## INFORMATION TO USERS

The most advanced technology has been used to photograph and reproduce this manuscript from the microfilm master. UMI films the original text directly from the copy submitted. Thus, some dissertation copies are in typewriter face, while others may be from a computer printer.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyrighted material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each oversize page is available as one exposure on a standard 35 mm slide or as a 17″ × 23″ black and white photographic print for an additional charge.

Photographs included in the original manuscript have been reproduced xerographically in this copy. 35 mm slides or 6″ × 9″ black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

Accessing the World's Information since 1938

300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA

Order Number 8801689

# Structural and functional analysis of the *MAL* loci of *Saccharomyces*

Charron, Maureen Joan, Ph.D.

City University of New York, 1987



U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

**PLEASE NOTE:**

In all cases this material has been filmed in the best possible way from the available copy. Problems encountered with this document have been identified here with a check mark ✓.

1. Glossy photographs or pages ✓
2. Colored illustrations, paper or print _____
3. Photographs with dark background ✓
4. Illustrations are poor copy _____
5. Pages with black marks, not original copy _____
6. Print shows through as there is text on both sides of page _____
7. Indistinct, broken or small print on several pages ✓
8. Print exceeds margin requirements _____
9. Tightly bound copy with print lost in spine _____
10. Computer printout pages with indistinct print _____
11. Page(s) _____ lacking when material received, and not available from school or author.
12. Page(s) _____ seem to be missing in numbering only as text follows.
13. Two pages numbered _____. Text follows.
14. Curling and wrinkled pages ✓
15. Dissertation contains pages with print at a slant, filmed as received ✓
16. Other _____

_____

_____

# STRUCTURAL AND FUNCTIONAL ANALYSIS OF THE *MAL* LOCI OF *SACCHAROMYCES*

by

Maureen Joan Charron

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

1987

This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

July 8, 1987
date

Corinne A. Michels
Chairman of Examining Committee
Prof. Corinne Michels, Queens College

July 9, 1987
date

Executive Officer
Prof. Peter C. Chabora

Prof. David Alsop, Queens College

Prof. Sharon D. Cosloy, City College

Prof. Marian Carlson, Columbia University

Prof. Carol Newlon, N.J. Med. School

Prof. Richard Needleman, Wayne State College of Med.

Supervisory Committee

The City University of New York

## Abstract

STRUCTURAL AND FUNCTIONAL ANALYSIS OF THE MAL LOCI OF SACCHAROMYCES

by

Maureen Joan Charron

Advisor: Professor Corinne A. Michels

Maltose fermentation by the Saccharomyces yeasts requires the presence of any one of five dominant multigene complexes (MAL1, MAL2, MAL3, MAL4 and MAL6) each of which encodes maltose permease (GENE 1), maltase (GENE 2) and the trans-acting MAL-activator (GENE 3). Four of these loci have been mapped (MAL1 - MAL4) and each is located at or near the telomeres of a different chromosome.

Using molecular cloning techniques each of the MAL loci were isolated. I describe the physical structure of the MAL loci and their flanking sequences. The MAL loci were shown to be both structurally and functionally homologous throughout an approximately 9.0 kb region. The orientation of the MAL loci was determined to be: CENTROMERE ... GENE 3 - GENE 1 - GENE 2 ...TELOMERE. Telomere-adjacent sequences were found flanking GENE 2 of the MAL1, MAL3 and MAL6 loci. No common repeated elements were found on the centromere-proximal side of all of the MAL loci. These results suggest that the MAL loci translocated to different chromosomes via a mechanism that involved the rearrangement(s) of chromosome termini.

In addition to the dominant MAL loci, several partially functional alleles have been identified which are linked to the MAL1 and MAL3 loci. Four naturally occurring alleles of MAL1 have been characterized (MAL1, MAL1p, MAL1g, $mal1^0$). MAL1 encodes all three genes needed for fermentation. The MAL1p allele functionally encodes only the MAL activator; the MAL1g allele functionally encodes a maltose permease and maltase; and the $mal1^0$ allele functionally encodes only maltase. Molecular analysis of these alleles and several kilobasepairs of flanking sequences indicates that MAL1p, MAL1g and $mal1^0$ have evolved from MAL1 by a series of rearrangements and/or deletions of this telomere-associated locus. I also describe the structure of a unique maltose transport gene contained within MAL1g.

Two alleles of MAL3 have been identified in yeast (MAL3, MAL3g). Similar analysis of these alleles has revealed that MAL3g is structurally and functionally homologous to MAL3 and MAL6 throughout a 6.3 kb region which contains the structural genes for maltase and permease. Analysis of flanking sequences demonstrates that MAL3g evolved from MAL3 via chromosomal rearrangement(s).

Additively, these results emphasize the high degree of fluidity of telomere-adjacent sequences.

## Dedication

This thesis is dedicated, in loving memory, to my grandmother Mary Magdalene Sena. Thank you for always believing in me. You are forever in my heart and in my prayers.

# Acknowledgements

I wish to thank the many people who have played influential roles in this thesis project.

First, I wish to express my deepest gratitude to my mentor, Dr. Corinne Michels for your careful guidance and support, and for teaching me the meaning and value of the word "independence", all of which have enabled me to grow as both a scientist and a woman.

Next, I wish to thank Dr. Richard Needleman for many discussions, critical comments on manuscripts, strains and assays. Here is to many more years of fruitful collaborations between your lab and Corinne's.

I also wish to extend my gratitude to Dr. Peter Chabora and Dr. Elizabeth Boylan for advice and support over the years. To Dr. Marian Carlson and the members of her lab for plasmids, yeast strains and discussion of results prior to publication.

Many thanks to my second examination and dissertation defense committee members namely, Dr. Richard Needleman, Dr. Marian Carlson, Dr. Carol Newlon, Dr. David Alsop, Dr. Sharon Cosloy and Dr. Amar Klar.

I am especially thankful to have been able to work in the lab with an excellent group of people whom I wish to acknowledge by name. First, I wish to thank my closest labmate Robert Dubin for all the strains, plasmids, discussions and especially for all the questions that always

kept me on my toes. To Sheryl "Right Arm" Haut, a superb assistant and very good friend, also the best media maker in town, thank you. To Joel Levine, a wonderful listener and person, may your love for the three S's never end. To Genevieve Cannon and George Bauries, your patience and persistance in tetrad dissections were of great help to me. I also wish to acknowledge the other students and assistants in our laboratory who have made for many colorful memories: Jeong "M.Y.O.B." Kim, Qi "I doubt it" Cheng, Esther Shiffman, Leanne Tanouye, Yolande Sylvestre, Marion Geras, Juan Guzman, Kathy Piscioneri and Marianne Violagis.

I would also like to thank the other graduate students, faculty and staff members of the Queens College Biology Department for their help and support. Many special thanks to Sylvia Schaffel for secretarial assistance with manuscripts.

To my best friends, Mary Ellen Calitri, Karen Dong, Ellen Katz, Roberta Koepfer and Helen Sabzevari you know how I feel; you were always there when I needed you most and that is what "true friendship" is all about.

Most of all, I wish to thank my family, my parents Joseph and Marie and my sister Joan and her husband Patrick for your endless support, encouragement and most important, your love; without you this would never have been possible.

Table of Contents

# List of Tables

Appendix 2

# List of Figures

# INTRODUCTION

Despite the fact that the utilization of sugars is a major aspect of the metabolism of *Saccharomyces* yeast, only recently has significant information become available concerning the various mechanisms of uptake and hydrolysis of some sugars and their role in the biochemistry of the yeast cell. These sugars include galactose, glucose, maltose, raffinose and sucrose. Much less is known about the utilization of $\alpha$-methylglucoside, melezitose and trehalose. Recent advances in recombinant DNA technology have facilitated both the cloning and *in-vitro* mutagenesis of yeast genes, resulting in a more detailed understanding of the structure, function and regulation of genes. These techniques make yeast an excellent model system for studying cellular functions in eukaryotes and have contributed greatly to our knowledge of the processes of sugar fermentation in yeast. This thesis focuses on the structure, evolution and regulation of the genes involved in the utilization of maltose by the *Saccharomyces* yeasts.

The classification of yeast is based, in part, upon the ability to utilize various sugars. Many, but not all strains of *Saccharomyces* yeasts are capable of fermenting maltose. "Bakers" yeast (many of which ferment maltose) and "brewers" yeast (all of which ferment maltose) are frequently classified as *Saccharomyces cerevisiae* which, according to Barnett (1981), includes the species *S.*

pastorianus, S. logos, S. bayanus, S. willianus, S. carlsbergensis, S. heterogenicus and S. diastaticus. Winge and Roberts (1948) were the first to study the genetic basis of maltose fermentation. Using several S. cerevisiae strains, they identified three MAL loci, MAL1, MAL2 and MAL3, each of which alone was capable of enabling the strain to ferment. While these loci appeared to be functionally equivalent, they mapped to different genomic sites and were unlinked. Winge and Roberts later went on to identify another MAL locus unlinked to the others, MAL4 locus, which was detected upon analyzing the results of a hybrid cross between an S. chevalieri strain and an S. cerevisiae strain (1950). The MAL6 locus was originally identified in S. carlsbergensis strains and again it is unlinked to the other MAL loci. Halvorson and Ellias (1958) studied the MAL1 locus of S. italicus strain Y1225. Therefore the MAL loci appear to represent a family of loci present in closely related species of Saccharomyces.

Four of the five MAL loci have been genetically mapped. All of these are located at or near a telomere: MAL1, the right arm of chromosome VII (Celenza and Carlson, 1985); MAL2, the right arm of chromosome III; MAL3, the right arm of chromosome II; and MAL4, the right arm of chromosome XI (Mortimer and Schild, 1980). The MAL6 locus has been mapped to chromosome VIII but the exact map position is unknown to date (R. Dubin, personal communication).

Upon analyzing various species of Saccharomyces isolated from the wild, Naumov (1971; 1972; 1976) determined that at least two complementation groups were involved in the fermentation of maltose. These complementation groups are known as MALp and MALg, which are both required in order to ferment maltose. From the eleven strains analyzed by Naumov, three alleles of the MAL1 locus were identified: MAL1, MAL1p and MAL1g. MAL1 was found in S. cerevisiae and S. italicus strains (Winge and Roberts, 1948; Halvorson and Ellias, 1958); MAL1p was present in S. paradoxus and MAL1g was found in S. globosus, S. carlsbergensis, S. chevalieri and S. paradoxus. In this analysis Naumov also detected a partially functional MALg allele of the MAL3 locus, namely MAL3g (originally characterized as MAL2g). MAL3g was detected in S. globosus, S. paradoxus and S. carlsbergensis strains.

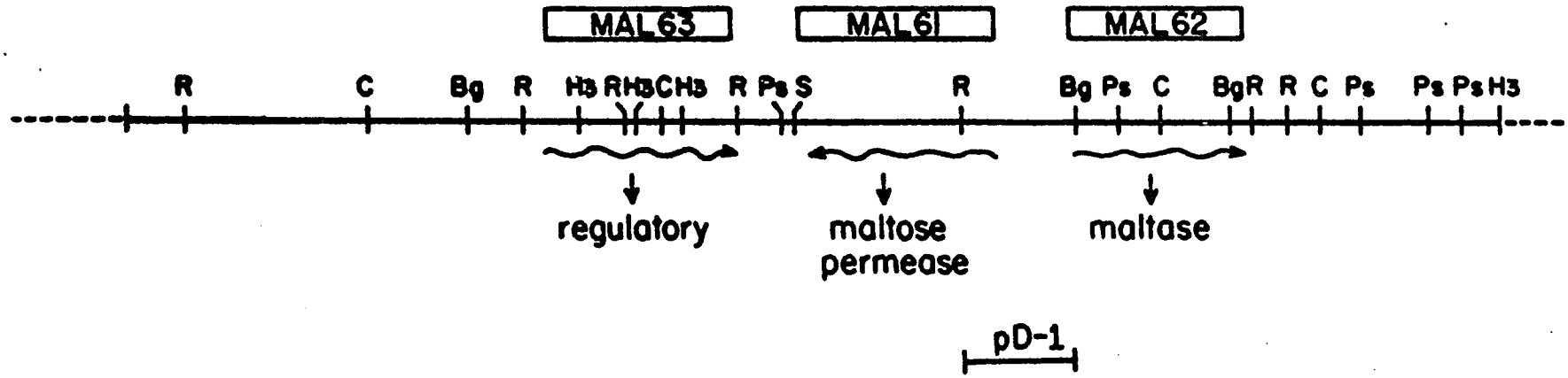
These strains were also functionally characterized and it was shown that MAL1p and MAL1g alleles when present singly are insufficient for maltose fermentation. Naumov (1976) then looked for complementation between the MAL1g or MAL1p and the maltose regulatory mutant mal6-17 isolated by ten Berge et al. (1974). The mal6-17 mutation was complemented by the MAL1p allele and not the MAL1g allele, implying that MAL1p codes for a regulatory protein that is functionally equivalent to that coded for by the MAL6 locus. Naumov (1976) speculated that MALg may encode

the structural genes (maltase and maltose permease). The presence of the MAL1 locus is enough to allow for maltose fermentation, suggesting that it is a complex locus having MALp and MALg function. Naumov also found strains which were not complemented by either the MAL1p or the MAL1g alleles. These were referred to as $mal^0$ strains. This allele was originally detected in S. paradoxus and S. chevaliere strains. Needleman and Michels (1983) were able to demonstrate the presence of MAL1-linked sequences in $mal^0$ strains thereby identifying a fourth MAL1 allele referred to as $mal1^0$.

Federoff et al. (1982) cloned a segment of DNA from the S. carlsbergensis strain CB11 (MAL6 MAL1g MAL3g) capable of complementing $mal6^-$ mutations. With this, the possibility presented itself of performing a detailed genetic and physical analysis of the naturally occurring maltose fermenting strains maintained by the Berkeley Yeast Stock Center. Using a subclone of this fragment (pD-1, see Figure 1), Needleman and Michels (1983) were able to demonstrate that this fragment was in fact derived from the MAL6 locus and that two additional homologies to this fragment were present in the genome of the standard MAL6 strain CB11. Genetic analysis, using MAL1p and MAL1g strains obtained from Naumov, enabled Needleman and Michels (1983) to demostrate that the two homologies were linked to MAL1 and MAL3. They also demonstrated that these loci alone were not fully functional MAL loci but

Figure 1: The MAL6 locus and its transcripts.

The restriction map of the MAL6 locus of strain CB11 is shown, along with the locations of the three transcribed gene regions MAL61, MAL62 and MAL63, their directions of transcription (~~>) and functions. Plasmid pD-1 is also shown.


MAL63
MAL6
MAL62
R
C
Bg
R
H3
R H3
C H3
R
Ps
S
R
Bg
Ps
C
Bg R
R
C
Ps
Ps
Ps H3
regulatory
maltose permease
maltase
pD-1

did complement MALp strains and thus represented the MAL1g and MAL3g loci.

Michels and Needleman (1983; 1984) then went on to characterize the other four standard maltose fermenting strains and showed that they too carried one dominant, fully functional, MAL locus and one or two additional MALg loci (homologous to plasmid pD-1) which were linked to either MAL1, MAL3, or both. Their results are summarized in Table 1. In summary, these results demonstrate that the MAL loci of the Saccharomyces yeasts represent a dispersed family of repeated loci. One of the main objectives of this thesis project is to examine the structure and genetic organization of the dominant and the partially functional MAL loci and to use these results to postulate a molecular mechanism for the evolution of the MAL family of loci.

Four mechanisms of genomic rearrangements can be postulated to explain the evolution of this small family of repeated genes. Firstly, transposable elements have been implicated in genomic rearrangements and duplications in both prokaryotes and eukaryotes. Many of these mobile elements contain transposase and resolvase activities which allow for replication and integration into various genomic locations. During the process of transposition, certain classes of prokaryotic transposable elements (class I) can carry with them sections of adjoining host chromosomal DNA (Kleckner, 1981). Thus, transposable

Table 1: Summary of the genetic and physical analysis of the standard MAL strains (Michels and Needleman, 1983; 1984).

Listed are the standard MAL strains which have been used in the various studies of maltose fermentation genetics and of maltase synthesis. The table summarizes the genotype of each of these strains including the dominant MAL locus associated with ability to ferment maltose as well as the partially functional MALg loci described in that report. Physical analysis of the genome of each of these strains showed multiple HindIII fragments with homology to the MAL6 probe pD-1 (Figure 1). These fragments are listed for each strain and the MAL locus shown to be linked to each of these fragments is indicated.

[a] Indicates fragments showing poor homology to the probe.

Table 1

Summary of the genetic and physical analysis of the standard MAL strains

| Strain (dominant MAL locus) | Genotype | Size (in kb) of HindIII fragments in each standard strain showing homology to the MAL6 probe (linked MAL locus) | |
|---|---|---|---|
| 4059 (MAL1) | MAL1 MAL3g | 7.3, (3.9)[a] | (MAL1) |
| | | 7.1 | (MAL3g) |
| 1453-3A (MAL2) | MAL2 MAL1g MAL3g | 7.6 | (MAL2) |
| | | 10.7 | (MAL1g) |
| | | 8.1 | (MAL3g) |
| | | 8.1 | ($mal^0$, unknown locus position) |
| 1412-4D (MAL3) | MAL3 MAL1g | 7.3 (4.7, 4.5)[a] | (MAL3) |
| | | 10.7 | (MAL1g) |
| 1403-7A (MAL4) | MAL4 MAL1g MAL3g | 7.6 | (MAL4) |
| | | 10.7 | (MAL1g) |
| | | 8.1 | (MAL3g) |
| | | 7.0 | ($mal^0$, unknown locus position) |
| CB11 (MAL6) | MAL6 MAL1g MAL3g | 7.3 | (MAL6) |
| | | 10.7 | (MAL1g) |
| | | 7.3 | (MAL3g) |

elements can act as vectors for translocating genes. It is unlikely that this mechanism can account for the dispersal of the MAL loci for the following reasons. Transposable elements similar to the prokaryotic class I elements have not yet been characterized in yeast. Additionally, the insertion of such elements into the prokaryotic genome occurs essentially at random, while the MAL loci apparently are not distributed randomly since all of those identified are located near the chromosome telomere.

A second proposed mechanism of transposition would utilize a special class of repeated elements called retrotransposons. The yeast Ty element is one such retrotransposon. Retrotransposons have been shown to transpose via the formation of an RNA intermediate, a mechanism reminiscent of retroviral transmission (Boeke et al., 1985; Garfinkel, et al., 1985). In transforming retroviruses, chromosomal genes are seen to become part of the retrovirus genome and can thus be transposed to new chromosomal locations by viral integration (Duesberg, 1983). The MAL6 locus has been shown to contain at least three divergently transcribed regions (Needleman et al., 1984). It is difficult to envision the MAL6 multigene complex as a member of this class of repeated elements because its translocation would necessitate the co-reverse transcription of these independent, divergently transcribed messages.

While the first two mechanisms are random with regard to the target site of the mobilized sequence, there are two other mechanisms that are more site specific. The well characterized mating type switching mechanism of S. cerevisiae involves the transposition of DNA from the silent cassettes HMR or HML into the MAT locus (Nasmyth and Tatchell, 1980; Nasmyth, 1983). This interconversion is initiated by the introduction of a double-strand break at the Y-Z junction of the MAT locus (Strathern et al., 1982). A site-specific endonuclease named YZ endonuclease generates this double-strand cut at the Y-Z junction in HO, SWI1 strains (Kostricken et al., 1983). It is possible that a similar mechanism is involved in the mobilization of the MAL loci.

The final mechanism of transposition, which has been referred to as telomeric translocation, involves the exchange of genetic information between non-homologous telomeres. For reasons that will be outlined, I hypothesize this as the most likely mechanism of translocation of the MAL loci.

As described previously, each of the mapped MAL loci are located at or near a telomere. Similar genomic arrangements are seen in other sugar fermentation genes. Of the six SUC loci (SUC1-5, and SUC7), which control sucrose fermentation, all those mapped have been found to be located at a telomere except SUC2 (Mortimer and Schild, 1980; Celenza and Carlson, 1985). The SUC1 and SUC3 loci

are tightly linked to the MAL1 and MAL3 loci, respectively. The MGL loci, which are involved in α-methylglucoside fermentation, are not as well characterized as the MAL or SUC loci. Three MGL loci (MGL1-3) have been studied in Saccharomyces carlsbergensis (ten Berge, 1972) and only the MGL2 locus has been mapped. MGL2 is tightly linked to the MAL3 locus (Mortimer and Schild, 1980) and is thus telomere linked. The genes coding for the two yeast isozymes hexokinase A and B (HXK1 and HXK2 respectively) also map to telomeres and have been shown to be highly sequence homologous (Frohlich et al., 1984). What is interesting to note in this case is that the HXK1 and HXK2 loci have acquired different functions. Both are structural genes for hexokinase but the enzymes appear to be differentially regulated. They serve different roles in glucose metabolism, HXK2 mutants relieve glucose repression while HXK1 mutants do not (Entian and Frohlich, 1984). Another example of a dispersed repeated gene family is found in S. diastaticus, a yeast that is closely related to S. cerevisiae. S. diastaticus contains three unlinked glucoamylase genes, (STA1, STA2, and STA3). All three of these genes have been cloned and are highly sequence homologous (Yamashita, et al., 1985; Pretorious et al., 1986a; 1986b). The map positions of the genes in this repeated family are, at present, undetermined. It will be interesting to see if the STA genes, which are required for starch utilization,

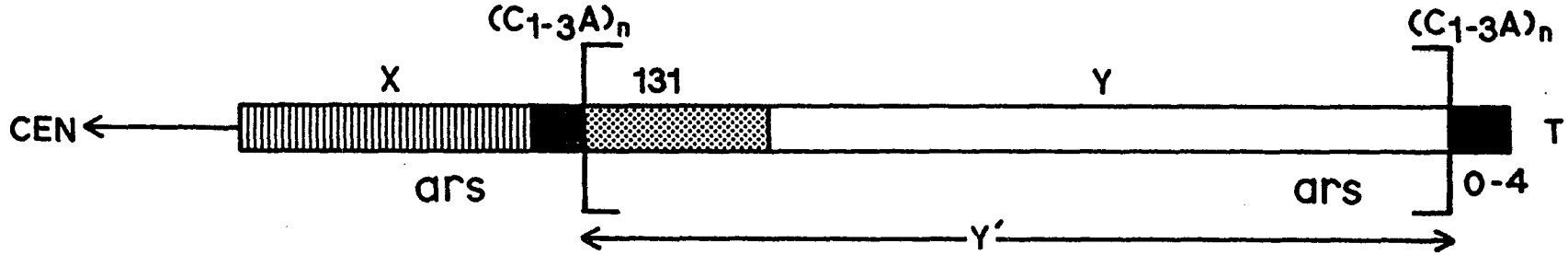
are also linked to telomeres.

These data collectively suggest that at least two themes may occur in the evolution of the yeast sugar fermentation gene families: (1) a common mode of dispersal is indicated by the telomeric location of the loci; (2) movement as a complex unit is suggested by the (MAL-SUC-MGL) linkage associations. Perhaps examining the structure of the telomere and adjacent DNA sequences will provide some insight into the mechanism of localization of these loci.

Telomeres are specialized structures required for the complete replication and segregation of the chromosome. The structure of the telomere of a number of microorganisms, including yeast, has been the subject of intensive research during the last few years. It has been shown that telomeres contain species-specific terminal repeat sequences, single-strand gaps and Z-DNA (Chan and Tye, 1983a; 1983b; Walmsley et al, 1983; Blackburn and Szostak, 1984; Walmsley et al., 1984). S. cerevisiae telomeres have been cloned (Szostak and Blackburn, 1984) and have been shown to contain ARS (autonomous replicating sequence) activity (Chan and Tye, 1983a; 1983b). Figure 2, adopted from Walmsley, et al. (1984), is a schemmatic representation of a typical yeast telomere containing the following repeated elements: X (0.3-3.75kb), Y (5.2kb), I31 (1.0-1.5kb) and $C_{1-3}A$ (0.33-0.6kb). Several classes of telomeres have been identified thus far, those which

Figure 2: Schemmatic representation of a typical yeast telomere.

Hatched bars represent X sequence. Solid bars indicate $C_{1-3}A$ sequences. Y sequence is represented by open bars and $131^3$ sequence by stipled bars. 131 sequence and Y sequence are the elements contained within a Y' element. Y' sequences may be present in 0-4 copies per telomere. The line indicates DNA sequences that are centromere-proximal to X sequence. T represents the chromosome terminus. This figure is adopted from Walmsley et al. (1984).


(C1-3A)n
(C1-3A)n
X
131
Y
CEN
T
ars
ars
0-4
Y'

contain between 1 and 4 copies of Y' sequences (Horowitz et al., 1984; Walmsley et al., 1984), and those which lack Y' sequences (Button and Astell, 1986). Recent reports suggest that several of the smaller yeast chromosomes have little or no sequence homology to X and/or Y' probes. This observation seems to be strain specific (Zakian et al., 1986). It has been shown that the length of the terminal $C_{1-3}A$ repeat sequence is somewhat variable (Walmsley and Petes, 1985) and is influenced by the products of the CDC17 (Carson and Hartwell, 1985), TEL1 and TEL2 genes (Lustig and Petes, 1986).

Strong sequence homology is observed among telomeres of S. cerevisiae in comparison to the poor homology between S. cerevisiae and other species of Saccharomyces, suggesting that a rapid exchange occurs between telomeres of non-homologous chromosomes within a species (Chan and Tye, 1983a). If telomere adjacent sequences (such as X and Y') do not end abruptly at the terminus of the chromosome but are found dispersed between structural genes mapping near the telomere, or if other as yet unidentified repeated squences common to several telomere-adjacent regions are present at the centromere-proximal side of a locus, then exchange processes between these sequences should result in the translocation of these structural genes to non-homologous chromosomes. Additionally, in the absence of any such centromere-proximal repeated sequences, random

breakage at these sites followed by the healing of this fragment to a non-homologous chromosomal end could also lead to translocation. We believe that one of these mechanisms is likely to account for the dispersal of the MAL loci. Given these mechanisms, certain predictions can be made. The organization of the genes within a complex locus such as a MAL locus should be identical when the different loci are compared. In addition, the orientation of the loci with regard to the telomere should be the same. Repeated telomere-adjacent sequences (such as X and Y') or some other repeated sequence could flank either one or both sides of these loci.

Results obtained with the SUC gene family suggest that such telomeric rearrangements may have played a role in the evolution of this repeated gene family (Carlson et al., 1985). Carlson et al. (1985) demonstrate, through the combined cloning and physical analysis of the dominant SUC loci and the $suc^0$ alleles, that a great deal of sequence homology exists among the SUC loci and in some instances this homology extends into the flanking DNA. Sequence homology to X and Y' probes is seen in the 5' and 3' flanking regions, respectively of some SUC loci demonstrating that the SUC loci have become embedded within the structure of the telomere (Carlson and Botstein, 1983; Carlson et al., 1985). These results suggest that the SUC loci could have become dispersed via a telomere - telomere recombination event between

non-homologous chromosomes perhaps involving X sequences located on the centromere-proximal side of these SUC loci.

Other examples of this type of transposition mechanism have been reported in a number of organisms. These include the sex reversion factor (Sxr) and the steroid sulphatase region (STS) of Mus (Jones and Singh, 1982; Craig and Tolley, 1986) and the sex realizer gene in Megaselia scalaris (Mainx, 1964; Green, 1980). The pseudoautosomal DNA sequences in the pairing region, including the MIC2 locus, of the human sex chromosomes may also undergo telomere transposition events (Buckle et al., 1985; Cooke et al., 1985). A variation on this theme may be acting in the expression of the variable surface glycoproteins (VSG) of trypanosomes (Englund et al., 1982). The VSG genes of T. brucei are either telomerically located or telomerically translocated and activated by splicing on a tandemly reiterated 35 bp leader sequence (DeLange, et al., 1983; van der Ploeg et al., 1984).

Cytological studies have shown that telomeres of homologous and non-homologous chromosomes interact during interphase and throughout meiosis (Wagenaar, 1969; Ashley, 1979), making it possible for telomeric exchanges to occur. In light of the above arguments, telomere-linked transposition of genes as a mechanism of gene dispersal could be important in the evolution of a number of gene families of diverse origin.

The second objective of this report is to compare the partially functional alleles of the MAL1 and MAL3 loci to the dominant allele. Horowitz et al. (1984) found extensive restriction endonuclease polymorphisms in chromosome termini of different strains of S. cerevisiae, demonstrating that telomeres undergo frequent rearrangements. Tetrad analysis revealed that these rearrangements occurred either before or during meiosis. The pseudoautosomal region of human sex chromosomes is not as polymorphic as yeast telomeres, however unique restriction fragment polymorphisms are detected upon pedigree analysis (Cooke et al., 1985; Simmler et al., 1985). The comparison of the alleles of the MAL1 and MAL3 loci was performed with the hope of determining the mutation process leading to the loss of selected functions, as well as, analyzing the structural heterogeniety of these telomere-associated sequences.

## OVERVIEW

This thesis is organized into two parts. First, I have prepared four chapters that address questions concerning the molecular organization and function of the genes present at all of the MAL loci, as well as the partially functional alleles of the MAL1 and MAL3 loci. Secondly, I have prepared a series of appendices. The first two appendices address questions concerning the molecular nature of two MAL-linked constitutive mutants, the last four appendices contain the results of the genetic analyses cited throughout the text.

Chapter one is entitled "Structural and functional analysis of the MAL1 locus of Saccharomyces". This manuscript co-written by myself, Robert A. Dubin and Corinne A. Michels appeared in Molecular and Cellular Biology Volume 6 (3891-3899). In this report we describe the molecular cloning and functional analysis of the MAL1 locus and its linkage to SUC1. We also compare MAL1 to the already characterized MAL6 locus.

The data presented in this paper represent the union of a series of experiments performed independently by Robert Dubin and myself (each contributing approximately equally to the project). My contribution to this work is presented in the first half of the paper which includes the cloning, characterization and complementation analysis of the MAL1 phage isolates, as well as linkage to SUC1.

Robert Dubin's contribution to this study is presented in the second half of the paper which includes construction and analysis (physical and functional) of MAL1 gene disruption strains, complementation of these mutations with MAL6 plasmids, and Northern analysis of MAL1 and MAL1 gene disruption strains. Also, Genevieve Cannon assisted in the tetrad analyses involved with these experiments.

Chapter two is entitled "Comparative structural and functional analysis of the alleles of the MAL1 locus of Saccharomyces". In this report we extend our study of the MAL1 locus to include the partially functional MAL1p, MAL1g and mal1$^{0}$ alleles. Most of the analyses done on the MAL1g allele was performed on plasmid pFE52 which was cloned in Dr. Marian Carlson's laboratory at Columbia University College of Physicians and Surgeons and was generously provided for these studies. At a later time, I also cloned the MAL1g locus and went on to repeat some, but not all of the experiments performed on pFE52. Since there was complete agreement between the results obtained with my clone and pFE52 in all preliminary experiments, further analysis of the second MAL1g clone isolated was not warranted. An interesting observation derived from these studies was the presence of a functionally equivalent yet structurally (physically) different maltose permease gene at this locus. On the other hand, the MALg allele that is linked to the MAL3 locus has been shown to contain sequences that are both structurally and

functionally homologous to MAL61 (Charron, 1983 and Chapter 3), thus implying that this other maltose permease is not common to all MALg alleles and may be unique to MAL1g. All of the experiments performed in this analysis were done by myself except that Robert Dubin assisted in constructing several MAL1g subclones and Genevieve Cannon aided in tetrad analysis.

Chapter three is entitled "Molecular cloning and physical analysis of the alleles of the MAL3 locus of Saccharomyces". This chapter represents an extension of my Masters thesis entitled "Molecular cloning and characterization of a MAL3g locus present in Saccharomyces cerevisiae". The major findings of this study demonstrate that the MAL3g locus is highly sequence homologous to both the MAL3 and MAL6 loci throughout a region of approximately 6.5 kb. Contained within this homologous region are the genes encoding both maltase and maltose permease. The results reported in this chapter represent the joint efforts of several individuals namely, Dr. Corinne Michels who constructed the 1453-2A library, Robert Dubin who screened this library and isolated phage λ26aq and Nancy Schorshinski who subcloned and performed partial restriction enzyme mapping on the isolated clone λ26aq. Assistance with heteroduplex mapping of pY6-R and pM1.1B, as well as interpretation of the electron micrographs was performed by Dr. Louise Chow of the University of Rochester Medical School. All other

analyses reported in this section were performed by myself.

Chapter four is entitled "Molecular evolution of the telomere-adjacent MAL loci of Saccharomyces". This report amplifies the findings of Chapter one which addresses only the relationship between the MAL1 and MAL6 loci. This chapter contains the complete comparison of the five dominant MAL loci (MAL1, MAL2, MAL3, MAL4 and MAL6) as well as several kilobasepairs of flanking DNA sequences. All the experiments performed in this section were done by myself except that Sheryl Haut aided in experiments dealing with the orientation of the MAL2 locus and the analysis of the MAL3 flanking DNA sequences during the final stages of this project.

