the gene-centromere distances for these five genes. This procedure estimates the gene-centromere distance using the numbers of Type I, II, and III asci observed in the following way: The maximum likelihood equation,

$$L = \prod_{\substack{\text{all} \\ i}} (P_i)^{N_i}$$

Figure 4. Segregation of alleles in a heterozygous tetraploid yeast strain.

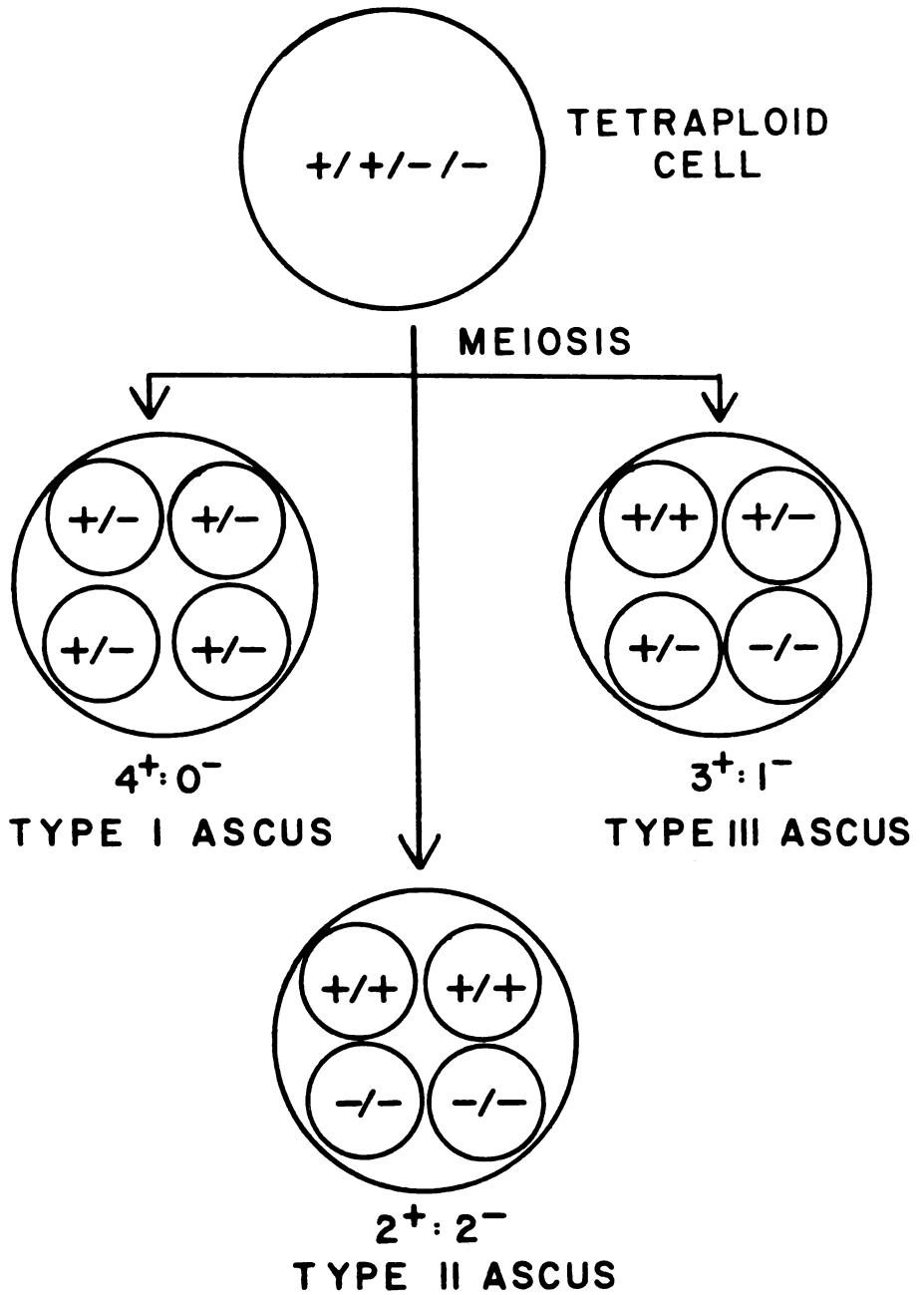
+/+/-/-
TETRAPLOID CELL
MEIOSIS
+/-
+/-
+/-
+/-
4+:0-
TYPE I ASCUS
+/+
+/-
+/-
-/-
3+:1-
TYPE III ASCUS
+/+
+/+
-/-
-/-
2+:2-
TYPE II ASCUS

Figure 4.

states that with respect to these data, the likelihood of observing Type I, II, and III asci is equal to the product of the probabilities of occurence of each tetrad-type raised to the power of the observed number of each tetrad type. The expected frequencies of Type I, II, and III asci are given by the following equations (102):

$$P(I) = 1/6\ (4 - 4x + 3x^2)$$

$$P(II) = 1/6\ (2 - 4x + 3x^2)$$

$$P(III) = 1/3\ (4x - 3x^2),$$

where x is the gene-centromere distance expressed as the frequency of second division segregation (SDS) asci. Therefore, the maximum likelihood function becomes:

$$L = P(I)^{NI} \cdot P(II)^{NII} \cdot P(III)^{NIII},$$

where N_I , N_{II}, and N_{III} are the numbers of Type I, II and III tetrads observed, respectively. Maximizing the value of L gives the minimun variance unbiased estimator of the distance. The estimates were substituted into the equations for P(I), P(II), and P(III), and used to calculate the expected numbers of Type I, II, and III asci. These numbers were compared to the observed numbers by a contingency chi square (Test 1). The degrees of freedom (df) is given by: df = number of observations - number of sums used in calculating the expectations - 1. When data for multiple loci were compared, the df are reduced by the number of loci. L was maximized using an interactive, time-sharing program for maximum likelihood and minimum chi-square estimation written by L. G. Robbins (101).

A significant difference between the observed and expected numbers of Type I, II, and III asci indicated aberrant tetraploid segregation. If the difference was not significant, a second statistical test was

done (Test 2). For Test 2, the estimated gene-centromere distance was compared to the distance derived from the analysis of diploids. To do this, the expected numbers of Type I, II and III asci were compared to the numbers predicted by the known gene-centromere distance. A significant difference between the two map distances was also interpreted as an indication of aberrant segregation in the tetraploid.

The numbers of Type I, II and III asci for the five loci examined are shown in Table 10, and the results of the two chi-square tests are shown in Table 11. It is apparent that for all three fusants, certain genes did not segregate as predicted for a tetraploid strain. In both SL641 and SL484 fusants, alleles for the MET4 and TRP5 loci segregated aberrantly, whereas in the SQMS7-1 fusant, only the alleles of the LYS2 gene exhibited aberrant segregation.

A close examination of the results led to the following hypotheses regarding the cause of nontetraploid segregation. With the SL641 fusant, all the asci were Type I for MET4. This suggests that the only MET4 allele present in the fusant was the wild-type allele and that the fusant was disomic rather than tetrasomic for chromosome XIV. In this case, both of the W66-8A chromosomes were lost. Only Type II asci were seen for the TRP5 locus. Again this can be explained by aneuploidy, in this case for chromosome VII. It is likely that a single chromosome VII from each parent was lost so that the fusant was heterozygous and disomic for chromosome VII.

The aberrant segregation of the MET4 and TRP5 alleles in the SL484 fusant can not be explained by the hypothesis of disomy for chromosomes XIV and VII since the observance of Type III asci precludes this explanation. However, if the fusant was trisomic for these chromosomes,

Table 10. Segregation of phenotypes in tetraploid fusants.

W66-8A fused with:	Ability to grow on medium lacking: [a]	Number of asci segregating as:		
		Type I	Type II	Type III
SL641	methionine	13	0	0
	tryptophan	0	11	0
	lysine	8	0	5
	histidine	9	0	4
	uracil	10	2	1
SL484	methionine	19	0	4
	tryptophan	22	0	1
	lysine	13	0	10
	histidine	10	5	8
	uracil	12	4	7
SCMS7-1	methionine	2	1	3
	tryptophan	5	0	1
	lysine	6	0	0
	histidine	5	0	1
	uracil	3	0	3

a. Ascospore colonies were patched to YEPD plates, incubated overnight at 30°C then replicated to minimal plates lacking the indicated nutrient.

Table 11. Analysis of patterns of segregation in tetraploid fusants.

W66-8A fused with:	Locus				
	MET4	TRP5	LYS2	HIS4	URA3
SL641	f(SDS)[a]	f(SDS)	f(SDS)	f(SDS)	f(SDS)
	estimated 0.00001	estimated 0.00001	estimated 0.67	estimated 0.48	estimated 0.069
	known 0.67 [b]	known 0.35	known 0.67	known 0.36	known 0.16
	Chi-square values: [c]				
	Test 1: 6.5 SD	Test 1: 22 SD	Test 1: 2.4 NSD	Test 1: 3.5 NSD	Test 1: 1.3 NSD
	Test 2: ND	Test 2: ND	Test 2: ND	Test 2: 3.2 NSD	Test 2: 2.0 NSD
SL484	f(SDS)	f(SDS)	f(SDS)	f(SDS)	f(SDS)
	estimated 0.21	estimated 0.045	estimated 0.67	estimated 0.32	estimated 0.30
	known 0.67	known 0.35	known 0.67	known 0.36	known 0.16
	Chi-square values:				
	Test 1: 8.5 SD	Test 1: 11 SD	Test 1: 3.3 NSD	Test 1: 0.548 NSD	Test 1: 0.008 NSD
	Test 2: ND	Test 2: ND	Test 2: ND	Test 2: 0.695 NSD	Test2: 2.2 NSD
SCMS7-1	f(SDS)	f(SDS)	f(SDS)	f(SDS)	f(SDS)
	estimated 0.67	estimated 0.19	estimated 0	estimated 0.19	estimated 0.67
	known 0.67	known 0.35	known 0.67	known 0.36	known 0.16
	Chi-square values:				
	Test 1: 0.38 NSD	Test 1: 2.2 NSD	Test 1: 3.0 NSD	Test 1: 2.2 NSD	Test 1: 0.75 NSD
	Test 2: ND	Test 2: 2.8 NSD	Test 2: 7.3 SD	Test 2: 3.1 NSD	Test 2: 4.8 NSD

a. f(SDS) is the frequency of second division segregation asci.
b. The known values of second division asci in diploids were obtained from Mortimer and Schild (87).
c. Each test had one degree of freedom. SD is significantly different ($p < 0.05$). NSD is not significantly different. When Test 1 indicated nontetraploid segregation, Test 2 was not done (ND) since the distances estimated in Test 1 were already shown to be incorrect.

with the genotype +/+/-, then Type III asci could be observed. In such a trisome, assuming that any two chromosomes are equally likely to pair during meiosis, Type I asci are produced when like chromosomes pair (p = 1/3), and when unlike chromosomes pair [a + chromosome with a - chromosome (p = 2/3)], the gene segregates in the first division (p = 1 - fSDS) and the third chromosome (a + chromosome) moves to the same pole as the - chromosome (p = 1/2). Type II asci are produced when the two unlike chromosomes pair (p = 2/3), the gene segregates in the first division (p = 1 - fSDS), and the third chromosome moves to the same pole as the other + chromosome (p = 1/2). Type III asci are formed when two unlike chromosomes pair and the gene segregates in the second division (p = fSDS). The frequencies of Type I, II, and III asci in a triploid are given by the following equations:

$$P(I) = 1/3\ (2 - fSDS)$$

$$P(II) = 1/3\ (1 - fSDS)$$

$$P(III) = 2/3\ fSDS$$

where fSDS is the frequency of second division segregations. fSDS was estimated by the maximum likelihood method and was 0.348 for the MET4 locus and 0.086 for the TRP5 locus. The fSDS estimates were substituted into the above equations and the expected numbers of Type I, II and III asci were calculated and compared to the observed numbers by a contingency chi-square. The chi-square values were 3.5716 (1 df, $p > 0.05$) and 4.426 (1 df, $p < 0.05$) for MET4 and TRP5 respectively. The estimated fSDS for the MET4 gene is greater than that for the TRP5 gene. This is consistent with their known gene-centromere distances so it was desirable to compare the estimated fSDS with the fSDS observed when heterozygous diploids are sporulated. The best method for

comparing these values is to first estimate the fSDS using a joint maximum likelihood procedure. In this procedure, fSDS is estimated using the numbers of Type I, II, and III asci observed when the fusant is sporulated and the numbers of first division (FDS) and SDS asci observed when diploids are analyzed. The estimate is then substituted into the formulae for Type I, II, and III asci and into the formulae for FDS and SDS asci and the expected numbers calculated. The expected numbers are compared to the observed numbers by a contingency chi square. Unfortunately, observed numbers of FDS and SDS asci are availble for the TRP5 locus only (87). The MET4 locus was mapped to Chromosome XIV by the observation of meiotic linkage of MET4 and PET8 (141). MET4 is not linked to its centromere. The theoretical frequency of SDS asci for a gene that is not linked to its centromere is 0.667. This value was used to calculate the numbers of Type I, II, and III asci, and these numbers were compared to the numbers calculated using the fSDS estimate (0.348). The difference between the expected numbers (calculated from the joint maximum likelihood estimate of fSDS) and the observed numbers of Type I, II, III, FDS, and SDS asci for the TRP5 gene was highly significant (chi square = 17.06, 3 df, $p < 0.01$). The difference between the numbers of Type I, II, and III asci calculated from the maximum likelihood estimate of fSDS and the theoretical value of fSDS was also significant (chi square = 5.182, 1 df, $p < 0.05$). These analyses indicate that MET4 was segregating in a trisomic fashion, however, the estimated fSDS was signigicantly less than expected for a gene that is unlinked to its centromere. The hypothesis of trisomy for the TRP5 chromosome was not suported by these analyses. Furthermore, the fSDS estimated by the joint maximum likelihood analysis

was significantly less than the fSDS observed when diploids are examined. The lower than expected fSDS values for both MET4 and TRP5 suggests that recombination events may have been repressed. In the case of the TRP5 gene repression of recombination may explain the results of the trisomy test which was only barely significant at the 0.05 level of probability.