The next section is composed of a series of appendices. Appendix one is entitled "The constitutive, glucose repression-insensitive mutation of the yeast MAL4 locus is an alteration of the MAL43 gene". This report describes the molecular cloning and genetic characterization of the gene responsible for constitutive synthesis of maltase and permease present at the MAL4 locus, namely the MAL43 gene. This report has appearred in Genetics Volume 116 (23-31) and was co-written by myself and Corinne A. Michels. All of the experiments contained within this section were performed by myself except that George Bauries aided in tetrad dissections.

Appendix two entitled "Interaction between the MAL64,

MAL64C, MAL63, mal63-13 and mal63-10 gene products". In this section I have summarized the major findings of a manuscript entitled "Constitutive expression of the maltose fermentative enzymes in Saccharomyces carlsbergensis is dependent upon the mutational activation of MAL64, a nonessential homologue of MAL63" which has been submitted for publication in Molecular and Cellular Biology by Robert A. Dubin, Maureen J. Charron, Sheryl R. Haut, Richard B. Needleman and Corinne A. Michels. This report focuses on the molecular cloning, localization and functional characterization of both the wild-type and constitutive MAL64 gene and its product. In addition to the results of this report I present some preliminary results from experiments that I have performed using the clones described in this manuscript.

My contribution to this work was in the construction of the integrative vectors used to clone the genes discussed; isolation of MAL64, MAL64-C2 and mal63-13 from the Saccharomyces genome; restriction enzyme mapping and Southern analysis of the clones; construction of plasmids pMAL64, pMAL64ΔXbaI, pMAL64-C2 and pMAL64-C2ΔXbaI and transformation of these plasmids into various strains followed by Southern analysis to determine the copy number of the integrated plasmid, and PNPGase (maltase) assays on selected transformants. These results are presented in this appendix. Robert Dubin's contribution to this work included the construction of the episomal derivatives of

the MAL64 and MAL64-C2 clones shown in Figures 1 and 2, as well as, the disruption plasmids utilized; physical and genetic analysis of all disruption strains; PNPGase (maltase) assays on all disruption strains; and all Northern analyses required for this study except one (strain A9 in YEP plus 2% galactose probed with plasmid pZ-1) which was done by Sheryl Haut, an undergraduate student in our laboratory. Edward Perkins of the Wayne State College of Medicine performed all of the maltose permease, maltase and α-methylglucosidase assays reported here.

Appendix three is entitled "Genetic demonstration of the presence of SUC1 in strain 600-1B". Genevieve Cannon and myself performed all the analyses included in this section. Contained within this appendix are the results of the genetic analyses which demonstrate that the MAL1 strain 600-1B contained the SUC1 locus. Results of this analysis are cited in Chapter one.

Appendix four is entitled "Genetic demonstration of the linkage of integrated plasmids pM1.4B, pY6ΔCΔH, pM3.2B, pY6-RΔC and pMJC6ΔC to various MAL loci". Again the work for this appendix was performed by Genevieve Cannon and myself in order to confirm the linkage of integrative plasmids used for cloning and analyzing the flanking DNA sequences at all of the MAL loci. Results from these analyses are cited in Chapters one, two and four, as well as in Appendices one and two.

Appendix five, entitled "Genetic demonstration of disruption sites", provides genetic evidence for the replacement of MAL43-C, MAL23 and MAL23-C by URA3 and MAL41/MAL42 by LEU2. Results of these analyses are cited in Appendix one. George Bauries and myself generated the data presented in this section.

Appendix six, entitled "Genetic demonstration of the random segregation of the maltase gene in strain 53-2C", summarizes the results of both tetrad and random spore analysis of diploid strain MCY148 [53-2C (MAL1p) x 100-1B (MAL13 MAL11 ΔMAL12)] which prove that the maltase structural gene present in strain 53-2C is unlinked to MAL1p. All of the work presented in this section was performed by myself.

# Chapter 1

## Structural and functional analysis of the *MAL1* locus of *Saccharomyces cerevisiae*

This chapter has been reproduced with the permission of the publisher of *Molecular and Cellular Biology*. It appeared in *Molecular and Cellular Biology* 6: 3891-3899, 1986.

## ABSTRACT

We describe the isolation of a 22.6 kb fragment of DNA containing the MAL1 locus of Saccharomyces cerevisiae. Our results demonstrate that the MAL1 locus, like the MAL6 locus, is a complex locus containing three genes. These genes are organized similarly to their MAL6-counterparts. We refer to them as MAL11, MAL12 and MAL13, and show that they are functionally homologous to the MAL61 (encoding maltose permease), MAL62 (encoding maltase) and MAL63 (encoding the positive regulator) genes of the MAL6 locus. Transcription from each of the three genes was analyzed in a strain carrying the undisrupted MAL1 locus and in strains carrying single disruptions in each of the MAL1 genes.

The MAL1 and MAL6 loci were found to be highly sequence homologous and conserved throughout the region containing these three genes. The strain used to isolate the MAL1 locus also carried the tightly linked SUC1 gene. The SUC1 gene was found to be located on the same 22.6 kb fragment containing the MAL1 locus and 5 kb from the 3' end of the MAL12 gene. The meaning of these results with regard to the mechanism of regulation of maltose fermentation is discussed.

# INTRODUCTION

The fermentation of maltose by the Saccharomyces yeasts requires the presence of at least one of a series of five unlinked loci: MAL1, MAL2, MAL3, MAL4 or MAL6 (Barnett, 1976). Several standard maltose fermenting laboratory strains have been genetically analyzed and shown to carry one dominant MAL locus and one or two additional, partially functional, MAL loci. Physical analysis of these strains revealed extensive sequence homology between the cloned MAL6 locus and the other MAL loci (Chow et al., 1983; Michels and Needleman, 1983; 1984; Needleman et al., 1984).

The maltose fermentative enzymes, maltase and maltose permease, are subject to both maltose induction and glucose repression. In the presence of maltose, both of these enzymes are induced approximately 30-fold while, in the non-induced state, only low basal levels of these enzymes are present (de Kroon and Koningsberger, 1970; Ouwehwnd and van Wijk, 1972). It has been postulated that a positive regulatory function is required for induction and that a gene encoding this regulatory function maps to each of the dominant MAL loci (ten Berge et al., 1973a; Zimmerman and Eaton, 1974). More recent genetic analysis of maltose fermentation has revealed other functions encoded by the MAL loci which are required for fermentation (Goldenthal et al., 1983; Needleman and

Michels, 1983; Cohen et al., 1984; Dubin et al., 1985; 1986; Chang et al., submitted). Federoff et al. (1982) cloned a segment of DNA from the Saccharomyces carlsbergensis strain CB11 which was shown to contain the MAL6 locus (1984). This cloned fragment contains three transcribed regions which we refer to as MAL61, MAL62 and MAL63 (1983). All three genes are required for maltose fermentation. Gene disruption experiments at the MAL6 locus have confirmed these results (Dubin et al., 1986; Chang et al., submitted). MAL63 has been shown to encode the positive regulatory gene product required for induction of maltase and maltose permease (Chang et al., submitted). Gene disruption experiments have shown that MAL62 is the structural gene encoding maltase (Dubin et al., 1985). MAL61 is believed to code for maltose permease (Cohen et al., 1985; Chang et al., submitted). Mutational analysis of the MAL1 locus indicated that this is also a complex locus (Cohen et al., 1984).

In this report we describe the molecular cloning and genetic dissection of the MAL1 locus. Our results demonstrate that MAL1 is a complex locus containing at least three genes which we refer to as MAL11, MAL12 and MAL13. These genes are organized similarly to their MAL6 counterparts and are structurally and functionally homologous to MAL61, MAL62 and MAL63, respectively. We also show that the MAL1 and MAL6 loci are highly sequence conserved throughout the region containing these three

genes and that this homology ends abruptly in the flanking DNA sequences. Physical linkage of the MAL1 and SUC1 loci is demonstrated.

# METHODS AND MATERIALS

## Strains and Growth Conditions

The yeast strains used in this study are listed in Table 1. Their MAL genotype was determined using the type of genetic analysis described in Michels and Needleman (1983). Plasmids were propagated in E. coli strain RR1.

Yeast strains were grown in YEP medium [(1%(wt/vol) yeast extract/1%(wt/vol) peptone] plus the indicated amount of a specified carbon source. Maltose fermentation is defined as the production of acid and gas in 1-3 days after inoculation and determined in 5ml of YEP plus 2%(wt/vol) maltose medium in Durham tubes. Sucrose fermentation was determined by the ability to produce gas and acid in 1-3 days after inoculation into 5ml YEP plus 2%(wt/vol) sucrose medium in Durham tubes and/or the ability to grow anaerobically on YEP plates containing 2%(wt/vol) sucrose at 30$^{o}$C.

## Gene Disruptions and Plasmid Rescue

Yeast transformation was performed by the method of Ito et al. (1983) using lithium acetate. For gene disruptions (1983) and for site-directed integration (1983), transformants which stably maintained the selective marker were further screened by Southern gel transfer analysis and by standard genetic analysis (1966).

Gene disruption plasmids were constructed using

Table 1

List of yeast strains

| Strain | Genotype |
|---|---|
| 4059 | MATα MAL1 MAL3g SUC1 leu1 ade1 ade2 |
| 600-1B | MATa MAL1 SUC1 SUC(?) ura3-52 leu2-3,112 |
| MCY621 | MAT MAL1g MAL3g $suc2^{am}$ ura3-52 ade |
| MCY619 | MATa MAL1g MAL3g $suc2^{am}$ ura3-52 $his4-339^{am}$ |
| DBY739 | MATα MAL1g MAL3g SUC1 ura1 |
| MCY106-3D | MATa MAL1g MAL3g SUC1 ura1 |
| 53-2C | MATα MAL1p met |
| 1-31 | MATa MAL1p met |
| 236-2A | MATa MAL1p leu2-3,112 lys2 |
| 345-4A | MATa MAL1p ura3-52 leu2-3,112 trp1 ade |
| 208-6D | MATα MAL1g lys ade |
| 340-1A | MATα MAL1g ura3-52 ade |
| JC27 | MATα MAL1g MAL3g leu2-3,112 his |
| 340-1C | MATa MAL1g ura3-52 lys ade |
| 340-2B | MATa MAL1g ura3-52 trp1 lys2 met |
| 303-2B | MATα $mal1^0$ leu2-3,112 ade |
| 303-3A | MATα $mal1^0$ leu2-3,112 ade1 |
| 208-1B | MATα $mal1^0$ lys ade |
| 328-4A | MATα $mal1^0$ ura3-52 trp1 met14 ade |
| 6-2A | MATα $mal1^0$ lys2 |
| 3-2B | MATa $mal1^0$ lys2 |

cloned MAL6 DNA sequences. Plasmids pDM2b and pDM3 have been previously described (Dubin et al., 1985; Chang et al., submitted). Plasmid pDM1b was constructed by subcloning the 3.0 kb PstI fragment containing the MAL61 gene from plasmid pY6 (Needleman et al., 1984) into a pBR322 derivative that had been deleted for its EcoRI and AvaI restriction sites. The resulting plasmid was restricted with EcoRI and AvaI, which cut only within the MAL61 gene, blunt-ended with T4 DNA polymerase, and EcoRI linkers were ligated on. The resulting plasmid, pB, contains a 900 bp deletion within the MAL61 gene and a single EcoRI site. A 3.0 kb EcoRI fragment containing the URA3 gene (along with approximately 350 bp of DNA from pBR322 and 1.7 kb of DNA from the yeast LEU2 locus) was subcloned into the EcoRI site of pB, creating pDM1b.

Plasmid pY6ΔCΔH contains 1.6 kb of MAL6 DNA and has been previously described (Dubin et al., 1986). This plasmid was integrated at MAL1 and plasmids pMJC1BamHI and pMJC1ΔH were isolated following digestion of genomic DNA of strain 600-1BΔCΔH#7 with BamHI and HindIII respectively, according to the method of Orr-Weaver (1983).

Measurement of p-nitrophenyl-α-D-glucopyranosidase Activity

Maltase activity was measured as the rate of release of p-nitrophenol from p-nitrophenol-α-D-glucopyranoside,

as previously described (Dubin et al., 1985).

DNA and RNA Isolation and Analysis

Plasmid DNA and yeast DNA and RNA isolations as well as Southern and Northern analyses were carried out as previously described except poly $[A]^+$ RNA was isolated following a single pass through oligo (dT) cellulose (Neeleman and Michels,1983; Dubin et al., 1985).

Large scale preparation of bacteriophage DNA was performed by the method of Blattner et el. (1977). Small scale DNA preparations were performed according to the method of Maniatis et al. (1982).

Bacteriophage Library Construction and Screening

Total genomic DNA from the strain 4059 was partially restricted with EcoRI and size fractionated on a sucrose density gradient. Fragments between 7 and 20 kb were pooled and ligated to gradient purified λgtWES B EcoRI arms. Packaging extracts were prepared by the method of Enquist and Sternberg (1979) using E. coli strains NS428 and NS433. This library was amplified in E. coli strain K803 (Maniatis et al., 1982) and $1.6 \times 10^5$ pfu were screened (Benton and Davis, 1977) using MAL6-derived probes.

# RESULTS

Cloning of the MAL1 locus. It has been previously shown that sequences homologous to the MAL6-derived probe pD-1 (see Figure 1) are present at other MAL loci including MAL1 (Chow et al., 1983; Michels and Needleman, 1983; 1984; Needleman and Michels, 1984). Based upon these results, we proceeded to clone the MAL1 locus from strain 4059. Strain 4059 has the MAL-related genotype MAL1 MAL3g (Michels and Needleman, 1983). A total genomic digest of strain 4059 shows three HindIII fragments having homology to probe pD-3 (Figure 1): a 7.3 kb MAL1-linked fragment, a 7.1 kb MAL3g-linked fragment, and an approximately 4.5 kb MAL1-linked fragment having weak homology (results not shown). A λgtWES B phage library was constructed from an EcoRI partial digest of strain 4059 genomic DNA. Five phage isolates homologous to the MAL6-derived probes pD-1, pD-3 or pP2.2 were selected for analysis (see Figure 1). The yeast inserts were digested with EcoRI and the fragment(s) showing homology to the pD-3 probe was determined by Southern analysis. The phage were found to contain either a 2.3kb, a 2.6kb or a 6.6kb EcoRI fragment homologous to the probe. One phage isolate, λMJC1.3, contained both the 2.3 kb and 2.6 kb fragments. Based upon a previous analysis of a cloned 11.0 kb BamHI fragment containing the MAL3g locus of strain 4059 (results to be reported elsewhere), the 6.6 kb

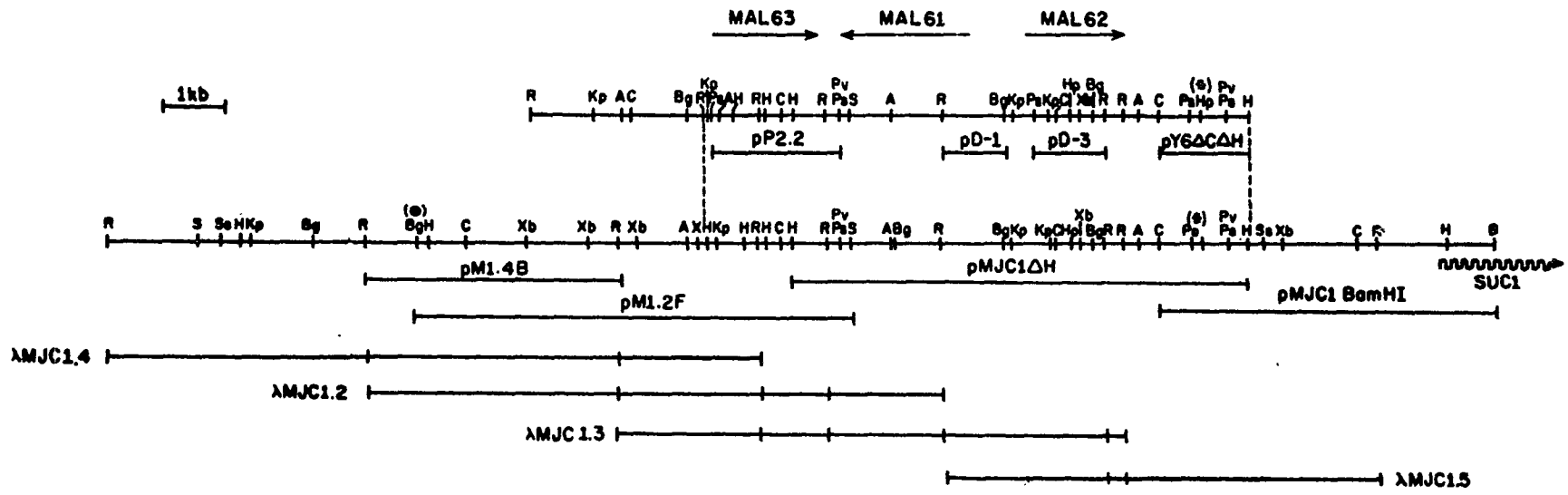
Figure 1: Restriction endonuclease maps of the MAL1 and MAL6 loci.

A partial restriction map of plasmid YEpMAL6, which contains the MAL6 locus of strain CB11, is shown at the top of the figure. The location and direction of transcription of the MAL61, MAL62 and MAL63 genes is indicated (Needleman et al., 1984). Regions of the MAL6 locus were subcloned into either pBR322 or pBR325 to form plasmids pP2.2, pD-1 and pD-3. Plasmid pY6ΔCΔH was constructed by subcloning the indicated ClaI - HindIII fragment from the MAL6 locus into YIp5 and then deleting the single HindIII site.

The restriction endonuclease map of the 22.6 kb sequence of DNA containing the MAL1 locus is shown below that of the MAL6 locus. The overlapping yeast DNA inserts contained in each of the phage isolates, λMJC1.2 through λMJC1.5, are shown at the bottom of the figure. Restriction mapping of these inserts was used to construct the composite map of the MAL1 locus shown. Plasmid pM1.4B contains the indicated EcoRI fragment subcloned into the vector YIp5. The BglII - SalI fragment of λMJC1.2 was subcloned into the vector YEp24 to form plasmid pM1.2F. The symbol (●) is used to indicate the site of integration of plasmid pM1.4B into strain 600-1B. The isolation of plasmids pMJC1Δ H and pMJC1BamHI is described in the Methods and Materials. The symbol (*) is used to indicate the site of integration of plasmid pY6ΔCΔH into strain 600-1B as the first step in the isolation of plasmids pMJC1ΔH and pMJC1BamHI.

The boundaries of the homology between the MAL1 and MAL6 loci are indicated by the vertical dashed lines. The region showing homology to the SUC2 gene probe pRB117 and the direction of transcription of the SUC1 gene is indicated by the wavy line.

Recognition sites of restriction endonucleases are abbreviated as follows: A, AvaI; B, BamHI; Bg, BglII; C, ClaI; H, HindIII; Hp, HpaI; Kp, KpnI; Ps, PstI; Pv, PvuII; R, EcoRI; S, SalI; Ss, SstI; Xb, XbaI and X, XhoI.


MAL63
MAL61
MAL62
1kb
pP2.2
pD-1
pD-3
pY6ΔCΔH
pM1.4B
pMJC1ΔH
pM1.2F
pMJC1 BamHI
SUC1
λMJC1.4
λMJC1.2
λMJC1.3
λMJC1.5

EcoRI fragment has been shown to be derived from the MAL3g locus of strain 4059. Thus, those phage isolates found to contain this 6.6 kb EcoRI fragment were not further analyzed.

Restriction enzyme mapping was performed on subclones of the remaining phage isolates, λMJC1.2 - λMJC1.5, and used to construct the composite map shown in Figure 1. This composite map is consistent with previously reported results (Michels and Neeldeman, 1983). The results reported below demonstrate that the 22.6 kb DNA region contained in these overlapping phage is derived from the MAL1 locus.

Structural comparison between the MAL1 and MAL6 loci.

Prior to this study the extent of the sequence homology between the MAL6 and MAL1 loci had not been determined. Southern analysis using probes spanning the entire cloned MAL6 locus was performed on λMJC1.2 - λMJC1.5. The results obtained show that the homology is quite extensive and includes the region containing the three MAL6 genes: MAL61, MAL62 and MAL63. Within this 9.0 kb region, the restriction maps of MAL1 and MAL6 are largely identical. The boundaries of homology between MAL6 and MAL1 are depicted in Figure 1 by vertical dashed lines. Homology diverges rapidly in the region 5' to MAL63. Preliminary analysis of MAL6 DNA sequences from the region flanking the 3' end of MAL62 suggests that the

homology between MAL6 and MAL1 ends near the HindIII site shown in Figure 1 (data not shown).

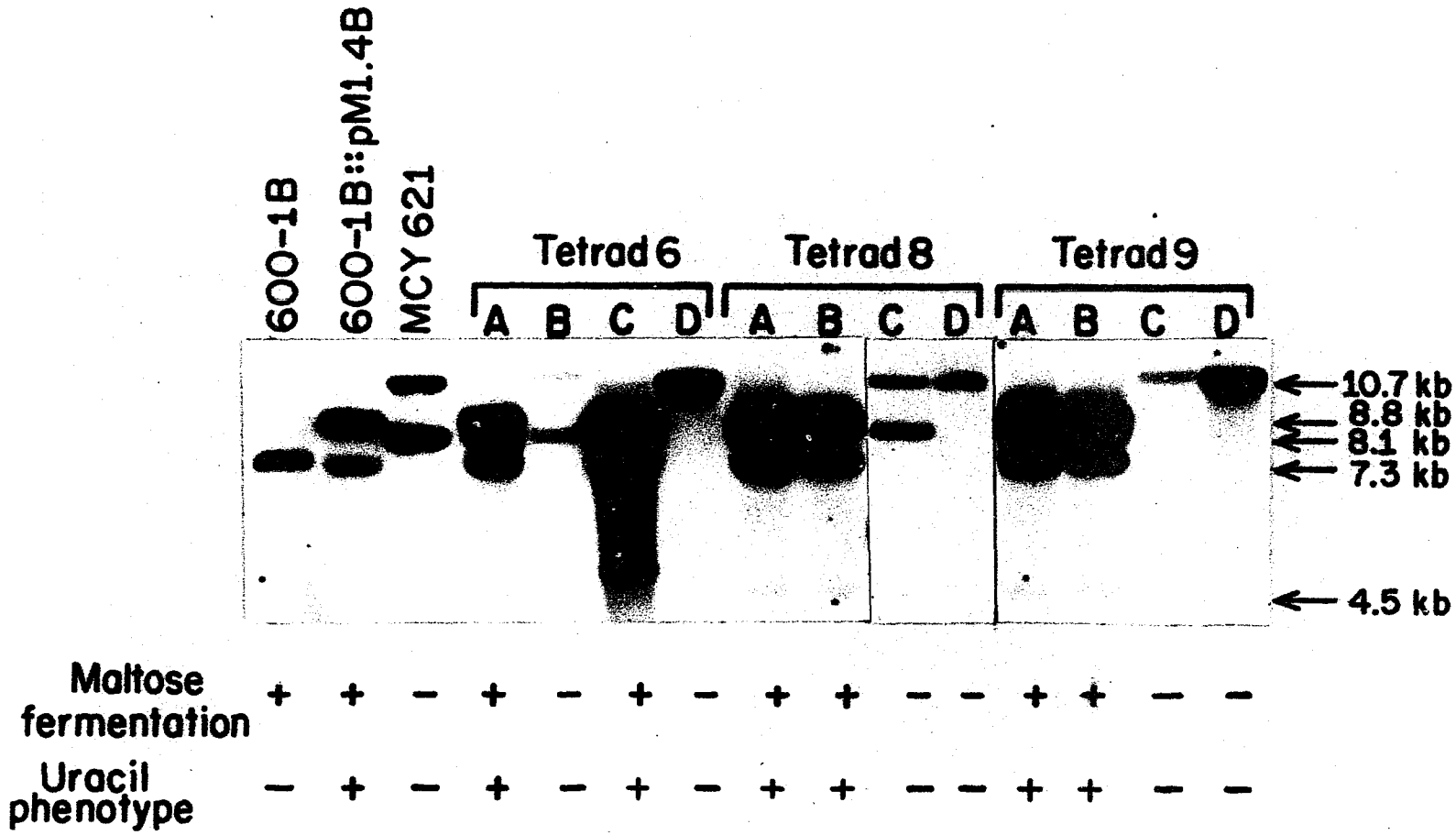
Analysis of MAL1 flanking DNA. In order to be assured that the EcoRI fragments contained in the phage clones actually flank the MAL1 locus, linkage association of these fragments to the MAL1 locus needs to be established. Also, we hoped to isolate the flanking region to the right of phage λMJC1.5. For this the MAL6-derived plasmid pY6ΔCΔH was used (Figure 1). Integration was directed to MAL1 by digesting the plasmid with HpaI prior to transforming strain 600-1B, a derivative of strain 4059 which contains only the MAL1 locus. Stable $Ura^+$ transformants were selected and integration at the MAL1 locus was confirmed for one strain, 600-1BΔCΔH#7, by Southern and genetic analyses (see Appendix 4). Genomic DNA from strain 600-1BΔCΔH#7 was used to isolate plasmid pMJC1BamHI as described above. The restriction endonuclease map of plasmid pMJC1BamHI is shown in Figure 1 and is consistent with the map derived from phage λMJC1.5 as well as genomic Southern analysis of this region.

Linkage of the EcoRI fragments contained in phage λMJC1.4 to the MAL1 locus was demonstrated in the following manner. Plasmid pM1.4B is described in Figure 1. Plasmid pM1.4B was linearized with BglII and used to transform strain 600-1B. Genetic and physical analyses

of one stable $Ura^+$ transformant, 600-1B::pM1.4B, (results described below) showed that the plasmid inserted at a site linked to the MAL1 locus. Strain 600-1B::pM1.4B was mated to strain MCY621 to form diploid MCY105, which was subsequently sporulated and subjected to tetrad analysis. The genotype of strain MCY621 is MAL1g MAL3g $suc2^{am}$ and thus it does not ferment either maltose or sucrose. In 13 tetrads analyzed, the ability to ferment maltose segregated 2:2 and the $Mal^+$ and $Ura^+$ phenotypes cosegrated (see Appendix 4). Genomic DNA was prepared from strain 600-1B, 600-1B::pM1.4B, MCY621 and three complete tetrads derived from diploid MCY105 (tetrads 6,8,9). These were digested with HindIII, subjected to Southern analysis and probed with the MAL6-derived plasmid pD-1. Results of this analysis are shown in Figure 2. The 7.3 kb HindIII fragment seen in all $Mal^+$ segregants corresponds to the 7.3 kb HindIII fragment of the MAL1 locus. The 4.5 kb HindIII fragment is also linked to the MAL1 locus. The 8.8 kb HindIII fragment corresponds to the plasmid pM1.4B sequences integrated outside of the MAL1 locus. As can be seen in Figure 2, all three of these HindIII fragments cosegrate with the $Mal^+/Ura^+$ phenotype. The 10.7 kb and 8.1 kb HindIII fragments correspond to the MAL1g and MAL3g loci respectively (Michels and Needleman, 1984). The MAL1g-linked HindIII fragment (10.7 kb) can be seen to segregate in repulsion to the three MAL1-linked fragments (8.8, 7.3 and 4.5 kb). The 8.1 kb MAL3g-linked

Figure 2: Southern analysis of tetrads from diploid MCY105 (600-1B::pM1.4B x MCY621).

Construction of plasmid pM1.4B is described in Figure 1. Plasmid pM1.4B was restricted with BglII and used to transform strain 600-1B (MAL1 SUC$^+$ ura3-52) to form strain 600-1B::pM1.4B. This transformant was mated to strain MCY621 (mal suc) to form diploid MCY105. Three tetrads from diploid MCY105, tetrads 6,7 and 8, were analyzed physically. Shown above is the result of Southern analysis of HindIII digested total genomic DNA from each of these strains probed with the MAL6-derived probe pD-1 (see Figure 1). The phenotype with respect to both maltose fermentation and uracil prototrophy is indicated.


600-1B
600-1B::pM1.4B
MCY 621
Tetrad 6
Tetrad 8
Tetrad 9
A B C D
A B C D
A B C D
10.7 kb
8.8 kb
8.1 kb
7.3 kb
4.5 kb
Maltose fermentation
+ + − + − + − + + − − + + − −
Uracil phenotype
− + − + − + − + + − − + + − −

fragment segregates in a Mendelian fashion and is unlinked to the other fragments in all three tetrads shown in Figure 2. Clearly, the integration of plasmid pM1.4B has occurred at a site linked to MAL1.

The restriction map derived from the phage isolates predicts that KpnI sites should flank the integration site of plasmid pM1.4B in strain 600-1B::pM1.4B. Based upon the restriction map of phage λMJC1.4, the genomic KpnI fragment homologous to plasmid pM1.4B is expected to be 6.95 kb in size. Southern analysis confirms this prediction (results not shown). Furthermore, integration of plasmid pM1.4B at the indicated BglII site is predicted to increase this KpnI fragment to 16.35 kb. Southern analysis of KpnI digested genomic DNA from strain 600-1B::pM1.4B gives the expected result (results not shown). Together these data confirm that the non-overlapping EcoRI fragment contained in phage λMJC1.4 flanks the MAL1 locus. It was concluded that the composite restriction endonuclease map obtained from the phage clones represents the genomic organization of the MAL1 locus along with several kilobasepairs of DNA sequences flanking this locus.

Linkage to SUC1. The MAL1 and SUC1 loci are tightly linked (Mortimer and Hawthorne, 1966; Carlson et al., 1985; Celenza and Carlson, 1985). The genotype of strain 4059 is reported as MAL1 SUC1 by the Berkeley Yeast

Stock Center. Thus, strain 600-1B should also carry the SUC1 gene. Analysis of 13 tetrads from diploid MCY105 (600-1B::pM1.4B x MCY621) described above indicated the presence of a two unlinked SUC genes in strain 600-1B. All of the $Mal^+$ segregants were $Suc^+$ supporting the presence of SUC1. Genetic analysis of one tetrad, tetrad 8, confirmed the presence of two SUC genes and demonstrated that one SUC gene was linked to MAL1 and allelic to SUC1 (see Appendix 3).

Based on the reported tight linkage between MAL1 and SUC1, the sequences contained in plasmid pMJC1BamHI were analyzed by Southern analysis using probe pRB117, which contains the 5' region of the SUC2 gene (Carlson and Botstein, 1982). The 800 bp BamHI-HindIII fragment indicated in Figure 1 showed homology to this SUC2 probe. This is consistent with previous evidence demonstrating physical linkage between SUC1 and a partially functional allele of the MAL1 locus, MAL1g (Carlson et al., 1985).

Functional characterization of the MAL1 clones. It has been proposed that a dominant MAL locus contains at least two functions, MALp and MALg (Naumov, 1976; Michels and Needleman, 1983). Two naturally occurring alleles of the MAL1 locus have been characterized, MAL1p and MAL1g. Neither alone is able to confer the ability to ferment maltose but they complement each other in diploids. A third naturally occurring allele, $mal1^0$, lacks both MALp

and MALg complementing activity. We have previously demonstrated that the MAL6 locus contains both of these functions by showing that cloned MAL6 sequences are able to complement either MAL1p or MAL1g standard tester strains and that these functions are physically separable. The MAL63 gene complements MAL1g strains and thus carries the MAL6p function. Plasmids carrying both the MAL61 and MAL62 genes complement strains containing the MAL1p locus and thus these two genes together represent the MAL6g function. We have obtained similar results for the cloned MAL1 locus. The 7.1kb BglII-SalI fragment of λMJC1.2 was subcloned into YEp24 to form the plasmid pM1.2F (see Figure 1). Plasmid pM1.2F contains only MALp activity as is evidenced by its ability to complement the MAL1g strain 340-2B, and its inability to complement a MAL1p strain, 345-4A, or a mal1$^{0}$ strain, 328-4A. More interestingly, plasmid pM1.2F was shown to complement strain 348-1B, a derivative of the mal6-10 mutant isolated by ten Berge et al. (1973a) which carries a mutation in the MAL63 gene (Chang et al., submitted). Thus plasmid pM1.2F contains the MALp function from MAL1.

A plasmid containing the 7.3kb HindIII fragment from the MAL1 locus, presumed to confer MALg function, could not be constructed from the phage clones isolated because none of the phage inserts covered this region in its entirety. MALg function was therefore cloned from strain 600-1B. Genomic DNA from strain 600-1BΔCΔH#7 was used to

isolate plasmid pMJC1ΔH (Figure 1) as described above. Restriction enzyme mapping of this plasmid showed that it is identical to the composite phage map seen in Figure 1. Plasmid pMJC1ΔH complements the MAL1p strain 345-4A but not a MAL1g strain (340-2B) or a mal1$^{0}$ strain (328-4A). Therefore, it contains MALg function from MAL1.

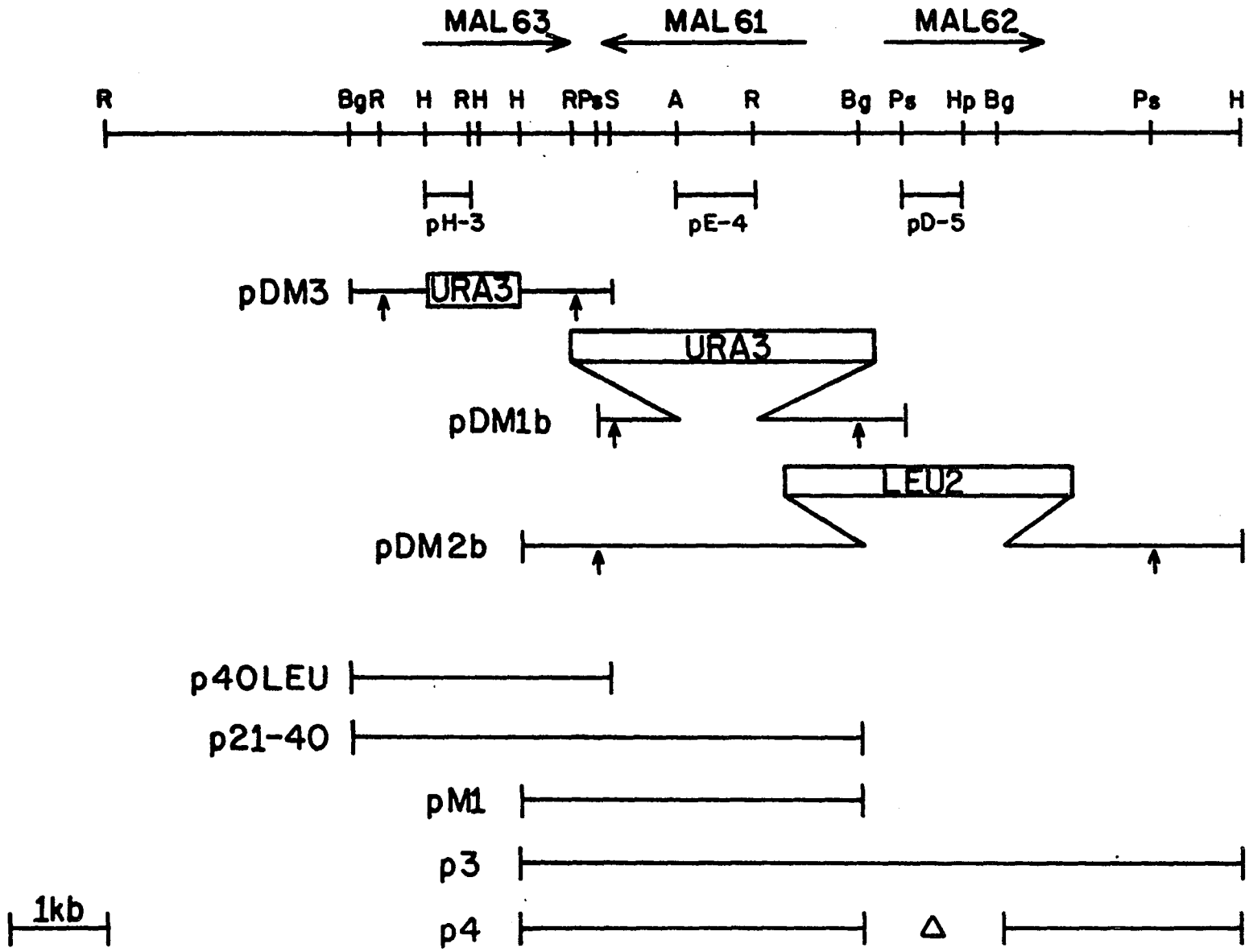
Gene disruptions at the MAL1 locus. While only two complementing functions are distinguishable using naturally occurring nonfermenting Saccharomyces strains, genetic and physical analysis of the MAL6 locus has demonstrated the presence of three genes (Needleman et al,,1984; Cohen et al., 1985; Dubin et al., 1985; Chang et al., submitted). In view of the functional similarities between MAL1 and MAL6 we took advantage of the sequence homology between these two loci and utilized MAL6-derived deletion/disruption plasmids to selectively mutate specific MAL1 regions (Rothstein, 1983). Creating these defined mutations has allowed us to confirm the mutagenesis analysis of Cohen et al. (1984) demonstrating the presence of three genes required for maltose fermentation at the MAL1 locus.

Disruption of MAL11. Sequences homologous to the MAL61 gene were deleted from the genomic MAL1 locus using disruption plasmid pDM1b (Figure 3). Plasmid pDM1b was restricted with SalI and BglII and used to transform the

Figure 3: Restriction map of MAL6-derived plasmids used for deletion/disruption of the MAL1 locus.

A partial restriction map of MAL6 and the location of the MAL61, MAL62 and MAL63 is shown. The pBR325 based plasmids pE-4, pD-5 and pH-3 contain DNA sequences internal to the MAL61, MAL62 and MAL63 genes, respectively. Plasmids p21-40 and pM1 contain the indicated regions subcloned into YEp13. Plasmid p40LEU contains the MAL63 gene on a BglII - SalI fragment, as well as the LEU2 gene from plasmid CV9, in a derivative of pBR322. Plasmid p3 contains the MAL61 and MAL62 genes as well as the LEU2 gene, in YIp5. Plasmid p4 is a derivative of plasmid p3 constructed by deleting the internal BglII fragment of the MAL62 gene. Both plasmids p3 and p4 contain an ARS sequence located in the HindIII - SalI region 3' to the MAL61 gene (Needleman et al., 1984). Disruption plasmids pDM1b, pDM2b and pDM3 (described in Methods and Materials) were digested with the indicated restriction endonucleases (↑) prior to transformation.

Recognition sites of restriction endonucleases are abbreviated as in Figure 1.


MAL63
MAL61
MAL62
R
BgR
H
RH
H
RPsS
A
R
Bg
Ps
Hp
Bg
Ps
H
pH-3
pE-4
pD-5
pDM3
URA3
URA3
pDM1b
LEU2
pDM2b
p40LEU
p21-40
pM1
p3
p4
Δ
1kb

MAL1 strain 600-1B to Ura$^+$. Two stable transformants were selected for further study. Southern analysis of both isolates and genetic analysis of one (strain 600-1BΔ11-1) confirmed integration at the MAL1 locus. Both isolates were unable to ferment maltose.

An isogenic series of strains carrying this MAL1 disruption but of different mating-types was constructed and mated to tester strains carrying the wild type MAL1 locus, or the naturally occurring MAL1g, MAL1p or mal1$^0$ alleles. While the undisrupted parental strain, when grown in the presence of maltose, exhibits a 90-fold increase in the level of maltase synthesized, the disruption strain does not induce for maltase (data not shown). The disruption strain is recessive to wild-type and is complemented by MAL1g strains but not by MAL1p or mal1$^0$ strains. These data are consistent with the following plasmid complementation results. Transformation of RDY100-1A (isogenic to strain 600-1BΔ11-1) with plasmid pM1, carrying the MAL61 gene, or plasmid pMJC1ΔH/YEp13, carrying the HindIII-BamH1 fragment from plasmid pMJC1ΔH cloned into YEp13, restored the ability to ferment maltose while the vector alone, YEp13, was unable to do so. Thus, this region of the MAL1 locus is functionally homologous to the MAL61 gene of MAL6 and appears to encode the MAL1-linked maltose permease. We refer to this gene as MAL11.

Transcription from this region of MAL1 was examined by

Northern analysis of poly $[A]^+$ RNA isolated from the undisrupted parent, strain 600-1B, and from strain RDY100-1A (MAL11::URA3 deletion/disruption) following growth in non-inducing (YEP + 2% galactose) or inducing (YEP + 2% maltose) medium. Plasmid pE-4 (Figure 2) contains a fragment internal to the MAL61 gene. In MAL6 strains it detects a 2.0 kb maltose inducible transcript and a 2.4 kb constitutively expressed transcript (strain 332-5A in Figure 4B). When plasmid pE-4 is used to probe for MAL61-homologous RNA sequences in a MAL1 strain, again both the 2.0 kb maltose inducible transcript and the 2.4 kb constitutive transcript are seen but the 2.4 kb transcript is of very low abundance (Figure 4B). The MAL11::URA3 deletion/disruption mutant is unable to produce either the inducible 2.0 kb transcript or the constitutive 2.4 kb transcript indicating that both are encoded by the MAL11 gene and suggesting that either multiple transcripts of different size originate from the MAL11 gene or that post-transcriptional modification is occurring.

Disruption of MAL12. Sequences homologous to the MAL62 gene were deleted from the genome at the MAL1 locus using disruption plasmid pDM2b (Dubin et al., 1985; Figure 3). PstI restricted pDM2b was used to transform strain 600-1B to $Leu^+$. Stable transformants were selected. Integration at MAL1 was confirmed by Southern analysis and

Figure 4: Transcriptional analysis of the MAL1 locus and the effects of deletion/disruption mutations at MAL1.

Poly[A]$^+$ RNA was prepared following growth on either YEP medium plus 2% galactose (G) or YEP medium plus 2% maltose (M). RNA was size fractionated on a formaldehyde/agarose gel, transferred to nitrocellulose, and probed with either plasmid pH-3 (panel A), pE-4 (panel B) or pD-5 (panel C). Plasmids pH-3, pE-4 and pD-5 (described in Figure 3) contain MAL63, MAL61 and MAL62 specific sequences, respectively. RNA integrity was confirmed by reprobing the filters with plasmid pYactI, which contains the yeast actin gene (Ng and Abelson, 1977). All lanes contain 5 ug of RNA except lanes 100-1A (G) and (M) in panel A, which contain 10 ug. Strain 332-5A is a MAL6 mal1$^0$ strain described previously (Dubin et al., 1986).

A.
1.6 kb
G 600-1B (MAL1)
M 600-1B (MAL1)
M 600-1BΔ13-14 (MAL13::URA3)
G 100-1A (MAL11::URA3)
M 100-1A (MAL11::URA3)
M 100-1B (MAL12::LEU2)
B.
2.4 kb
2.0kb
M 332-5A (MAL6 mal1°)
G 600-1B (MAL1)
M 600-1B (MAL1)
M 600-1BΔ13-14 (MAL13::URA3)
M 100-1A (MAL11::URA3)
M 100-1B (MAL12::LEU2)
C.
1.9 kb
M 332-5A (MAL6 mal1°)
G 600-1B (MAL1)
M 600-1B (MAL1)
M 600-1BΔ13-14 (MAL13::URA3)
M 100-1A (MAL11::URA3)
M 100-1B (MAL12::LEU2)

by genetic analysis for one transformant, 600-1BΔ12-2. All were maltose nonfermenters. An isogenic series of strains carrying this MAL1 disruption but of different mating types was constructed and mated to tester strains carrying the wild-type MAL1 locus or the naturally occurring MAL1g, MAL1p or mal1$^0$ alleles. The disruption strain did not induce for maltase (data not shown), was recessive to the wild-type MAL1 locus and was complemented by MAL1g strains and by most mal1$^0$ strains tested. Complementation by mal1$^0$ strains was not entirely unexpected. Although previously considered completely non-functional, at least some mal1$^0$ alleles contain a functional MAL1-linked maltase structural gene (Dubin et al., 1985). This disruption strain did not complement the MAL1p tester strains with one exception, strain 53-2C. Complementation by strain 53-2C was unexpected and demonstrates previously unrecognized distinctions among MAL1p alleles. Most likely these distinctions result from differences in the gene encoding maltase. That is, in certain MAL1p strains, the MAL1 locus encodes a functional maltase and in others it does not.

Plasmid complementation in strain RDY100-1B (isogenic to 600-1BΔ12-2) was examined in detail. Transformation of this strain with plasmid p3 (Figure 3), a plasmid containing the MAL61 and MAL62 genes was able to restore the ability to ferment maltose while plasmid p4 (Figure 3), a derivative of p3 which lacks the MAL62 gene, was

unable to do so. In addition, pMJC1Δ H/YEp13 which contains the MAL11 and MAL12 genes was able to complement strain RDY100-1B while the vector alone was not. The complementation patterns described above indicate that the region deleted in strain 600-1BΔ12-2 is structurally and functionally homologous to the MAL62 gene and thus encodes the MAL1 linked maltase. We refer to this gene as MAL12.

Plasmid pD-5 (Figure 3) contains a fragment internal to the MAL62 gene. This probe detects a 1.9 kb maltose inducible transcript in MAL6 strains (Dubin et al., 1985; Needleman et al., 1984). Plasmid pD-5 also detects a 1.9 kb maltose inducible transcript in the MAL1 strain 600-1B (Figure 4C). Strain RDY100-1B (MAL12::LEU2) was examined for its ability to transcribe MAL specific genes. No MAL12 specific transcripts were detected, however the MAL11 and MAL13 specific transcripts continue to be expressed (Figure 4). These results show that, in a MAL1 strain lacking maltase, induction of the MAL11 2.0 kb transcript is normal. Similar results were observed in a MAL6 strain lacking all maltase structural genes (results not shown).

Disruption of MAL13. Sequences homologous to the MAL63 gene were deleted from the genome at the MAL1 locus using disruption plasmid pDM3 (Chang et al., submitted; Figure 3). EcoRI restricted pDM3 was used to transform strain 600-1B to $Ura^+$. Five stable $Ura^+$ transformants

were examined by Southern analysis and integration at MAL1 was confirmed. For one isolate, 600-1B Δ 13-14, integration at MAL1 was confirmed genetically. All transformants were maltose non-fermenters.

An isogenic series of strains carrying this MAL1 disruption but of different mating-types was constructed and mated to tester strains carrying the wild type MAL1 locus or its naturally occurring MAL1g, MAL1p or mal1$^0$ alleles. The deletion/disruption strain did not induce for maltase (data not shown). It was recessive to MAL1 and was complemented by various MAL1p strains but not by MAL1g or mal1$^0$ strains. Transformation of strain 600-1BΔ13-14 with plasmid p21-40 (Needleman et al., 1984), a plasmid containing both the MAL61 and MAL63 genes, was able to restore the ability to ferment maltose while plasmids pM1 (Figure 3), which contains only the MAL61 gene, and vector plasmid YEp13 could not. Additionally, integration of plasmid p40LEU (Figure 3) at the LEU2 gene complements the MAL13::URA3 disruption. Plasmid pM1.2F also complements this disruption. This was shown by transforming strain 328-4A, carrying the mal1$^0$ allele, with plasmid pM1.2F and mating this transformant to strain 600-1B Δ 13-14. This diploid fermented while the diploid of the untransformed strains 328-4A and 600-1BΔ13-14 did not. As described above, plasmid pM1.2F was also found to complement a mutation in the MAL63 gene isolated by ten Berge et al. (1973a). These results

indicate that this region of MAL1 is structurally and functionally homologous to the MAL63 gene. We refer to this gene as MAL13.

Plasmid pH-3 (Figure 3) contains a fragment of the MAL63 gene. MAL63 has been demonstrated to encode a positive, trans-acting regulatory function required for the induction of maltase, maltose permease and the MAL61 and MAL62 maltose inducible transcripts (Needleman et al.,1984; Cohen et al., 1985; Chang et al., submitted). In MAL6 strains, probes containing the MAL63 gene detect a 1.6 kb and a 2.0 kb transcript both of which are constitutively expressed however the larger transcript appears to be slightly induced by maltose (Needleman et al., 1984; Chang et al., submitted) . When plasmid pH-3 is used to probe for MAL63-homologous RNA sequences in a MAL1 strain a 1.6 kb constitutively expressed transcript is detected (Figure 4A). Northern analysis has revealed that the MAL13 disruption strain 600-1BΔ13-14 was unable to synthesize the MAL13 specific transcript. Additionally, this mutant was unable to synthesize the maltose inducible MAL11 and MAL12 specific transcripts. The 2.4 kb MAL11 transcript was however detected (Figure 4A). Strain 600-1BΔ13-14 also does not induce for maltase (data not shown). These results are consistent with the proposal that the MAL13 gene product, like the MAL63 gene product, contains a positive regulatory function required for maltose fermentation, the induction of maltase and

maltose permease, and the transcription and / or accumulation of their specific mRNAs. It should also be noted that a strain carrying a disruption of the MAL11 gene (RDY100-1A) is also unable to induce the transcription of MAL12 (and thus synthesis of maltase) but this deletion does not affect the expression of the MAL13 gene (Figure 4B). MAL11 is likely to encode the MAL1-linked maltose permease. Thus, the loss of this activity appears to restrict the transport of maltose sufficiently enough to prevent the induction of other maltose regulated genes, confirming that transport is an important rate limiting step in controlling maltose fermentation.

MAL11, MAL12, and MAL13 represent three genes. In order to conclusively demonstrate disruption of three separate genes at the MAL1 locus, the disruption strains described above were mated to each other in all possible pairwise combinations. Each disruption mutant was capable of complementing the other two but not itself. This confirms the presence at the MAL1 locus of three independent genes, each essential for maltose fermentation. These results support similar findings by Cohen et al. (1984), who demonstrated the presence of three genes located at the MAL1 locus by the use of standard mutagenic analysis. The clear complementation patterns seen for MAL1 mutations contrast with similar

experiments performed at the MAL6 locus. At MAL6 it was found that strains disrupted for MAL63 and MAL61 fail to complement each other (Chang et al., submitted). This difference between the MAL6 and MAL1 loci remains unexplained and may represent valid distinctions among the dominant MAL loci or may simply result from subtle background differences in these strains.

# DISCUSSION

We have cloned the MAL1 locus from a lambda phage library of strain 4059 DNA using MAL6-derived probes. The cloned region includes about 22.6 kb of DNA. As shown in Figure 1, only 9.0 kb of this region is homologous to the MAL6 locus. This homology is quite extensive as evidenced by the almost identical restriction maps of these two regions, as well as by comparative Southern analysis.

The loci are also functionally homologous. The MAL6 locus has been shown to be a cluster of three genes all required for the fermentation of maltose: MAL61, encodes maltose permease; MAL62, encodes maltase; and MAL63 encodes a positive regulatory protein required for the induction of the structural genes (Needleman et al., 1984; Dubin et al.,1985; Chang et al., submitted). Similarly, the MAL1 locus is shown here to contain three genes required for maltose fermentation. This was shown by deletion/disruption of three regions of MAL1 homologous to the three MAL6 genes using plasmids constructed from MAL6-derived subclones. Each of the three disruptions produced a maltose nonfermenting phenotype. Each disruption could be complemented by plasmids containing the appropriate MAL1-derived subclones as well as by plasmids containing the appropriate MAL6-derived subclone. In addition, as was shown for the MAL6 locus, subclones of the MAL1 locus complement the naturally occurring,

partially functional alleles of MAL1: MAL1p and MAL1g. Matings between the various MAL1 disruption strains showed that they are complemented by each other. Taken together, these results clearly demonstrate that the MAL6 and the MAL1 loci are both structurally and functionally homologous. We refer to the three genes of the MAL1 locus as MAL11, MAL12 and MAL13 and propose that these are functionally equivalent to their MAL6 counterparts MAL61, MAL62 and MAL63, respectively.

MAL6-derived probes were used to study transcription from the MAL1 locus. Both the MAL62 and the MAL12 genes encode a single 1.9 kb maltose inducible transcript. Deletion of the MAL12 gene results in the loss of this transcript with no apparent effect on the transcription of the MAL11 or MAL13 genes. Like MAL61, two transcripts homologous to the MAL11 gene are synthesized: a 2.4 kb constitutive message and a 2.0 kb maltose inducible message. Disruption of the MAL11 gene leads to the loss of both messages. It has not yet been determined if the MAL11 gene is transcribed as two differently sized mRNAs or if post-transcriptional modification is occurring. MAL11 disruption also leads to the loss of inducibility of the MAL12 transcript. Like MAL61, MAL11 is likely to encode permease. Thus, we believe that the lack of induction of maltase and the MAL12 transcript in this disruption strain probably results from insufficient transport of maltose. MAL13 transcription is unaffected

by the MAL11 disruption. A single, constitutively expressed 1.6 kb transcript is synthesized by the MAL13 gene. In MAL6 strains, MAL63-derived probes hybridize to two messages: a 1.6 kb constitutive mRNA and a 2.0 kb constitutive mRNA that is slightly induced by maltose (Needleman et al., 1984). The exact nature of this difference is not yet understood. Disruption of the MAL13 gene in the MAL1 strain 600-1B produces a nonfermenter which is uninducible for maltase and for both the 2.0 kb MAL11 and 1.9 kb MAL12 maltose inducible transcripts. The 2.4 kb constitutive MAL11 transcript is unaffected. These results are similar to those obtained for disruptions of the MAL63 gene, indicating that the product of the MAL13 gene is also a positive regulator of the structural genes for maltose fermentation.

The results presented here are consistent with previous studies of the MAL1 locus. Cohen et al. (1984) demonstrated, using standard genetic analysis, the presence of three genes at the MAL1 locus. They later showed that these mutants could be complemented by discrete fragments of the cloned MAL6 locus (Cohen et al., 1985). The isolation of MAL1-linked, temperature sensitive mutations for maltose permease (Goldenthal et al., 1983) and maltase (Cohen et al., 1984) is also consistent with our results indicating that both structural genes are part of the MAL1 complex locus. The results reported here fully demonstrate the extent of the

homology between the MAL1 and MAL6 loci.

One of the most interesting findings reported here is the fact that little or no homology exists between the DNA sequences flanking the 9.0 kb MAL1 and MAL6 complex loci to the left of the regulatory gene. We have recently identified a second trans-acting regulatory gene located 2.3 cM to the left of MAL63, that regulates the expression of the two structural genes (Dubin, et al., 1986). In view of the lack of homology between MAL6 and MAL1 flanking sequences, the possibility arises that this second regulatory gene is a peculiar feature of the MAL6 locus and not common to all dominant MAL loci. Detailed analysis of the MAL1 and MAL6 flanking sequences as well as those sequences flanking the other MAL loci are in progress (Charron and Michels, in preparation-Chapter four). Preliminary evidence suggests that the homology among the MAL loci is limited to the approximately 9.0 kb region encoding the three characterized genes. It is hoped that these comparative studies will provide further insight into regulation of maltose fermentation and into the evolutionary mechanisms which have led to the development of this and other telomere-linked polygenic systems in the Saccharomyces yeasts.

# Chapter 2

## Comparative structural and functional analysis of the alleles of the *MAL1* locus of *Saccharomyces*

## ABSTRACT

In order for a yeast strain to ferment maltose it must contain any one of the five dominant MAL loci. Each MAL locus contains three genes: GENE 1, encoding maltose permease, GENE 2 encoding maltase and GENE 3 encoding a positive trans-acting regulatory protein. In addition to these dominant MAL loci, several partially functional alleles have been identified. These have been shown to be linked to the MAL1 and MAL3 loci. Four naturally occurring alleles of MAL1 have been characterized. MAL1 which encodes all three genes needed for fermentation. The MAL1p allele which functionally encodes only the MAL activator; the MAL1g allele which functionally encodes a maltose permease and maltase, and the $mal1^0$ allele which functionally encodes only maltase. Molecular analysis of these alleles and several kilobasepairs of flanking sequences indicates that MAL1p, MAL1g and $mal1^0$ have evolved from MAL1 by a series of rearrangements and/or deletions of this yeast telomere-associated locus. We also describe the structure of a unique maltose transport gene contained within the MAL1g locus.

# INTRODUCTION

Saccharomyces strains capable of fermenting maltose carry any one of five unlinked MAL loci: MAL1, MAL2, MAL3, MAL4, and MAL6 (reviewed by Barnett, 1976). The genetic organization of the MAL1 and MAL6 loci has been described in detail (Needleman et al., 1984; Dubin et al., 1985; Charron et al., 1986-Chapter one; Chang et al., submitted). Both are complex loci consisting of three genes: GENE 1 appears to encode the maltose permease (Goldenthal et al., 1983; Cohen et al., 1984; Chang et al., submitted); GENE 2 encodes maltase (Dubin et al., 1985); and GENE 3 encodes a trans-acting activator required for maltose induction of the transcription of GENES 1 and 2 (Charron et al., 1986-Chapter one; Chang et al., submitted). We refer to the three genes at the MAL1 locus as MAL11 (GENE 1), MAL12 (GENE 2), and MAL13 (GENE 3), whereas, at MAL6, these genes are called MAL61 (GENE 1), MAL62 (GENE 2) and MAL63 (GENE 3). The genetic number can thereby be used to designate both the locus position and the gene function. Based on Southern analysis and restriction mapping, MAL1 and MAL6 are extensively sequence homologous throughout the region encoding the three genes (Charron et al., 1986-Chapter one).

Both the work of Naumov (1971, 1972, and 1976) and Oshima (1967) described the existence of naturally occurring partially functional MAL loci. Upon analyzing eleven Saccharomyces strains isolated from the wild, Naumov

identified two alleles of the MAL1 locus which he referred to as MAL1p and MAL1g. Strains carrying only one of these alleles are unable to ferment maltose. In heterozygous diploids, the MAL1p and MAL1g alleles complemented and allowed for fermentation. Naumov also found strains which were unable to ferment maltose and were not complemented in diploids by either the MAL1p or the MAL1g alleles. He referred to these as $mal^0$ strains. Needleman and Michels (1983) were able to demonstrate the presence of MAL1-linked sequences in one $mal^0$ strain thereby identifying a fourth MAL1 allele referred to as $mal1^0$.

The MAL1 alleles have been functionally characterized. Strains carrying the MAL1p allele are complemented by cloned MAL1 and MAL6 sequences containing both GENE 1 and GENE 2 (that is, MAL11 and MAL12 or MAL61 and MAL62) (Needleman et al., 1984; Charron et al., 1986-Chapter one). Thus, the MAL1p allele encodes an activator functionally homologous to the MAL13 or MAL63 gene product. Strains carrying the MAL1g allele are complemented by plasmids containing the MAL13 or MAL63 gene encoding the trans-acting activator, and thus the MAL1g allele appears to encode both structural gene functions. Deletion/disruption analysis of the MAL62-homologous region of the $mal1^0$ allele demonstrated that this MAL1 allele encodes a temperature labile form of maltase (Dubin et al., 1985). Based on the lack of complementation by MAL1p or MAL1g it has been assumed that this is the only MAL function encoded by the $mal1^0$ allele.

In this report we describe the cloning and structural analysis of the three partially functional alleles of MAL1. Our results indicate that these alleles appear to have been derived from the MAL1 locus by the deletion and/or rearrangement of regions of the MAL1 locus as well as the accumulation of "point mutations". In addition, physical linkage of the MAL1 alleles to SUC1, $suc1^0$ and telomere adjacent X and Y' sequences is demonstrated.

# MATERIALS and METHODS

## Strains and Growth Conditions

The S.cerevisiae strains used in this study are listed in Table 1. Many of the MAL1p, MAL1g and mal1$^0$ tester strains were constructed by R. Needleman from homothallic strains kindly provided by G. Naumov (Moscow). The revelant MAL genotype was determined using the genetic analysis described in Michels and Needleman (1983) and is shown in Table 1.

Plasmids were propagated in E.coli strain C600 or strain RR1.

Yeast strains were grown on YEP medium [1% (wt/vol) yeast extract/ 1% (wt/vol) peptone] plus 2% (wt/vol) glucose. Maltose fermentation is defined as the production of acid and gas in 1-3 days after inoculation and determined in 5 ml of YEP plus 2% (wt/vol) maltose medium in Durham tubes.

## Preparation and Analysis of DNA

Plasmid DNA's, yeast DNA's and phage DNA's were prepared as described previously (Needleman et al., 1984 and Charron et al., 1986-Chapter one). Restriction endonuclease mapping, gel electrophoresis and DNA subcloning were performed using standard methods (Maniatis et al., 1982). Southern gel transfer analysis was performed as described previously (Dubin et al., 1985). Hybridizations were

Table 1

List of S. cerevisiae strains used in this study

| Strain | Genotype |
|---|---|
| 600-1B | MATa MAL1 SUC1 ura3-52 leu2-3,112 |
| 4059 | MATα MAL1 MAL3g SUC1 leu1 ade1 ade2 |
| W15-7D | MATa MAL1 leu ade |
| 100-1A | MATa MAL11::URA3 leu2-3,112 |
| 100-1B | MATa MAL12::LEU2 ura3-52 |
| 600-1BΔ13-14 | MATa MAL13::URA3 leu2-3,112 |
| 6-2A | MATα $mal1^0$ lys2 |
| 303-3A | MATα $mal1^0$ leu2-3,112 ade1 |
| 303-2B | MATa $mal1^0$ leu2-3,112 ade |
| 328-4A | MATα $mal1^0$ ura3-52 trp1 met14 ade |
| 3-2B | MATa $mal1^0$ lys2 |
| 53-2C | MATα MAL1p met |
| 236-2A | MATa MAL1p leu2-3,112 lys2 |
| 345-4A | MATa MAL1p leu2-3,112 ura3-52 trp1 ade |
| JC27 | MATα MAL1g MAL3g leu2-3,112 his |
| 340-2B | MATa MAL1g ura3-52 trp1 lys2 met |
| 340-2A | MATα MAL1g ura3-52 ade |
| DBY939 | MATα MAL1g MAL3g suc2-215 ade2 gal2 |
| DBY782 | MATα MAL1g MAL3g SUC2 ade2 gal2 |
| 340-3B | MATα MAL1g ura3-52 trp1 met14 ade |
| 628-5B | MATa MAL62::LEU2 MAL12::LEU2 ura3-52 trp1 his |

carried out at 65°C in 0.6M NaCl/ 0.06M Na Citrate (pH 7.0)/ 0.2% Ficoll/ 0.2% polyvinylpyrrolidine/ 0.2% BSA/ 0.1% SDS/ 0.1 mg/ml salmon sperm DNA unless otherwise noted. DNA's were labelled with [$\gamma$ -$^{32}$P]-dCTP (Amersham) by nick translation (Rigby et al., 1977).

Yeast Transformation and Plasmid Rescue

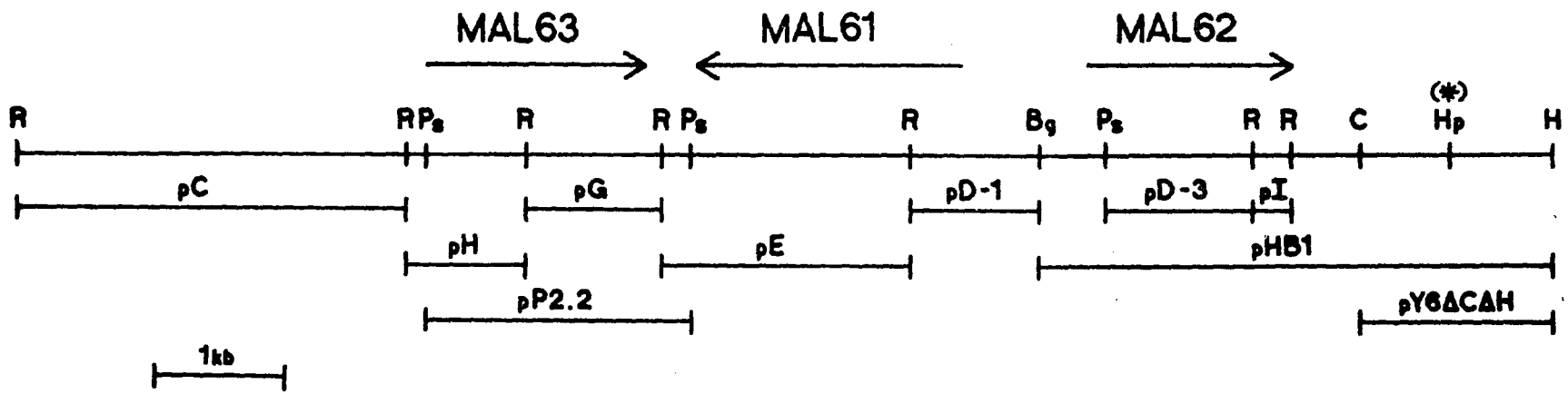
Yeast transformation was performed by the method of Ito et al. (1983) using lithium acetate. Many transformants were screened for plasmid stability and "ARS" function by passage through non-selective media [YEP medium plus 2% (wt/vol) glucose].

All plasmid constructs were assayed for functional MAL genes by the ability to complement standard MAL1p, MAL1g, or mal1$^0$ tester strains and/or ΔMAL11, ΔMAL12, or ΔMAL13 strains (Charron et al., 1986-Chapter one), (Table 1). In each case, maltose fermentation was scored for approximately 50 transformants using the criteria described above.

Plasmid pY6ΔCΔH was used for site-directed integration (Orr-Weaver et al., 1983) into the mal1$^0$ locus of strain 328-4A and the MAL1g locus of strain 340-2B following linearization with HpaI (see Figure 1 (*) and Dubin et al., 1986). Linkage of the plasmid marker (URA3) to the mal1$^0$ and MAL1g loci was determined using both physical and genetic analysis as follows. The plasmid was shown to have integrated into the appropriate HindIII fragment using Southern analysis. Each of the integrative transformants

Figure 1: Subclones of the (wild-type) MAL6 locus from strain CB11.