In the SCMS7-1 fusant, the LYS2 gene segregated only 4+ : 0- (Type I ascus). This is consistant with an aneuploid condition in which the two mutation-bearing chromosomes were missing so that the strain was a homozygous disomic for the wild-type LYS2 allele.

Segregation of the SAG defect. The colonies which arose from the spores produced by the SL641 fusant were also tested for their ability to sporulate and produce SAG. Initially, an attempt was made to grow small 5-10 ml cultures in PSP prior to incubation in SPM. It was immediately apparent however, that many of the ascospore colonies only grew well in a rich medium such as YEPacetate. Therefore, all of the spore colonies were tested after pregrowth in YEPacetate followed by incubation in liquid SPM for 24-48 hours. The results are shown in Table 12. The frequencies of Type I, II and III asci produced by the SL641 fusant were consistent with the hypothesis that a single sporulation defect was segregating in a tetraploid strain of genotype +/+/-/- (see Linkage analysis of the SL641 defect). In the SCMS7-1 fusant, only Type I asci were observed since this strain did not contain any defective SAG alleles.

Linkage analysis of the SL641 defect. If two genes are unlinked, the segregation of alleles at each locus is independent. In diploid strains, this results in three tetrad types: parental ditype, nonparental ditype, and tetratype, with parental ditypes and nonparental

Table 12. Segregation of the ability to sporulate and to produce SAG.[a]

W66-8A fused with:	Number of asci segregating as:		
	Type I	Type II	Type III
SL641	6	3	3
SCMS7-1	6	0	0

a. Five to 10 ml cultures of ascospore colonies were sporulated in SPM at 34° C after pregrowth in YEPacetate. SAG was assayed using the whole cell assay and the ability to sporulate was determined by microscopic examination after 24 and 48 hours of incubation.

ditypes being produced in equal frequency. In a tetraploid, in which alleles for two genes, gene a and gene b, are segregating, each ascus can be Type I, II or III for each gene. Therefore, nine different combinations of the three ascus types are possible when both genes are considered (Table 13). If genes a and b are unlinked, the probability of, for instance, an ascus being Type I for gene a and Type I for gene b is equal to the probability of a Type I ascus for gene a multiplied by the probability of a Type I ascus for gene b. As stated earlier, the probability of a Type I ascus for gene a is dependent on the distance of gene a from its centromere and likewise the probability of a Type I ascus for gene b is dependent on the distance of gene b from its centromere. If genes a and gene b are linked, the probability of an ascus being Type I for both genes is not equal to the product of the individual probabilities since these two events are not independent. Thus, if two genes are linked the frequencies of the nine possible ascus types will differ from the frequencies predicted by independent segregation. Linkage of the SL641 defect to any of the other genes segregating in the SL641 fusant was determined by using the numbers of the nine ascus types to estimate the distance of each gene to its centromere by the maximum likelihood method. The estimates were used to calculate the expected numbers of the nine ascus types. The expected numbers were then compared to the observed numbers. If the expected numbers did not differ significantly from the observed numbers of ascus types, then two conclusions were reached. First that the two genes under consideration were segregating independently and so were unlinked, and second, that each gene was segregating in a tetraploid fashion.

Table 13. Linkage analysis of the SAG defect in the SL641 fusant.

Tetrad Type		Number of asci	
SAG gene	LYS2 gene		
I	I	2	
I	II	0	
I	III	4	
II	I	3	Chi-square= 6.7
II	II	0	df = 6[a]
II	III	0	NSD
III	I	2	
III	II	0	
III	III	1	
SAG gene	URA3 gene		
I	I	5	
I	II	0	
I	III	1	
II	I	3	Chi-square=6.9
II	II	0	df = 6
II	III	0	NSD
III	I	1	
III	II	2	
III	III	0	
SAG gene	HIS4 gene		
I	I	5	
I	II	0	
I	III	1	
II	I	1	Chi-square=5.2
II	II	0	df = 6
II	III	2	NSD
III	I	2	
III	II	0	
III	III	1	

a. df is degrees of freedom. df = number of observations - number of parameters estimated - 1.

Since the SL641 fusant was probably disomic for the MET4 and TRP5 chromosomes, the SAG defect was tested for linkage only to LYS2, HIS4 and URA3. As can be seen in Table 13, the observed numbers of tetrads did not differ significantly from the expected for any of the three genes. Therefore, by this test, the SAG defect in SL641 is not linked to LYS2, HIS4 or URA3, and is segregating as expected for a single gene in a tetraploid. The map distance between the SL641 defect and its centromere expressed as the frequency of SDS tetrads was estimated to be 0.214.

Complementation tests. Spheroplasts of SL641 and the three sporulation mutants spo7, spo1 and spo3 were fused to construct trans-heterozygotes for complementation analyses. In each experiment, the culture was shifted to SPM, half was incubated at 34°C and the other half was incubated at the permissive temperature as a positive control. The results of the 34°C incubations are shown in Table 14. All three fusants produced SAG and sporulated. Although the percent sporulation was low for the spo7 and spo1 fusants, none of the parents sporulate to this degree at 34°C. In all cases, SAG was produced, and the percent sporulation was of the same magnitude as that observed at the permissive temperature. Thus the SL641 mutation does not appear to be allelic to spo1, spo3 or spo7.

Evidence for the lack of allelism between SL641 and SL484. Ideally, a trans-heterozygote constructed from SL641 and SL484 should be tested for SAG production and the ability to sporulate. Complementation would indicate that the two mutations are not different alleles of the same gene. Unfortunately, such a trans-heterozygote was never successfully constructed. However, the results of the linkage analysis of the two

Table 14. Complementation tests: SL641 vs spo1, spo3 and spo7 strains.[a]

SL641 fused with:	Sporulation defect	Percent sporulation	SAG Activity
spo7	DNA synthesis	16.3	+
spo1	Meiosis I	4.2[b]	+[b]
spo3	Spore formation	55.1[c]	+[c]

a. Unless otherwise noted, a culture of cells growing in YEPacetate was shifted into SPM and half of the culture was incubated at 34°C and the other half was incubated at the permissive temperature. After 48 hours of incubation, the cultures were examined for sporulation and SAG was assayed using the whole cell assay described in Materials and Methods. All cultures sporulated and produced SAG at the permissive temperature.
b. Results after 24 hours of incubation.
c. Cells were pregrown in PSP then shifted into SPM and incubated as above.

mutations strongly suggests that they are not allelic. The SL484 defect was found to be linked to the TRP5 locus on chromosome VII. In the SL641 fusant, though aberrant segregation of the TRP5 chromosome was observed, indicating aneuploidy for that chromosome, the SAG defect segregated normally. This precludes the possibility that SL641 is located on chromosome VII. Since SL484 is located on Chromosome VII and SL641 is not, the two mutations must be located in different genes.

Stability of the fusants. In the course of these experiments, a number of unexpected difficulties arose which hampered and somewhat limited the genetical analyses of the various fusants constructed. These difficulties raised questions about the stability of the fusants and will be discussed below.

One problem encountered was poor vegetative growth. The fusants were isolated as prototrophs by plating the fusion mixes onto MIN-sorbitol medium. Yet, frequently, when the fusants were picked from these plates and streaked onto MIN they failed to grow or grew very slowly. Furthermore, many grew poorly on PSP and so the sporulation protocol was altered and the cultures were pregrown on the rich medium YEPacetate.

Another problem was that many of the fusants sporulated poorly. As indicated earlier, a third mutant, SL382, was not characterized well. This was because the SL382 x W66-8A fusant sporulated extremely poorly. Out of six different experiments, the highest degree of sporulation was the 8.3% observed in the first experiment. In four of the six experiments, the fusant failed to sporulate. With fusants constructed from SL641 and W66-8A poor sporulation was also a problem, though less severe. The SL641 fusant was sporulated a total of 10 times and

averaged 11.4% sporulation. The average of the final six experiments was 4.3% and in many of these final experiments predominately two-spored asci were observed.

These observations suggested that the fusants were losing chromosomes and that this reduced their viability and ability to sporulate. There are two ways chromosome loss might have occured. First, it may be that the cytoplasms of the two strains fused but that there was no karyogamy or incomplete karyogamy, or second, it may be that karyogamy occured but some of the chromosomes failed to segregate properly during mitosis and meiosis. Three observations suggest that karyogamy occured. First, the fusants of W66-8A with SL484, SL641, SL382 and SCMS7-1 were examined using the fluorescent stain DAPI and were found to have a single nucleus. Second, when examined for the presence of various genetic markers, the fusants were found to harbor mutant alleles from both parents. Lastly, the generation of aneuploids, which exhibited disomic and trisomic combinations of chromosomes from both parents, is inconsistent with failure of karyogamy. Unfortunately, not all of the fusants constructed were examined using DAPI nor were all tested for the presence of parental alleles, so it is not known if all the fusants did indeed undergo karyogamy as well as cytoplasmic fusion.

Although most of the fusants exhibited the troublesome behavior described above, at least two fusants appeared to be genetically stable. These fusants, the products of SL641 and the spo3 strain were isolated on MIN-sorbitol medium and grew as visible colonies within two to three days. When picked and streaked to MIN they also grew well. Single colonies of the fusants were then isolated on YEPD and tested for their ability to grow on MIN. Twenty-three colonies from one fusant,

and 30 colonies from the second fusant were tested. All were able to grow on MIN. The two SL641 x spo3 fusants were used for complementation analyses. Both grew well in PSP and sporulated well.

PRELIMINARY EXPERIMENTS WITH MUTANT STRAIN SL572

Introduction. One of the first SAG-deficient isolates indentified in the SAG screen was able to sporulate at low levels and so was amenable to standard genetic analyses. Reported below are the characteristics of the strain and the results of two preliminary experiments. This isolate, called SL572, is thought to have a defect in conjugation.

Preliminary experiments on the nature of the SL572 defect. SL572 exhibited low levels of SAG activity, but sporulated sufficiently to make genetic analyses relatively simple (Table 15). Spores derived from SL572 were crossed to a wild type heterothallic MATa strain, A364a. Three prototrophic progeny were isolated, sporulated and their tetrads dissected. The hybrid was heterozygous for HO, so each tetrad consisted of two HO spores and two ho spores which would give rise to two diploid and two haploid colonies, respectively. Only the diploids could be tested for SAG activity so the tetrads were first examined for their ability to mate. It was expected that in each tetrad there would be 2 nonmaters : 2 maters. All segregants were tested at least twice for mating ability and the results of the analysis of tetrads of one of the hybrids are shown in Table 16. Similar results were found with the other two hybrids. There were three unexpected results. First, 2+ : 2- segregation of mating ability was not observed. Second, some spores behaved differently in successive experiments. The variable behavior

Table 15. Specific activity of SAG in mutant SL572.[a]

Experiment[b]	Specific activity at following time in SPM (h)			
	0	24	48	72
1	0.114	3.25 (3.3)[c]	3.00	2.40 (2.3)
2	0.00876	8.16	6.08 (19.6)	8.66 (21.3)
3	ND[d]	37.8	ND	ND

a. Cells were incubated in SPM at 34°C for the indicated times after pregrowth in PSP.
b. Experiments are listed in chronological order.
c. Values in parentheses are the percentage of cells that sporulated.
d. ND is not determined.

Table 16. Segregation of mating ability in a SL572 x A364a diploid.

Genotype of diploid:

HO/ho MATa/MAT

expected segregation of mating ability:

2 nonmaters : 2 maters

Spore	Mating behavior/ mating type[a]	Spore	Mating behavior/ mating type
2A	NM[b]	12A	wMATα → Bi → wMATα
B	NM	B	NM
C	MATa	C	MATα
D	NM → wMATα[c]	D	wMATα → MATα
3A	NM	13A	wMATα → MATα → NM
B	NM → wMATα	B	MATα
C	MATα → wMATα	C	wMATα → NM
D	NM → wMATa	D	wMATα → NM
5A	wMATα	17A	wMATα → NM
B	NM	B	wMATα → MATα
C	MATα	C	MATa
D	NM	D	wMATα → wBi → wMATα
7A	wMATa → NM	20A	MATα → Bi (sMATα, wMATa)[e]
B	NM	B	wMATa → MATa
C	MATa	C	MATα
D	NM	D	wMATα → NM
8A	MATα	21A	MATα
B	NM	B	MATα
C	NM → wMATα	C	wMATα
D	wMATa → wMATα	D	wMATα
10A	MATα → wMATα	22A	wMATα → Bi
B	wMATa	B	wMATa
C	wMATα → wMATa → NM → wBi[d]	C	MATα
D	wMATa → MATa	D	wMATα → Bi (sMATα, wMATa)
11A	MATa → wMATa	24A	MATα
B	wMATα → Bi → NM	B	wMATα
C	MATα	C	Bi (sMATα, wMATa)
D	wBi → wMATα → NM	D	MATα
12A	wMATα → Bi → wMATα		
B	NM		
C	MATα		
D	wMATα → MATα		

a. Mating ability was tested by cross-replicating the colonies on a minimal medium to MATa and MATα tester strains with complementary markers. The arrow separates the results of successive experiments. All spores were tested at least twice and only changes in mating behavior are shown.
b. NM is nonmater.
c. w is signifies a weak mating response.
d. Bi is Bimater; mating both as MATa and MATα.
e. s signifies a strong mating response.

took the form of weak mater→nonmater (spores 7A, 13A, 13C, 13D, 17A, 20D) and nonmater→weak mater (spores 2D, 3B, 3D, 8C). MATα→MATa (spore 10C) and MATa→MATα (spore 8D) conversions were also observed. Third, some spores mated both as MATa and MATα in a single test for mating ability (spores 10C, 11B, 11D, 12A, 17D, 20A, 22A, 22D, 24C).