A partial restriction map of the MAL6 locus of strain CB11 is presented along with the locations and directions of transcription of the MAL61, MAL62 and MAL63 genes. All fragments shown were subcloned into pBR325 except pY6ΔCΔH which was subcloned into a derivative of YIp5 as described previously (Dubin et al.,1985; Charron et al.,1986-Chapter one). The symbol (*) represents the site of linearization of plasmid pY6ΔCΔH in site-directed integration/plasmid rescue experiments. Restriction enzymes are abbreviated as follows: Bg, BglII; C, ClaI; H, HindIII; Hp, HpaI; Ps, PstI; R, EcoRI.


MAL63
MAL61
MAL62
R
RPs
R
R Ps
R
Bg
Ps
R R
C
(*)
Hp
H
pC
pG
pD-1
pD-3
pI
pH
pE
pHB1
pP2.2
pY6ΔCΔH
1kb

was also mated to a MAL1 ura3-52 strain and linkage of the URA3 marker to the nonfermenting phenotype determined by tetrad analysis (see Appendix 4). The mal1$^0$ and mal1$^0$ adjacent DNA sequences were isolated from the genome of the transformant following digestion of genomic DNA with either HindIII, SalI or BamHI individually, ligation of the digested DNA at low concentration, followed by selection of plasmid clones following transformation of E.coli strain RR1 to ampicillin resistance.

Isolation of Cloned MAL DNA.

Bacteriophage library construction and screening was as described previously (Charron et al.,1986-Chapter one).

(i) Cloning the mal1$^0$ allele. Total genomic DNA from strain 3-2B was partially restricted with EcoRI, size fractionated (5.0-17.0 kb) and ligated into λgt WES.B EcoRI arms, packaged, and amplified as described in Charron et al. (1986). The resultant library was screened using MAL6-derived probes (see Figure 1). Two phage clones that were isolated using probes pP2.2 and pD-3 (λMJC0.1 and λMJC0.2) and one clone that was isolated using pHB1 (λMJC0.3) (Figure 2) were saved for further analysis. All other clones containing smaller insert DNA's were not analyzed further. Additional mal1$^0$-linked sequences were cloned from strain 328-4A by the plasmid rescue technique of Orr-Weaver et al.(1983) using plasmid pY6ΔCΔH as described above. Results of restriction endonuclease analysis of

these cloned sequences is shown in Figure 2.

(ii) Cloning the MAL1g allele. The MAL1g containing plasmid pFE52 (gift of M. Carlson) was isolated from a total genomic DNA library of strain DBY939 (congenic to S288C) using a $suc1^0$ probe as described in Carlson et al. (1985).

The MAL1g locus and adjacent flanking DNA sequences were also isolated from strain 340-2B using plasmid pY6ΔCΔH as described above.

(iii) Cloning MAL1p-linked sequences. Total genomic DNA from strain 236-2A, partially restricted with EcoRI (6.0 - 20.0 kb), was ligated into the lambda phage vector EMBL3. The resulting library was screened with the MAL6-derived probe pH (Figure 1).

(iv) Cloning of a putative mal pseudogene. Total genomic DNA from strain W15-7D was digested with BamHI and ligated into EMBL3 BamHI arms. The resultant library was screened with the MAL6-derived probes pD-1 and pP2.2 (Figure 1) and two identical clones were isolated (λMJC1.7 and λMJC1.8). λMJC1.7 was saved for further characterization.

# RESULTS

Unlike most cloned yeast sequences which encode proteins, sequences derived from the three genes of the MAL6 or MAL1 loci are not unique in the genome. When these MAL sequences are used in Southern analysis to probe strains carrying 1 or 2 MAL loci, several more than 1 or 2 homologous fragments will be seen. While these additional sequences do not appear to encode functional enzymes utilized for maltose fermentation, their origin and role are as yet undetermined. The presence of these repeated copies complicates efforts to isolate the DNA fragments encoding the MAL loci and their alleles since probing a library for homologous sequences will lead to the isolation of a variety of different fragments derived from several genomic sites. For this reason, the results described here utilize several physical and genetic methods to unequivocably demonstrate that the desired locus has in fact been isolated.

Cloning of the mal1$^0$ allele. Strains 3-2B and 328-4A carry the mal1$^0$ allele and no other MAL genes. Southern analysis of both strains shows a single 7.0 kb mal1$^0$-linked HindIII fragment with homology to the MAL6-derived plasmid pD-1 (Michels and Needleman, 1983). A λgtWES.B library was constructed from strain 3-2B as described in Materials and Methods. Two phage clones were isolated using the MAL6-derived plasmids pP2.2 and pD-3 ( λMJC0.1 and λMJC0.2)

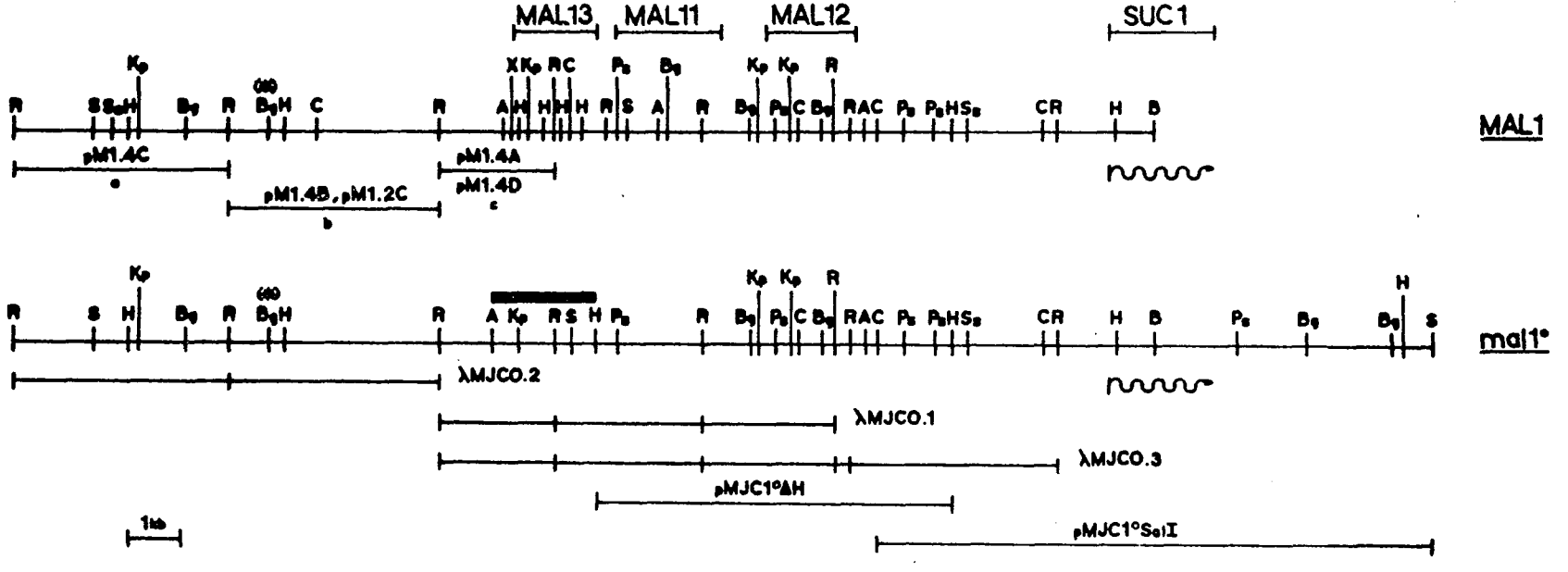
and one clone was isolated using plasmid pHB1 ( λMJC0.3) (see Figure 1 for the MAL6 plasmids). Restriction maps of the yeast inserts are shown in Figure 2. Phage λMJC0.1 and λMJC0.3 contain overlapping yeast inserts and, based on Southern analysis, phage λMJC0.3 contains a 7.0kb HindIII fragment which is homologous to plasmid pD-1. Both phage clones would appear to be derived from the $mal1^0$ locus of strain 3-2B.

The $mal1^0$ allele of strain 328-4A was also isolated along with several kilobases of flanking DNA using plasmid pY6ΔCΔH and the technique of plasmid rescue of genomically integrated plasmids as described in the Materials and Methods. Both strains 3-2B and 328-4A were derived from the same $mal^0$ strain of Naumov (S. chevalieri strain 407) by crosses to S. carlsbergensis and S. cerevisiae strains. Since our standard laboratory strains used for these crosses carry the MAL1g allele of MAL1, it is expected that the $mal1^0$ alleles of both 3-2B and 328-4A were derived from strain 407. Plasmids $pMJC1^0\Delta H$ and $pMJC1^0$SalI were isolated from the $mal1^0$ allele of strain 328-4A (Figure 2). The restriction endonuclease map of $pMJC1^0\Delta H$ was identical to that of λMJC0.3, supporting our assumption that 3-2B and 328-4A contain identical $mal1^0$ alleles. Based on these results the restriction endonuclease map of the $mal1^0$ allele diagrammed in Figure 2 is a composite of the 3-2B and 328-4A clones.

To further demonstrate that these sequences contained

Figure 2: Restriction endonuclease maps of the MAL1 and mal1$^0$ loci.

The complete restriction map of the MAL1 locus is shown at the top of the figure. The approximate size and location of the MAL11, MAL12 and MAL13 genes are indicated. Regions of the MAL1 locus were subcloned into pBR325 to form plasmids pM1.2C and pM1.4D or YIp5 to form plasmids pM1.4A, pM1.4B and pM1.4C (Charron et al., 1986-Chapter one). The restriction map of the 27.3 kb sequence of DNA containing the mal1$^0$ locus is shown below that of the MAL1 locus. Overlapping yeast inserts contained in the phage isolates $\lambda$MJC0$_0$1 through $\lambda$MJC0.3 and the plasmids pMJC1$^0\Delta$H and pMJC1$^0$SalI (see Materials & Methods) are shown at the bottom of the figure. The symbol (*) represents the site of integration of plasmid pM1.4B into strain 328-4A. The solid horizontal bar represents a region of poor homology to MAL13-derived probes. The region showing homology to the SUC2 gene probes pRB117 and pRB59 and the direction of transcription of the SUC1 gene are indicated by the wavy line. Restriction enzymes are abbreviated as in Figure 1 with the following additions: A, AvaI; B, BamHI; Kp, KpnI; S, SalI; Ss, SstI; X, XhoI.


MAL13
MAL11
MAL12
SUC1
MAL1
pM1.4C
pM1.4A
pM1.4D
pM1.4B, pM1.2C
mal1°
λMJCO.2
λMJCO.1
λMJCO.3
pMJC1°ΔH
pMJC1°SalI
1kb

the mal1$^0$ allele, the 7.0kb pD-1 homologous HindIII fragment of phage λMJC0.3 was subcloned into both YIp5 and YEp13 to produce plasmids pM0.3A and pM0.3B, respectively. These plasmids and pMJC1$^0$ΔH (from strain 3-2B) (Figure 2) were transformed independently into MAL1p (236-2A and 345-4A), MAL1g (340-2B and JC27) and mal1$^0$ (303-3A and 328-4A) tester strains and, as expected, complementation was not observed. However, when plasmids pM0.3A and pMJC1$^0$ΔH are transformed into strain 100-1B (Charron et al., 1986-Chapter one), a maltose non-fermenting strain that carries a deletion/disruption of MAL12 (maltase), complementation is observed. Similarly, pM0.3A and pMJC1$^0$ΔH complement the MAL62/MAL12 double disruption strain 628-5B (Dubin et al., 1985). Taken together, the physical and functional evidence described above clearly demonstrate the clones isolated are derived from the mal1$^0$ allele.

Prior to this study the extent of sequence homology between the mal1$^0$ locus and either the cloned MAL6 or MAL1 locus had not been determined. The MAL6 and MAL1 loci show extensive homology over a 9.0kb BglII to HindIII region containing the MAL61-62-63 and MAL11-12-13 genes, respectively (Charron et al., 1986-Chapter one). Southern gel transfer analysis, using MAL6-derived probes spanning this region of homology, was performed on the phage clones λMJC0.1 and λMJC0.3 (Figures 1 and 2). Extensive homology is observed within the MAL structural gene region (MAL11 and MAL12) eventhough our plasmid complementation

results described above clearly indicate that a functional maltose permease is not encoded at this locus.

Homology to sequences derived from the activator gene region (MAL13) are poor (shown as a solid bar in Figure 3). Comparative restriction enzyme mapping reveals several restriction site polymorphisms between the MAL1 and mal1$^0$ alleles in the region of the MAL11 and MAL13 genes (Figure 2). The overall size of the region remains the same as that in MAL1 and certain restriction sites (particularly the EcoRI sites) are retained. The results of Southern gel transfer analysis show that little or no sequences homologous to MAL13- and MAL63-derived probes are present at the mal1$^0$ locus (data not shown). Taken together these results imply that extensive sequence divergence possibly due to the accumulation of "point mutations" rather than chromosomal rearrangement has led to the formation of the partially functional mal1$^0$ allele. Only DNA sequence analysis will allow us to determine the extent of the sequence divergence between these two alleles.

To be sure that the non-overlapping sequences contained in phage clone λMJC0.2 actually flanked the mal1$^0$ locus, linkage association between these fragments and the mal1$^0$ locus had to be established. This was accomplished as follows. Plasmid pM1.4B (Figure 2) was linearized with BglII and used to transform strain 328-4A to Ura$^+$. Genetic and physical analysis of one stable Ura$^+$ transformant, similar to that described for the MAL1 locus (Charron et

al., 1986-Chapter one), showed that the plasmid had inserted at a site linked to the mal1$^0$ allele of the MAL1 locus (data not shown). Briefly, integration of plasmid pM1.4B at the mal1$^0$ locus was shown in the following way. The restriction map derived from the phage clones ($\lambda$MJC0.1 - $\lambda$MJC0.3) predicted that SalI and KpnI sites should flank the integration site of plasmid pM1.4B in strain 328-4A::pM1.4B. Based on the restriction map of phage $\lambda$MJC0.2, an approximately 6.95 kb pM1.4B homologous KpnI fragment is expected. In the transformed strain this KpnI fragment should increase in size to about 16.35 kb. Additionally, an approximately 9.3 kb pM1.4B homologous SalI fragment is predicted if sequences contained in the non-overlapping phage clone $\lambda$MJC0.2 are actually adjacent to sequences cloned into phage $\lambda$MJC0.1 and $\lambda$MJC0.3. Southern analysis confirmed these predictions and in the pM1.4B transformed strain the 9.3 kb SalI fragment is replaced by two SalI fragments of the expected sizes (approximately 12.1 kb and 9.3 kb) (data not shown).

Restriction endonuclease analysis of phage $\lambda$MJC0.2 was performed. As can be seen in Figure 2, the MAL1 and mal1$^0$ restriction maps are largely identical in this region. This observation is supported by the results of Southern analysis using the MAL1 flanking DNA probes pM1.4C, pM1.2C and pM1.4D, demonstrating good sequence homology to the EcoRI fragments a, b, and c, respectively, in phage $\lambda$MJC0.2. The sequences diverge near the AvaI site in fragment c (Figure

2). When probes derived from the MAL61-MAL62 intergenic region (pD-1 and pD-3, Figure 1) are used to probe the mal1$^0$ phage clones, poor homology to fragments c and b of λMJC0.2 is seen in addition to the expected homology to the 2.5 kb EcoRI fragment of λMJC0.1 and λMJC0.3 (data not shown). Unusual sequence homologies similar to the results obtained here within the mal1$^0$ allele have been noted within the MAL1 locus itself (Charron et al., 1986-Chapter one). The exact meaning of this apparent repeated sequence is not known.

DNA sequences flanking the other side of the MAL1 and mal1$^0$ alleles were analyzed for sequences homologous to the telomere-adjacent repeated SUC genes. The MAL1 strain used to clone the MAL1 locus is a sucrose fermenter and has been shown to contain the SUC1 locus (Charron et al., 1986-Chapter one). The mal1$^0$ strains used in this study also ferment sucrose. DNA sequences flanking this side of the mal1$^0$ locus were isolated in plasmid pMJC1$^0$SalI as described above (Methods and Materials). As can be seen in Figure 2, SUC-homologous sequences are found flanking both the MAL1 and mal1$^0$ alleles (Charron et al., 1986-Chapter one). The restriction map of plasmid pMJC1$^0$SalI closely resembles the map of the SUC1 locus and its flanking DNA sequences (Carlson et al., 1985). Together these results suggest that plasmid pMJC1$^0$SalI also contains the telomere-adjacent SUC1 locus.

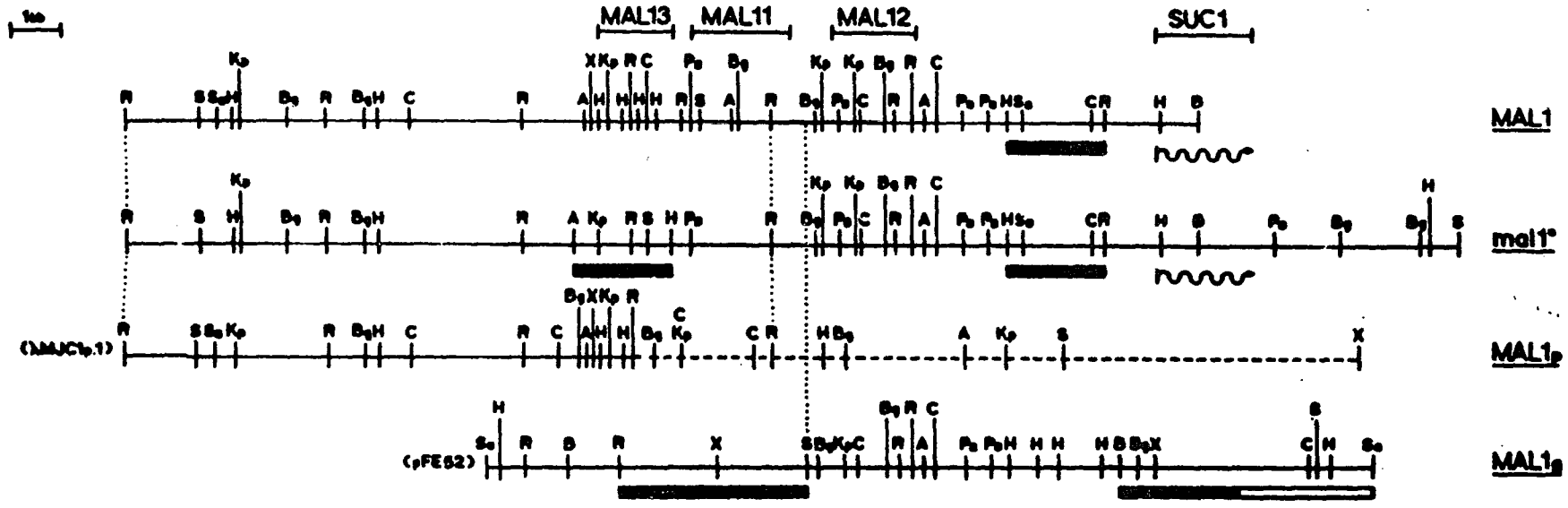
Analysis of MAL1g clones. Physical analysis of MAL1g strains revealed that a 10.7 kb HindIII fragment,

homologous to the MAL6-derived probe, D-1, is linked to the MAL1g locus (Michels and Needleman, 1983; 1984; Needleman and Michels, 1983). This 10.7 kb HindIII fragment is found within plasmid pFE52 (Figure 3). Briefly, plasmid pFE52 was isolated by homology to $suc1^0$ probes from a YEp24/Sau3A partial library of a strain that is congenic to strain S288C (DBY939) (Carlson et al., 1985). Plasmid pFE52 contains MALg function (permease and maltase activity) as evidenced by its ability to complement a MAL1p (345-4A) strain but not MAL1g (340-2B) or $mal1^0$ (328-4A) strains. This complementing activity is localized to the 9.2 kb BamHI / HindIII fragment which is also able to complement a ΔMAL11 (100-1A) and a ΔMAL12 strain (100-1B) (Charron et al., 1986-Chapter one). To further localize the maltase activity at this allele of the MAL1 locus, the 1.5 kb BglII fragment was deleted from the BamHI/HindIII subclone. The resulting plasmid no longer complements strain 100-1B (Δ MAL12) but does complement strain 100-1A (ΔMAL11). Therefore, based on these results, maltose permease is encoded by the 5.4 kb BamHI/BglII fragment and maltase is encoded by the sequences near the 1.5 kb BglII fragment.

The location of the structural genes present at the MAL1g allele is not surprizing in view of the organization of the genes present at a dominant MAL locus (Needleman et al., 1984; Charron et al., 1986-Chapter one). To confirm the fuctional analysis and to determine the extent of the sequence homology between MAL1g and the other cloned MAL

Figure 3: Structural comparison of the MAL1 alleles.

The restriction endonuclease map of the MAL1, mal1$^0$, MAL1p and MAL1g alleles are diagrammed. The approximate size and location of the MAL11, MAL12 and MAL13 genes are shown. Horizontal solid lines indicated cloned sequences and horizontal dashed lines indicate results obtained from genomic Southern analysis. The boundaries of homology between MAL1 and the three partially functional alleles are indicated by vertical dotted lines. The solid bar designates poor homology to probes derived from the trans-acting activator, and the stipled bar indicates the region of poor homology to maltose permease-derived probes. Homology to telomere-derived X sequences (YRp120 and YRp131A) are diagrammed with vertical hatched bars and the Y' sequences (YRp131B) with open bars. Homology to SUC1 (pRB117 and pRB59) is also indicated. Recognition sites for restriction endonucleases are abbreviated as in Figures 1 and 2.


1kb
MAL13
MAL11
MAL12
SUC1
MAL1
mal1°
(λMUC1p.1)
MAL1p
(pFE52)
MAL1g

loci, Southern analysis using probes derived from both the MAL1 and MAL6 loci was performed (Figures 1 and 2). The results of this analysis are summarized in Figure 3. Sequences derived from the maltase structural gene (MAL62) and approximately 2.0 kb of DNA 3' to the maltase gene are highly conserved in the MAL1g clone. Divergence between the MAL1g and MAL1 locus is seen in the region of the right end of the clone (Figure 2). Note that the MAL1 and $mal1^0$ strains used in this study carry the SUC1 locus (Charron et al., 1986-Chapter one), whereas the MAL1g strain DBY939 does not (Carlson et al., 1985). Since all $suc^0$ alleles are null, except for $suc2^0$ (Carlson et al., 1985), strain DBY939 would be expected to lack SUC homologous sequences linked to the MAL1g locus. The sequence divergence in the region 3' to the MAL12 gene between the flanking DNA sequences of the MAL1g, MAL1 and $mal1^0$ alleles is consistent with the $suc1^0$ genotype.

In the sequences 5' to the maltase structural gene, several interesting obervations can be made. Firstly, based on restriction endonuclease mapping, MAL1g and MAL1 are quite divergent in this region, yet MAL1g encodes a functional maltose permease. Secondly, Southern analysis shows that there is little or no homology between MAL61 (permease)-derived probes and this region of the MAL1g locus which was shown to contain functional permease. (This region is indicated by the stipled bar in Figure 3). Thirdly, sequences homologous to probes derived from the 5'

region of the activator MAL63 are completely absent from plasmid pFE52 and no homology is found between this region of plasmid pFE52 and MAL1 flanking sequences.

The MAL1g allele from strain 340-2B was also isolated using the integrating plasmid pY6ΔCΔH as described in Materials and Methods. Both restriction endonuclease analysis and Southern gel transfer analysis, gave results identical to those obtained for plasmid pFE52 (data not shown).

Structural analysis of the MAL1p allele. Sequences linked to the MAL1p allele of strain 236-2A were isolated in phage λMJC1p.1. This clone was selected using the MAL63-derived probe pH (Figure 1) as described in Materials and Methods. Restriction mapping and Southern analysis using plasmid probes pM1.2C, pM1.4A, pM1.4B and pM1.4C derived from sequences flanking MAL13 of the MAL1 locus (see Figure 2 for probes) clearly demonstrates the homology between the yeast insert contained in phage λMJC1p.1 and the MAL1 locus (Figure 3). Further demonstration that the cloned sequences found in λMJC1p.1 were in fact linked to MAL1p, plasmid pM1.4B was targeted into the genome of strain 345-4A following digestion with BglII (see (*) Figure 2). One stable $Ura^+$ transformant (345-4A::pM1.4B) was analyzed using physical and genetic methods. Briefly, results of physical analysis can be summarized as follows. The restriction map of λMJC1p.1 shown in Figure 3 as a solid line predicts that KpnI sites should flank the site of

integration of plasmid pM1.4B. In the transformed strain 345-4A::pM1.4B the approximately 7.0 kb pM1.4B-homologous KpnI fragment should increase in size to approximately 16.45 kb. Southern gel transfer analysis confirmed this prediction (data not shown). Also, when 345-4A::pM1.4B is crossed to a MAL1 Ura$^-$ strain, Ura$^+$ was shown to cosegregate with MAL1p (see Appendix 4). Together, these results indicate that the phage clone λMJC1p.1 contains MAL1p-linked sequences.

Southern analysis of λMJC1p.1 using MAL63-derived probes suggests that only the sequences from the 5' half of the regulatory gene are present in λMJC1p.1 (Figure 3). We therefore attempted to clone the remainder of the MAL1p allele using a variety of methods. First, the MAL1p phage library was rescreened with the MAL6-probe pH (Figure 1). Several clones were isolated, none, however, were linked to MAL1p. Next, total genomic DNA from transformant 345-4A::pM1.4B#1 (described above) partially resticted with EcoRI, was ligated and used to transform E.coli to ampicillin resistance. None of the clones isolated extended the sequences through the region of the activator gene (and) towards the telomere. Finally, plasmid pM1.4A (Figure 1) was targeted to the MAL1p allele by linearization independently with KpnI and XbaI prior to transformation. Integration of these sequences at a site linked to the MAL1p allele was not achieved. Therefore, despite intense efforts, the cloning of this MAL1 allele remains incomplete.

In view of the fact that these same methods were easily successful in cloning other MAL genes (Dubin et al., 1985, Charron et al., 1986-Chapter one, Charron and Michels, 1987 -Appendix one, Dubin et al., submitted-Appendix two; Charron and Michels, in preparation-Chapter four) it would seem reasonable to assume that such methods would also enable us to isolate the MAL1p locus. This, however, was not the case. Perhaps, for reasons that are not presently apparent, the cloning of the remainder of the MAL1p locus requires very different cloning strategies than those described above.

The restriction map of the remainder of the MAL1p locus, shown in Figure 3 as a dashed line, was derived from Southern analysis of total genomic DNA from strain 236-2A using probes spanning the MAL6 and MAL1 loci (Figures 1 and 2). The results of this analysis are summarized in Figure 3. Homology to only the plasmid probe pE and pG (see Figure 1) could clearly be demonstrated. The vertical dotted line indicates the extent of homology between the MAL1 and MAL1p loci.

Comparative Southern Gel Transfer Analysis of the Flanking DNA Sequences of the MAL1 Alleles. Linkage of MAL1 and SUC1 to the distal end of the right arm of chromosome VII has been demonstrated (Celenza and Carlson, 1985; Carlson et al., 1985). We wished to determine the relationship between the telomere - associated repeated

sequences, X, $(C_{1-3}A)_n$ and Y' and the MAL1 alleles. Homology to X sequences was determined using plasmid YRp120 (Chan and Tye, 1983) and is detected in the cloned MAL1, $mal1^0$ and MAL1g flanking DNA sequences (see Figure 3 shown as vertically hatched bars). Y' sequences, homologous to YR131B (Chan and Tye, 1983), were only found in the sequences flanking MAL1g ( Figure 3 ). Poly (deoxyguanylate-deoxythymidylate) (Boehringer Mannheim) was used to detect $(C_{1-3}A)_n$ repeat sequences flanking the cloned MAL1 alleles. Homology was only detected on the putative telomere-proximal side of each of the isolated loci but was not precisely localized (data not shown). Physical linkage of MAL1 and $mal1^0$ to SUC1 is demonstrated here and in our previous studies (Charron et al., 1986-Chapter one; Figure 2). The results of these analyses define the telomere proximal side of the MAL1 locus and its alleles and clearly demonstrates that all are oriented in the same way with regard to the centromere and telomere of chromosome VII.

Restriction mapping and Southern analysis using probes derived from the centromere-proximal side of the MAL1 locus (pM1.4D, pM1.2C and pM1.4C in Figure 2: fragment c, b, a, respectively) were used to determine the boundaries of homology present among the MAL1, MAL1p and $mal1^0$ alleles (Figure 3). It is clear that homology extends to the end of the isolated fragments. The only observed difference, other than a few polymorphisms, is a small insert (approximately 100 bp) into the MAL1p locus.

It was also of interest to know whether any of the centromere-proximal flanking sequences were present at multiple genomic locations. The results of Southern blots performed on genomic EcoRI digested DNA from MAL1, mal1$^0$, MAL1p and MAL1g strains probed with fragments a, b and c derived from the MAL1 locus (Figure 2) are shown in Figure 4. Fragment a is unique in the genome. In contrast, fragments b and c are repeated several times in all of the strains analyzed (2-9 EcoRI fragments depending upon the strain analyzed). Interestingly, hybridization to all but one or two of the homologous fragments is dramaticallly reduced when hybridizations are carried out at increased stringency (2.4% salt) (data not shown). Thus, these sequences are imperfectly repeated in the genome of these strains. The function of this moderately repeated element, if any, is unclear.

Cloning putative mal pseudogene sequences. Upon screening the MAL1 library (W15-7D:EMBL3) with probes pP2.2 and pD-1 (Figure 1) phage λMJC1.7 was isolated (Materials and Methods). Preliminary restriction endonuclease mapping of the 16.0 kb BamHI insert suggested that something other than the MAL1 locus had been isolated. Southern gel transfer analysis shows that the yeast insert is homologous to pD-1 (Figure 5). When the MAL62-derived plasmid pD-1 is used to probe BamHI digested MAL1 genomic DNA, a highly homologous fragment (approximately 24.0 kb) is detected as

Figure 4: Southern analysis of MAL1, mal1$^{0}$, MAL1p and MAL1g strains probed with MAL1-derived centromere-proximal flanking DNA sequences.

Total genomic DNA digested with EcoRI probed in panel A with fragment a (pM1.4C), in panel B with fragment b (pM1.2C) and in panel C with fragment c (represents results obtained when a replica of the filter from panel B is probed with fragment c (pM1.4A)). The arrows indicate the size of the fragment used in the hybridizations. Lane 1: 600-1B (MAL1); lane 2: 328-4A (mal1$^{0}$); lane 3: 3-2B (mal1$^{0}$); lane 4: 6-2A (mal1$^{0}$); lane 5: 236-2A (MAL1p); lane 6: 345-4A (MAL1p); lane 7: 53-2C (MAL1p); lane 8: DBY782 (MAL1g MAL3g); lane 9: 340-3B (MAL1g) and lane 10: 340-2B (MAL1g).

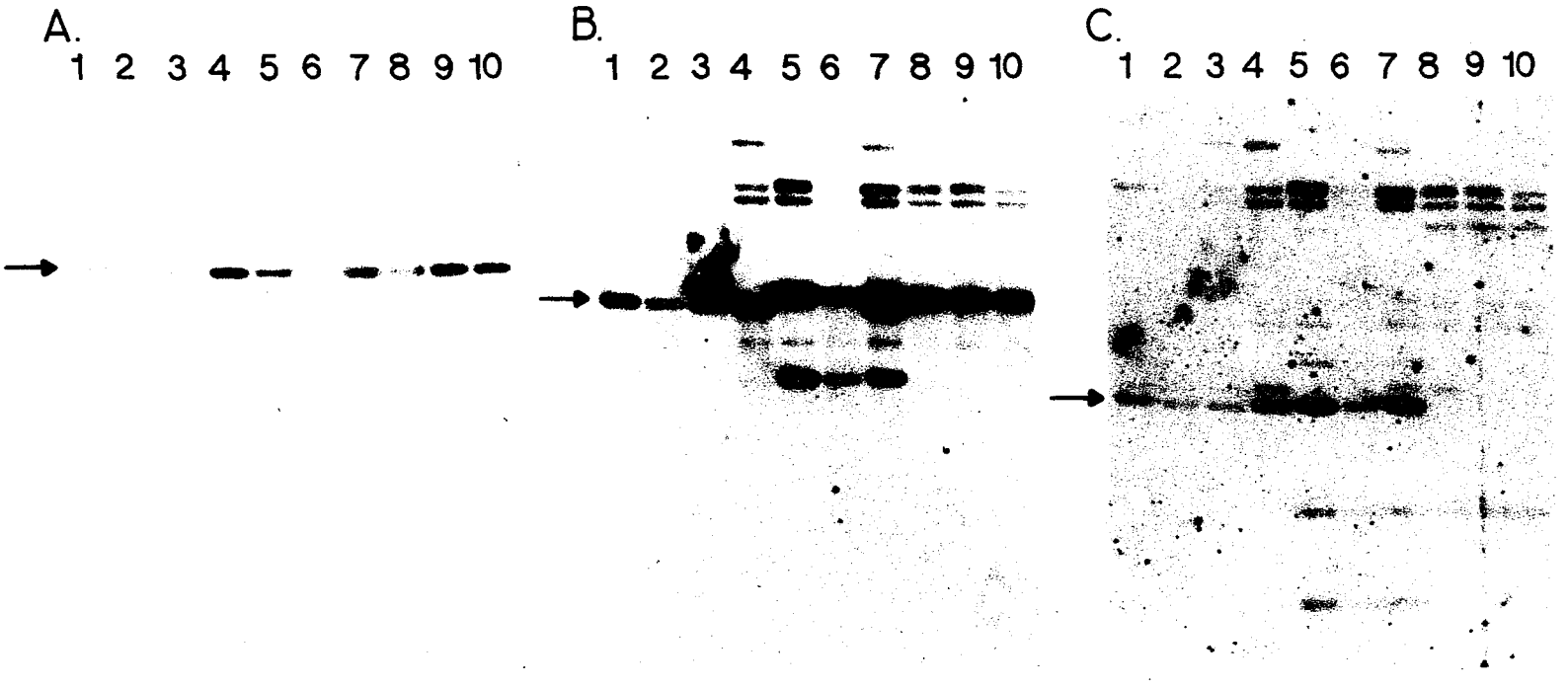
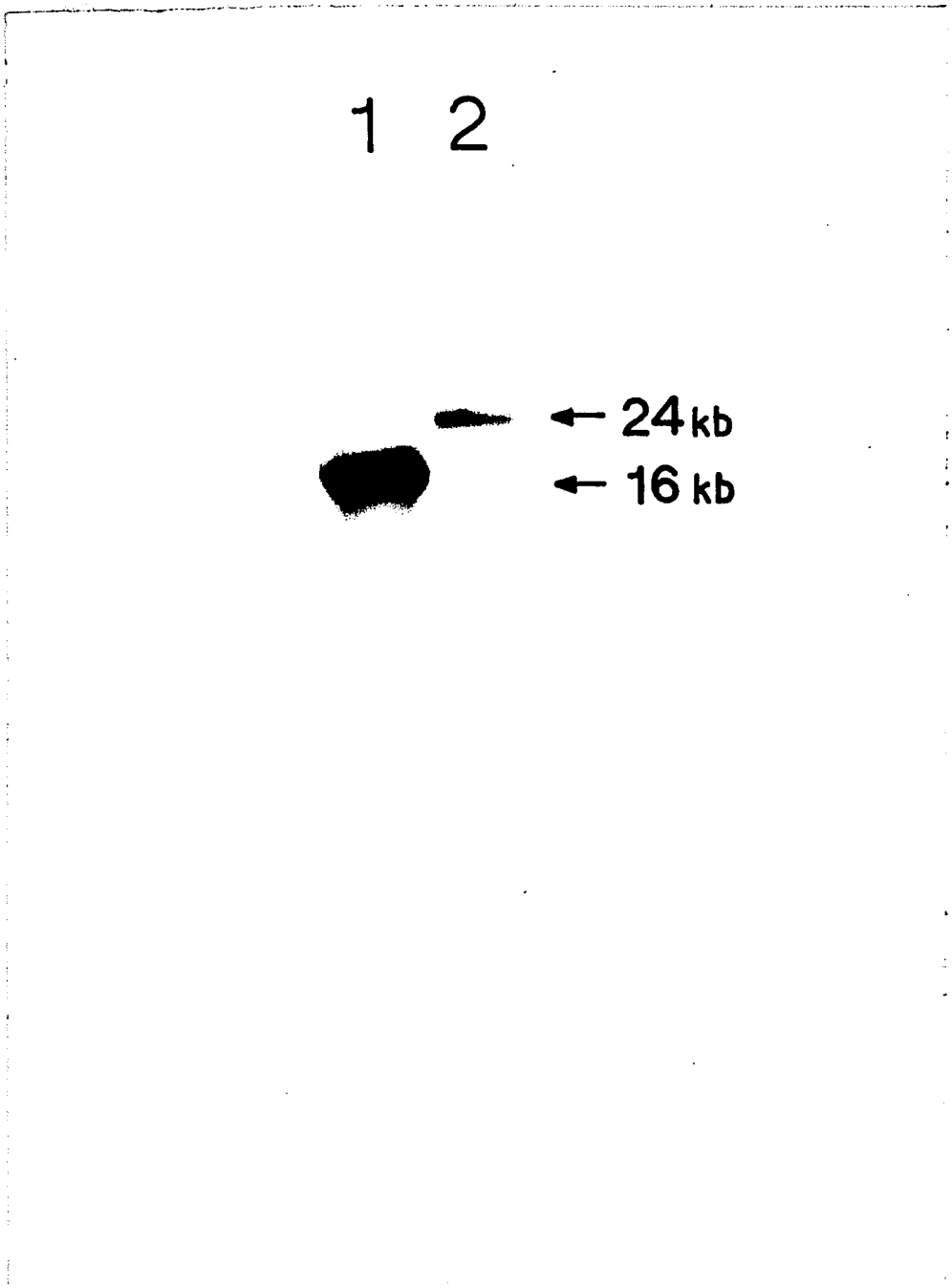

A.
1 2 3 4 5 6 7 8 9 10
B.
1 2 3 4 5 6 7 8 9 10
C.
1 2 3 4 5 6 7 8 9 10
FRAGMENT a
FRAGMENT b
FRAGMENT c

Figure 5: Homology between probe pD-1 and λMJC1.7.

Southern gel transfer analysis of BamHI digested DNA from phage clone λMJC1.7 and MAL1 strain W15-7D probed with the MAL6-derived plasmid pD-1 (Figure 1). Strain W15-7D (lane 2) contains an approximately 24.0kb BamHI fragment displaying homology to pD-1 and a 16.0 kb fragment with poorer homology to the MAL6 probe. Phage λMJC1.7 (lane 1) contains this 16.0 kb BamHI fragment. Approximately twenty times more λMJC1.7 DNA was loaded than W15-7D DNA in order to easily detect pD-1 homology.


1 2
24 kb
16 kb

well as a 16.0 kb fragment with significantly less homology. The 24.0 kb BamHI fragment corresponds to the MAL1 locus. The 16.0 kb fragment present in λMJC1.7 is the smaller BamHI fragment having poor homology to plasmid pD-1 (Figure 5).

Phage λMJC1.7 was further analyzed for homology to other MAL6- and MAL1-derived sequences (Figures 1 and 2), as well as, telomere-associated X and Y' sequences. Results of these studies show that λMJC1.7 has significant homology to probe pHB1 (Figure 1) and very poor homology to X sequences (YRp120) (data not shown). These results suggest that λMJC1.7 contains sequences homologous to maltase and maltase 3' flanking DNA sequences and may also be located at or near to a telomere.

This fragment does not encode functional maltase. The insert DNA of λMJC1.7 was subcloned into YIp5 and tested for its ability to complement the defect in the Δ MAL12 strain 100-1B. The phenotype of the resulting transformant remained unchanged. This result is consistent with the genetic analysis of strain W15-7D showing that it contains only one functional maltase gene, that which is present at the MAL1 locus (R. Needleman, personal communication). Whether this fragment contains a non-functional copy of the maltase gene or simply encodes a gene which is homologous to MAL12 but whose function is unrelated to maltose fermentation is undetermined.

# DISCUSSION

In a previous report we describe the cloning and functional analysis of the MAL1 locus (Charron et al., 1986 -Chapter one). Results of this study have shown that MAL1, like MAL6, contains three genes (MAL11, MAL12 and MAL13) within an approximately 9.0 kb region. These MAL1 genes are both structurally and functionally homologous to their MAL6 counterparts (MAL61, MAL62 and MAL63). Additionally, the telomere-associated SUC1 locus was found to be located 5.0 kb from the 3' end of MAL12. Here we extend our study of the MAL1 locus to include all four alleles of MAL1 (MAL1, $mal1^0$, MAL1p and MAL1g). Through the combined cloning and physical analysis of the three partially functional alleles of MAL1 ($mal1^0$, MAL1p and MAL1g) we demonstrate that the MAL1 alleles appear to be derived from the MAL1 locus by both rearrangements and/or deletions of the MAL1 locus as well as the accumulation of "point mutations".

Genetic analysis of the MAL1 alleles had shown that MAL1p and MAL1g strains complement each other, whereas $mal1^0$ strains complemented neither MAL1p nor MAL1g strains. Further studies by Dubin et al. (1985) were able to demonstrate that the $mal1^0$ locus encodes a functional, slightly thermolabile maltase. In this report we show that $mal1^0$ is highly homologous to MAL1 throughout the entire cloned region (approximately 22.6 kb) with the exception of the MAL13 gene region. Sequence divergence in the region of

the gene encoding the activator (MAL13) is observed through both restriction site polymorphisms and poorer hybridization to MAL63-derived probes (Figure 2). Good homology to maltose permease (MAL61)-derived probes is observed, eventhough the restriction maps are divergent in this region and a functional permease is not present. This data suggests that $mal1^0$ may have evolved from MAL1 through a series of "point mutations" in the region of MAL11 and MAL13 which served to inactivate these genes. The exact mechanism by which a region accumulates large numbers of "point mutations" is not clear. One possible explanation is that this occurred by gene conversion between this locus and another nonfunctional partially sequence homologous region. Our studies clearly demonstrate that such regions are present in the genome.

Results of the physical analysis of the MAL1g allele demonstrate that the cloned sequences completely lack homology to sequences derived from the 5' region of the trans-acting regulatory gene MAL13 and displays poor homology to the MAL63-derived probe pG. MAL1g is highly sequence homologous to MAL12 (maltase) but interestingly, has little sequence homology to MAL11 (maltose permease) eventhough MAL1g was shown to encode a functional maltose permease. This unique maltose permease gene is contained within the 5.4 kb BamHI/BglII fragment (Figure 3). Southern gel transfer analysis of total genomic DNA from MAL1, $mal1^0$, MAL1p and MAL1g strains digested with EcoRI all show similar

patterns of fragments having homology to the 5.4 kb BamHI/BglII fragment derived from the "permease" encoding region of MAL1g (data not shown) demonstrating that several sequences homologous to this "permease" gene are present in each of these strains. The formation of the MAL1g allele from the MAL1 locus appears to have occurred by means of a deletion(s) or rearrangement(s) of the MAL1 region of chromosome VII, resulting in the loss of MAL11 sequences and the positioning of this new "maltose permease" adjacent to the MAL12 gene in such a way as to place the expression under the control of maltose and the maltose activator. Analysis of two different MAL3g alleles reveals that this type of alteration of maltose permease is unique to MAL1g and is not common to all naturally occurring MALg loci (Charron and Michels, in preparation-Chapter three). Perhaps these unusual "maltose permease" sequences are derived from the structural gene of another sugar transport system, the most likely candidates being alpha-methylglucoside permease and maltotriose permease (ten Berge, 1971). More detailed analysis of this allele will be required in order to fully understand the origin of this "maltose permease".

Lastly, we examine the structure of the MAL1p allele. Here, for reasons that are not entirely apparent, only the 5' end of the MAL13 gene and about 9.5 kb of flanking sequences were isolated. The remainder of the map shown in Figure 3 was derived from genomic Southern analysis. The

combined results of cloning and genomic Southern gel transfer analysis revealed that MAL1 and MAL1p are highly sequence homologous in the region encoding the activator MAL13 through the putative centromere-proximal sequences.

We also examined another MAL1p strain, 53-2C. Strain 53-2C has been shown to encode a functional maltase gene by its ability to complement the Δ MAL12 strain 100-1B, in addition to a Δ MAL13 strain (Charron et al., 1986 -Chapter one). Therefore, the MAL1p allele present in strain 53-2C appeared to differ from the other MAL1p strains analyzed. Genetic analysis of a cross between strain 53-2C and the ΔMAL12 strain 100-1B demonstrates that the maltase gene in strain 53-2C is not linked to the MAL1p locus as evidenced by the presence of $Mal^+$ spores (see Appendix 6). The origin and location of the maltase structural gene in this strain are questions for future examination.

MAL1 maps to the distal region of chromosome VII and is tightly linked to SUC1 (Celenza and Carlson, 1985; Carlson et al., 1985). Structural analysis of cloned yeast telomeres has shown that they contain a number of repeated elements: X (0.3 - 3.75 kb), Y' (6.7 kb) and $C_{1-3}A$ (0.33-0.6 kb) (Walmsley et al., 1984). Not all yeast telomeres contain both X and Y' sequences (Horowitz et al., 1984; Zakian et al., 1986). The telomere-linked SUC1 locus, as well as the other telomere-associated SUC loci were shown to be embedded between the telomere repeated X and Y' sequences (Carlson et al., 1985). DNA sequences flanking the MAL1

alleles were examined for telomere-adjacent repeated elements such as X, Y' and SUC. A summary of this analysis is presented in Figure 3. The MAL12 gene of MAL1 and mal1$^0$ is flanked by X sequences and SUC1 and the MAL12 gene of MAL1g is flanked by X, Y' and suc1$^0$ sequences. Due to the repeated nature of telomere-associated sequences it was impossible to determine linkage of MAL1p flanking DNA sequences to either X or Y' by Southern gel transfer of genomic DNA.

It has been shown that extensive restriction site polymorphisms exist in telomeres of different strains, as well as within the same strain of S. cerevisiae and these regions undergo frequent rearrangements (Horowitz et al., 1984). The highly polymorphic nature of the MAL1 alleles is quite evident in the results of these analyses. The polymorphisms are structural as well as functional. Perhaps the telomere-adjacent location of the MAL1 locus has contributed to the apparent mutability of this locus which have resulted in the formation of three partially functional alleles (MAL1p, MAL1g and mal1$^0$). The MAL1 locus is the only MAL locus known to have four alleles. The telomere-associated MAL3 locus has two known alleles: the fully functional MAL3 allele and the partially functional MAL3g allele. A complete molecular analysis of the MAL3 alleles will be presented elsewhere (Charron and Michels, in preparation-Chapter three). The other MAL loci (MAL2, MAL4 and MAL6) are also linked to chromosome termini, however

partially functional alleles of these loci have not been found in the laboratory strains used (Needleman and Michels, 1983; Michels and Needleman, 1984). Perhaps genetic and physical analysis of strains isolated from the wild is needed to determine if MAL2, MAL4 and/or MAL6 have partially functional alleles similar to those linked to MAL1 and MAL3.

All yeast strains that have been analyzed genetically have been shown to contain one of the alleles of the MAL1 locus. Therefore, it has been proposed that MAL1 may be the progenitor MAL locus. To examine this possibility DNA sequences flanking the centromere-proximal side of the MAL1 locus were also analyzed. No telomere associated sequences, such as X, Y' or $(C_{1-3}A)_n$ were detected. It has been proposed that polygenic families such as the SUC and MAL loci were produced by inter-chromosomal homologous recombination events involving sequences found at several (or all) telomeres. Fragments b and c (Figure 2) were shown to be repeated several times in most MAL1 strains analyzed (Figure 4) and therefore are candidates for mediating such recombination events. DNA sequences flanking the other MAL loci have been cloned and show no homology to these fragments suggesting that fragments b and c were not influential in the mobilization of the MAL loci (Charron and Michels, in preparation-Chapter four). Our results do not exclude the possibilities that a small sequence in fragment b and/or c is common to all MAL loci and that the probes used were just too large to detect this small repeat, or

that sequence differences could have arisen subsequent to the translocation event.

It is interesting to note that in most yeast strains analyzed homology to probes derived from GENE 1, GENE 2 and GENE 3 are observed at several genomic locations outside the approximately 9.0 kb boundary of the MAL locus (data not shown). Exactly what these other homologies are and how they originated is not known. In the course of screening libraries with MAL-gene probes it is expected that several different unlinked fragments will be isolated. We describe the cloning and partial functional analysis of one such sequence (λMJC1.7) from a MAL1 library. As seen in Figure 5 the insert in λMJC1.7 has poor homology to probe pD-1. The sequences contained within this cloned fragment do not allow us to decide whether these sequences represent a degenerate maltase gene or homologous sequences that have no relationship to maltose fermentation. Li (1984) and Hall et al. (1983) discuss the role of cryptic genes in microbial populations and suggest that these elements add to the "genetic reservoir" of the species because they may become mutationally activated at sometime in the future. Whether or not these other MAL homologous sequences (ie: λMJC1.7) can serve a similar role is a question that remains unanswered.

# Chapter 3

## Molecular cloning and physical analysis of the alleles of the *MAL3* locus of *Saccharomyces*

## ABSTRACT

The maltose fermentation loci (*MAL1* - *MAL4*, *MAL6*) represent a dispersed family of repeated genes present in *Saccharomyces*. A *MAL* locus encodes three gene functions: maltose permease, maltase and a trans-acting activator protein. Partially functional *MALg* alleles, which encode maltase and maltose permease, linked to the *MAL1* and *MAL3* loci have been identified. In this report we describe the molecular cloning of three *MAL3g* loci. The *MAL3g* clones were compared to cloned sequences from the *MAL3* and the *MAL6* loci using restriction enzyme mapping, Southern blotting and heteroduplex analysis. Results demonstrate that *MAL3g* is structurally and functionally homologous to *MAL3* and *MAL6* throughout a 6.3 kb region which contains the structural genes for maltase and permease. Analysis of flanking DNA sequences demonstrates that *MAL3g* is located next to a telomere and has probably evolved from *MAL3* via a deletion(s) or rearrangement of sequences on the centromere -proximal side of the locus which include *MAL33*.

The fermentation of maltose by the Saccharomyces yeasts requires the presence of any one of five unlinked, telomere-associated loci: MAL1, MAL2, MAL3, MAL4 or MAL6 (reviewed by Barnett, 1976). Molecular cloning and mutational analysis of the MAL1 and MAL6 loci has identified a cluster of three genes at each locus, all of which are needed for fermentation. GENE 1 which encodes maltose permease, GENE 2 which encodes maltase and GENE 3 which encodes a trans-acting activator (Needleman et al., 1984; Dubin et al., 1985; Charron et al., 1986-Chapter one; Chang et al., submitted). Genetic and physical analyses of standard maltose fermenting Saccharomyces strains revealed that, in addition to the dominant MAL locus, each strain contained either one or two partially functional MAL loci. These partially functional loci were shown to be allelic to MAL1 and MAL3 (Michels and Needleman, 1983; 1984; Needleman and Michels, 1983). Three partially functional alleles of the MAL1 locus were identified (MAL1p, MAL1g and mal1$^{0}$) and have been structurally and functionally analyzed (Charron and Michels, manuscript in preparation-Chapter two). The only allele of MAL3 identified by these studies is MAL3g. More recent studies (Needleman et al., 1984; Dubin et al., 1985; Chang et al., submitted) have demonstrated that the MALg class of partially functional alleles encode the structural gene functions (maltase and maltose permease) while the MALp alleles encode a functional activator.

Needleman and Michels (1983) and Michels and Needleman

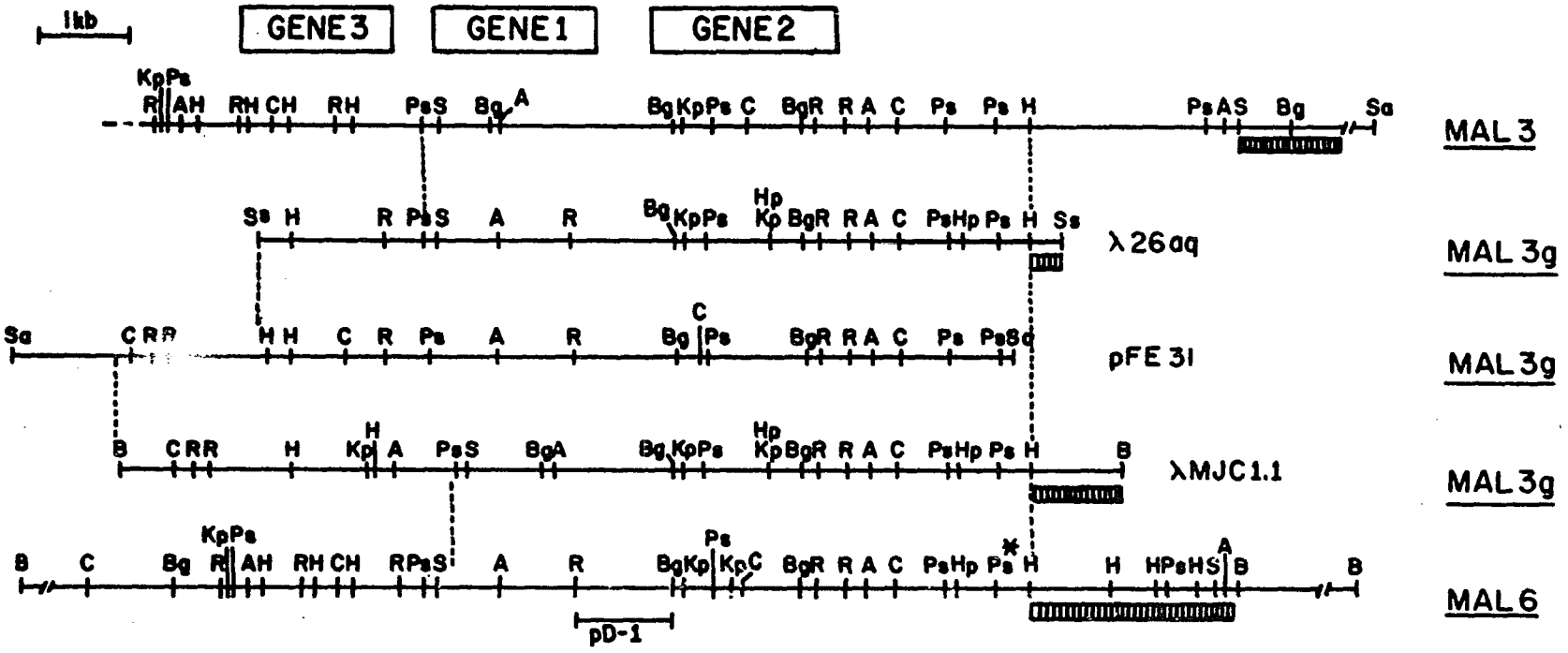
(1983;1984) demonstrate that the two known alleles of the MAL3 locus (MAL3 and MAL3g) of several Saccharomyces strains are sequence homologous to the MAL6-derived probe pD-1 (Figure 1). The MAL3 locus has been cloned and shown to be both structurally and functionally homologous to MAL6 throughout an approximately 9.0 kb region (Charron et al., manuscript in preparation-Chapter four; Figure 1). Here we describe the molecular cloning and comparative structural and functional anlaysis of three cloned MAL3g loci.

The MAL3g locus was cloned from strain 28A (MAL1g MAL3g) (Hicks et al., 1979) which was derived from strain 2180-1A (a derivative of S288C). SstI restricted DNA from strain 28A was ligated into phage λgt WES.B using methods previously described (Charron et al., 1986 - Chapter one). The resultant library was screened using the MAL6-derived probe pD-1 and clone λ26aq was isolated. The restriction enzyme map of the yeast insert in λ26aq is shown in Figure 1. Phage λ26aq contains the MAL3g locus of strain 28A since the 8.1 kb HindIII fragment, when subcloned into YIp5, is able to complement a MAL1p strain (strain 345-4A described in Charron et al., 1986-Chapter one) which has been shown to encode only a functional activator protein.

Another MAL3g clone was analyzed, plasmid pFE31 (gift of M. Carlson). To isolate this plasmid, DNA from strain DBY939 (MAL1g MAL3g congenic to strain S288C) was partially restricted with Sau3A and ligated into YEp24. Clone pFE31 was isolated using a SUC1 - derived probe as described

Figure 1: Restriction endonuclease maps of the MAL3 alleles and MAL6.

Complete restriction endonuclease maps of the MAL3 locus, the MAL3g loci and the MAL6 locus are shown. Boundaries of sequence homology are indicated by the vertical dotted lines. Homology to telomere-associated X sequences (YRp120; Chan and Tye, 1983) are indicated by vertical hatched bars. Abbreviations for restriction endonucleases are as follows: A, AvaI; B, BamHI; Bg, BglII; C, ClaI; H, HindIII; Hp, HpaI; Kp, KpnI; Ps, PstI; R, EcoRI; S, SalI; Sa, Sau3A; Ss, SstI. Not all Sau3A or HpaI sites are shown.


1kb
GENE3
GENE1
GENE2
MAL3
λ26aq
MAL3g
pFE31
MAL3g
λMJC1.1
MAL3g
MAL6
pD-1

previously (Carlson et al., 1985). Results of Southern and genetic analysis demonstrated that the sequences contained in plasmid pFE31 were linked to the suc3$^0$ locus and therefore contain suc3$^0$ flanking DNA sequences (Carlson et al., 1985). The restriction enzyme map of the yeast insert in plasmid pFE31, as shown in Figure 1, is very similar to that of λ26aq suggesting that they may be of common origin (S288C). This clone also contains the previously described MAL3g-linked 8.1 kb HindIII fragment homologous to plasmid pD-1 (Michels and Needleman, 1983). Similarly, plasmid pFE31 only complements the MAL1p strain 345-4A.

Lastly, the MAL3g locus from strain 4059 of the Berkeley Yeast Stock Center (MAL1 MAL3g) (Michels and Needleman, 1983) was cloned. BamHI digested DNA from strain 4059 was ligated into phage EMBL4. Clone λMJC1.1 was isolated from the resultant library using probe pD-1. The restriction endonuclease map of the 11.0 kb BamHI insert in λMJC1.1 is presented in Figure 1. Phage λMJC1.1 was shown to contain the MAL3g locus of strain 4059 based on the finding that the 7.1 kb HindIII fragment of the yeast insert, when subcloned into YIp5, complements the MAL1p strain 345-4A demonstrating that it contains functional maltase and permease genes.

As seen in Figure 1, restriction site polymorphisms are found in each of the MAL3g loci analyzed, most note worthy the HindIII polymorphism. In their studies of various maltose fermenting strains, Needleman and Michels (1983;

Michels and Needleman, 1983;1984) found that both the 7.1 kb and the 8.1 kb HindIII fragments homologous to the MAL6 -derived probe pD-1 were linked to the MAL3g locus depending upon the strain analyzed. These results demonstrate that the MAL3g loci obtained from unrelated strains (derived from strain S288C vs. strain 4059) are quite heterogeneous in nature and contrasts with those obtained from the analysis of two MAL1g loci, both of which had the same restriction endonuclease map (Charron and Michels, manuscript in preparation-Chapter two). The MAL3g restriction endonuclease maps diverge from that of the MAL3 and MAL6 loci in the region of GENE 3 (MAL regulator).

The MAL3g clones were next subjected to Southern gel transfer analysis using probes derived from GENEs 1, 2 and 3 of the MAL6 locus. The results of this analysis are summarized in Figure 1. Sequences homologous to maltose permease (GENE 1) and maltase (GENE 2) but not to the regulator (GENE 3) are present in each clone and are contained within either the 7.1 kb or 8.1 kb HindIII fragment found between the vertical dotted lines. Results of heteroduplex analysis (described below) of the 7.1 kb HindIII fragment from λMJC1.1 (MAL3g) and the 7.3 kb HindIII fragment from MAL6 confirmed these results (Figure 2). Whether the loss of the regulatory gene MAL33 was the result of a deletion event, a rearrangement or an accumulation of point mutations in this region cannot be determined from these results.

Next, the flanking DNA sequences of the MAL3g clones were analyzed. The MAL3 locus maps close to the telomere of the right arm of chromosome II (Mortimer and Schild, 1980) therefore the clones were screened for the presence of known telomere-associated sequences such as X, Y' and SUC (Chan and Tye, 1983; Carlson et al., 1985). As shown in Figure 1, X sequences flank the right side of GENE 2 in MAL3, MAL3g and MAL6. X sequences are also found flanking the MAL3g locus contained in strain DBY939 as reported by Carlson et al. (1985) but are not present on plasmid pFE31. The position of the X-homology is different in MAL3 as compared to the MAL3g loci. The significance of this is unknown.

To complete the comparative Southern analysis, probes derived from the centromere-proximal side of MAL33 were tested for homology to MAL3g sequences. One sequence derived from the region approximately 7 kb to the left of MAL33 was shown to be homologous to the 3.8 kb BamHI - SalI fragment of λMJC1.1. This region of λMJC1.1 is also homologous to the 4.5 kb Sau3A - PstI fragment of plasmid pFE31 and the 1.9 kb SstI - SalI fragment of phage λ26aq (data not shown). This result may be suggesting that MAL3g evolved from MAL3 via a deletion of MAL33 and several kilobasepairs of flanking sequences. However, the restriction enzyme maps of the homologous regions are divergent thus suggesting that mutation(s) subsequent to the rearrangement may have occurred.

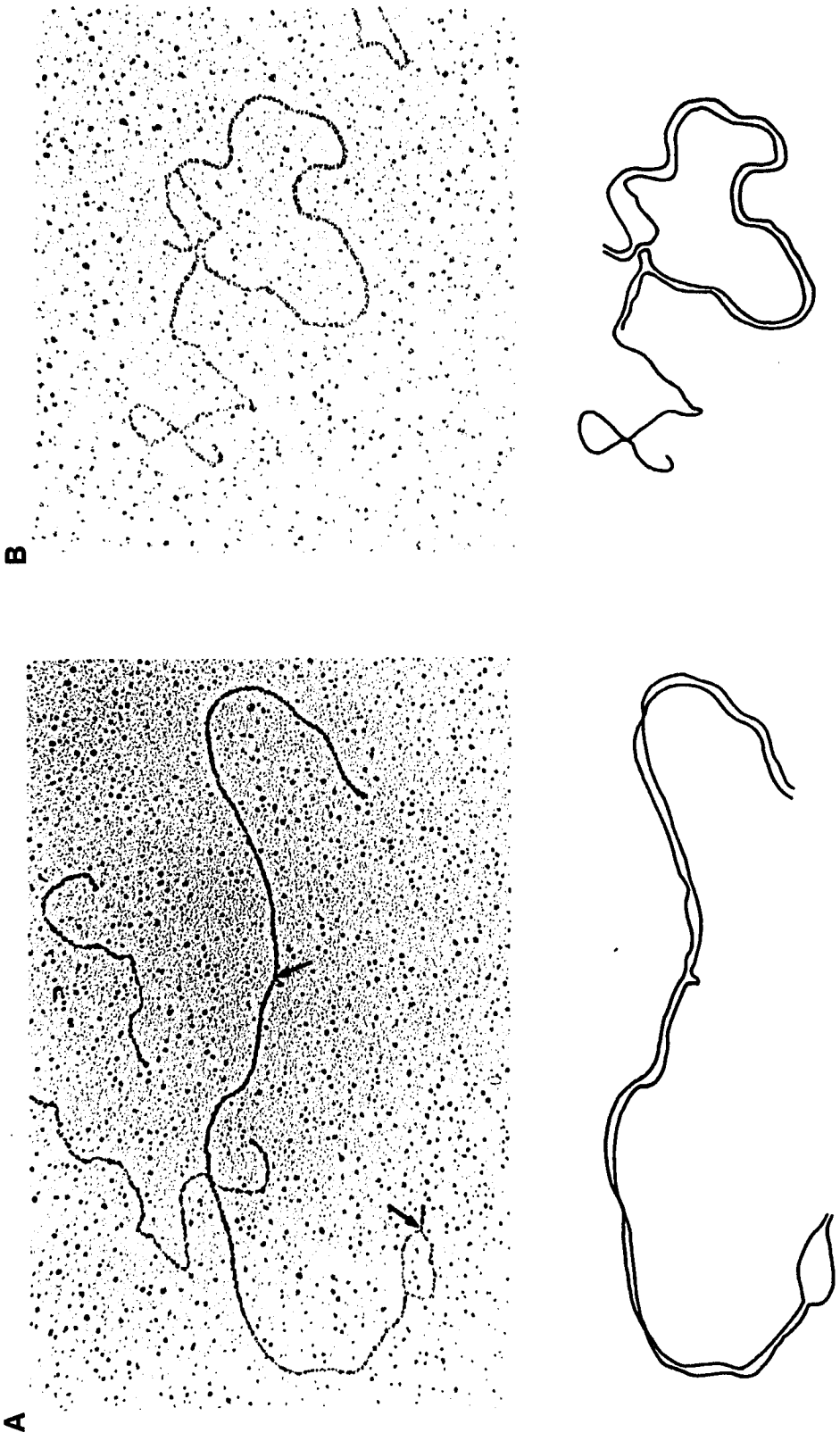
Lastly, as stated above, the structural genes from the

MAL3g clone λMJC1.1, contained within the 7.1 kb HindIII fragment, were compared with those of the MAL6 locus, contained within the 7.3 kb HindIII fragment, by heteroduplex analysis. Electron micrographs of two such heteroduplexes are shown in Figure 2 with the schemmatic tracings of the hybrid DNA molecules below. The ends of the yeast insert are indicated by the arrows in Figure 2A. It is clear from this electron micrograph that the MAL6 clone contains an insertion of approximately 300 bp near to the end of the yeast insert (approximate location indicated by the * in Figure 1). At the other end of the yeast insert a denaturation bubble is present, indicating a lack of sequence homology between MAL3g and MAL6. This defines the boundary of the sequence homology between these two clones (shown as vertical dotted lines in Figure 1). In another electron micrograph (Figure 2B), sequence homology between the small insertion loop and the distal end of the denaturation loop was detected. The repeated homology present here extends approximately 150-300 bp and was not detected or localized by Southern analysis. The meaning of this apparent repeat sequence flanking the functional "MAL6g" unit, consisting of the MAL61 and MAL62 genes, remains to be determined.