The above results suggest that the SL572 mutation affects mating behavior. Some of the colonies that exhibited variable mating ability were examined for ascospore production, and were found to sporulate. Thus the mutation did not completely block conjugation. There are several classes of mutants defective in mating. These include mutations in the MAT locus, pheromone production, pheromone sensitivity, and other mating functions (Table 3). Some of these mutations affect both mating types, whereas others are specific to a single mating type. The spores were not characterized further, so an assignment of genotype assuming any one of these mutations was not possible.

Tetrads derived from SL572 were tested for mating ability on successive days to determine the pattern of aberrant mating and to determine if the defect segregated in all spores. If all segregants exhibited variable mating behavior it would rule out the possibility that the mutation was in the MAT locus. There are two striking features of the results shown in Table 17. One is that the mating phenotype of each spore, except 1D, varied, and the second is that a strong MATa mating response was never observed. The cells always varied between a weak MATα, a strong MATα and a nonmating phenotype. This suggests that the SL572 mating defect is specific to MATa cells. The mating behavior of the spores could be explained in the following way. Since mating-type conversion was normal, a colony could consist of MATa and

Table 17. Mating behavior of SL572 spore-derived colonies.[a]

Spore	Mating behavior on day: 4	5	6	7	8	9	11
1A	wMATα	NM	MATα	wMATα	MATα	wMATα	NM
B	wMATα	MATα	wMATα	wMATα	MATα	wMATα	wMATα
C	Bi*[b]	MATα	wMATα	wBi	wMATα	wBi	wMATα
D	NM	NM	NM	NM	NM	NM	NM
2A	MATα	MATα	NM	MATα	wMATα	NM	NM
B	MATα	MATα	wBi	MATα	MATα	wMATα	NM
C	NM	wMATα	NM	MATα	NM	NM	NM
D	wBi	Bi*	wMATα	wBi	wMATα	wMATα	wMATα
3A	wMATα	wBi	NM	NM	NM	NM	NM
B	wMATα	Bi*	NM	NM	wMATα	NM	NM
C	MATα	NM	NM	MATα	NM	wMATα	NM
D	MATα	wBi	wMATα	wMATα	wMATα	wMATα	wMATα

a. Mating ability was tested as described in Table 16.
b. Bi* indicates that the colony exhibited a strong MATα response and a weak MATa response. All other abbreviations are the sames as those in Table 16.

MATα cells. The leaky nature of the defect would permit some mating within the colony so that diploids would be present in the colony as well. Thus nonmating colonies would consist of diploids and possibly some MATa cells. The wMATα response would be observed when the colony consisted of relatively few MATα cells. The remainder of the colony would be MATa and diploid cells. The strong MATα response would be observed when MATα cells predominated in the colony. Three MATa-specific conjugation genes have been identified, STE2, STE6 and STE14 (124). STE6 and STE14 mutants are deficient for a-factor production and so are candidates for the a-factor structural gene or genes functioning in a-factor precursor processing. STE2 mutants synthesize a-factor, but are unresponsive to α-factor and so STE2 is thought to code for an α-factor receptor protein. The colonies derived from tetrad 1 were also tested for their ability to sporulate when the mating experiments were completed. They sporulated < 0.1%, 49.0%, 45.8% and 24.5% for spores 1A, B, C, and D, respectively. STE2, STE6 and STE14 mutants also sporulate normally.

Summary. SL572 is apparently defective in conjugation. The nature of the mutation has not been determined but the results suggest that the mutation is not in the MAT locus, since all four SL572 spores in a tetrad exhibit aberrant mating behavior. The results also suggest that the defect is specific to MATa cells, since a MATa mating response is never observed in SL572 spores. The mutation does not completely block conjugation, since diploid cells capable of sporulating are eventually formed.

It seems likely that the SAG-deficient phenotype of SL572 was due to the large number of asporogenous haploid cells in the colony.

Successive subculturing led to the isolation of colonies consisting of many diploid cells which sporulated. Similar leaky conjugation defects could account for the detection of presumptive mutants in the initial screen that later exhibited a SAG-positive phenotype.

In order to study the SL572 mating defect it must first be isolated in both MATa and MATα heterothallic backgrounds. The SL572 x A364a cross was the first step towards isolating such strains. Tetrads isolated from the SL572 x A364a diploid exhibiting particular segregation patterns of mating ability would be examined further. Assuming that the defect is MATa-specific, tetrads segregating 3 nonmaters : 1 MATα would include the desired strains. If the defect is nonspecific, then tetrads segregating 4 nonmaters : 0 maters, or 2 nonmaters : 1 weak MATα : 1 weak MATa, or 3 nonmaters : 1 weak mater would include the desired strains. In either case, two of the nonmaters would sporulate, producing spores that were also nonmaters and never exhibted aberrant mating behavior. Once in a heterothallic background the mutation could be described more thoroughly and its relationship to other known conjugation mutants examined.

DISCUSSION

I have described the isolation and characterization of two SAG-deficient mutants of S. cerevisiae, SL484 and SL641. A third mutant, SL382 was also isolated but was not characterized in as much detail. SL484 and SL641 had similar phenotypes. Neither synthesized DNA, completed Meiosis I, or formed asci when incubated in sporulation medium at 34°C. SL484 differed from SL641 by being temperature-sensitive.

The isolation of only three mutants out of approximately 2,000 screened was surprising since in the initial round of screening almost 300 colonies had a SAGless phenotype. With every retest, however, many of the presumptive mutants were found to produce SAG. It is unlikely that the whole cell assay was unreliable since control strains always exhibited the correct phenotype with respect to SAG in each round of screening and rescreening. The gradual loss of presumptive mutants suggests that the isolates harbored mutations that were unstable. However, an alternative explanation is that some of the isolates had leaky defects either in conjugation genes or in the homothallism gene, HO. Initially such mutants would be SAGless because the colonies would consist predominantly of haploid cells. Over time, mating could occur to form a SAG-producing diploid colony. The isolation of SL572 supports this possibility . It was expected that a number of mutations in HO, MAT and conjugation loci would be detected in this screen. Therefore,

after screening and retesting for SAG the isolates were tested for mating pheromone production. The isolation of leaky conjugation mutants like SL572 and the unnecessary screening of HO and MAT mutants could be avoided by first testing for mating pheromone production and then testing for SAG production.

It has not been determined whether the defects in SL484 and SL641 are in the structural gene for SAG. SAG has been purified from wild-type strains and antibodies against it have been made (M. J. Clancy, personal communication). Anti-SAG antibodies could be used in a Western Blot (133) procedure to detect SAG. In a Western Blot, proteins are separated by gel electrophoresis then transferred electrophoretically to nitrocellulose sheets. The protein of interest is detected immunologically on the nitrocellulose. If SL484 or SL641 produced an antibody-reacting protein during sporulation that migrated differently than the wild-type protein this would suggest that the mutation was in the structural gene.

A number of early sporulation defects are known (16, 41). These have been characterized in terms of DNA synthesis, recombination-competence and the ability to form various meiotic structures such as spindle pole bodies and synaptonemal complexes. Many radiation-sensitive mutants either do not sporulate or sporulate poorly due to their defects in DNA repair or recombination functions (41). Recombination, ultrastructural development and radiation sensitivity have not been examined in SL484 or SL641. These experiments are necessary to describe their phenotype more completely. SL484 and SL641 are not defective in cell-cycle genes since they grow normally at the temperatures tested. With the exception of spo1, spo3 and spo7 which

were complemented by both SL484 and SL641, the relationship to other known sporulation genes has not been examined. Complementation tests will help establish the relationship between SL641 and SL484 and these other sporulation mutants.

The 2+ : 2- segregation pattern of the SAGless phenotype in cells heterozygotic for the SL484 defect shows that the phenotype is caused by a mutation in a single gene. The mutation maps 8.8 map units from the TRP5 gene on chromosome VII. SL641 was a nonconditional mutant so genetic analyses were done using tetraploid strains constructed by fusing spheroplasts of SL641 with spheroplasts of other homothallic diploid strains. The segregation pattern in the tetraploid heterozygotes indicated that the SL641 phenotype was the result of a single gene defect. It is unlikely that SL641 and SL484 are different alleles of the same locus because the SL641 tetraploid exhibited aberrant segregation of Chromosome VII due to a disomy for that chromosome, while at the same time exhibiting normal tetraploid segregation of the SAGless phenotype.

The aberrant segregation of alleles in the SL641, SL484 and SCMS7-1 derived tetraploid fusants was explained by chromosome loss. Intraspecific (1, 2, 9, 15, 23, 42, 45, 51, 54, 55, 63, 64, 75, 76, 109-111, 118-120, 128, 135, 136, 145, 147, 149, 150) interspecific (19, 20, 28, 109, 117, 122, 123, 126, 140, 150) and intergeneric (21, 98, 117, 123, 130) spheroplast fusion experiments have been done with yeasts. Many intraspecific fusions were reported to produce homokaryons carrying genetic information from both parents (2, 9, 15, 23, 42, 45, 51, 54, 75, 111, 119, 120, 126, 128, 135, 136). Meiotic or mitotic segregation of alleles was examined for some fusants. Although euploidy

was generally indicated (9, 15, 45, 51, 54, 75, 111, 120, 126, 136), some cases of aneuploidy were reported. Sarachek, et. al. (111) fused protoplasts of two different Candida albicans strains and observed that karyogamy occured. C. albicans has no known sexual cycle and is thought to be diploid by some investigators (90, 139). In their study, Sarachek et. al. (111) considered C. albicans to be haploid, and based on DNA measurements, they hypothesized that karyogamy had occured but was followed by an attempted haploidization that resulted in aneuploidy. Arima and Takana (2) examined diploid and polyploid fusants that were produced by simple and multiple fusions of two or three different S. cerevisiae strains. They observed unexpected phenotypes that suggested the fusion products were aneuploid. This possibility was not examined further. Sipiczki and Ferenczy (119) also reported evidence of aneuploidy after karyogamy in fusants constructed from two haploid Rhodosporidium strains.

Most intraspecific fusion experiments with S. cerevisiae have fused haploid strains of like mating type (2, 9, 15, 23, 42, 51, 54, 75, 76, 109, 128, 136, 145, 150). There has been only one report of an attempted fusion of two MATa/MATα strains (55). The two strains differed in their nuclear and mitochondrial genetic make-up. The fusant was diploid, having the nucleus of only one of the parents, but it harbored the mitochondria of the other parent. Thus cytoplasmic but not nuclear fusion had occured. Stewart and Russell (109) reported the fusion of a MATa strain with a polyploid brewing strain that carried both MAT alleles. The fusant sporulated well and the alleles at eight loci segregated 2 : 2 suggesting that the fusant was diploid. The

formation of a diploid from a fusion of polyploid and haploid strains must have involved the loss of genetic material.

There are also reports of fusions of S. cerevisiae and the closely related S. diastaticus (20, 122). S. cerevisiae and S. diastaticus are interfertile and are differentiated solely on the ability of S. diastaticus to secrete a starch-degrading enzyme. de Figueroa et. al. (20) fused a highly flocculent, glycerol-respiring MATa/MATα S. cerevisiae strain with a nonflocculent spontaneously-arising petite MATa/MATα strain of S. diastaticus. The S. diastaticus parent was unable to sporulate or grow on glycerol because of its respiratory deficiency. All the fusants should have been flocculent, able to sporulate, degrade starch and grow on glycerol. Most fusants exhibited this phenotype. However one fusant was nonflocculent but able to sporulate, degrade starch and grow on glycerol, and four other fusants were nonflocculent, degraded starch and grew on glycerol but did not sporulate. de Figueroa et. al. (20) suggested that the phenotypes of the exceptional fusants were the result of chromosome loss during post-fusion vegetative growth.

An alternative possibility is that the aneuploidy observed in the tetraploid fusants isolated in this study was the result of chromosomal transfer from one parental nucleus to the other nucleus in response to continued selective pressure. Internuclear chromsome transfer has been reported between haploid nuclei in a karyogamy defective, kar1/KAR1 background (30). In the SL641 fusant, at least six chromosomes, and in the SL484 and SCMS7-1 fusants, at least eight chromosomes would have been transferred. Dutcher (30) reported that, in her studies, only one chromosome was transferred. It seems unlikely that internuclear

chromosome transfer could account for the recombination of alleles and aneuploidy observed in the SL484, SL641 and SCMS7-1 derived fusants.

In addition to the classical genetic approach described here, new findings suggest a molecular approach for identifying the SAG structural gene. S. cerevisiae harbors a gene, called INH1, that inhibits the synthesis of the S. diastaticus starch-degrading glucoamylase (148). Interestingly, glucoamylase synthesis is also controlled by mating type, as it is repressed in MATa/MATα diploids of S. diastaticus (147). Glycogen and starch both contain α1,4- and α1,6-linkages of glucose moieties. Both glucoamylase and SAG are able to cleave these bonds starting at the non-reducing ends of the molecule. These observations suggest a relationship of the two enzymes at the gene level. Erratt and Stewart have identified three genes DEX1 (35), DEX2 (36) and DEX3 (37) that confer starch-degrading ability in S. diastaticus. Tamaki (129) also identified three genes, STA1, STA2 and STA3. DEX3 and STA3 are allelic (37) but the relationship of DEX1 and DEX2 to STA1 and STA2 has not been reported. Erratt and Nasim (34) recently cloned one or more of the DEX genes by complementation in S. cerevisiae. Restriction maps of the clones were compared to the map of the STA1 gene which has also been cloned (146). Experiments are now being done to determine if S. cerevisiae contains any sequences homologous to the DEX clones (34). If sequence homologies do exist, one might be the SAG structural gene. In addition, the sporulation-specific clones isolated by Clancy, et. al. (12) are being screened for DEX-hybridizing sequences (P. T. Magee, personal communication). If SAG is detected, mutant strains could be isolated by in vitro mutagenesis (108). Both the isolated SAG gene and SAG mutants could be used to study the regulation of this

sporulation-specific gene and its epistatic relationship to other known sporulation genes, thus furthering our understanding of the sporulation pathway and its regulation.