In summary, we have demonstrated through the functional and structural analysis of several MAL3g clones that the MAL3g allele is both structurally and functionally homologous to the MAL3 and MAL6 loci throughout an

Figure 2: Heteroduplex mapping of the MAL3g locus of strain 4059 and the MAL61 and MAL62 genes of strain CB11.

The 7.1 kb HindIII fragment of λMJC1.1 was subcloned into YIp5, linearized with BamHI and hybridized under conditions that favor heteroduplex formation with a plasmid containing the MAL61 and MAL62 genes on the 7.3 kb HindIII fragment also subcloned into YIp5 and linearized with BamHI (Chow and Broker, 1981). Electron micrographs of two heteroduplexes identified are shown at the top. Schemmatic tracings depicting the interaction between the two DNA strands are shown below. Arrows indicate the ends of the yeast insert (approximate location of the HindIII sites).


B
A

approximately 6.3 kb region. Contained within this region are the structural genes for maltose permease (MAL31) and maltase (MAL32). Sequences homologous to the MAL33 gene are lacking from all three cloned MAL3g alleles. Analysis of the flanking DNA sequences demonstrates that MAL3g, like MAL3 and MAL6, is located next to a telomere and that MAL3g has probably evolved from MAL3 via a deletion(s) or rearrangement event involving the MAL33 gene of MAL3 but not the MAL31 and MAL32 genes. The results of this study contrast with those of the MAL1g allele (Charron and Michels, manuscript in preparation-Chapter two) in two respects. Firstly, the MAL1g alleles of different strains appear to be very similar. All are contained on a 10.7 kb HindIII fragment and the two cloned MAL1g loci are highly homologous with regard to their restriction map (Charron and Michels, manuscript in preparation-Chapter two). Secondly, the MAL1g allele contains a maltose permease gene which is not structurally homologous to MAL61. Together these results underscore the variable nature of sequences located near yeast telomeres.

# Chapter 4

## Molecular evolution of the telomere-associated *MAL* loci of *Saccharomyces*

## ABSTRACT

The *MAL* gene family of *Saccharomyces* consists of five multigene complexes (*MAL1*, *MAL2*, *MAL3*, *MAL4* and *MAL6*) each of which encodes maltose permease (GENE 1), maltase (GENE 2) and the trans-acting *MAL*-activator (GENE 3). Four of these loci have been mapped and each is located at or near the telomeres of a different chromosome. We describe the physical structure of the *MAL* loci and their flanking sequences. The *MAL* loci were shown to be both structurally and functionally homologous throughout an approximatly 9.0 kb region. The orientation of the *MAL* loci was determined to be: CENTROMERE ... GENE 3 - GENE 1 - GENE 2 ... TELOMERE. Telomere-adjacent sequences were found flanking GENE 2 of the *MAL1*, *MAL3* and *MAL6* loci. No common repeated elements were found on the centromere-proximal side of all of the *MAL* loci. These results suggest that the *MAL* loci translocated to different chromosomes via a mechanism that involved the rearrangement(s) of chromosome termini.

# INTRODUCTION

The fermentation of the disaccharide maltose by the Saccharomyces yeasts requires the presence of any one of a family of five unlinked loci (MAL1, MAL2, MAL3, MAL4 and MAL6) (reviewed by Barnett, 1976). Four of the five MAL loci have been genetically mapped and are located at or near to a telomere: MAL1, right arm chromosome VII (Celenza and Carlson, 1985); MAL2, right arm chromosome III; MAL3, right arm chromosome II; and MAL4, right arm chromosome XI (Mortimer and Schild, 1980). MAL6 is linked to chromosome VIII, however its exact map position is unknown (R. Dubin, 1987). A similar genomic arrangement is observed in the SUC gene family encoding invertase (Mortimer and Schild, 1980; Celenza and Carlson, 1985). In fact, the MAL1 and MAL3 loci are tightly linked to the SUC1 and SUC3 loci, respectively.

Genetic and physical analyses of strains containing each of the MAL loci show that the MAL loci are highly sequence homologous (Michels and Needleman, 1983; 1984; Needleman and Michels, 1983). Physical comparison of the cloned MAL1 and MAL6 loci by restriction mapping and Southern analysis reveals extensive homology over an approximately 9.0 kb region (Charron et al., 1986-Chapter one). Functional analysis of this region demonstrates the presence of three genes (Needleman et al., 1984; Dubin et al., 1985; Charron et al., 1986-Chapter one; Chang et al.,

submitted). GENE 1 appears to encode maltose permease; GENE 2 encodes maltase and GENE 3 encodes a positive trans-acting regulator of the structural genes and is refered to as the MAL activator.

In this study we extend our comparative analysis of the MAL loci to MAL2, MAL3 and MAL4, as well as to the DNA sequences flanking each of the five MAL loci. Our results demonstrate that all of the MAL loci are both structurally and functionally homologous throughout an approximately 9.0 kb region containing the three genes encoding the fermentative enzymes and the activator protein. Additional sequence homology extending beyond this 9.0 kb region to the centromere-proximal side of the MAL2 and MAL4 loci is detected. Telomere-adjacent sequences are found in the region flanking GENE 2 of the MAL1, MAL3 and MAL6 loci. No common repeated elements flank the centromere-proximal region of all the MAL loci. The orientation of the MAL loci was determined to be: CENTROMERE ... GENE 3 - GENE 1 - GENE 2 ... TELOMERE. The implication of these results in regard to the mechanism of translocation of the MAL loci is discussed.

# MATERIALS and METHODS

## Strain Growth Conditions

Yeast strains were grown on YEP medium (1% (wt/vol) yeast extract/ 1% (wt/vol) peptone) plus 2% (wt/vol) glucose. Maltose fermentation is defined as the production of acid and gas in 1-3 days after inoculation and is determined in 5ml YEP plus 2% (wt/vol) maltose medium in Durham tubes.

Plasmids were propagated in E.coli strain C600 or RR1.

## Preparation and Analysis of DNA

Plasmid, yeast and phage DNA's were prepared as described previously (Needleman et al., 1984; Charron et al., 1986-Chapter one). Standard methods for restriction mapping, gel electrophoresis and DNA subcloning were utilized (Maniatis et al., 1982). Southern gel transfer analysis was as described in Dubin et al. (1985).

## Yeast Transformation and Plasmid Rescue

Yeast transformations were carried out by the method of Ito et al. (1983) using lithium acetate. Transformants were screened for functional "ARS" sequences by assaying plasmid stability following passage through non-selective media (YEP medium plus 2% (wt/vol) glucose).

All plasmids generated were assayed for functional

MAL genes by the ability to complement standard MAL1p, MAL1g and/or mal1$^0$ tester strains (Michels and Needleman, 1983; Charron et al., 1986-Chapter one) (Table 1). In most cases, 50 transformants were assayed for maltose fermentation using the criteria described above.

Plasmids pMJC6ΔC, pY6-RΔC and pY6ΔCΔH were used for site-directed integration (Orr-Weaver et al., 1983) into several of the MAL loci following linearization with ClaI, BglII and HpaI, respectively, prior to transformation (Figure 1 (*); Dubin et al., 1986; Charron and Michels, 1987-Appendix one; Dubin et al., submitted-Appendix two). Linkage of the plasmid marker (URA3) to a particular MAL locus was determined using both physical and genetic analysis similar to that described in Charron et al. (1986-Chapter one) (see Appendix four).

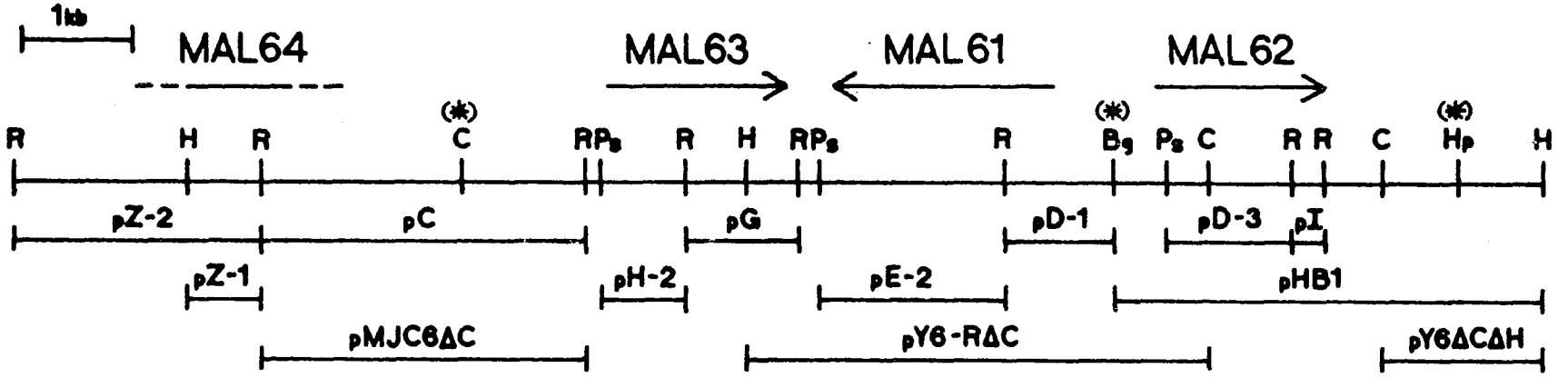
MAL-linked DNA sequences were isolated from pY6-RΔC transformed strains following digestion with BamHI; from pY6ΔCΔH transformed strains following digestion with HindIII, BamHI or SalI; and from pMJC6ΔC transformed strains digested with BamHI. The restricted DNA's were then ligated under dilute conditions and used to transform E. coli strain RR1 to ampicillin resistance as described previously (see above).

Cloning the MAL3 locus of strain 48-2C

Total genomic DNA from strain 48-2C was partially restricted with Sau3A, size fractionated (9.0 kb - 20.0

Figure 1: Restriction endonuclease map of the MAL6 locus from strain CB11 and subclones used in this study.

A partial restriction endonuclease map of the MAL6 locus along with the locations of the MAL61, MAL62, MAL63 and MAL64 genes is presented. Direction of transcription of MAL61, MAL62 and MAL63 is shown. All subclones diagramed are contained in plasmid pBR325 except pY6ΔCΔH (in YIp5 ΔHindIII), pY6-RΔC (in YIp5) and pMJC6ΔC (in YIp5 ClaI) (Charron et al., 1986-Chapter one; Charron and Michels, 1987-Appendix one; Dubin et al., submitted- Appendix two, respectively). The symbol (*) represents the site of linearization of plasmids pY6ΔCΔH, pY6-RΔC and pMJC6ΔC used in site-directed integration experiments. Restriction endonucleases are abbreviated as follows: Bg, BglII; C, ClaI; H, HindIII; Hp, HpaI; Ps, PstI; R, EcoRI.


1kb
MAL64
MAL63
MAL61
MAL62
R
H
R
(*)
C
RPs
R
H
RPs
R
(*)
Bg
Ps
C
R
R
C
(*)
Hp
H
pZ-2
pC
pG
pD-1
pD-3
pI
pZ-1
pH-2
pE-2
pHB1
pMJC6ΔC
pY6-RΔC
pY6ΔCΔH

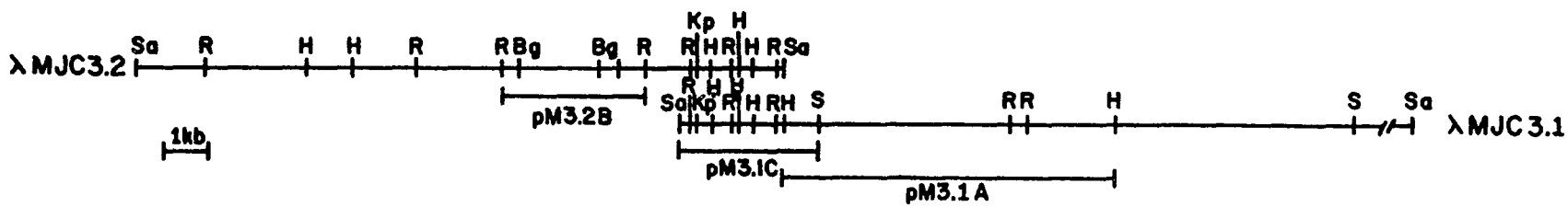
kb) and ligated into EMBL3 BamHI arms, packaged and amplified as described in Charron et al. (1986-Chapter one). The resultant library was screened with MAL6-derived probes. One phage clone, λMJC3.1, was isolated using probe pD-1 and a second, partially overlapping, clone (λMJC3.2) was isolated using pH (see Figure 1 for MAL6 probes). Figure 2 shows a composite restriction map of these two phage clones.

Combined physical and genetic analysis of the MAL3 locus has shown that a 7.3 kb, a 4.7 kb and a 4.5 kb pD-1 homologous HindIII fragment is linked to this locus (Michels and Needleman, 1983). Phage clone λMJC3.1 has been shown to contain a 7.3 kb HindIII fragment and phage λMJC3.2 contains a 5.9 kb HindIII fragment (originally sized as 4.7 kb by Michels and Needleman, 1983) that is homologous to pD-1, thus suggesting that they are derived from the MAL3 locus (data not shown). Phage λMJC3.1 was shown to contain functional MAL31, MAL32 and MAL33 genes through plasmid subcloning and complementation studies (described in Results and Table 1).

As can be seen in Figure 2, λMJC3.1 and λMJC3.2 overlap only in the region of MAL33. To be sure that the non-overlapping sequences contained in phage clone λMJC3.2 actually flank the MAL3 locus, linkage association between these fragments and the MAL3 locus had to be established. Plasmid pM3.2B (Figure 2) was linearized by partial digestion with BglII and used to transform strain

Figure 2: MAL3 phage clones and subclones used in this study.

The MAL3 locus of strain 48-2C (MATα MAL3 mal1$^0$ ade1 lys2) was cloned into phage EMBL3 (see Materials and Methods). Partial restriction maps of the yeast inserts contained within phage clones λMJC3.1 and λMJC3.2 are shown. Plasmid pM3.1C contains the 3.1 kb SalI fragment from λMJC3.1 in YEp13 and plasmid pM3.1A contains the 7.3 kb HindIII fragment of λMJC3.1 in YIp5. The 3.2 kb EcoRI fragment flanking MAL33 was subcloned into YIp5 ClaI (a YIp5 derivative from which the ClaI site has been deleted) to form plasmid pM3.2B. Restriction enzymes are abbreviated as in Figure 1 with the following additions: Kp, KpnI; S, SalI.


λMJC3.2
Sa
R
H
H
R
RBg
Bg
R
Kp
H
RHRHRSa
pM3.2B
SaKpRHRHRH
S
RR
H
S
Sa
λMJC3.1
1kb
pM3.1C
pM3.1A

MCY102-5A to Ura$^+$. One stable Ura$^+$ transformant was selected, MCY102-5A::pM3.2B#13, and this was crossed to the mal1$^0$ strain 347-2A. The plasmid marker (URA3) was shown to cosegregate with the Mal$^+$ phenotype (see Appendix four). Southern gel transfer analysis of the untransformed strain MCY102-5A, the parent strains (MCY102-5A::pM3.2B#13 and 347-2A) and one complete tetrad supports the conclusion that the site of integration of plasmid pM3.2B is linked to the MAL3 locus and, therefore, that the sequences contained in plasmid pM3.2B are linked to MAL3. The restriction map shown in Figure 2 represents a composite of the yeast inserts in the two phage λMJC3.1 and λMJC3.2. Further analysis of the MAL33 and MAL32 flanking regions by plasmid rescue of genomic sequences, is currently underway in an effort to demonstrate that the map shown in Figure 2 represents the actual genomic arrangement of sequences flanking MAL33 and to extend the MAL3 sequences toward the telomere in the hope of cloning SUC3.

Construction of plasmids pL3-4 and pL5-15

In order to determine the orientation of the MAL2 locus with respect to the centromere and telomere, plasmids pL3-4 and pL5-15 were constructed (Figure 4). These plasmids were formed by ligating the HindIII/SalI digested HMR plasmid p15C (Klar et al., 1981) to the MAL63 gene disruption plasmid pDM3 (Charron et al., 1986-Chapter

one; Chang et al., submitted) which was partially restricted with HindIII and completely digested with SalI. Plasmid pL3-4 contains the 4.1 kb HindIII/EcoRI fragment from plasmid p15C containing the HMR locus, a 900bp HindIII/SalI fragment from plasmid pDM3 (Charron et al., 1986) containing the 5' end of MAL63 along with some vector sequences and the 1.1 kb HindIII fragment containing the URA3 gene from YIp30. The organization of these fragments is shown in Figure 4 (ORIENTATION 2). Plasmid pL5-15 is identical to plasmid pL3-4 except that it contains the 900 bp HindIII/SalI fragment from the 3' end of the MAL63 gene instead of the 5' MAL63 sequences (see Figure 1), thus defining ORIENTATION 1 shown in Figure 4. Both plasmids pL3-4 and pL5-15 were digested with EcoRI prior to transformation of strain MCY101-3A to $Ura^+$.

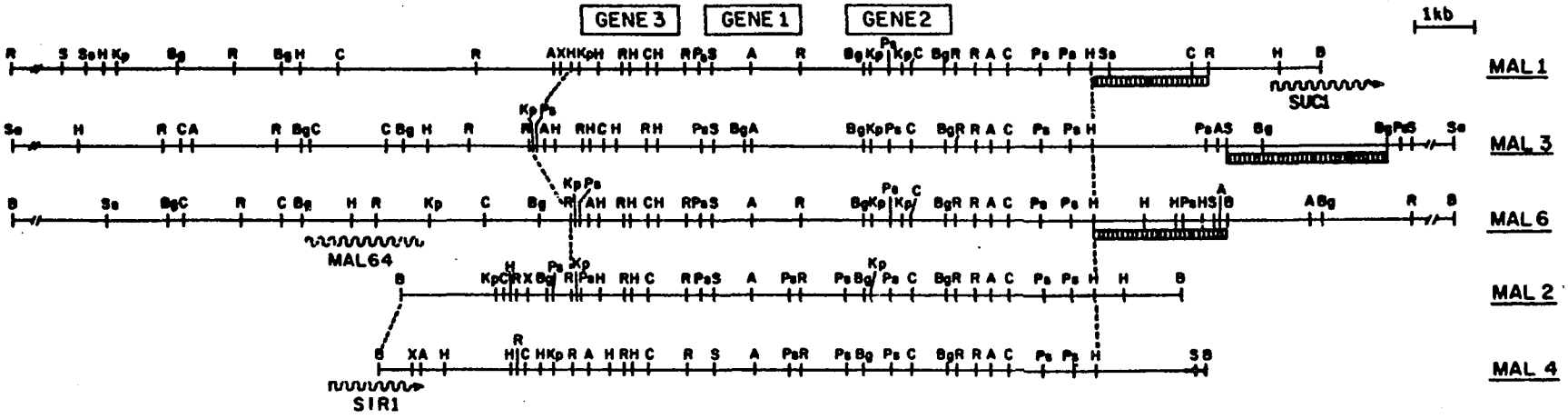
# RESULTS

Structural and functional comparison of the coding region of the MAL loci. Federoff et al. (1982) describe the isolation of a 12.5 kb DNA fragment which was shown to contain the MAL6 locus (Needleman and Michels, 1983). We have extended the size of this cloned region by isolating chromosomal fragments flanking the MAL6 locus. This was done using the technique of plasmid rescue, which involves the recovery from the genome of chromosomally integrated plasmids (described in Materials and Methods; Dubin et al., submitted). This same technique was used to isolate the MAL2 and MAL4 loci along with flanking DNA sequences (see Materials and Methods; Charron and Michels, 1987 -Appendix one). The isolation of the MAL1 locus has been described previously (Charron et al., 1986-Chapter one). The MAL3 locus was isolated from a genomic phage library as described in the Materials and Methods. Figure 3 shows the restriction map of each of these loci along with their flanking DNA sequences. Indicated above the map is the position of the genes encoding maltose permease (GENE 1), maltase (GENE 2) and the MAL-activator (GENE 3) that have been identified at the MAL1 and MAL6 loci.

Based on a comparison of the restriction maps the cloned sequences appear to be highly conserved throughout the coding regions with only a few restriction site polymorphisms (Figure 3). The results of Southern

Figure 3: Comparison of the MAL loci.

The restriction endonuclease map of the MAL1, MAL2, MAL3, MAL4 and MAL6 loci and their flanking sequences. The approximate location of the three genes needed for maltose fermentation are diagramed above the maps. The boundaries of homology between the MAL loci are indicated by vertical dotted lines. Homology to telomere-derived X sequences was detected using plasmid YRp120 (Chan and Tye, 1983) and regions of homology are diagramed with vertical hatched bars. Homology to SUC sequences (pRB117- containing the 5' region of the SUC2 gene; Carlson and Botstein, 1983) and to SIR1 sequences (pJH570- containing the entire SIR1 gene; Ivy et al., 1985) are indicated. Recognition sites for restriction endonucleases are abbreviated as in Figures 1 and 2 with the following additions: A, AvaI; X, XhoI. Not all ClaI, HindIII, KpnI, or PstI sites are shown in MAL3 flanking DNA sequences.


GENE 3
GENE 1
GENE2
1kb
MAL 1
MAL 3
MAL 6
MAL 2
MAL 4
SUC1
MAL64
SIR1

analysis using probes spanning the entire MAL6 locus (see Figure 1) demonstrate that all of the MAL loci are highly sequence homologous throughout the region encoding GENEs 1, 2, and 3 and this homology extends about 2 kb beyond GENE 2 into noncoding sequences. In addition, homology between the MAL2 and MAL4 loci extends approximately 3 kb beyond GENE 3 into flanking sequences (discussed below). The vertical dotted line indicate the approximate boundary of the homology (Figure 3).

Based upon these results we have devised a system of nomenclature that utilizes a two digit gene number which identifies both the locus position and the gene number of each MAL gene (Charron and Michels, 1987-Appendix one). The first digit designates the locus position and the second digit indicates the gene function. With this in mind, MAL21 is the maltose permease gene present at the MAL2 locus, and MAL32 is the maltase structural gene contained within the MAL3 locus.

Plasmid complementation studies were performed using cloned sequences from each of the MAL loci to determine if the MAL loci have remained functionally homologous. The results of this analysis are presented in Table 1. Table 1 indicates the MAL genes contained in each plasmid as well as the restriction site end-point of the MAL-derived DNA fragment. Each plasmid functioned as an episomal plasmid either because a yeast 2 micron vector or a yeast ARS vector was used, or as was found in this study and in

previous work, the cloned MAL sequences themselves provided the ARS element (Charron and Michels, manuscript in preparation-Chapter two). Detailed analysis of the MAL6 locus localized an ARS element to the 500bp HindIII - EcoRI fragment between the 3' end of the MAL61 and the MAL63 genes (Sylvestre and Michels, unpublished data). The plasmids were transformed into strains carrying partially functional alleles of MAL1. A detailed structural and functional analysis of these alleles is presented in Charron and Michels (manuscript in preparation-Chapter two) and Table 1 indicates the genotype of each strain as deduced from this analysis.

In summary, the results presented in Table 1 demonstrate that the MAL loci are not only highly homologous on the sequence level but have maintained functional homology. The structural genes (GENE 1 and GENE 2) from each MAL locus complement the MAL13 gene of the MAL1p locus. With one exception, the activator gene product of each of the loci is capable of activating the expression of the $MAL11^{*}$ and MAL12 genes of the MAL1g and $mal1^{0}$ loci. Plasmid p21-40, containing the MAL63 and MAL61 genes, complements the MAL1g locus (encoding the $MAL11^{*}$ and MAL12 genes) yet does not complement the $mal1^{0}$ locus (encoding MAL12) (Needleman et al., 1984). This result implies that the MAL12 genes of these two loci differ and that the MAL63 gene product, unlike the GENE 3 proteins encoded by the other MAL loci (namely MAL2 and

Table 1: Functional homology of the MAL loci.

As indicated in columns two and three, clones containing each of the genes from the MAL loci were tested for the ability to complememt maltose non-fermenting tester strains. Plasmid pM1.2F (Charron et al., 1986 -Chapter one) contains the indicated yeast in the URA3 vector YEp24; the MAL3 sequences in plasmid pM3.1C and MAL6 sequences in plasmid p21-40 (Needleman et al., 1984) are in the LEU2 vector YEp13; MAL3 and MAL6 sequences in plasmids pM3.1A and pY6 (Needleman et al., 1984) respectively are in the URA3 vector YIp5; and the MAL43-containing plasmid pM43BS (Charron and Michels, 1987-Appendix four) and the MAL63-containing plasmid pDF-1 contain the indicated yeast in the TRP1 vector pLC544. Plasmids pMJC2B and pMJC4B (Charron and Michels, 1987-Appendix one) were isolated from strains MCY101-3A (MATa MAL2 mal1$^0$ ura3-52 leu2-3,112 ade1) and MCY100-3A (MAT$\alpha$ MAL4 mal1$^0$ ura3-52 lys, Charron and Michels, 1987 -Appendix one) respectively using plasmid rescue techniques decribed previously (Charron and Michels, 1987-Appendix one). Both plasmids pMJC2B and pMJC4B contain an 800 bp BglII - ClaI fragment derived from MAL62 (Charron and Michels, 1987-Appendix one). Plasmids pMJC1$\Delta$H, pMJC2$\Delta$H and pMJC4$\Delta$H were isolated from strains 600-1B (Charron et al., 1986 -Chapter one), MCY101-3A (this work) and MCY100-2C (MAT$\alpha$ MAL4 mal1$^0$ ura3-52 leu2-3,112 ade) respectively using plasmid rescue techniques described in Charron et al. (1986-Chapter one). All three plasmids (pMJC1$\Delta$H, pMJC2$\Delta$H and pMJC4$\Delta$H) contain the 2.4 kb MAL6- derived ClaI - HindIII fragment of plasmid pY6$\Delta$C$\Delta$H (Charron et al., 1986-Chapter one). In another study we describe the MAL1 tester strains used in this study. We demonstrate that MAL1p only encodes the regulator (GENE 3), MAL1g encodes maltase and permease (GENES 2 and 1, respectively) and mal1$^0$ only encodes a functional maltase (GENE 2) (Charron and Michels, manuscript in preparation; Chapter 2). MAL1p strains used were 345-4A and 236-2A (Charron et al., 1986-Chapter one) mal1$^0$ strains used were 328-4A and 303-3A (Charron et al., 1986 -Chapter one) and MAL1g strains used were 340-2B and JC27 (Charron et al., 1986-Chapter one). The MAL11 gene encodes a maltose permease defined in previous studies (Charron et al., 1986 -Chapter one; Charron and Michels, manuscript in preparation -Chapter two). Plasmid complementation of the defective/deficient MAL function(s) is determined by the ability of the transformed strain to ferment maltose as described in Materials and Methods.

Table 1

Functional homology of the *MAL* loci

| Plasmid | *MAL* GENE(s) contained | Fragment kb | (end points) | Ability to complement maltose non-fermenting strains of the given genotype: *MAL13* *mal11* *mal12*$^0$ (*MAL1p* allele) | *mal13* *MAL11*$^*$ *MAL12* (*MAL1q* allele) | *mal13* *mal11* *MAL12* (*mal1*$^0$ allele) |
|---|---|---|---|---|---|---|
| pM1.2F | *MAL13* | 7.1 | (*Bgl*II-*Sal*I) | - | + | - |
| pMJC1ΔH | *MAL11* *MAL12* | 7.3 | (*Hind*III-*Hind*III) | + | - | - |
| pMJC2B | *MAL23* *MAL21* | 7.7 | (*Bam*HI-*Cla*I) | - | + | + |
| pMJC2ΔH | *MAL21* *MAL22* | 7.6 | (*Hind*III-*Hind*III) | + | - | - |
| pM3.1C | *MAL33* | 3.1 | (*Sal*I-*Sal*I) | - | + | - |
| pM3.1A | *MAL31* *MAL32* | 7.3 | (*Hind*III-*Hind*III) | + | - | - |
| pMJC4B | *MAL43* *MAL41* | 9.0 | (*Bam*HI-*Cla*I) | - | + | + |
| pM43BS | *MAL43* | 5.8 | (*Bam*HI-*Sal*I) | - | + | - |
| pMJC4ΔH | *MAL41* *MAL42* | 7.6 | (*Hind*III-*Hind*III) | + | - | - |
| pDF-1 | *MAL63* | 2.6 | (*Bgl*II-*Sal*I) | - | + | - |
| pY6 | *MAL61* *MAL62* | 7.3 | (*Hind*III-*Hind*III) | + | - | - |
| p21-40 | *MAL63* *MAL61* | 6.8 | (*Bgl*II-*Bgl*II) | - | + | - |

MAL4), is unable to activate the expression of the MAL12 gene of the $mal1^0$ locus. This finding is consistent with the results reported by Dubin et al. (1985) that the temperature labile maltase encoded by the MAL12 gene of the $mal1^0$ locus is not synthesized in the MAL6 $mal1^0$ strain 332-5A. Interestingly, deletion of the MAL62 gene in this strain allows the MAL12 gene to be expressed, albeit poorly (Dubin et al., 1985).

Orientation of the MAL loci:

Homology to telomere adjacent sequences and to SUC sequences in MAL flanking DNA. All of the MAL loci, except MAL6, have been mapped in the Saccharomyces genome and have been shown to be near a chromosomal telomere (Mortimer and Schild, 1980; Celenza and Carlson, 1985). Studies done on the MAL1 locus and its alleles have shown that telomere adjacent X sequences, SUC1 and $suc1^0$ and, in the case of MAL1g, Y' sequences flank one side of the locus (adjacent to GENE 2) and not the other side of the locus (Carlson et al., 1985; Charron and Michels, manuscript in preparation-Chapter two). Additionally, tight linkage between the MAL3 and SUC3 loci has been demonstrated (Mortimer and Schild, 1980; Carlson et al., 1985). With these results in mind we set out to determine if any of the other sequences cloned from the MAL loci contain homology to known telomere adjacent sequences (ie: X, Y' and SUC).

The results of Southern gel transfer analyses are summarized in Figure 3. Telomere adjacent X sequences are found flanking GENE 2 of the MAL1, MAL3 and MAL6 loci and the approximate location is indicated. Significant homology to Y' sequence was not detected in the cloned sequences from these three loci but this result is not surprising. Y' sequences are found immediately at the chromosomal terminus. Both MAL1 and MAL3 strains used in our studies have linked SUC genes (SUC1 and SUC3 respectively) and these map closer to the telomere than the MAL locus (Mortimer and Schild, 1980; Carlson et al., 1985). In fact, as indicated in Figure 3 and previously reported by us (Charron et al., 1986-Chapter one), sequences derived from the SUC1 locus are present in the GENE 2 flanking DNA of MAL1. Unfortunately, no homology to SUC2 probes was detected in the cloned MAL3-flanking DNA sequences. MAL3 and SUC3 are not as tightly linked as MAL1 and SUC1 and most likely additional flanking sequences will have to be isolated to reach the SUC3 locus. Attempts to do this are in progress. The absence of significant Y' homology in the cloned MAL6 sequences might be explained by the fact that the MAL6 locus originated in S. carlsbergensis strains and it has been shown the Y' homology is not highly conserved in species of Saccharomyces other than S. cerevisiae (Chan and Tye, 1983).