LIST OF REFERENCES

LIST OF REFERENCES

1. Allmark, B. M., A. J. Morgan, and P. A. Whittaker. 1978. The use of protoplast fusion in demonstrating chromosomal and mitochondrial inheritance of respiratory-deficiency in Kluveromyces lactis, a petite-negative yeast. Mol. Gen. Genet. 159:297-299.

2. Arima, K. and I. Takano. 1979. Multiple fusions of protoplasts in Saccharomyces yeasts. Mol. Gen. Genet. 173:271-272.

3. Bedard, D. P., G. C. Johnston, and R. A. Singer. 1981. New mutations in the yeast Saccharomyces cerevisiae affecting completion of "Start". Curr. Genet. 4:205-214.

4. Byers, B. and L. Goetsch. 1975. Behavior of spindles and spindle plaques in the cell cycle and conjugation of Saccharomyces cerevisiae. J. Bacteriol. 124:511-523.

5. Byers, B. and L. Goetsch. 1982. Reversible pachytene arrest of Saccharomyces cerevisiae at elevated temperature. Mol. Gen. Genet. 187:47-53.

6. Calvert, G. R. and I. W. Dawes. 1984. Initiation of sporulation in Saccharomyces cerevisiae. Mutations preventing initiation. J. Gen. Microbiol. 130:605-613.

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

8. Carlson, M., B. C. Osmond, and Botstein, D. 1981. Mutants of yeast defective in sucrose utilization. Genetics 98:25-40.

9. Christensen, B. E. 1979. Somatic hybridization in Saccharomyces cerevisiae: Analysis of products of protoplast fusion. Carlsberg. Res. Commun. 44:225-233.

10. Ciriacy, M. 1977. Isolation and characterization of yeast mutants defective in intermediary carbon metabolism and in carbon catabolite derepression. Mol. Gen. Genet. 154:213-220.

11. Ciriacy, M. 1978. A yeast mutant with glucose-resistant formation of mitochondrial enzymes. Mol. Gen. Genet. 129:329-335.

12. Clancy, M. J., B. Buten-Magee, D. J. Straight, A. L. Kennedy, R. M. Partridge and P. T. Magee. 1983. Isolation of genes expressed preferentially during sporulation in the yeast Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA 80:3000-3004.

13. Colonna, W. J. and P. T. Magee. 1978. Glycogenolytic enzymes in sporulating yeast. J. Bacteriol. 134:844-853.

14. Cooper, T. G. 1982. Nitrogen metabolism in Saccharomyces cerevisiae. In The Molecular Biology of the Yeast Saccharomyces. Metabolism and Gene Expression, eds. Strathern, J. N., et. al. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) p.39.

15. Curran, B. P. G. and B. L. A. Carter. 1983. α-factor enhancement of hybrid formation by protoplast fusion in the yeast Saccharomyces cerevisiae. J. Gen. Microbiol. 129:1589-1591.

16. Davidow, L. S. and B. Byers. 1984. Enhanced gene conversion and postmeiotic segregation in pachytene-arrested Saccharomyces cerevisiae. Genetics 106:165-183.

17. Dawes, I. W. and G. R. Calvert. 1984. Initiation of sporulation in Saccharomyces cerevisiae. Mutations causing derepressed sporulation and G1 arrest in the cell division cycle. J. Gen. Microbiol. 130:605-613.

18. Dawes, I. W., S. Donaldson, R. Edwards, and J. Dawes. 1983. Synthesis of a spore-specific surface antigen during sporulation of Saccharomyces cerevisiae. J. Gen. Microbiol. 129: 1103-1108.

19. de Figueroa, L. I., M. F. de Richard, and M. R. de van Broock. 1984. Interspecific protoplast fusion of the baker's yeast Saccharomyces cerevisiae and Saccharomyces diastaticus. Biotech. Letts. 6:269-274.

20. de Figueroa, L. I., M. F. de Richard, and M. R. de van Broock. 1984. Use of the somatic fusion method to introduce the flocculation property into Saccharomyces diastaticus. Biotech. Letts. 6:587-592.

21. de Richard, M. S. and M. R. de van Broock. 1984. Protoplast fusion between a petite strain of Candida utilis and Saccharomyces cerevisiae respiratory-competent cells. Curr. Microbiol. 10:117-120.

22. de van Broock, M. R., M. Sierra, and L. de Figueroa. 1981. Intergeneric fusion of yeast protoplasts. In Current Developments in Yeast Research. Advances in Biotechnology, eds. Stewart, G. G. and I. Russell. (Pergamon Press) p. 171.

23. de van Broock, M. R., M. Sierra, and L. I. de Figueroa. 1983. Ploidy reduction using p-fluorophenylalanine of fusion products of Saccharomyces cerevisiae. Curr. Microbiol. 8:13-16.

24. del Ray, F., T. Santos, I. Garcia-Acha, and C. Nombela. 1979. Synthesis of 1,3-β-glucanases in Saccharomyces cerevisiae during the mitotic cycle, mating and sporulation. J. Bacteriol. 139:924-931.

25. del Ray, F., T. Santos, I. Garcia-Acha, and C. Nombela. 1980. Synthesis of -glucanases during sporulation in Saccharomyces cerevisiae: Formation of a new sporulation-specific 1,3-β-glucanase. J. Bacteriol. 143:621-627.

26. del Ray, F., T. G. Villa, T. Santos, I. Garcia-Acha, and C. Nombela. 1982. Purification and partial characterization of a new, sporulation-specific, exo-β-glucanase from Saccharomyces cerevisiae. Biochem. Biophys. Res. Commun. 105:1347-1353.

27. Denis, C. L., M. Ciriacy, and E. T. Young. 1981. A positive regulatory gene is required for accumulation of the functional messenger RNA for the glucose-repressible alcohol dehydrogenase from Saccharomyces cerevisiae. J. Mol. Biol. 148:355-368.

28. Dhawall, M. R. and W. M. Ingledew. 1983. Interspecific protoplast fusion of Schwannomyces yeasts. Biotech. Letts. 5:825-830.

29. Dickinson, J. R., I. W. Dawes, A. S. F. Boyd, and R. L. Baxter. 1983. 13C-NMR studies of acetate metabolism during sporulation of Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA 80:5847-5851.

30. Dutcher, S. K. 1981. Internuclear transfer of genetic information in kar1-1/KAR1 heterokaryons in Saccharomyces cerevisiae. Mol. Cell. Biol. 1:245-253.

31. Dutcher, S. K. and L. H. Hartwell. 1982. The role of S. cerevisiae cell division cycle genes in nuclear fusion. Genetics 100:175-184.

32. Entian, K.-D. and K.-U. Frolich. 1984. Saccharomyces cerevisiae mutants provide evidence of hexokinase as a bifunctional enzyme with catalytic and regulatory domains for triggering carbon catabolite repression. J. Bacteriol. 158:29-35.

33. Entian, K.-D., F. K. Zimmermann, and I. Scheel. 1977. A partial defect in carbon catabolite repression in mutants of Saccharomyces cerevisiae with reduced hexose phosphorylation. Mol. Gen. Genet. 156:99-105.

34. Erratt, J. A. and A. Nasim. 1984. Cloning of the glucoamylase gene from Saccharomyces diastaticus to S. cerevisiae by functional complementation. Abstr. XII Int. Conference on Yeast Genetics and Molecular Biology, G13.

35. Erratt, J. A. and G. G. Stewart. 1978. Genetic and biochemical studies on yeast strains able to utilize dextrins. J. Amer. Soc. Brew. Chem. 36:151-161.

36. Erratt, J. A. and G. G. Stewart. 1981. Genetic and biochemical studies on glucoamylase from Saccharomyces diastaticus. In Current Developments in Yeast Research, eds. Stewart, G. G. and I. Russell. (Pergamon Press) p.177.

37. Erratt, J. A. and G. G. Stewart. 1981. Fermentation studies using Saccharomyces diastaticus yeast strains. Developments in Ind. Microbiol. 22:577-589.

38. Esposito, M. S. and R. E. Esposito. 1969. The genetic control of sporulation in Saccharomyces. I. The isolation of temperature-sensitive sporulation-deficient mutants. Genetics 61:79-89.

39. Esposito, M. S. and R. E. Esposito. 1974. Genes controlling meiosis and spore formation in yeast. Genetics 78:215-225.

40. Esposito, M. S. and R. E. Esposito. 1975. Mutants of meiosis and ascospore formation. Methods Cell Biol. 11:303-326.

41. Esposito, R. E. and S. Klapholz. 1981. Meiosis and ascospore development. In The Molecular Biology of the Yeast Saccharomyces. Life Cycle and Inheritance, eds. Strathern, J. N., et. al. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) p. 211

42. Ferenczy, L. and A. Maraz. 1977. Transfer of mitochondria by protoplast fusion in Saccharomyces cerevisiae. Nature 268:524-525.

43. Fink, G. R. 1970. The biochemical genetics of yeast. Methods Enzymol. 17A:59-78.

44. Fogel, S., R. Mortimer, K. Lusnak, and F. Travares. 1979. Meiotic gene conversion: A signal of the basic recombination event in yeast. Cold Spring Harbor Symp. Quant. Biol. 43:1325-1341.

45. Fournier, P., A. Provost, C. Bourguignon, and H. Heslot. 1977. Recombination after protoplast fusion in the yeast Candida tropicalis. Arch. Microbiol. 115:143-149.

46. Fowell, R. R. 1975. Ascospores of yeast. In Spores, eds. Gerhardt, P., et. al. (American Society for Microbiology, Washington, DC) Vol. 6, p. 124.

47. Fraenkel, D. G. 1982. Carbohydrate metabolism. In The Molecular Biology of the Yeast Saccharomyces. Metabolism and Gene Expression, eds. Strathern, J. N., et. al. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) p.1.

48. Freese, E. B., M. I. Chu, and E. Freese. 1982. Initiation of yeast sporulation by partial carbon, nitrogen, or phosphate deprivation. J. Bacteriol. 149:840-851.

49. Fritz, H., G. Hartwich, and E. Werle. 1966. Uber protease inhibitoren. I. Isolierang and charakterisierung des trypsininhibitors aus pankreasgewebe und pankreassekret. Hoppe-Seylers Z. Physiol. Chem. 345:150-157.

50. Garel, A. and R. Axel. 1976. Selective digestion of transcriptionally active ovalbumin genes from oviduct nuclei. Proc. Natl. Acad. Sci. USA 73:3966-3970.

51. Gunge, N. and A. Tamari. 1978. Genetic analysis of products of protoplast fusion in Saccharomyces cerevisiae. Japan. J. Genet. 53:41-49.

52. Haber, J. E., M. S. Esposito, P. T. Magee, and R. E. Esposito. 1975. Current trends in genetic and biochemical study of yeast sporulation. In Spores, eds. Gerhardt, P., et. al. (American Society for Microbiology, Washington, DC) Vol. 6, p.132.

53. Haber, J. E. and H. O. Halvorson. 1975. Methods in sporulation and germination of yeasts. Methods Cell Biol. 11:45-69.

54. Halfmann, H. J., C. C. Emeis, and U. Zimmermann. 1983. Electrofusion of haploid Saccharomyces yeast cells of identical mating type. Arch. Microbiol. 134:1-4.

55. Halfmann, H. J., W. Rochen, C. C. Emeis and U. Zimmermann. 1982. Transfer of mitochondrial function into a cytoplasmic respiratory-deficient mutant of Saccharomyces yeast by electro-fusion. Curr. Genet. 6:25-28.

56. Haynes, R. H. and B. A. Kunz. 1981. DNA repair and mutagenesis in yeast. In The Molecular Biology of the Yeast Saccharomyces. Life Cycle and Inheritance, eds. Strathern, J. N., et. al. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) p.371.

57. Herskowitz, I. and Y. Oshima. 1981. Control of cell type in Saccharomyces cerevisiae: Mating type and mating-type interconversion. In The Molecular Biology of the Yeast Saccharomyces. Life Cycle and Inheritance, eds. Strathern, J. N., et. al. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) p.181.

58. Hesse, G., R. Lindner, and D. Krebs. 1975. Schnelle fluorotometrische deoxribosespezifische DNS-bestimmung an nicht desintegrierten mikroorgismen mit 3,5-diaminobenzoesaure (DABA). I. Chemisch-analytische methodik und anwendung auf hefepopulationen. Z. Allg. Mikrobiol. 15:9-18.

59. Hien, N. H. and G. H. Fleet. 1983. Separation and characterization of six (1,3)-β-glucanases from Saccharomyces cerevisiae. J. Bacteriol. 156:1204-1213.

60. Hien, N. H. and G. H. Fleet. 1983. Variation of (1,3)-β-glucanases in Saccharomyces cerevisiae during vegetative growth, conjugation, and sporulation. J. Bacteriol. 156:1214-1220.

61. Hopper, A. K. and V. L. MacKay. 1980. Control of sporulation in yeast: SAD1--a mating type specific, unstable alteration that uncouples sporulation from mating-type control. Mol. Gen. Genet. 180:301-314.

62. Johnston, L. H., D. H. Williamson, A. L. Johnson, and D. J. Fennell. 1982. On the mechanism of premeiotic DNA synthesis in the yeast Saccharomyces cerevisiae. Exp. Cell Res. 141:53-62.

63. Kakar, S. N. and P. T. Magee. 1982. Genetic analysis of Candida albicans: Identification of different isoleucine-valine, methionine, and arginine alleles by complementation. J. Bacteriol. 151:1247-1252.

64. Kakar, S. N., R. M. Partridge, and P. T. Magee. 1983. A genetic analysis of Candida albicans: Isolation of a wide variety of auxotrophs and demonstration of linkage and complementation. Genetics 104:241-255.

65. Kassir, Y., J. B. Hicks, and I. Herskowitz. 1983. SAD mutation of Saccharomyces cerevisiae is an extra a cassette. Mol. Cell. Biol. 3:871-880.

66. Kelly, R. and S. L. Phillips. 1983. Comparison of the levels of 21S mitochondrial rRNA in derepressed and glucose-repressed Saccharomyces cerevisiae. Mol. Cell. Biol. 3:1949-1957.

67. Kempthorn, O. 1969. An Introduction to Genetic Statistics. Iowa State University Press, Ames, Iowa.

68. Klebl, F., T. Huffaker, and W. Tanner. 1984. A temperature sensitive N-glycosylation mutant of S. cerevisiae that behaves like a cell-cycle mutant. Exp. Cell Res. 150:309-313.

69. Kraig, E. and J. E. Haber. 1980. Messenger ribonucleic acid and protein metabolism during sporulation of Saccharomyces cerevisiae. J. Bacteriol. 144:1098-1112.

70. Krebs, E. G. 1972. Protein kinases. Curr. Top. Cell. Reg. 5:99-133.

71. Kurtz, S. and S. Lindquist. 1984. Changing patterns of gene expression during sporulation in yeast. Proc. Natl. Acad. Sci. USA 81:7323-7327.

72. Lauer, G. D., T. M. Roberts, and L. C. Klotz. 1977. Determination of the nuclear DNA content of Saccharomyces cerevisiae and implications for the organization of DNA in yeast chromosomes. J. Mol. Biol. 114:507-526.

73. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.

74. Magasanik, B. 1976. Classical and postclassical modes of regulation of the synthesis of degradative bacterial enzymes. Prog. Nucleic Acid Res. Biol. 17: 99-115.

75. Maraz, A., M. Kiss, and L. Ferenczy. 1978. Protoplast fusion in Saccharomyces cerevisiae strains of identical and opposite mating types. FEMS Microbiol. Letts. 3:319-322.

76. Maraz, A. and J. Subik. 1981. Transmission and recombination of mitochondrial genes in Saccharomyces cerevisiae after protoplast fusion. Mol. Gen. Genet. 181:131-133.

77. Marian, B. and U. Wintersberger. 1982. Modification of histones during the mitotic and meiotic cycle of yeast. FEBS Letts. 139:72-76.

78. Marmiroli, N., C. Ferrari, F. Tedeschi, P. P. Puglisi, and C. Bruschi. 1981. Ultrastructural analysis of the life cycle of an apomictic (Apo) strain of Saccharomyces cerevisiae. I. Meiosis and ascospore development. Biol. Cell. 41:79-84.

79. Marmiroli, N., F. Tedeschi, C. Ferrari, P. P. Puglisi, and C. Bruschi. 1981. Ultrastructural analysis of the life cycle of an apomictic (Apo) strain of Saccharomyces cerevisiae. II. Mitotic recombination. Biol. Cell. 41:85-90.

80. Matsumoto, K., I. Uno, and T. Ishikawa. 1983. Control of cell division in Saccharomyces cerevisiae mutants defective in adenylate cyclase and cAMP-dependent protein kinase. Exp. Cell Res. 146:151-161.

81. Matsumoto, K., I. Uno, and T. Ishikawa. 1983. Initiation of meiosis in yeast mutants defective in adenylate cyclase and cyclic AMP-dependent protein kinase. Cell 32:417-423.

82. Matsumoto, K., I. Uno, Y. Oshima, and T. Ishikawa. 1982. Isolation and characterization of yeast mutants deficient in adenylate cyclase and cAMP-dependent protein kinase. Proc. Natl. Acad. Sci. USA 79:2355-2359.

83. Matsumoto, K., I. Uno, A. Toh-E, T. Ishikawa, and Y. Oshima. 1982. Cyclic AMP may not be involved in catabolite repression in Saccharomyces cerevisiae: Evidence from mutants capable of utilizing it as an adenine source. J. Bacteriol. 150:277-285.

84. Matsumoto, K., T. Yoshimatsu, and Y. Oshima. 1983. Recessive mutations conferring resistance to carbon catabolite repression of galactokinase synthesis in Saccharomyces cerevisiae. J. Bacteriol. 153:1405-1414.

85. Michels, C. A. and A. Romanowski. 1980. Pleiotropic glucose repression-resistant mutation in Saccharomyces carlsbergensis. J. Bacteriol. 143:674-679.

86. Moens, P. B. 1982. Mutants of yeast meiosis (Saccharomyces cerevisiae). Can. J. Genet. Cytol. 24:243-256.

87. Mortimer, R. K. and D. Schild. 1980. Genetic map of Saccharomyces cerevisiae. Microbiol. Rev. 44:519-571.

88. Mowshowitz, D. B. 1976. Permeabilization of yeast for enzyme assays: An extremely simple method for small samples. Analyt. Biochem. 70:94-99.

89. Okamoto, S. and T. Iino. 1981. Selective abortion of two non-sister nuclei in a developing ascus of the hfd1-1 mutant in Saccharomyces cerevisiae. Genetics 99:197-209.

90. Olaiya, A. F. and S. J. Sogin. 1979. Ploidy determination of Candida albicans. J. Bacteriol. 140:1043-1049.

91. Ota, A. 1982. Enzyme activities during early ascosporulation in Saccharomyces cerevisiae. Int. J. Biochem. 14:111-118.

92. Parry, E. M. and B. S. Cox. 1970. The tolerance of aneuploidy in yeast. Genet. Res., Camb. 16:333-340.

93. Peberdy, J. F. 1979. Fungal protoplasts: Isolation, reversion and fusion. Ann. Rev. Microbiol. 33:21-39.

94. Percival-Smith, A. and J. Segall. 1984. Isolation of DNA sequences preferentially expressed during sporulation in Saccharomyces cerevisiae. Mol. Cell. Biol. 4:142-150.

95. Peterson, J. G. L. 1981. SDS polyacrylamide gel electrophoresis of chromatin proteins from yeast during vegetative growth and sporulation. Carlsberg. Res. Commun. 46:107-119.

96. Pringle, J. 1975. Methods for avoiding proteolytic artifacts in studies of enzymes and other proteins from yeast. Methods Cell Biol. 12:149-184.

97. Pringle, J. R. and L. H. Hartwell. 1981. The Saccharomyces cerevisiae cell cycle. In The Molecular Biology of the Yeast Saccharomyces. Life Cycle and Inheritance, eds. Strathern, J. N., et. al. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) p. 97.

84. Matsumoto, K., T. Yoshimatsu, and Y. Oshima. 1983. Recessive mutations conferring resistance to carbon catabolite repression of galactokinase synthesis in Saccharomyces cerevisiae. J. Bacteriol. 153:1405-1414.

85. Michels, C. A. and A. Romanowski. 1980. Pleiotropic glucose repression-resistant mutation in Saccharomyces carlsbergensis. J. Bacteriol. 143:674-679.

86. Moens, P. B. 1982. Mutants of yeast meiosis (Saccharomyces cerevisiae). Can. J. Genet. Cytol. 24:243-256.

87. Mortimer, R. K. and D. Schild. 1980. Genetic map of Saccharomyces cerevisiae. Microbiol. Rev. 44:519-571.

88. Mowshowitz, D. B. 1976. Permeabilization of yeast for enzyme assays: An extremely simple method for small samples. Analyt. Biochem. 70:94-99.

89. Okamoto, S. and T. Iino. 1981. Selective abortion of two non-sister nuclei in a developing ascus of the hfd1-1 mutant in Saccharomyces cerevisiae. Genetics 99:197-209.

90. Olaiya, A. F. and S. J. Sogin. 1979. Ploidy determination of Candida albicans. J. Bacteriol. 140:1043-1049.

91. Ota, A. 1982. Enzyme activities during early ascosporulation in Saccharomyces cerevisiae. Int. J. Biochem. 14:111-118.

92. Parry, E. M. and B. S. Cox. 1970. The tolerance of aneuploidy in yeast. Genet. Res., Camb. 16:333-340.

93. Peberdy, J. F. 1979. Fungal protoplasts: Isolation, reversion and fusion. Ann. Rev. Microbiol. 33:21-39.

94. Percival-Smith, A. and J. Segall. 1984. Isolation of DNA sequences preferentially expressed during sporulation in Saccharomyces cerevisiae. Mol. Cell. Biol. 4:142-150.

95. Peterson, J. G. L. 1981. SDS polyacrylamide gel electrophoresis of chromatin proteins from yeast during vegetative growth and sporulation. Carlsberg. Res. Commun. 46:107-119.

96. Pringle, J. 1975. Methods for avoiding proteolytic artifacts in studies of enzymes and other proteins from yeast. Methods Cell Biol. 12:149-184.

97. Pringle, J. R. and L. H. Hartwell. 1981. The Saccharomyces cerevisiae cell cycle. In The Molecular Biology of the Yeast Saccharomyces. Life Cycle and Inheritance, eds. Strathern, J. N., et. al. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) p. 97.

98. Provost, A., C. Bourguignon, P. Fournier, A. M. Ribet, and H. Heslot. 1978. Intergeneric hybridization in yeasts through protoplast fusion. FEMS Microbiol. Letts. 3:309-312.

99. Rine, J., G. F. Sprague, Jr., and I. Herskowitz. 1981. rme1 mutation of Saccharomyces cerevisiae: Map position and bypass of mating-type locus control of sporulation. Mol. Cell. Biol. 1:958-960.

100. Resnick, M. A., A. Sugino, J. Nitiss, and T. Chow. 1984. DNA polymerases, deoxyribonucleases and recombination during meiosis in Saccharomyces cerevisiae. Mol. Cell. Biol. 4:2811-2817.

101. Robbins, L. G. 1977. The meiotic effect of a deficiency in Drosophila melanogaster with a model for the effects of enzyme deficiency on recombination. Genetics 87:655-684.

102. Roman, H., M. M. Phillips, and S. M. Sands. 1955. Studies of polyploid Saccharomyces I. Tetraploid Segregation. Genetics 40:546-561.

103. Rose, A. H. and J. S. Harrison. 1969. The Yeasts. Vol. 1 Biology of Yeasts. Academic Press.

104. Roth, R. 1973. Chromosome replication during meiosis: Identification of gene functions required for premeiotic DNA synthesis. Proc. Natl. Acad. Sci. USA 70:3087-3091.

105. Roth, R. 1976. Temperature-sensitive yeast mutants defective in meiotic recombination and replication. Genetics 83:675-686.

106. Roth, R. and S. Fogel. 1971. A system selective for yeast mutants deficient in meiotic recombination. Mol. Gen. Genet. 112:295-305.

107. Roth, R. and H. O. Halvorson. 1969. Sporulation of yeast harvested during logarithmic growth. J. Bacteriol. 98:831-832.

108. Rothstein, R. J. 1983. One-step gene disruption in yeast. Methods Enzymol. 101:202-211.

109. Russell, I. and G. G. Stewart. 1978. Spheroplast fusion of brewer's yeast strains. J. Inst. Brew. 85:95-98.

110. Sarachek, A. and D. D. Rhoads. 1981. Production of heterokaryons of Candida albicans by protoplast fusions: Effects of differences in proportions and regenerative abilities of fusion partners. Curr. Genet. 4:221-222.

111. Sarachek, A., D. D. Rhoads, and R. H. Schwarzhoff. 1981. Hybridization of Candida albicans through fusion of protoplasts. Arch. Microbiol. 129:1-8.

112. Schild, D. and B. Byers. 1980. Diploid spore formation and other meiotic effects of two cell-division-cycle mutations of Saccharomyces cerevisiae. Genetics 96:859-876.

113. Schroeder, R., M. Breitenbach, and R. J. Schweyen. 1983. Mitochondrial circular RNAs are absent in sporulating cells of Saccharomyces cerevisiae. Nucs. Acids Res. 11:1735-1746.

114. Sherman, F., G. R. Fink, and J. B. Hicks. 1981. Methods in Yeast Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

115. Shuster, J. R. 1982. "Start" mutants of Saccharomyces cerevisiae are suppressed in carbon catabolite-derepressing medium. J. Bacteriol. 151:1059-1061.

116. Singer, R. A. and G. C. Johnston. 1981. Nature of the G1 phase of the yeast Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA 78:3030-3033.

117. Sipiczki, M. 1979. Interspecific protoplast fusion in fission yeasts. Curr. Microbiol. 3:37-40.

118. Sipiczki, M. 1983. Diploid protoplasts in Schizosaccharomyces pombe: Formation, growth, and sporulation. Can. J. Microbiol. 29:593-595.

119. Sipiczki, M. and L. Ferenczy. 1977. Fusion of Rhodosporidium (Rhodotorula) protoplasts. Fems Microbiol. Letts. 2:203-205.

120. Sipickzi, M. and L. Ferenczy. 1977. Protoplast fusion of Schizosaccharomyces pombe auxotrophic mutants of identical mating-type. Mol. Gen. Genet. 151:77-81.

121. Sledziewski, A. and E. T. Young. 1982. Chromatin conformational changes accompany transcriptional activation of a glucose-repressed gene in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA 79:253-256.

122. Spencer, J. F. T., P. Laud, and D. M. Spencer. 1980. The use of mitochondrial mutants in the isolation of hybrids involving industrial yeast strains. Mol. Gen. Genet. 178:651-654.

123. Spencer, J. F. T. and D. M. Spencer. 1981. The use of mitochondrial mutants in hybridization of industrial yeasts. III. Restoration of mitochondrial function in petites of industrial yeast strains by fusion with respiratory-competant protoplasts of other yeast species. Curr. Genet. 4:177-180.

124. Sprague, G. F., Jr., L. C. Blair, and J. Thorner. 1983. Cell interactions and regulation of cell type in the yeast Saccharomyces cerevisiae. Ann. Rev. Microbiol. 37:623-660.