No significant homology to any of the telomere

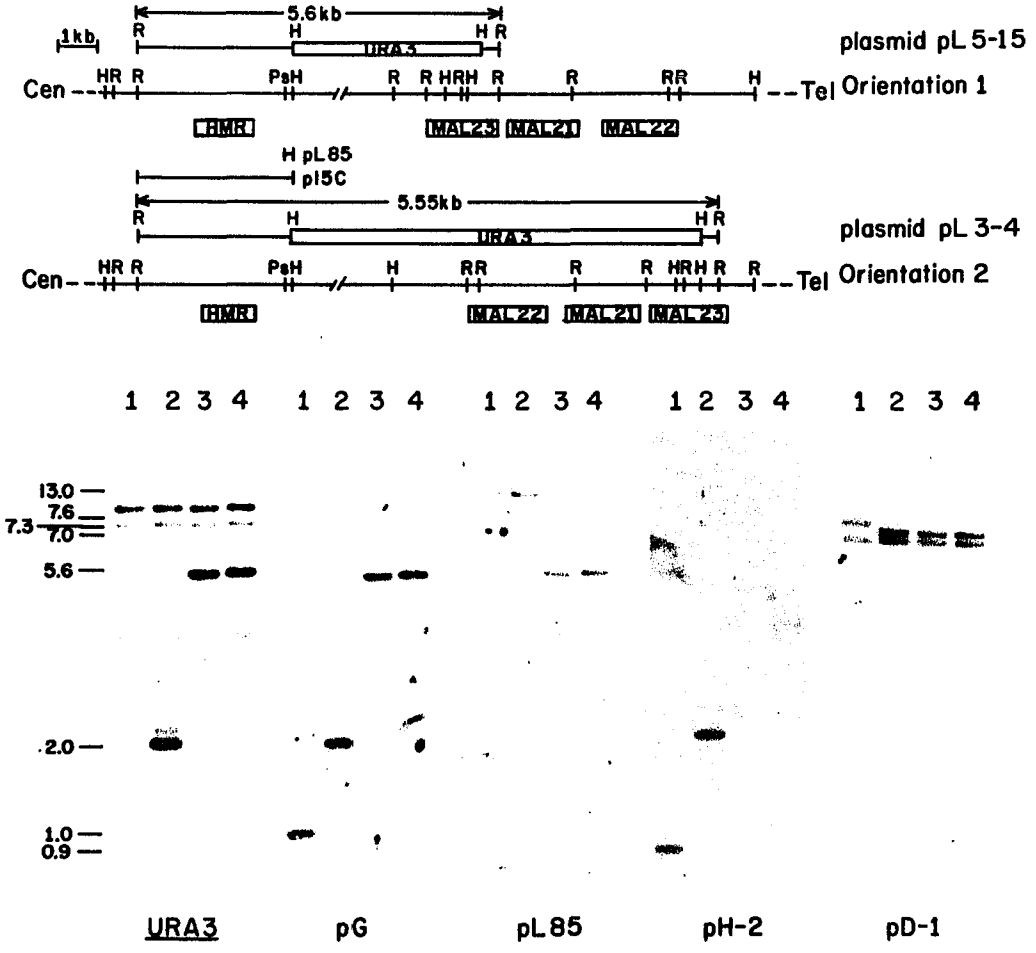
associated probes used was detected in the cloned MAL2 and MAL4 flanking DNA sequences. Both loci are located on smaller yeast chromosomes and according to Zakian et al. (1986), X and Y' sequences appear to be absent from several of the smaller S. cerevisiae chromosomes (chromosomes I, III, VI and XI).

In summary, three of the MAL loci contain known telomere adjacent DNA sequences flanking one side of the locus (adjacent to GENE 2) and not the other. These results allow us to conclude that: (1) MAL6, like the other four MAL loci, maps close to a chromosome terminus; and (2) the orientation of MAL1, MAL3 and MAL6 is CENTROMERE ... GENE 3 - GENE 1 - GENE 2 ... TELOMERE.

Orientation of MAL2 by chromosomal deletion. In order to determine the orientation of the MAL2 locus with respect to the centromere and telomere, disruption plasmids pL5-15 and pL3-4 were constructed to delete the region of chromosome III from HMR to the MAL23 gene (see Materials and Methods; Figure 4). By determining which MAL2 sequences are deleted during the transplacement we will be able to determine the orientation of MAL2. If ORIENTATION 1 is correct, disruption by the 5.6 kb EcoRI fragment of plasmid pL5-15 will leave the MAL21 and MAL22 genes intact but will delete the MAL23 gene resulting in a nonfermenting strain which should be complemented by a MAL1p strain. If ORIENTATION 2 is correct, disruption by

Figure 4: Orientation of MAL2 by chromosomal deletion.

The yeast inserts of disruption plasmids pL5-15 and pL3-4 are diagramed above along with the HMR-linked plasmids pL85 and p15C. Location of HMR, MAL21, MAL22 and MAL23 are shown in proposed Orientations 1 and 2. Centromere and telomere are abbreviated as Cen and Tel respectively. Results of Southern gel transfer analysis of strains MCY101-3A (lane 1), MCY101-3A::pDM3 (Δ MAL23) (lane 2), L5-15#1 (lane 3) and L5-15#17 (lane 4) are shown. Panels #1 through #4 contain EcoRI digested DNA probed with URA3, pG, pL85 and pH-2 respectively. Panel #5 contains HindIII digested DNA probed with pD-1. Sizes of fragments homologous to the probes used are indicated as kilobasepairs.


5.6kb
1kb
R H URA3 H R
plasmid pL 5-15
Cen HR R PsH R R HRH R R RR H Tel
Orientation 1
HMR MAL23 MAL21 MAL22
H pL85
pl5C
5.55kb
R H URA3 H R
plasmid pL 3-4
Cen HR R PsH H RR R R HRH R R Tel
Orientation 2
HMR MAL22 MAL21 MAL23
1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
13.0
7.6
7.3
7.0
5.6
2.0
1.0
0.9
URA3
pG
pL85
pH-2
pD-1

the 5.55 kb EcoRI fragment of plasmid pL3-4 will delete almost all MAL2 coding sequences and the resulting disruption strain will be a nonfermenter and will not be complemented by any of the MAL tester strains (MAL1p, MAL1g or $mal1^0$). As diagramed in Figure 4, the 5.6 kb EcoRI disruption fragment of plasmid pL5-15 will not mediate any viable transplacement events if ORIENTATION 2 is correct. Similarly, the 5.55 kb EcoRI disruption fragment of plasmid pL3-4 will not lead to any viable transplacement events if ORIENTATION 1 is correct thus, any stable $Ura^+$ transformants will have resulted from other types of events (R. Rothstein, personal communication). The most likely of these events, and this was born out by our experimental results, was the establishment of the URA3 gene as an episomal plasmid as follows. The 5.6 kb EcoRI fragment of plasmid pL5-15 and the 5.55 kb EcoRI fragment of plasmid pL3-4 contain the complete HMR locus including the E and I elements described by Abraham et al. (1982) both of which have ARS activity. These fragments were able to circularize and establish themselves as episomal plasmids.

Strain MCY101-3A, a haploid strain carrying the MAL2 locus, was transformed using plasmids pL3-4 and pL5-15 and stable $Ura^+$ transformants were isolated. These were tested for their ability to ferment maltose. Two pL5-15 transformants (L5-15#1 and L5-15#17), out of 228 screened, did not ferment maltose. Results of Southern gel transfer

analysis using MAL63-derived probes, pH-2 and pG; the HMR-specific probe, pL85 (Strathern et al., 1980); a fragment from the URA3 gene and pD-1 (Figure 1) demonstrates the expected deletion/disruption has occurred, that is, that MAL23 sequences have been deleted from strain MCY101-3A leaving the MAL21 and MAL22 genes intact (Figure 4). Additionally, in these transformants the HMR locus is tightly linked to MAL sequences as evidenced by the fact that transformants L5-15#1 and L5-15#17 contain a 5.6 kb EcoRI fragment homologous to pL85, pG and URA3 sequences. The intensity of hybridization indicates that this fragment is present in single copy and not as an episomal plasmid in several copies. Strains L5-15#1 and L5-15#17 lack the 900 bp pH-2 homologous EcoRI fragment seen in MCY101-3A and shown to be derived from the MAL23 gene (the Δ MAL23 strain shown in Figure 4 also lacks this fragment). The 13.0 kb pL85 homologous EcoRI fragment containing the HMR locus and the 1.0 kb pG homologous EcoRI fragment derived from the MAL23 gene shift in size to that of the pL5-15 disruption fragment, 5.6 kb. When these strains are digested with HindIII and probed with pD-1 a decrease of approximately 300 bp is detected in the disruption strains (7.6 kb --> 7.3 kb). This can be accounted for by the presence of a HindIII restriction site polymorphism between MAL6 (donor sequences) and MAL2 (acceptor sequences) as shown in Figure 3. Strains L5-15#1 and L5-15#17 were further

analyzed and shown to be complemented by the MAL1p tester strain 53-2C (Charron et al., 1986-Chapter one; Charron and Michels, manuscript in preparation-Chapter two).

An almost equal number of pL3-4 transformants (214) were assayed for maltose fermentation and all were $Mal^+$. Southern analysis of five pL3-4 transformants demonstrated that the HMR and MAL2 loci remain undisrupted in these $Ura^+$, $Mal^+$ transformants. In four out of five of these transformants a 5.55 kb (pL85-, pG- and URA3-homologous) EcoRI fragment is detected in what appears to be a high copy number of copies. The fifth transformant analyzed lacked this plasmid and appeared to be a URA3 gene convertant (data not shown). These results support the hypothesis that ORIENTATION 1 is the correct orientation for MAL2.

Only the MAL4 locus has not been unambiguously oriented with regard to the telomere but, based on the following results, we have diagrammed the MAL4 locus in Figure 3 in the same orientation as the other MAL loci. MAL4 and SIR1 are tightly linked and we have detected SIR1-homologous sequences flanking the MAL43 gene (see below; Ivy et al., 1985). Recent genetic and physical analysis of the BAS1 gene has shown that it is tightly linked to both SIR1 and MAL4 but, preliminary results indicate that BAS1 is more tightly linked to SIR1 than to MAL4 suggesting that the order is BAS1 - SIR1 - MAL4 (K. Arndt and G. Fink, personal communication). Currently, a

four point test cross including the centromere-proximal MET14 gene, BAS1, SIR1 and MAL4 is in progress and will provide the necessary information to orient the BAS1 - SIR1 - MAL4 cluster with respect to the telomere.

Taken together, results of the analyses described above suggest that all of the MAL loci are found next to a chromosome terminus and that all appear to be oriented as follows: CENTROMERE...GENE 3 - GENE 1 - GENE 2 - [SUC]...TELOMERE.

Centromere-proximal sequences flanking the MAL loci.

One of the proposed mechanisms leading to the formation of polygenic families of loci, such as MAL and SUC, is an inter-chromosomal recombination process involving homologous sequences proposed to be present at the centromere-proximal side of each member of the polygenic family. For this reason, it became important to analyze the region of each MAL locus flanking GENE 3 for sequences common to all of the loci.

One of the four MAL1 alleles has been found in all strains examined by our laboratory, and for this reason it has been proposed that it is the progenitor MAL locus. With this in mind we used probes containing the three EcoRI fragments upstream of MAL13 (Figure 3; Charron et al., 1986-Chapter one; Chapter two) to probe all the other cloned MAL sequences. No sequence homology was detected, suggesting that these sequences are unique to MAL1.

A fourth MAL gene has been identified at the MAL6 locus (MAL64). This gene has been shown to be functional only in MAL6 constitutive mutants (see Appendix two). We have shown that MAL64 constitutive mutations lie in a region which shows significant homology to MAL63 and MAL61 and looks like a tandem duplication of these sequences (Dubin et al., submitted-Appendix two; Figure 3). Probes derived from this region (pZ-1 and pZ-2 in Figure 1) were used to examine the other MAL loci for potential MAL64 homologues. Sequences containing some homology to pZ-2 were found in the region upstream of MAL23 and MAL33. The sequences flanking MAL33 hybridized to pZ-1 (a small subclone of pZ-2) as well as to plasmids derived from MAL coding regions (pH-2, pG, pE-2, pD-1). These results indicate that MAL3, like MAL6, has a linked duplication. More detailed analysis of this region is in progress. Thus, in summary a MAL64 equivalent is not found at any of the other MAL loci with the possible exception of MAL3.

Because of the tight genetic linkage between MAL4 and SIR1 (Ivy et al., 1985), MAL4 flanking sequences were screened for homology to SIR1 sequences contained in plasmid pJH570 (Ivy et al., 1985). Significant homology was detected and the location is indicated in Figure 3. Additionally, it was determined that the cloned SIR1 fragment lacks the linked MAL4 homologous sequences and therefore appears to have been isolated from a null ($mal4^0$) strain. Similar observations have been made for

the null SUC loci (ie: $suc1^0$, etc.). It is interesting to note that these MAL43 flanking sequences are homologous to those flanking MAL23, but MAL2 homology to SIR1 sequences is poor. Other features of the restriction endonuclease maps of the MAL2 and MAL4 loci suggest that they are more closely related to each other.

Finally, we examined subclones of the DNA sequences flanking GENE 3 from each of the MAL loci and probed the other cloned MAL-related sequences to detect homologies. These probes were also used to scan genomic EcoRI and/or HindIII digested DNA to determine if these sequences are repeated in the Saccharomyces genome. Results of this analysis suggest that the sequences flanking each of the MAL loci seem to be unique to that MAL locus (with the exceptions noted above). No clearly conserved sequence was detected in the MAL flanking DNA sequences. Genomic Southerns revealed that several fragments flanking both MAL13 and MAL33 contain sequences that are repeated several times throughout the genome (Charron and Michels, manuscript in preparation-Chapter two; data not shown). The significance of this repeated homology is not clear but may be a function of the telomere-adjacent location of these sequences.

# DISCUSSION

The MAL loci present in the Saccharomyces yeasts are a repeated family of polygenic loci that map to chromosome termini (Mortimer and Schild, 1980; Michels and Needleman, 1983; 1984; Needleman and Michels, 1983). Studies on the cloned MAL6 and MAL1 loci demonstrated that both are complex loci and contain three genes. GENE 1 encodes the transport enzyme, maltose permease; GENE 2 encodes maltase; and GENE 3 encodes a trans-activator required for the expression of GENEs 1 and 2 (Needleman et al., 1984; Dubin et al., 1985; Charron et al., 1986-Chapter one; Chang et al.,submitted).

In this study we extend our comparative analysis of the MAL loci to include the MAL2, MAL3 and MAL4 loci. Through comparative restriction enzyme mapping and Southern analysis of the coding regions and several kilobasepairs of flanking DNA sequences we demonstrate that the MAL loci are structurally and functionally homologous throughout an approximately 9.0 kb region containing the three genes needed for fermentation.

The MAL flanking sequences were analyzed and it was shown that known telomere-associated X sequences were found flanking GENE 2 at MAL1, MAL3 and MAL6. These regions were also screened for homology to SUC sequences and the SUC1 locus was detected approximately 5.0 kb from MAL12. SUC3 was not found in the more than 9.0 kb

sequences flanking MAL32, eventhough strain 48-2C is believed to have the genotype MAL3 SUC3. These results demonstrate that linkage between MAL1 and SUC1 is tighter than that between MAL3 and SUC3 and this finding is consistent with the results reported by Mortimer and Hawthorne (1966). It is interesting to note that approximately 2.0 kb of sequences flanking GENE 2 (EcoRI - HindIII in Figure 3) are highly conserved at all the MAL loci. These sequences play no essential role in the fermentative pathway as chromosomal deletions of this region have no apparent phenotypic effect (R. Dubin and C. Michels, unpublished results). The presence of this sequence at all MAL loci may be fortuitous or may be a result of the mechanism of the translocation process occurring at telomeres.

We determined that the orientation of all of the MAL loci, with respect to the centromere and telomere, is CENTROMERE ... GENE 3 - GENE 1 - GENE 2 - [SUC] ... TELOMERE. MAL1, 3 and 6 were oriented based upon the finding of known telomere adjacent sequences flanking one side of the locus, the GENE 2 side; MAL2 was oriented through the use of chromosomal deletion/disruption; and MAL4 was oriented based upon the finding of SIR1 homologous sequences in the DNA sequences flanking the left of MAL43 and the genetic mapping of BAS1 by Arndt and Fink (personal communication).

We also examined the nature of the DNA sequences on

the centromere-proximal side of the locus (adjacent to GENE 3). Results of Southern analysis demonstrate that the sequences flanking each locus are, by and large, unique to that locus. Additional homology was detected between the MAL4 - SIR1 intergenic region and MAL2. These results suggest that MAL2 and MAL4 are more closely related, a point that is supported by the presence of several restriction site polymorphisms common to these loci (Figure 3).

The telomeric translocation of the MAL loci among closely related Saccharomyces species can be accounted for by several mechanisms. It is possible that a small sequence on the centromere-proximal side of the MAL locus can mediate a conversion or recombination event that could mobilize the MAL loci among telomeres. This sequence would have to be small and possibly located very close to GENE 3 as no common sequences were detected with the large centromere-proximal probes used (2.0 kb - 4.8 kb size range). Another possible mechanism that could account for the translocation of this polygenic family is one that involves a random break in the chromosome on the centromere-proximal side of the MAL locus during chromosome disjunction followed by the healing of this broken end onto a new chromosome. Previous studies have shown that chromosome ends interact during interphase and throughout meiosis (Wagenaar, 1969; Ashley, 1979) and the work of Haber and Thorburn (1984) has shown that broken

chromosomes have a tendency to "heal" themselves by adding on a new telomere or telomere-like structure. Events of this nature would not be of common occurrance and therefore it would not be expected to find MAL loci on every yeast telomere.

The results presented above underscore the unique nature of this system. This is the first report on the translocation of a multigene complex, spanning approximately 9.0 kb, among chromosome termini. The mobilization of genes among telomeres is not unique to the MAL system or to Saccharomyces. Other sugar utilization gene families such as SUC (encoding invertase) (Carlson et al., 1985), MGL (encoding ∝-methylglucosidase) (ten Berge 1972; Mortimer and Schild, 1980), and HXK (encoding hexokinase) (Frohlich et al., 1984) also represent repeated, telomere-associated gene families. Examples of similar types of translocations are seen in other eukaryotes. The pseudoautosomal region of the human X and Y chromosomes (Buckle et al., 1985; Cooke et al., 1985), the VSG genes of the trypanosomes (Englund et al., 1982; van der Ploeg et al., 1984), the sex reversion factor (Sxr) (Jones and Singh, 1982) and the steroid sulphatase region (STS) of Mus (Craig and Tolley, 1986) and the sex realizer gene of Megaselia scalaris (Mainx, 1964; Green, 1980) are examples. The telomeric translocation of genes and gene families may represent a common mechanism of gene dispersal utilized by a variety of eukaryotic organisms

thus warranting a more detailed examination of the exact process(es) involved.

# Appendix 1

The constitutive, glucose repression-insensitive mutation of the yeast MAL4 locus is an alteration of the MAL43 gene

This chapter has been reproduced with the permission of the publisher of Genetics. It appeared in Genetics 116: 23-31, 1987.

## ABSTRACT

Mutations resulting in constitutive production of maltase have been identified at each of the five *MAL* loci of *Saccharomyces* yeasts. Here we examine a dominant constitutive, glucose repression insensitive allele of the *MAL4* locus (*MAL4-C*). Our results demonstrate that *MAL4-C* is an alteration in the *MAL43* gene, which encodes the positive regulator of the *MAL* structural genes, and that its product is trans-acting. The *MAL43* gene from the *MAL4-C* strain was cloned and integrated into a series of non-fermenting strains lacking a functional regulatory gene but carrying copies of the maltose permease and maltase structural genes. Expression of the maltase structural gene was both constitutive and insensitive to glucose repression in these transformants. The *MAL4-C* allele also results in constitutive expression of the unlinked *MAL12* gene (encoding maltase) in this strain. In addition, the cloned *MAL43* gene was shown to be dominant to the wild type *MAL63* gene. We also show that most of the glucose repression insensitivity of strains carrying the *MAL4-C* allele results from alteration of *MAL43*.

# INTRODUCTION

The fermentation of maltose by Saccharomyces strains utilizes two enzymes: maltose permease, and maltase, which cleaves the disaccharide into two molecules of glucose. The synthesis of these enzymes is subject to dual regulatory control: synthesis of both enzymes is induced approximately 30-fold over basal levels by growth in the presence of maltose (de Kroon and Koningsberger, 1970; Ouwehand and van Wijk, 1972); in the presence of glucose, maltase expression is repressed (catabolite or glucose repression) (van Wijk et al., 1969; Gorts, 1969).

Saccharomyces strains able to ferment maltose contain at least one of five unlinked MAL loci (reviewed in Barnett, 1976). Genetic and physical analyses of the MAL6 locus of Saccharomyces carlsbergensis have demonstrated that the locus includes three genes: MAL61, encoding maltose permease; MAL62, encoding maltase; and MAL63, encoding a positive trans-acting regulator required for induction of the two structural genes (Needleman et al., 1984; Cohen et al. 1985; Dubin et al. 1985; Chang et al., submitted). Mutations in MAL63 result in a maltose nonfermenting phenotype, and such strains are unable to induce the expression of the structural genes above basal levels (ten Berge et al., 1973a; Chang et al.,submitted). Several MAL6-linked constitutive mutations were isolated as maltose fermenting revertants of mal63 mutations (ten

Berge et al. 1973b; ten Berge et al., 1974; Dubin et al., 1986). All but one of these constitutive revertants are recessive to the wild type MAL6 allele and to mal63 nonfermenting mutations and are sensitive to glucose repression. Surprisingly, detailed genetic analysis of two MAL6 constitutive revertants revealed that they map to MAL64, a gene lying outside the MAL61-MAL62-MAL63 complex (Dubin et al., 1986).

Constitutive mutations of other MAL loci appear to be fundamentally different from those at MAL6 (Winge and Roberts, 1950; Kahn and Eaton, 1971; Needleman and Eaton, 1974; Zimmerman and Eaton, 1974). All five MAL2-linked constitutive revertants of a mal2 mutant are dominant to the wild type allele, and three are glucose repression insensitive (Zimmerman and Eaton, 1974). Similarly, a constitutive allele of MAL4 is dominant to the wild type and glucose repression insensitive (Kahn and Eaton, 1971). The dominant nature of these MAL2- and MAL4-linked constitutive mutations as well as their resistance to glucose repression implies that their genetic basis is fundamentally different from that of the MAL6-linked constitutives (described by ten Berge et al., 1973b; ten Berge et al., 1974; Dubin et al., 1986).

In order to investigate the genetic basis of the dominant constitutive, glucose repression insensitive type of MAL regulatory mutation, we undertook an analysis of the MAL4-constitutive strain described by Kahn and Eaton

(1971). We demonstrate that the alteration(s) leading to both the constitutive and glucose repression insensitive phenotypes of this strain lies in the MAL43 gene. Based on the results presented here on the functional and structural homology of MAL43 to the MAL63 gene, we conclude that MAL43 encodes a positive trans-acting regulatory protein.

# METHODS AND MATERIALS

## Strains and Growth Conditions

Yeast strains used in this study are listed in Table 1. Plasmids were propagated in E.coli strain RR1. Strain MCY100-3A was derived from the constitutive MAL4-C strain 1403-7A originally described by Kahn and Eaton (1971). Utilizing the type of genetic analysis described in Michels and Needleman (1983), the MAL1g and MAL3g loci were found to be present in 1403-7A and were crossed out of the strain. The MAL genotype of MCY100-3A is MAL4-C $mal1^0$. In addition to containing the MAL4-linked constitutive mutation MCY100-3A also contains a partially functional allele of the MAL1 locus which has been shown to contain only a functional MAL12 gene encoding maltase. Strain MCY111-2 was isolated, following selection of $Mal^-$, $Ura^+$, $Trp^-$, $Leu^-$ isolates, from a random spore analysis (Dawes and Hardie, 1974) of diploid MCY111 (MCY100-3AΔ43-1 and 332-5A).

Yeast strains were grown on YEP medium [1% (wt/vol) yeast extract/1% (wt/vol) peptone] plus the indicated amount of a specified carbon source. Maltose fermentation is defined as the production of acid and gas in 1-3 days after inoculation and determined in 5 ml of YEP plus 2% (wt/vol) maltose medium in Durham tubes.

Selection of the ura3 mutant MCY111-2R4 was performed by the method of Boeke et al. (1984).

TABLE 1

List of strains

| Strain | (Relevant MAL) Genotype | Source |
|---|---|---|
| 1403-7A | MATa MAL4-C MAL1g MAL3g trp1 ura3 | Berkeley Stock Center |
| 212-3B | MATα MAL4-C mal1$^0$ lys | R. Needleman |
| MCY100-3A | MATα MAL4-C mal1$^0$ lys ura3-52 | This work |
| MCY133-1D | MATα MAL4-C mal1$^0$ trp1 | This work |
| MCY133-1C | MATa MAL4-C mal1$^0$ ura3-52 leu2-3,112 lys | This work |
| 8-2B | MATa MAL6-C2 mal1$^0$ ura3-52 leu2-3,112 trp1 ade | Dubin et al. (1986) |
| 332-5A | MATa MAL6 mal1$^0$ ura3-52 leu2-3,112 trp1 his | Dubin et al. (1985) |
| 348-1B | MATa mal63-10 mal1$^0$ ura3-52 leu2-3,112 trp1 | Chang et al. (submitted) |
| 340-2B | MATa MAL1g ura3-52 trp1 lys met | Needleman et al. (1984) |
| 347-2A | MATa mal1$^0$ ura3-52 trp1 leu2-3,112 | R. Needleman |
| 349-6A | MATa mal1$^0$ ura3-52 leu2-3,112 trp1 | R. Needleman |
| MCY111-2 | MATa MAL43::URA3 mal1$^0$ leu2-3,112 trp1 | This work |
| A9 | MATa MAL63::URA3 mal1$^0$ leu2-3,112 trp1 his | Chang et al. (submitted) |
| 332-5A/Δ61/62-9 | MATa MAL61/62::LEU2 mal1$^0$ ura3-52 trp1 his | Dubin et al. (1986) |
| MCY100-2C | MATα MAL4-C mal1$^0$ ura3-52 ade leu2-3,112 | This work |
| 328-4A | MATα mal1$^0$ ura3-52 trp1 ade | Needleman et al. (1984) |
| 1-31 | MATa MAL1p met | R. Needleman |
| 345-4A | MATa MAL1p ura3-52 trp1 ade leu2-3,112 | Needleman et al. (1984) |

## Yeast Transformation

Yeast transformation was performed by the method of Ito et al. (1983) using lithium acetate. All transformants were screened for the stability of the selective marker by a modification of the method of Grunstein and Hogness (1975) as described by Hinnen et al.(1978) and by passage through non-selective media (YEP medium plus 2% (wt/vol) glucose) to determine whether the plasmid was being maintained in an integrated state or episomally. Only those transformants containing single copy integrants of the plasmid as determined by Southern gel transfer analysis were selected for further analysis.

## Measurement of p-nitrophenyl-α-D-glucopyranoside (PNPGase) Activity

Determination of maltase activity, measured as the rate of release of p-nitrophenol from p-nitrophenol-α-D-glucopyranoside, was performed by a modification of the method of Kahn and Eaton (1967) as described in Dubin et al. (1986).

## DNA Isolation and Analysis

DNA isolation as well as Southern analysis were carried out as previously described (Needleman et al., 1984).

## Gene Disruptions and Plasmid Rescue

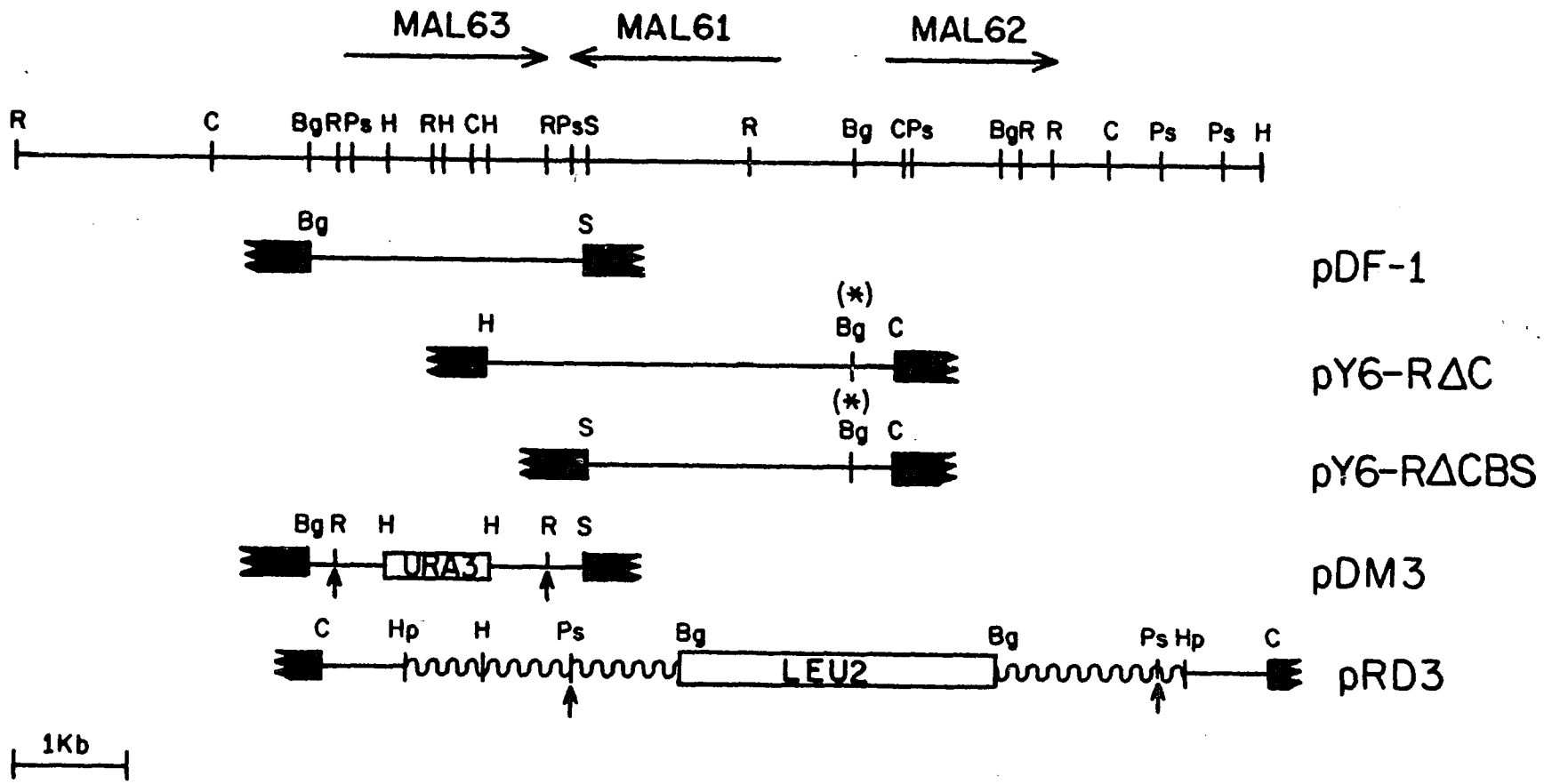
For gene disruptions (Rothstein, 1983) and for site-directed integration (Orr-Weaver et al., 1983), transformants which stably maintained the selective marker were further screened by Southern gel transfer analysis and by standard genetic analysis (Mortimer and Hawthorne, 1966) (see Appendices 4 and 5).

Gene disruption plasmids were constructed using cloned MAL6 DNA sequences. Plasmids pDM3 and pRD3 have been previously described and used in the construction of strain A9 ( a MAL63 deletion / disruption ) and 332-5A/Δ61/62-9 (a MAL61/MAL62 deletion/disruption strain) (Dubin et al., 1986; Charron et al., 1986-Chapter one; Chang et al., submitted). These plasmids are used here for the deletion/disruption of MAL4-linked sequences. The construction of a MAL43::URA3 strain utilizing pDM3 is described in the Results. Deletion of MAL41-MAL42 was carried out using pRD3. Plasmid pRD3 was constructed from MAL6 sequences by replacing a portion of the 5' end of the MAL61 and MAL62 genes along with the intergenic sequences with the LEU2 gene as described in Dubin et al. (1986). Because of the homology between the MAL loci we can use this plasmid to delete MAL4 sequences. Plasmid pRD3 (see Figure 1) was digested with PstI and used to transform strain MCY133-1C. Stable $Leu^+$, maltose non-fermenting transformants were isolated and shown by Southern analysis to have disrupted the 7.6 kb HindIII fragment of the MAL4 locus (Needleman and Michels, 1983; data not shown).

Figure 1: Restriction endonuclease map of MAL6-derived plasmids used for deletion/disruption of the MAL4 locus.

A partial restriction map of MAL6 and the location of MAL61, MAL62 and MAL63 is shown. Plasmid pDF-1 contains the BglII - SalI fragment subcloned into pLC544 (Needleman et al., 1984). Plasmids pY6-RΔC and pY6-RΔCBS contain the indicated regions subcloned into YIp5. Plasmid pY6-RΔCBS was formed by digesting plasmid pY6-RΔC with BamHI and SalI. The ends were filled-in with T4 DNA Polymerase prior to self-ligation and plasmid pY6-RΔCBS was isolated following transformation of E.coli strain RR1. Disruption plasmids pDM3 and pRD3 (described in Methods and Materials) were digested with the indicated restriction endonucleases (↑) prior to transformation. Wavey lines indicate DNA sequences derived from the MAL6 constitutive strain 8-2B (Dubin et al., 1986). The symbol (*) is used to indicate the site of linearization of plasmids pY6-RΔC and pY6-RΔCBS.