125. Sprague, G. F., Jr. and I. Herskowitz. 1981. Control of yeast cell type by the mating type locus. I. Identification and control of expression of the a-specific gene, BAR1. J. Mol. Biol. 153:305-321.

126. Stahl, U. 1978. Zygote formation and recombination between like mating types in the yeast Saccharomycopsis lipolytica by protoplast fusion. Mol. Gen. Genet. 160:111-113.

127. Svoboda, A. 1966. Regeneration of yeast protoplasts in agar gels. Exp. Cell Res. 44:640-642.

128. Svoboda, A. 1978. Fusion of yeast protoplasts induced by polyethylene glycol. J. Gen. Microbiol. 109:169-175.

129. Tamaki, H. Genetic studies of ability to ferment starch in Saccharomyces: Gene polymorphism. Mol. Gen. Genet. 164:205-209.

130. Tamaki, H. 1982. Genetic properties of abortive products resulting from the protoplast fusion in yeast. Mol. Gen. Genet. 187:177-179.

131. Thorner, J. 1981. Pheromonal regulation of development in Saccharomyces cerevisiae. In The Molecular Biology of the Yeast Saccharomyces. Life Cycle and Inheritance, eds. Strathern, J., et. al. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) p.143.

132. Thorner, J. 1982. An essential role for cyclic AMP in growth control: The case for yeast. Cell 30:5-6.

133. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. USA 76:4350-4354.

134. Tsuboi, M. 1983. The isolation and genetic analysis of sporulation-deficient mutants in Saccharomyces cerevisiae. Mol. Gen. Genet. 191:17-21.

135. Vallin, C. and L. Ferenczy. 1978. Diploid formation of Candida tropicalis via protoplast fusion. Acta. Microbiol. Acad. Sci. hung. 25:209-212.

136. van Solingen, P. and J. B. van der Plaat. 1977. Fusion of yeast spheroplasts. J. Bacteriol. 130:946-947.

137. Weintraub, H. and Groudine, M. 1976. Chromosomal subunits in active genes have an altered conformation. Science 193:848-856.

138. Weir-Thompson, E. M. and I. W. Dawes. 1984. Developmental changes in translatable RNA species associated with meiosis and spore formation in Saccharomyces cerevisiae. Mol. Cell. Biol. 4:695-702.

139. Whelan, W. L., R. M. Partridge, and P. T. Magee. 1980. Heterozygosity and segregation in Candida albicans. Mol. Gen. Genet. 180:107-113.

140. Whittaker, P. A. and S. M. Leach. 1978. Interspecific hybrid production between the yeasts Kluveromyces lactis and Kluveromyces fragilis by protoplast fusion. FEMS Microbiol. Letts. 4:31-34.

141. Wickner, R. B., F. Boutelet, and F. Hilger. 1983. Evidence for a new chromosome in Saccharomyces cerevisiae. Mol. Cell. Biol. 3:415-420.

142. Williamson, D. H. and D. J. Fennell. 1975. The use of fluorescent DNA-binding agent for detecting and separating yeast mitochondrial DNA. Methods Cell Biol. 12:335-351.

143. Williamson, D. H., L. H. Johnston, D. J. Fennell, and G. Simchen. 1983. The timing of the S phase and other nuclear events in yeast meiosis. Exp. Cell. Res. 145:209-217.

144. Wright, J. F., N. Ajam, and I. W. Dawes. 1981. Nature and timing of some sporulation-specific protein changes in Saccharomyces cerevisiae. Mol. Cell. Biol. 1:910-918.

145. Yamamoto, M. and S. Fukui. 1977. Fusion of yeast protoplasts. Agric. Biol. Chem. 41:1829-1830.

146. Yamashita, I. and S. Fukui. 1983. Molecular cloning of a glucoamylase-producing gene in the yeast Saccharomyces. Agric. Biol. Chem. 47:2689-2692.

147. Yamashita, I. and S. Fukui. 1983. Mating signals control expression of both starch fermentation genes and a novel flocculation gene FLO8 in the yeast Saccharomyces. Agric. Biol. Chem. 47:2889-2896.

148. Yamashita, I. and S. Fukui. 1984. Genetic background of glucoamylase production in the yeast Saccharomyces. Agric. Biol. Chem. 48:137-141.

149. Yamashita, I. and S. Fukui. 1984. Isolation of glucosamylase-non-producing mutants in the yeast Saccharomyces diastaticus. Agric. Biol. Chem. 48:131-135.

150. Yoshida, K. 1979. Interspecific and intraspecific mitochondria-induced cytoplasmic transformation in yeast. Plant Cell Physiol. 20:851-856.

151. Zitomer, R. S. and D. L. Nichols. 1978. Kinetics of glucose repression of yeast cytochrome C. J. Bacteriol. 135:39-44.

APPENDIX

MOLECULAR AND CELLULAR BIOLOGY, Feb. 1982, p. 171-178
0270-7306/82/020171-08$02.00/0

Vol. 2, No. 2

Developmental Regulation of a Sporulation-Specific Enzyme Activity in *Saccharomyces cerevisiae*†

MARY J. CLANCY, LINDA M. SMITH, AND P. T. MAGEE*

Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan 48824

Received 18 May 1981/Accepted 11 September 1981

An α-glucosidase activity (SAG) occurs in a/α *Saccharomyces cerevisiae* cells beginning at about 8 to 10 h after the initiation of sporulation. This enzyme is responsible for the rapid degradation of intracellular glycogen which follows the completion of meiosis in these cells. SAG differs from similar activities present in vegetative cells and appears to be a sporulation-specific enzyme. Cells arrested at various stages in sporulation (DNA replication, recombination, meiosis I, and meiosis II) were examined for SAG activity; the results show that SAG appearance depends on DNA synthesis and some recombination events but not on the meiotic divisions.

It is well documented that as cells undergo developmental processes the proteins synthesized change (1-3, 41). Although this phenomenon has been observed and analyzed in higher and lower eucaryotes and in procaryotes, the molecular basis is poorly understood. Sporulation in the yeast *Saccharomyces cerevisiae* is a useful system for studying development for several reasons. Initiation of sporulation is easily manipulated experimentally, and one can obtain relatively synchronous populations of cells in various stages of sporulation. During the process of sporulation, the cells undergo DNA synthesis, meiosis, and spore formation (6, 10, 30, 35), and conditional mutants which arrest development at particular stages under restrictive conditions are available (8). In addition, the genetic system of *S. cerevisiae* is well understood, making sophisticated genetic analysis possible.

Several reports of attempts to identify sporulation-specific gene products by two-dimensional gel analysis have appeared (21, 40). In general, they have revealed few proteins which are made only during sporulation, although a few such sporulation-specific proteins were observed in one study (I. Dawes, personal communication). Although two-dimensional gel analysis reveals when proteins are made, it does not provide information about their function in development or their regulation. An alternative approach, which is more difficult but could be more fruitful in the long run, is to look for proteins which mediate specific sporulation events and to analyze their regulation by biochemical and genetic techniques. The sporulation amyloglucosidase (SAG) (first described in this laboratory [5]) is such a protein. It is easily detected and amenable to such analysis. The enzyme appears in sporulating cells at about 8 to 10 h after the shift to sporulation medium (SPM) and is responsible for the extensive glycogen degradation which occurs in the cells at the time of completion of meiosis (5). Nonsporulating cells, by contrast, accumulate large amounts of glycogen, but do not degrade it (5). SAG is distinct from glycogen phosphorylase (12) and α-glucosidase (maltase) (16, 28), which are found in vegetative cells; it may be a sporulation-specific enzyme. Understanding the regulation of SAG appearance in sporulating cultures may therefore provide insight into the way that other sporulation events are regulated.

As a first step toward understanding SAG regulation, we have analyzed its appearance in cells arrested at a series of stages in sporulation, either by the presence of inhibitors or because of a genetic constitution which renders them asporogenous (i.e., haploidy, mating type homozygosity, or temperature sensitivity in functions required for DNA replication and meiosis). The purpose of this analysis was to determine whether SAG appearance in sporulating cells depends on normal progress of the cells through DNA replication and meiosis or whether it is regulated in some other way independent of the meiotic process. The results demonstrate that premeiotic DNA replication and possibly some recombination events are necessary for the appearance of SAG but that completion of the meiotic divisions is not. We have also examined vegetative cells under conditions where glycogen catabolism occurs to determine whether SAG is

† Michigan Agricultural Experiment Station journal article no. 9961.

expressed. The absence of the activity in these cells suggests that SAG is a sporulation-specific enzyme.

MATERIALS AND METHODS

Yeast strains. The standard yeast strains used in this study were AP1a/α, AP1α/α (20), AP3a/α, AP3a/a, AP3α/α, and X2180-1A which was obtained from The Yeast Genetic Stock Center in Berkeley, Calif. The AP3 strains were obtained from A. Hopper. *AP3*a/α was derived from a cross between A36A4 and $\alpha_3$131-20. AP3a/a and AP3α/α were derived from UV irradiation of the diploid AP3. Temperature-sensitive diploid homozygous *spo* mutants (*spo1 spo3 spo7*) (8, 11) and their parent strain S41 were obtained from R. E. Esposito. A diploid homozygous for the temperature-sensitive cell division cycle mutation, *cdc4* (18), was obtained from Breck Byers. RD5, a diploid homozygous for the *rad52-1* (33) allele was constructed from haploid *rad52-1* strains obtained from R. Malone.

Growth and sporulation of cells. Cells were ordinarily pregrown in the acetate-containing presporulation medium (PSP) of Roth and Halvorson (34) or in YEP (10 g of yeast extract and 10 g of peptone) + 10 g potassium acetate per liter of distilled water. Cell cultures (100 to 1,000 ml) were grown at 30 or 22°C (*cdc4*) with shaking until the cell density reached 1×10^7 to 2×10^7 cells per ml. The cells were harvested by centrifugation at 4,000 × *g* for 5 min, washed twice by centrifugation in sterile distilled water, suspended in SPM (3 g of potassium acetate and 0.2 g of raffinose per liter of distilled water) to a concentration of 2×10^7 to 3×10^7 cells per ml, and incubated with shaking at the sporulation temperature (22, 30, or 36°C, depending on the experiment). Potassium chloride (4.5%) was added to the SPM in some experiments with the sporulation mutants, and adenine (40 μg/ml) or arginine (40 μg/ml) was included in the medium when the strains used contained these auxotrophic markers. In some experiments, cells were grown in YEPD (10 g of yeast extract, 10 g of peptone, and 20 g of dextrose per liter of distilled water).

Preparation of cell extracts. Washed cells were suspended to a density of 1×10^9 to 3×10^9 cells per ml in 0.1 M Na-citrate buffer (pH 6.2) containing the protease inhibitors phenylmethylsulfonyl fluoride (0.3 mg/ml, predissolved in 95% ethanol [32]) and aprotinin (Sigma Chemical Co.) (13). When 5×10^9 cells or more were to be broken, the Bronwill homogenizer was used. The cell suspension was transferred to a Bronwill flask containing 2 to 10 g of Glasperlen glass beads (0.45 mm diameter; B. Braun Melsungen AG, Germany), and the cells were cooled with compressed CO_2 during homogenization (90 to 120 s). When fewer than 5×10^9 cells were used, they were broken by blending in a Vortex mixer with glass beads by a method similar to that of Kraig and Haber (21). Breakage with either method was always greater than 90%. The broken cell suspension was immediately centrifuged at 12,000 × *g* (10,000 rpm in a Sorvall SS34 rotor) for 20 min and then at 50,000 rpm for 2 h in a Beckman Type 65 rotor (R_{max}, 218,000 × *g*). The supernatant was dialyzed for 12 to 18 h against two changes of 0.1 M sodium citrate buffer at pH 6.2 and then either stored at −20°C or assayed immediately for SAG and protein. Control experiments demonstrated that SAG activity was very low in particulate fractions in both nonsporulating cells and in cells undergoing sporulation.

Nuclear staining. Progress through meiosis and the percentage of sporulation were monitored by use of the fluorescent stain 4,6-diamidino-2-phenylindole (43). Epifluorescence of the cells was observed with a Zeiss fluorescence phase-contrast microscope, and cell types were counted with a phase-contrast hemocytometer. At least 300 cells were counted for each time point.

Analytical methods. SAG was assayed by measuring the rate of glucose release from glycogen using a coupled assay system containing glucose oxidase (EC 1.1.3.4), peroxidase (EC 1.11.1.7), and *o*-dianisidine, as described previously (5), except that 0.33 mM *p*-chloromercuribenzoate (PCMB) was included in the assay mixture in some of the experiments. D-Glucose was used as a standard. Specific activity was expressed in milliunits per milligram of protein, where one unit is defined as 1 μmol of glucose released per minute. Protein was determined by the method of Lowry et al. (26).

RESULTS

SAG appearance in sporulating cells. Cycloheximide arrests sporulation if it is added to the cells at any time before ascus formation is complete (20). Glycogen degradation becomes insensitive to the drug only shortly before this event occurs (20), suggesting that it might be mediated by proteins which are synthesized at this time. To determine whether SAG appearance depended on continued protein synthesis, we examined AP1a/α cells incubated in SPM in the presence and absence of cycloheximide. The addition of cycloheximide (100 μg/ml) after 5 h of incubation in SPM completely prevented SAG appearance (Fig. 1). When the inhibitor was added at 9 h, when SAG specific activity is increasing, no further increase was observed and the level of SAG activity remained constant. In the control culture, SAG appeared normally. This experiment suggests that SAG may be synthesized de novo during sporulation, but it is also possible that its appearance depends on the synthesis of an activator or other proteins.

Sporulation specificity of SAG. Many yeast strains grown under the appropriate condition contain enzymes which are capable of releasing glucose from α-1,4-glucosides and α-1,6-glucosides of various lengths. These include maltase, which can comprise up to 2% of the soluble protein in some strains (28), isomaltase (α-methylglucosidase) (22), and glucoamylase (19). Release of glucose from glycogen could also be due to an amylase in combination with maltase or to glycogen phosphorylase and a phosphatase. Although little or no soluble α-1,4-glucosidase activity is present in AP1a/α cells during pregrowth in PSP (Table 1), it was possible that SAG might be present in some vegetative cells but that this activity had been attributed to one

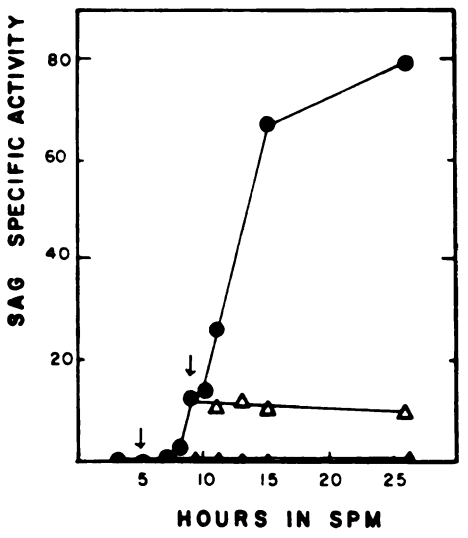

FIG. 1. Inhibition of the appearance of glycogenolytic activity by cycloheximide. A vegetatively growing PSP culture of AP1a/α (~3 × 10^7 cells per ml) was shifted into SPM and incubated at 30°C (●). At 5 (▲) and 9 h (△), cycloheximide (100 μg/ml) was added to portions of the culture as indicated by the arrows. At the indicated times, 50-ml samples were harvested from the three cultures, the cells were stored at −20°C until crude extracts were made by blending in a Vortex mixer with glass beads as described in Materials and Methods. SAG activity was assayed in the presence of 0.33 mM PCMB by measuring the release of glucose from glycogen with glucose oxidase, as described above.

of these other enzymes. SAG activity can be distinguished from other activities observed in crude extracts by sensitivity to inhibitors, particularly the sulfhydryl reagent, PCMB (0.33 mM) (M. J. Clancy and P. T. Magee, manuscript in preparation). We therefore assayed amyloglucosidase activity in the presence and absence of this inhibitor in extracts from AP1a/α cells harvested in the exponential phase of growth in YEP acetate, PSP, and YEPD, in stationary phase in YEPD, and in stationary-phase cells shifted to fresh YEPD for the presence of an enzyme insensitive to this inhibitor. Significant activity against glycogen was observed in extracts from YEP-acetate-grown cells and in those from stationary-phase cells shifted to fresh YEPD, but these activities were about 85 to 90% inhibited by 0.33 mM PCMB (Table 1). The activity in extracts from sporulating cells was not inhibited at this concentration (Table 1), showing that the sporulation activity was distinct from those present in vegetative cells, and that if SAG is present in the vegetative cultures examined, its level must be at least 20 to 100 times lower than in sporulating cells.

Appearance of SAG in sporulating and nonsporulating cells. Cells which are either haploid or diploid and homozygous at the mating type locus (α/α or a/a) undergo the physiological changes associated with starvation which are presumably involved in the initiation of meiosis in a/α cells, but fail to undergo premeiotic S or meiosis (20). To determine whether SAG appearance depended on entry into meiosis or resulted merely from prolonged starvation in SPM, we shifted cultures of the sporulation-proficient AP1a/α and the asporogenous diploid AP1α/α and haploid X2180-1A to SPM and examined them for SAG activity and sporulation at 0, 24, 48, and 72 h after the shift. The specific activity of SAG after 24 h of incubation in SPM was about 200-fold higher in the AP1a/α culture than in the AP1α/α culture and 400-fold higher than in the haploid X2180-1A (Table 2). The SAG activity in the sporulated culture declined after the completion of sporulation, whereas the slight activity observed in the α/α and haploid cultures increased marginally. At 72 h, when the activity in the AP1a/α culture had declined to 50% of the value obtained at 24 h, the activity in the α/α culture was still at least eightfold lower than in the a/α cells. The extent of sporulation was 48% in the a/α culture, and no asci were observed in the other cultures. A similar experiment was performed with the diploid strains AP3a/α, AP3a/a, and AP3α/α (Table 2). As in experiment 1, glycogen-degrading activity was low in the nonsporulating cells (a/a and α/α) but high in the sporulating a/α culture. These results demonstrate that the physiological adaptations to starvation occurring in asporogenous (α/α, a/a, and haploid) cells are not sufficient for SAG expression and suggest that events specific to a/α cells may also be necessary.

Appearance of SAG in cells unable to complete DNA synthesis and recombination. To determine whether premeiotic DNA synthesis is required for SAG appearance, we measured the

TABLE 1. SAG specific activity in AP1a/α cells with glycogen as substrate

Medium	Sp act	
	−PCMB	+PCMB
YEP acetate	2.25	0.221
YEPD		
Exponential growth	0.0381	ND[a]
Stationary phase	0.261	ND
Fresh YEPD[b]	7.66	1.08
PSP	0.309	0.0387
Sporulating cells	23.3	23.7

[a] ND, Not determined.

[b] Cells were grown to stationary phase in YEPD then shifted to fresh YEPD and incubated at 30°C for 30 min.

TABLE 2. SAG specific activity in strains of *S. cerevisiae*[a]

Strain	Sp act at following time in sporulation medium (h):			
	0	24	48	72
AP1a/α	0.202	16.2[b] (ND)[c]	12.5[b] (48)[d]	8.45[b] (43)
AP1α/α	0[b]	0.104[b] (0)	0.697[b] (0)	1.67[b] (0)
X2180-1A	0.0487	0.0382[b] (0)	0.540[b] (0)	1.24[b] (0)
AP3a/α[e]	0.681	9.77 (58.0)	11.3 (ND)	8.07 (78.0)
AP3a/a[e]	0.418	0.369 (0)	0.282 (0)	0.124 (0)
AP3α/α[e]	0.234	0.562 (0)	1.19 (0)	0.446 (0)

[a] Unless otherwise noted, all extracts were made from cells incubated at 30°C in SPM after pregrowth in PSP, and SAG activity was assayed in the presence of 0.33 mM PCMB.
[b] Assayed in the absence of 0.33 mM PCMB.
[c] ND, Not determined.
[d] Numbers in parentheses indicate the percentage of sporulation.
[e] YEP acetate preculture.

specific activity of SAG in AP1a/α cells incubated in SPM in the presence of DNA synthesis inhibitors and in a strain containing a temperature-sensitive mutation affecting DNA synthesis (*cdc4* [39]). Table 3 shows the effect of hydroxyurea (6 mg/ml) (38) and sulfanilamide (12 mg/ml) (4) on the level of SAG in AP1a/α cells incubated for 24 h in SPM after pregrowth in PSP. The specific activity of SAG was about 65-fold lower in the hydroxyurea-treated culture and 220-fold lower in the sulfanilamide-treated culture than in the untreated AP1a/α cells. Cells treated with these inhibitors failed to sporulate and remained mononucleate.

A culture of the homozygous diploid strain *cdc4* (temperature sensitive for vegetative and premeiotic DNA synthesis) was pregrown in YEP acetate at 22°C and shifted to SPM at 34°C (the restrictive temperature) or at 22°C. In this experiment and in those experiments with *spo* mutants (to be described below) the cells were precultured in YEP acetate because these strains grew poorly in PSP. As a result, sporulation was slower than that observed with PSP-grown cells. Samples were removed at 0, 24, 48, and 72 h after the shift, and the cells were monitored for SAG appearance and progress through meiosis. *cdc4* cells at 22°C progressed normally through meiosis and spore formation, as indicated by a high percentage of bi- and tetranucleate cells (data not shown) and by the amount of sporulation at 48 and 72 h (46.7 and 50.3%, respectively). The 34°C cells, however, were generally arrested at the mononucleate stage, and the specific activity of SAG was at least 10 times lower than in the 22°C cells (Table 4). We conclude from these experiments that premeiotic DNA synthesis is necessary for SAG expression in sporulating cells. Further experiments were then performed to determine whether later meiotic events (i.e., recombination and meiotic divisions) were also required for SAG expression.

TABLE 3. SAG specific activity in a/α diploid strain AP1 arrested at premeiotic DNA synthesis[a]

Inhibitor	% Sporulation	Sp act
None	70%	37.6
Hydroxyurea	<0.5%	0.578
Sulfanilamide	<0.3%	0.171

[a] A 100-ml culture of AP1a/α was grown in PSP to a concentration of 3.7×10^7 cells per ml and shifted to 150 ml of SPM. This culture was divided immediately into three subcultures, and hydroxyurea (6 mg/ml) or sulfanilamide (12 mg/ml) was added to two subcultures. The third culture contained no inhibitor (control). After 24 h of incubation in SPM, cells were harvested by centrifugation and broken by blending in a Vortex mixer with glass beads (18). Extracts were prepared and assayed for SAG in the presence of 0.33 mM PCMB as described in Materials and Methods.

AP1a/α cells harbor a mutation, *pac1*, which prevents completion of meiosis at 36°C (7); these cells undergo DNA synthesis, though slightly later than do those at 30°C, form synaptonemal complexes, and become committed to recombination at high levels, but they fail to complete recombination or to undergo the meiotic division. They complete recombination normally if returned to vegetative medium at either 30 or 36°C (unpublished data). To determine whether SAG appears in AP1a/α cells arrested in pachytene at 36°C, extracts were prepared from AP1a/α cells sampled at intervals during incubation in SPM at 30 and 36°C and assayed for this activity. Only the 30°C (control) cells showed a high specific activity of SAG, although some activity was present in 36°C cells at later times (Fig. 2A). This may be due to a slight leakiness of the 36°C block, since a few cells (~5%) were able to progress through meiosis to form asci in some experiments (not shown). The low level of activity observed in the 36°C cells cannot be

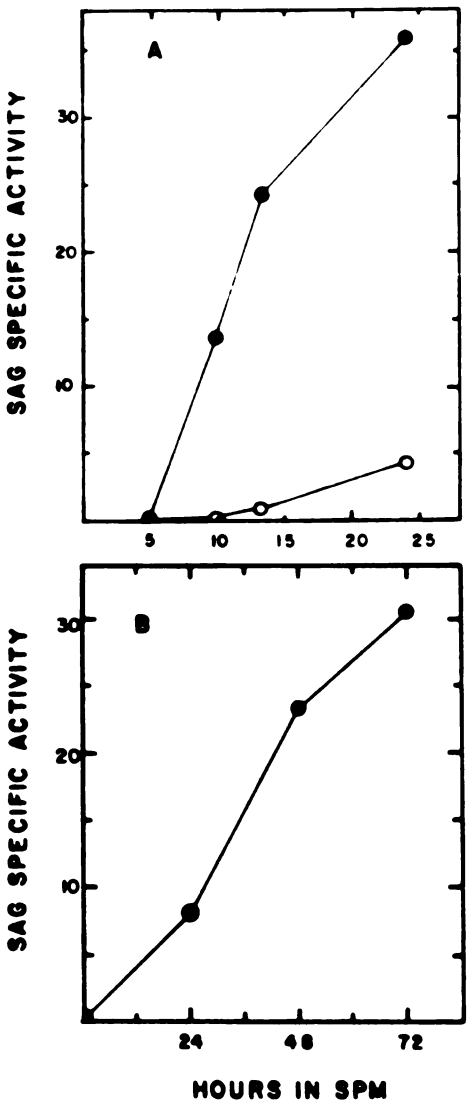

FIG. 2. SAG activity in cells blocked in pachytene or in cells unable to complete recombination. PSP cultures were shifted into SPM at a concentration of 2.5×10^7 cells per ml. (A) AP1*a*/α incubated at either 30°C (●) or 36°C (○). At various times, 50-ml portions were harvested, broken by blending in a Vortex mixer with glass beads, and assayed as in Fig. 1. Ascus formation was 63 and 0% at 24 h in the 30 and 36°C cultures, respectively. (B) PSP preculture of RD-5 was shifted to SPM at 2×10^7 cells per ml and incubated at 30°C. At the indicated times, 250-ml portions were harvested, broken by Bronwell homogenation and assayed as in Fig. 1.

due to loss of viability, since these cells had normal levels of SAG if they were returned to 30°C (not shown).

The failure of cells blocked in DNA synthesis or pachytene to express SAG suggests that the appearance of this activity may require completion of recombination. To examine this possibility, the appearance of SAG was monitored in RD-5, a diploid strain homozygous for the *rad52-1* allele, which increases X-ray sensitivity and reduces sporulation (14, 33). The *RAD52* gene product may be involved in generalized recombination, since mutations in the *RAD52* gene reduce both meiotic (15, 31) and mitotic (27) recombination. RD-5 cells incubated in SPM at 30°C were sampled at 0, 24, 48, and 72 h and examined for progress through meiosis and for SAG activity. Although mature asci were not observed, the appearance of bi- and tetranucleate cells indicated that entry into meiosis had occurred (data not shown). SAG activity was present in these cells at essentially normal levels (Fig. 2B). This shows that successful completion of recombination is not necessary for the appearance of SAG.

SAG appearance in cells unable to complete meiosis. The specific activity of SAG was also determined in sporulation mutants which are blocked at meiosis I (*spo1*) or in nuclear migration after meiosis II (*spo3*) (8). Cultures of *spo1* and *spo3* were grown in YEP acetate at 30°C and shifted to SPM at 22 and 34°C. Samples were taken at intervals for determination of the percentage of sporulation and the specific activity of SAG. In both mutants SAG occurred at roughly the same specific activity in the 22°C cells as in the 34°C cells (Table 4). The specific activity in *spo1* (Table 4) at both temperatures, however, was considerably lower than in *spo3* or its parent strain S41 (Table 4). This was probably due to a failure of many of the *spo1* cells to initiate sporulation even at 22°C, as suggested by the low percentage of asci (9.2%).

We conclude from the experiments with mutants and inhibitors that SAG appearance is a developmental event which depends on DNA synthesis and some pachytene steps but not on completion of the meiotic divisions.

DISCUSSION

The evidence presented here indicates that the amyloglucosidase activity which appears in sporulating *S. cerevisiae* cells at the time of completion of meiosis is developmentally regulated and is probably unique to sporulating cells. The temporal regulation of this activity may be achieved by coordination with the meiotic process. SAG falls into the class of functions which are under the control of mating type, since it is not expressed in **a** cells or α/α and **a**/**a** cells in sporulation medium. Neither does it occur in **a**/α cells under vegetative conditions in which accumulation or degradation of glycogen occurs (23). The low level of activity seen in the nonsporulat-

TABLE 4. SAG specific activity in mutants of *S. cerevisiae*[a]

Strain	Stage of arrest	Sp act at following time in sporulation medium (h):				
		0	24	48	72	96
S41		ND[b] 22°C	2.18 (10.5)[c]	78.4 (48)	64.9 (54)	ND
		34°C	3.00 (4.4)	53.8 (34.0)	51.8 (29)	ND
cdc4	DNA synthesis	0.154 22°C	3.63 (18.0)	23.0 (46.7)	24.6 (50.3)	ND
		34°C	1.69 (0)	1.18 (0)	2.82 (0)	ND
spo1[d]	Meiosis[d]	0.421 22°C	ND	5.54 (7.1)	9.80 (9.2)	12.5 (9.2)
		34°C	ND	9.47 (0)	9.82 (0)	10.5 (0)
spo3[d]	Spore formation	0.151 22°C	1.37 (0)	13.2 (ND)	ND	61.6 (51.0)
		34°C	3.19 (0)	55.7 (ND)	ND	95.5 (9.5)

[a] All strains were preincubated in YEP acetate at 22°C (*cdc4*) or 30°C (S41 and *spo* mutants). SAG-specific activity was determined in the presence of 0.33 mM PCMB after incubation in SPM at 22°C (permissive temperature) and 34°C (restrictive temperature) as described in Materials and Methods.

[b] ND, Not determined.

[c] Numbers in parentheses indicate the percentage of sporulation.

[d] Incubation in SPM was done in the presence of 4.5% KCl.

ing cells may be attributed to another enzyme, since it is sensitive to the addition of PCMB, a compound to which SAG is relatively insensitive. It could also be due to glycogen phosphorylase in combination with a phosphatase. It would thus seem that SAG is a true sporulation-specific enzyme, the only one so far identified, although numerous sporulation-specific activities (such as nucleases and DNA repair enzymes) have been postulated to exist (40). Developmentally specific proteins have also been observed in other systems, particularly in *Dictyostelium discoideum*, in which a series of stage-specific enzyme activities has been detected (24, 25, 36).

The programmed appearance of a substantial enzyme activity and its apparent restriction to sporulating cells is in apparent contradiction to the results of Trew et al. (40) and Kraig and Haber (21), who have looked for sporulation-specific proteins on two-dimensional gels with no success. Several possible explanations for this discrepancy may be considered. For example, the SAG protein might be a very small fraction of the protein synthesis at any one time, it might have properties which make it difficult to detect on a gel (i.e., be very basic), or it might be synthesized in vegetative cells and activated during sporulation by a proteolytic modification. Kraig and Haber have estimated that only the most prominent 10% of proteins synthesized by sporulating cells are observed by two-dimensional analysis; if SAG is not a member of this prominent group of proteins, it could easily have been undetected on the gels (21). These possibilities can be resolved by immunoprecipitation of labeled extracts. Our preliminary experiments with antibody against purified SAG suggest that SAG is, in fact, synthesized de novo during sporulation (Clancy and Magee, unpublished data). The requirement for protein synthesis until the time of SAG appearance (Fig. 1) also supports this notion.

Genetic analysis of mutants defective in the cell cycle (*cdc*) (17, 31, 37, 39) and sporulation (*spo*) (8, 9) and similar mutants in other developmental systems (*D. discoideum* [25], *Polysphondylium violaceum* [42], and *Caulobacter crescentus* [29]) has led to models for development in which the order of events (i.e. in this case, DNA replication, nuclear division, etc.) is fixed; as a consequence particular events fail to occur when early events are blocked. The existence of these developmental mutants in *S. cerevisiae* has enabled us to ask whether SAG appearance is coordinated with the meiotic process and to characterize relatively precisely the stage in sporulation upon which it is dependent. Our results demonstrate that SAG activity fails to appear in asporogenous cells and in cells in which sporulation is arrested before or during premeiotic DNA synthesis (by the *cdc4* mutation or the inhibitors hydroxyurea or sulfanilamide). When meiosis is arrested at the first division (*spo1*) or after the second division (*spo3*), however, SAG appearance occurs normally. This indicates that SAG appearance in sporulating cells depends on premeiotic DNA synthesis or later steps, but not on completion of the meiotic divisions. The results obtained when SAG appearance was measured in cells arrested during recombination in *pac1* or *rad52* cells indicate that SAG expression depends on some recombination steps but not on the successful completion of recombination. These experiments demonstrate that SAG appearance depends on progress through meiosis and that SAG

is a developmentally regulated enzyme. An understanding of the molecular basis of this regulation will require isolation and a detailed characterization of the SAG gene and its product; these experiments are currently in progress.

ACKNOWLEDGMENTS

The authors acknowledge the assistance of the departmental clerical staff, especially B. Schmidt, in the preparation of this manuscript and D. Lee for help with some of the experiments. We also thank L. Snyder and R. Patterson for critically reading the manuscript and R. Malone, R. Esposito, and B. Byers for sending their strains.

This manuscript was supported by National Science Foundation grant PCM 7812581-04.

LITERATURE CITED

1. **Alton, T. H., and H. F. Lodish.** 1977. Developmental changes in messenger RNA and protein synthesis in *Dictyostelium discoideum*. Dev. Biol. **60**:180–206.
2. **Barnett, T., C. Pachl, J. P. Gergen, and P. C. Wensick.** 1980. The isolation and characterization of *Drosophila* yolk protein genes. Cell **21**:729–738.
3. **Cheung, K. K., and A. Newton.** 1977. Patterns of protein synthesis during development of *Caulobacter crescentus*. Dev. Biol. **56**:417–425.
4. **Colonna, W. J., J. M. Gentile, and P. T. Magee.** 1977. Inhibition by sulfanilamide of sporulation in *Saccharomyces cerevisiae*. Can. J. Microbiol. **23**:659–671.
5. **Colonna, W. J., and P. T. Magee.** 1978. Glycogenolytic enzymes in sporulating yeast. J. Bacteriol. **134**:844–853.
6. **Crees, A. F.** 1967. Induction of meiosis in yeast. I. Timing of cytological and biochemical events. Planta **76**:209–226.
7. **Davidow, L., L. Goetsch, and B. Byers.** 1980. Preferential occurrence of nonsister spores in two-spored asci of *Saccharomyces cerevisiae*: evidence for regulation of spore-wall formation by the spindle pole body. Genetics **94**:581–595.
8. **Esposito, M. S., and R. E. Esposito.** 1974. Genes controlling meiosis and spore formation in yeast. Genetics **78**:215–225.
9. **Esposito, M. S., and R. E. Esposito.** 1978. Aspects of the genetic control of meiosis and ascospore development inferred from the study of *spo* (sporulation-deficient) mutants of *Saccharomyces cerevisiae*. Biol. Cellulaire **33**:93–102.
10. **Esposito, M. S., R. E. Esposito, M. Arnaud, and H. O. Halvorson.** 1969. Acetate utilization and macromolecular synthesis during sporulation of yeast. J. Bacteriol. **100**:180–186.
11. **Esposito, R. E., N. Frink, P. Bernstein, and M. S. Esposito.** 1972. The genetic control of sporulation in *Saccharomyces*. II. Dominance and complementation of mutants of meiosis and spore formation. Mol. Gen. Genetics **114**:241–248.
12. **Fosset, M., L. W. Muir, L. O. Nielsen, and E. H. Fischer.** 1971. Purification and properties of yeast glycogen phosphorylase a and b. Biochemistry **10**:4105–4113.
13. **Fritz, H., G. Hartwich, and E. Werle.** 1966. Uber Protease inhibitoren. I. Isolierang and Charakterisierung des Trypsininhibitors aus Pankreasgewebe und Pankreassekret. Hoppe-Seylers Z. Physiol. Chem. **345**:150–157.
14. **Game, J. C., and R. K. Mortimer.** 1974. A genetic study of x-ray sensitive mutants in yeast. Mutat. Res. **24**:281–292.
15. **Game, J. C., T. J. Zamb, R. J. Braun, M. Resnick, and R. M. Roth.** 1980. The role of radiation (rad) genes in meiotic recombination in yeast. Genetics **94**:51–68.
16. **Halvorson, H.** 1966. α-Glucosidase from yeast. Methods Enzymol. **8**:559–562.
17. **Hartwell, L. H.** 1974. *Saccharomyces cerevisiae* cell cycle. Bacteriol. Rev. **39**:164–198.
18. **Hereford, L. M., and L. H Hartwell.** 1974. Sequential gene function in the initiation of *Saccharomyces cerevisiae* DNA synthesis. J. Mol. Biol. **84**:445–461.
19. **Hopkins, R. D., and D. Kulka.** 1957. The glucamylase and debrancher of *S. diastaticus*. Arch. Biochem. Biophys. **69**:45–55.
20. **Hopper, A. K., P. T. Magee, S. K. Welch, M. Friedman, and B. D. Hall.** 1974. Macromolecule synthesis and breakdown in relation to sporulation and meiosis in yeast. J. Bacteriol. **119**:619–629.
21. **Kraig, E., and J. E. Haber.** 1980. Messenger ribonucleic acid and protein metabolism during sporulation of *Saccharomyces cerevisiae*. J. Bacteriol. **144**:1098–1112.
22. **Lai, H. L., and B. Axelrod.** 1975. The specificity of the synthetic reaction of two yeast α-glucosidases. Biochim. Biophys. Acta **391**:121–128.
23. **Lillie, S. H., and J. R. Pringle.** 1980. Reserve carbohydrate metabolism in *Saccharomyces cerevisiae*: responses to nutrient limitation. J. Bacteriol. **143**:1384–1394.
24. **Loomis, W. F., Jr.** 1969. Developmental regulation of alkaline phosphatase in *Dictyostelium discoideum*. J. Bacteriol. **100**:417–422.
25. **Loomis, W. F., Jr., S. White, and R. Dimond.** 1976. A sequence of dependent stages in the development of *Dictyostelium discoideum*. Dev. Biol. **53**:171–177.
26. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
27. **Malone, R. E., and R. E. Esposito.** 1980. The RAD52 gene is required for homothallic interconversion of mating types and spontaneous mitotic recombination in yeast. Proc. Natl. Acad. Sci. U.S.A. **77**:503–507.
28. **Needleman, R. B., H. J. Federoff, T. R. Eccleshall, B. Buchferer, and J. Marmur.** 1978. Purification and characterization of an α-glucosidase from *Saccharomyces carlsbergensis*. Biochemistry **17**:4657–4661.
29. **Osley, M. A., and A. Newton.** 1980. Temporal control of the cell cycle in *Caulobacter crescentus*: roles of DNA chain elongation and completion. J. Mol. Biol. **138**:109–128.
30. **Piñon, R., Y. Salts, and G. Simchen.** 1974. Nuclear and mitochondrial DNA synthesis during yeast sporulation. Exp. Cell Res. **83**:231–238.
31. **Prakash, S., L. Prakash, W. Burke, and B. A. Montelcone.** 1980. Effects of the RAD52 gene on recombination in *Saccharomyces cerevisiae*. Genetics **94**:34–50.
32. **Pringle, J.** 1975. Methods for avoiding proteolytic artifacts in studies of enzymes and other proteins from yeast. Methods Cell Biol. **12**:149–184.
33. **Resnick, M. A.** 1969. Genetic control of radiation sensitivity in *Saccharomyces cerevisiae*. Genetics **62**:519–531.
34. **Roth, R., and H. O. Halvorson.** 1969. Sporulation of yeast harvested during logarithmic growth. J. Bacteriol. **98**:831–832.
35. **Roth, R., and K. Lusnak.** 1970. DNA synthesis during yeast sporulation: genetic control of an early developmental event. Science **168**:493–494.
36. **Roth, R., and M. Sussman.** 1968. Trehalose 6-phosphate synthetase (uridine diphosphate glucose:D-glucose-6-phosphate 1-glucosyl transferase) and its regulation during slime mold development. J. Biol. Chem. **243**:5081–5087.
37. **Schild, D., and B. Byers.** 1978. Meiotic effects of DNA-defective cell division cycle mutations of *Saccharomyces cerevisiae*. Chromosoma **70**:109–130.
38. **Silva-Lopez, E., T. J. Zamb, and R. Roth.** 1975. Role of premeiotic replication in gene conversion. Nature (London) **253**:212–214.
39. **Simchen, G., and J. Hirschberg.** 1977. Effects of the mitotic cell-cycle mutation cdc4 on yeast meiosis. Genetics **86**:57–72.
40. **Trew, B. J., J. Friesen, and P. Meens.** 1979. Two-dimensional protein patterns during growth and sporulation in *Saccharomyces cerevisiae*. J. Bacteriol. **138**:60–69.
41. **Waring, G. L., and A. P. Mahowald.** 1979. Identification

and time of synthesis of chorion proteins in *Drosophila melanogaster*. Cell 16:599–607.
42. Warren, A. J., W. D. Warren, and E. C. Cox. 1976. Genetic and morphological study of aggregation in the cellular slime mold *Polysphondylium violaceum*. Genetics 83:25–47.
43. Williamson, D. H., and D. J. Fennell. 1975. The use of fluorescent DNA-binding agent for detecting and separating yeast mitochondrial DNA. Methods Cell Biol. 12:335–351.