Recognition sites of restriction endonucleases are abbreviated as follows: Bg, BglII; C, ClaI; H, HindIII; Ps, PstI; R, EcoRI and S, SalI.


MAL63
MAL61
MAL62
R C BgRPs H RH CH RPsS R Bg CPs BgR R C Ps Ps H
Bg S
pDF-1
(*)
H Bg C
pY6-RΔC
(*)
S Bg C
pY6-RΔCBS
Bg R H H R S
URA3
pDM3
C Hp H Ps Bg Bg Ps Hp C
LEU2
pRD3
1Kb

Plasmid pY6-RΔC contains a 3.9 kb HindIII - ClaI fragment derived from the MAL6 locus containing the MAL61 gene and the 5' end of the MAL62 gene cloned into YIp5. This plasmid was integrated at the MAL4 locus following linearization of the plasmid with BglII. Plasmid pMJC4B was later isolated following digestion of genomic DNA from the strain MCY100-3A:pY6-RΔC-2 with BamHI according to the method of Orr-Weaver et al., (1983).

## RESULTS

Nomenclature. Our comparative studies involving isolated sequences from the five MAL loci (MAL1, MAL2, MAL3, MAL4 and MAL6) indicate that each is organized identically to the MAL6 locus, as described in Needleman et al. (1984), Charron et al. (1986-Chapter one) and Charron and Michels (in preparation-Chapter four). In view of the homology to MAL6 (which extends for approximately 9.0 kb and includes the MAL61, MAL62 and MAL63 genes) we have devised a system for naming the homologous genes at each of the loci. Gene 1 encodes maltose permease, gene 2 encodes maltase and gene 3 encodes the positive trans-acting regulatory protein. To designate the locus position of the particular gene, the locus number is inserted before the gene number. For example, the gene encoding maltase at the MAL6 locus is MAL62. At the MAL4 locus, it is the MAL42 gene. In this way, information regarding both the locus position and the function encoded by a particular gene is given.

Disruption of MAL43. Because of the extensive homology among the MAL loci, one can utilize MAL6 sequences to alter MAL4 chromosomal sequences. Sequences homologous to the MAL63 gene were deleted from the genomic MAL4-C locus using plasmid pDM3 (Figure 1). Plasmid pDM3 was cleaved with EcoRI and used to transform the MAL4

strain MCY100-3A to Ura$^+$. Southern analysis of three transformants indicated disruption of the MAL43 gene (Charron et al., 1986-Chapter one). Genetic analysis of one of these confirmed disruption at the MAL4-C locus as follows. Strain MCY111-2 (MAL43::URA3 mal1$^0$ (see Methods and Materials)) was mated to a strain (MCY100-3A:Y6-RΔC-2) that carries an integrated URA3 gene at the MAL4 locus (see below). The 4:0 segregation of Ura$^+$ to Ura$^-$ confirmed disruption at the MAL4 locus (see Appendix 5). All three isolates are unable to ferment maltose, and one of these, MCY100-3A Δ 43-1, neither constitutively expresses nor induces the synthesis of maltase (Table 2). The disruption mutation is therefore epistatic to the parental MAL4-C allele. This contrasts to the situation at the MAL6 locus, where deletion of MAL63 in MAL6-constitutive strains has no effect on the ability to constitutively express the maltose fermentative enzymes (Dubin et al., 1986).

Disruption strain MCY100-3A Δ 43-1 was mated to strains carrying the naturally occurring partially funtional alleles of the MAL1 locus: MAL1g, MAL1p and mal1$^0$. A complete structural and functional analysis of these MAL1 alleles has been carried out (Charron and Michels, in preparation-Chapter two). For the purposes of this study, it is sufficent to state that: the MAL1p allele encodes a functional activator but lacks functional structural genes for maltose fermentation; the MAL1g

allele encodes functional structural genes but lacks the activator; and the mal1$^0$ allele encodes only functional maltase (Dubin et al. 1985; Charron et al., 1986-Chapter one). The MAL43 disruption mutation was complemented in strains carrying the MAL1p but not mal1$^0$ or MAL1g alleles indicating that only the regulatory function present at MAL4 had been altered by the disruption.

Transformation of MCY111-2 with the MAL63 plasmid pDF-1 (see Figure 1) (Needleman et al., 1984), targeted to integrate at TRP1, restores the ability to ferment maltose, while the vector alone, pLC544, does not (Clarke and Carbon, 1980). While pDF-1 restores the ability to ferment, the transformant is inducible for maltase (Tables 2 and 3). For comparison purposes, the MAL63 plasmid pDF-1 was inserted into a MAL6 strain carrying a deletion of the MAL63 gene (strain A9). Maltase synthesis in the resulting transformants is inducible (see Table 2, line 8). Taken together the results described above strongly suggest that the constitutive mutation lies in the MAL43 gene. (A low but reproducible glucose insensitive synthesis of maltase is seen in maltose plus glucose grown MAL63-transformed cells. The significance of this is not clear).

The undisrupted MAL4-C mal1$^0$ strain MCY133-1D was also transformed with a single integrated copy of plasmid pDF-1 containing the MAL63 gene. The resulting strain is constitutive for the production of maltase and insensitive

Table 2: Maltase synthesis in MAL43 and MAL63 deletion strains transformed with the MAL63 gene.

Cells were pregrown in uninducing medium (YEP plus 2% galactose), inducing medium (YEP plus 2% maltose or repressing medium (YEP plus 2% maltose/ 5% glucose), diluted into fresh YEP medium containing the indicated sugar additions and allowed to grow 8-9 hours (mid to late log phase). Maltase activity is determined by measuring the rate of hydrolysis of PNPG and is expressed as nmoles substrate split/min/mg protein at 30°C.

For these experiments and all others requiring a wild-type allele of the regulatory gene, we used plasmid pDF-1 (see Figure 1).

All transformants were shown by Southern analysis to contain a single integrated copy of the plasmid.

TABLE 2

Maltase synthesis in MAL43 and MAL63 deletion strains transformed with the MAL63 gene

| Host Strain | MAL Genotype | Integrated Plasmid (Site) | Plasmid MAL Gene | Maltase Activity (nM PNPG/minute/mg protein) 2% Galactose Grown | 2% Maltose Grown | 2% Maltose/ 5% Glucose Grown |
|---|---|---|---|---|---|---|
| MCY100-3A | MAL4-C mal1$^0$ | - | - | 338 | 282 | 102 |
| MCY100-3A-Δ43-1 | MAL43::URA3 | - | - | 9 | 2 | 0 |
| MCY111-2 | MAL43::URA3 mal1$^0$ | - | - | 1 | 4 | 0 |
| MCY111-2 | MAL43::URA3 mal1$^0$ | pDF-1 (TRP1) | MAL63 | 2 | 304 | 17 |
| MCY111-2 | MAL43::URA3 mal1$^0$ | pLC544 (TRP1) | - | 1 | 4 | 0 |
| 332-5A | MAL6 mal1$^0$ | - | - | 5 | 249 | 0 |
| A9 | MAL63::URA3 mal1$^0$ | - | - | 2 | 2 | 0 |
| A9 | MAL63::URA3 mal1$^0$ | pDF-1 (TRP1) | MAL63 | 7 | 357 | 6 |
| A9 | MAL63::URA3 mal1$^0$ | pLC544 (TRP1) | - | 3 | 2 | 0 |
| MCY133-1D | MAL4-C mal1$^0$ | - | - | 178 | 348 | 57 |
| MCY133-1D | MAL4-C mal1$^0$ | pDF-1 (TRP1) | MAL63 | 226 | 387 | 42 |
| MCY133-1D | MAL4-C mal1$^0$ | pLC544 (TRP1) | - | 442 | 355 | 58 |

to glucose repression, thus confirming the dominant nature of this MAL4-linked mutation (Table 2).

Cloning MAL41 and MAL43. The possibility that the MAL43 gene interacts with additional components in this strain to produce the constitutive phenotype could not be ruled out. To clarify this issue and to localize the mutation within MAL4, the MAL43 gene was isolated from the MAL4-C mutant strain MCY100-3A. This was done by integrating a selectable plasmid at the MAL4 locus and recovering this plasmid from the genome along with its flanking MAL4 DNA. Based upon the demonstrated homology between the 7.3kb HindIII fragment of the MAL6 locus and that of the 7.6kb HindIII fragment of the MAL4-C constitutive mutant, a subclone of the MAL6 locus (see pY6-RΔC in Figure 1) was used to direct the integration of a yeast selectable plasmid to the MAL4 locus of constitutive strain MCY100-3A (Needleman and Michels, 1983; M.J. Charron and C.A. Michels, unpublished results). Plasmid pY6-RΔC was directed to integrate at MAL4 by digesting the plasmid with BglII (see (*) Figure 1) prior to transformation. Integration at MAL4 was confirmed for one $Ura^+$ transformant (strain MCY100-3A:Y6-RΔC-2) by Southern analysis. In strain MCY100-3A:Y6-RΔC-2 a 7.6 kb HindIII fragment having homology to MAL6 structural gene sequences and shown to be linked to MAL4 is altered in size in a way consistent with the integration of the

plasmid at the MAL4 locus (data not shown). Plasmid pMJC4B and flanking MAL4 DNA (see Figure 2) was then recovered from the genome of MCY100-3A:pY6-RΔC-2 as described in Materials and Methods.

Restriction enzyme mapping of this cloned MAL4 region showed that it is similar to the MAL61 and MAL63 genes of strain CB11 except for a few restriction site polymorphisms. The restriction map shown in Figure 2 is similar to the map of a DNA fragment from a MAL4 constitutive strain isolated by Rodicio and Zimmerman (1985). Hybridization between cloned DNA derived from the MAL6 locus of CB11 and the MAL4 locus of strain MCY100-3A confirmed the sequence homology between the two loci and enabled us to localize the MAL43 and MAL41 genes (see Figure 2).

Alteration(s) causing both the constitutivity and glucose repression insensitivity lie in MAL43. To determine if the MAL43 gene cloned from the constitutive mutant is sufficient by itself to confer both the constitutive and the glucose repression insensitive phenotypes we constructed plasmids (pM43BS and pM43S) which contain only the MAL43 gene (see Figure 2).

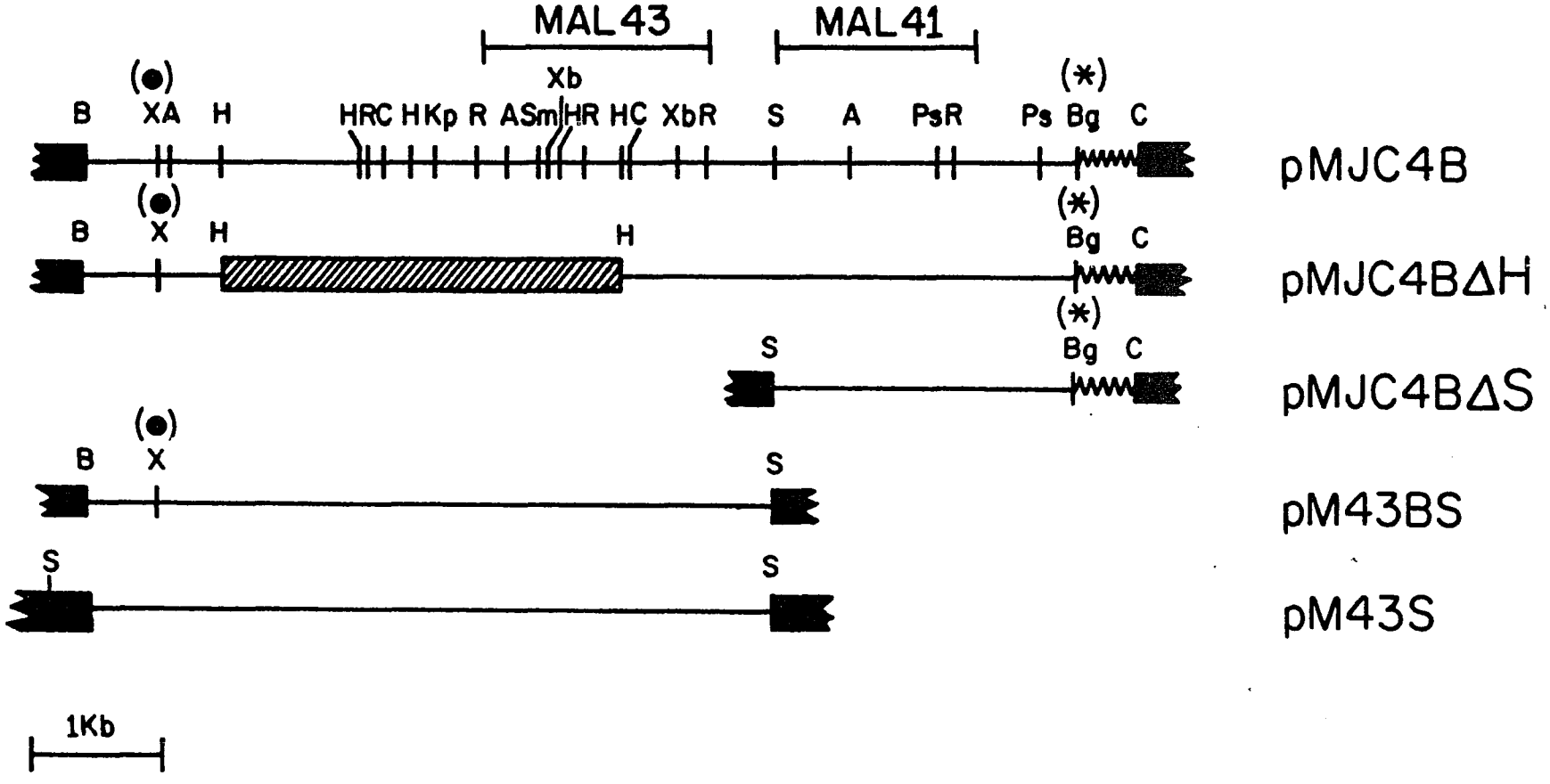
The plasmid pM43BS or pM43S was introduced into a series of strains carrying the structural genes encoding maltose permease and maltase but lacking a functional positive regulator. Prior to transformation, the plasmid

Figure 2: Restriction endonuclease map of part of the MAL4 locus.

The approximate location of the MAL41 and MAL43 genes are indicated above the restriction map. Plasmid pMJC4B was isolated as described in Methods and Materials. Plasmids pMJC4BΔH and pMJC4BΔS were isolated after digestion of plasmid pMJC4B with HindIII and SalI respectively followed by self ligation. Plasmid pM43BS contains the 6.1 kb BamHI - SalI fragment subcloned into pLC544 and plasmid pM43S contains the 6.4 kb SalI fragment of plasmid pMJC4B subcloned into YEp13 SalI. Jagged lines indicate sequences derived from the MAL6 locus.

The symbols (*) and (●) are used to indicate the site of linearization of various plasmids used in yeast transformations (described in text).

Recognition sites for restriction endonucleases are abbreviated as in Figure 1 with the following additions: A, AvaI; B, BamHI; Kp, KpnI; Sm, SmaI; X, XhoI and Xb, XbaI.


MAL43
MAL41
(●)
Xb
(*)
B XA H HRC HKp R ASm HR HC XbR S A PsR Ps Bg C
pMJC4B
(●)
(*)
B X H H Bg C
pMJC4BΔH
(*)
S Bg C
pMJC4BΔS
(●)
B X S
pM43BS
S S
pM43S
1Kb

DNA was linearized in order to direct the integration of the plasmid near LEU2, TRP1, or MAL4. Several integrative transformants of each type were screened by Southern analysis and only strains carrying a single copy of the plasmid were analyzed further. Table 3 shows the level of maltase activity of the various strains and transformants following growth under uninduced, induced and glucose repressed conditions. Maltase synthesis is fully constitutive and at least partially glucose repression insensitive in each of the MAL43-C transformants (Table 3, lines 8, 12, 15, 19). On the other hand transformation of each of the strains with the isolated wild type allele of MAL63, leads to inducible maltase synthesis (Tables 2 and 3). Thus the isolated MAL43 gene from the MAL4-linked constitutive strain is sufficient to confer both constitutivity and glucose repression insensitivity. In addition, the MAL43 gene product is clearly trans-acting.

As further proof of the trans-acting nature of this constitutive and glucose repression insensitive MAL43 mutant allele, we attempted to determine whether or not the MAL12 gene in strain MCY133-1C is also constitutively expressed, as was observed in the MAL64 constitutive mutants (Dubin et al. 1986). Additionally, we wished to confirm that the glucose repression insensitivity is also trans-acting. For this, the MAL41 and MAL42 genes were deleted from strain MCY133-1C utilizing plasmid pRD3 (see Materials and Methods). One transformant selected for

Table 3: Maltase synthesis in strains transformed with the cloned MAL43 gene of the MAL4-C strain.

Growth and assay conditions were performed as described in Table 2. All transformants were shown by Southern analysis to contain a single integrated copy of the plasmid. In most cases, two independent transformants were screened and both gave similar results. The variablity seen in the degree of glucose repression insensitivity and in the basal levels of activity of maltase appear to result from differences in genetic background.

Strains MCY111-2 and A9 are MAL4-C and MAL6 strains in which the linked regulatory gene, MAL43 and MAL63, has been deletion/disrupted (Chang et al., submitted). Strain 348-1B is a MAL6 strain carrying a point mutation in the MAL63 gene (ten Berge et al., 1973a; Chang et al., submitted). Strain 340-2B contains the partially functional MAL1g allele encoding the MAL structural genes only (M.J. Charron and C.A. Michels, in preparation -Chapter two).

Plasmid pM43S was digested with BglII which opens the plasmid in the Ty element flanking the LEU2 gene and directs integration to a Ty (Roeder, 1983). Digestion of plasmids pM43BS and pDF-1 with BglII was used to target integration to the TRP1 gene (Clarke and Carbon, 1980). Digestion of plasmid pM43BS with XhoI, which cuts at a site flanking MAL43 to the left, directs integration to this chromosomal site in MAL4 containing strains. In other strains, such as MAL6 or MAL1g strains, this MAL4 chromosomal region is presumably lacking. Nonetheless, integration does occur at a reasonable rate implying some homologous sequences are present in these strains. In these cases, the exact site of integration is not known.

TABLE 3

Maltase synthesis in strains transformed with the cloned *MAL43* gene of the *MAL4-C* strain

| Host Strain | MAL Genotype | Integrated Plasmid (Site) | Plasmid MAL Gene | Maltose Activity (nM PNPG/minute/mg protein) 2% Galactose Grown | 2% Maltose Grown | 2% Maltose/ 5% Glucose Grown |
|---|---|---|---|---|---|---|
| MCY100-3A | *MAL4-C mal1*$^0$ | - | - | 338 | 282 | 102 |
| MCY111-2 | *MAL43::URA3 mal1*$^0$ | - | - | 1 | 4 | 0 |
| MCY111-2 | *MAL43::URA3 mal1*$^0$ | pM43BS(N.D.) | *MAL43-C* | 176 | 267 | 57 |
| MCY111-2 | *MAL43::URA3 mal1*$^0$ | pLC544(*TRP1*) | - | 1 | 4 | 0 |
| MCY111-2 | *MAL43::URA3 mal1*$^0$ | pM43S(*Ty*) | *MAL43-C* | 211 | 317 | 63 |
| MCY111-2 | *MAL43::URA3 mal1*$^0$ | YEp13ΔS(*Ty*) | - | 1 | 2 | 0 |
| MCY133-1D | *MAL4-C mal1*$^0$ | - | - | 178 | 348 | 57 |
| MCY133-1D | *MAL4-C mal1*$^0$ | pM43BS(*TRP1*) | *MAL43-C* | 360 | 283 | 85 |
| MCY133-1D | *MAL4-C mal1*$^0$ | pLC544(*TRP1*) | - | 442 | 355 | 58 |
| 332-5A | *MAL6 mal1*$^0$ | - | - | 5 | 249 | 0 |
| A9 | *MAL63::URA3 mal1*$^0$ | - | - | 2 | 2 | 0 |
| A9 | *MAL63::URA3 mal1*$^0$ | pM43S(*Ty*) | *MAL43-C* | 382 | 422 | 100 |
| A9 | *MAL63::URA3 mal1*$^0$ | YEp13ΔS(*Ty*) | - | 4 | 6 | 0 |

TABLE 3 (continued)

| | | | | | | |
|---|---|---|---|---|---|---|
| 348-1B | *mal63-10 mal1*$^0$ | - | - | 10 | 3 | 1 |
| 348-1B | *mal63-10 mal1*$^0$ | pM43BS (N.D.) | *MAL43-C* | 188 | 339 | 80 |
| 348-1B | *mal63-10 mal1*$^0$ | pDF-1 (*TRP1*) | *MAL63* | 8 | 210 | 2 |
| 348-1B | *mal63-10 mal1*$^0$ | pLC544 (*TRP1*) | - | 10 | 1 | 1 |
| 340-2B | *MAL1g* | - | - | 1 | 2 | 2 |
| 340-2B | *MAL1g* | pM43BS (N.D.) | *MAL43-C* | 170 | 204 | 60 |
| 340-2B | *MAL1g* | pDF-1 (*TRP1*) | *MAL63* | 16 | 324 | 2 |
| 340-2B | *MAL1g* | pLC544 (*TRP1*) | - | 15 | 3 | 0 |

further analysis (strain MCY133-1C/Δ41/42-8) was shown to carry the MAL41/42::LEU2 disruption (see Appendix 5). As can be seen by the results reported in Table 4, deletion of the MAL41 and MAL42 genes produces a nonfermenter with reduced, but significant levels of constitutively expressed maltase. Strain MCY133-1C/Δ41/42-8 was then mated to the MAL63::URA3 disruption strain A9 and to the MAL43::URA3 disruption strain MCY111-2. Both diploids ferment maltose and constitutively produce high levels of maltase. In addition, they are glucose repression insensitive (see Table 4). In summary, these results clearly demonstrate that both constitutivity and glucose repression insensitivity at the MAL4 locus are trans-acting functions.

Analysis of the MAL43 flanking DNA sequences. To rule out the possibility that sequences outside of the MAL43 gene contribute to the MAL4-C phenotype, the entire MAL41 gene including flanking sequences was inserted into a MAL6 strain deleted for sequences upstream of both MAL61 and MAL62, (using plasmid pRD3, see Figure 1 and Dubin et al. 1986). This strain (332-5A/Δ61/62-9) therefore contains an intact copy of the MAL63 gene and a functional MAL12 gene (encoding maltase) and is a nonfermenter because it lacks functional maltose permease.

Strain 332-5A/Δ61/62-9 was transformed separately with three different plasmids: pMJC4BΔH, containing the MAL41 gene, the MAL41 - MAL42 intergenic region (and a

TABLE 4

Maltase synthesis in strains containing the MAL41/42 deletion disruption[a]

| Strain | MAL Genotype | Maltase Activity (nM PNPG/minute/mg protein) 2% Galactose Grown | 2% Maltose Grown | 2% maltose/ 5% Glucose Grown |
|---|---|---|---|---|
| MCY133-1C | MAL4-C mal1$^0$ | 365 | 426 | 76 |
| MCY133-1C/Δ41/42-8 | MAL41/42::LEU2 mal1$^0$ | 209 | 226 | 31 |
| A9 | MAL63::URA3 mal1$^0$ | 2 | 2 | 0 |
| MCY111-2 | MAL43-C::URA3 mal1$^0$ | 1 | 4 | 0 |
| MCY111-2 x MCY133-1C/Δ41/42-8 | MAL41 MAL42 MAL43-C::URA3 mal1$^0$ / MAL41/42::LEU2 MAL43-C mal1$^0$ | 393 | 583 | 95 |
| MCY133-1C/Δ41/42-8 x A9 | MAL41/42:LEU2 MAL43-C MAL6ø mal1$^0$ / MALø MAL61 MAL62 MAL63::URA3 mal1$^0$ | 343 | 582 | 82 |

[a]Growth and assay conditions were carried out as described in Table 2. Construction of strain 332-5A/Δ61/62-9 is described in Dubin et al. (1986).

portion of the 5' end of MAL62) (see Figure 2); plasmid pMJC4BΔS, containing the MAL41 gene, the MAL41 - MAL42 intergenic region (and a portion of the 5' end of MAL62) (see Figure 2); and plasmid pY6-PΔCBS, containing the MAL61 gene, the MAL61 - MAL62 intergenic region (and a portion of the 5' end of MAL62) (see Figure 1). In each case, the plasmid was directed to integrate at the MAL12 gene, which was confirmed by Southern analysis. Each of the above plasmids encodes maltose permease (MAL41 or MAL61) and, as expected, all transformants are maltose fermentors. One can now ask is the expression of MAL12 inducible or constitutive? Maltase synthesis in three independent transformants containing the MAL41 gene and flanking sequences is fully inducible, as is also found for two independent transformants containing the MAL61 gene and its flanking sequences. This indicates that the constitutive phenotype is mediated by the MAL43 gene alone with little or no contribution from structural gene upstream sequences. This is again confirmed by the results shown in the last two lines of Table 5. Plasmid pMJC4B, containing the MAL43-C gene, the MAL41 gene and sequences extending into the MAL42 gene was transformed into strain 332-5A/Δ61/62-9 and integrated at either the MAL12 gene of the $mal1^0$ locus or at an undetermined site unlinked to either MAL1 or MAL4 (by linearization at the XhoI site in the DNA sequence flanking MAL43 to the left). In both sets of

TABLE 5

Maltase synthesis in the MAL61/62 deletion disruption strain 332-5A/Δ61/62-9[a] transformed with MAL41, MAL61 and MAL41 MAL43-C containing plasmids[b]

| Host Strain | MAL Genotype | Integrated Plasmid (Site) | Plasmid MAL gene | Maltase Activity (nM PNPG/minute/mg protein) 2% Galactose Grown | 2% Maltose Grown | 2% Maltose/ 5% Glucose Grown |
|---|---|---|---|---|---|---|
| 332-5A | MAL6 mal1$^0$ | - | - | 5 | 249 | 0 |
| 332-5A/Δ61/62-9 | MAL61/62::LEU2 mal1$^0$ | - | - | 2 | 7 | 0 |
| " | " | pMJC4BΔH (mal1$^0$) | MAL41 | 4 | 357 | 2 |
| " | " | pMJC4BΔS (mal1$^0$) | MAL41 | 4 | 150 | 8 |
| " | " | pY6-RΔCBS (mal1$^0$) | MAL61 | 14 | 247 | 0 |
| " | " | pMJC4B (mal1$^0$) | MAL41 MAL43-C | 332 | 315 | 74 |
| " | " | pMJC4B (N.D.*) | MAL41 MAL43-C | 170 | 300 | 28 |

N.D.* Site of integration not determined; unlinked to MAL1 or MAL4

[a] Construction of strain 332-5A/Δ61/62-9 is described in Dubin et al. (1986).

[b] Growth and assay conditions were performed as described in Table 2.

transformants, the strains are constitutive and glucose repression insensitive. These results clearly indicate that the glucose repression insensitive phenotype of the MAL4-C mutant is mediated by the MAL43-C allele. Since these transformants contain both the MAL43-C gene and the genomic MAL63 gene, these results also confirm that the mutation(s) in the MAL43-C gene is (are) dominant and trans-acting.

# DISCUSSION

Kahn and Eaton (1971) described a yeast strain carrying an allele of the MAL4 locus causing constitutive MAL gene expression. They showed the constitutive phenotype to be dominant to the wild type inducible glucose repression sensitive phenotype, with both traits being tightly linked. The origin of this MAL4-C mutation is not clear but other reports indicate that it was originally isolated by Winge and Roberts (1950) either spontaneously or by X-ray mutagenesis. Nevertheless, the dominant constitutivity and glucose repression insensitivity of this mutation make it important for us to understand the genetic basis of these phenotypes in order to more fully understand the mechanisms controlling maltose fermentation in Saccharomyces.

For this, we isolated DNA sequences from the MAL4 locus extending from the coding region of the MAL42 gene to the DNA sequences flanking MAL43 (including both MAL41 and MAL43 (see Figure 2)). Using these sequences, in a variety of strain constructions, we demonstrate the following. 1) The alteration(s) in this MAL4-C locus which lead to the constitutive phenotype clearly map to the MAL43 gene (encoding the MAL4-linked positive trans-acting regulatory protein homologous to the MAL63 gene of the MAL6 locus). Preliminary results of similar experiments performed on a MAL2 - linked dominant,

constitutive mutant are in agreement with those described above (M.J. Charron and C.A. Michels, unpublished results-Appendix five). In addition, an alteration(s) responsible for the glucose repression insensitivity of this strain maps to MAL43. 2) Both the constitutive and the glucose repression insensitive phenotypes caused by this MAL43-C mutation are dominant to the wild type MAL63 allele. 3) The mutation is trans-acting. Our results do not exclude the possibility that additional mutations at this MAL4-C locus also could be contributing to the glucose repression insensitivity but the results do not support this possibility.

These results are in contrast to those of Dubin et al. (1986) regarding the MAL6-linked constitutive mutations. In their study, two constitutive revertants of mal63 mutations were analyzed in detail and shown to map to a gene, called MAL64, which lies 2.3 centamorgans to the left of MAL63 and not to MAL63 itself which encodes the positive regulator of maltose fermentation. These and other studies have shown that MAL64 constitutive mutations are recessive to the wild type MAL64 allele and to various mal63 mutant alleles and that they are glucose repression sensitive (ten Berge et al., 1973b; ten Berge, 1974). Clearly, the genetic basis of the constitutive mutations at MAL6 is different from that found here for the MAL4-C constitutive allele.

MAL4-C mutants are fully constitutive but only partly

insensitive to glucose repression. It is not clear whether the partial insensitivity to glucose repression is a characteristic of this particular mutant allele of MAL43 or if the glucose repression of maltase synthesis is mediated by a number of independent systems. Genetic analysis indicates that three control circuits are involved in the glucose repression of the galactose fermentative enzymes (Matsumoto et al., 1983). We feel that glucose may have similar multiple pathways in controlling maltose fermentation.

Identification of dominant constitutive mutations in the MAL43 gene is not surprising. The product of the MAL63 gene, and therefore, by homology, the product of the MAL43 gene, is a positive regulator controlling the expression of the maltose fermentative enzymes (Chang et al., submitted). Similar dominant constitutive mutations have been reported in other yeast positive regulators. Douglas and Hawthorne (1966) and Matsumoto et al., (1980) describe extensive genetic analyses of mutations in the GAL4 gene, encoding the positive activator controlling the synthesis of the galactose fermentative enzymes. Dominant constitutive mutations were obtained, many by reverting noninducible gal4 mutations. None of the GAL4-constitutive mutations described show resistance to glucose repression. The glucose repression insensitive phenotype of the MAL43-C mutation described here is therefore unique and makes the detailed genetic analysis

of this mutation, as well as the MAL2-linked constitutive glucose repression insensitive mutations described by Zimmerman and Eaton (1974) important for any analysis of the mechanisms of glucose repression of the maltose fermentative enzymes in yeast. It is interesting to note that sequence analysis of a dominant mutation of the ADR1 gene, which encodes a positive regulator controlling the glucose sensitive expression of ADH2, has found the alteration to lie within a putative recognition site for a cyclic AMP-dependent kinase (Ciriacy, 1979; Denis and Gallo, 1986). Analysis of the sequence of the MAL regulatory genes is underway in this laboratory and should prove quite fruitful.

One of the most interesting issues raised by the results reported here is why MAL64-like constitutives are not obtained at MAL4. Comparisons by Southern analysis between the cloned MAL4 and MAL6 loci including several kb of flanking DNA sequences, show that the homology is limited to the approximately 9.0 kb region containing the structural genes and the activator (MAL61-62-63 and MAL41-42-43) (M.J. Charron and C.A. Michels, in preparation-Chapter four). Thus, a gene equivalent to MAL64 does not appear to be present at the MAL4 locus. The MAL64 gene has been localized to a site about 3.0 kb from MAL63 in a region showing significant sequence homology to MAL63 and like, MAL63, MAL64- constitutive mutants encode an activator of MAL structural gene

transcription (Dubin et al., submitted-Appendix two). Obtaining dominant constitutive mutations similar to MAL43-C in genes encoding activators is not surprising. What is unusual is that MAL63 constitutive mutations have not yet been isolated. A comparison of the isolated MAL63, MAL64 and MAL64-C sequences should give some insight into the reason for the absence of this class of mutations.

# Appendix 2

## Interaction between the MAL64, MAL64C, MAL63, mal63-13 and mal63-10 gene products

In a recent study, Dubin et al. (1986) demonstrate that the constitutive expression of maltose permease and maltase in the constitutive strains 8-2B (a constitutive revertant of the nonfermenting mutant mal63-13 isolated by ten Berge et al. (1973)) and R10 (a constitutive revertant of a nonfermenting ΔMAL63 deletion/disruption isolated by Dubin et al. (1986)) is due to a mutation(s) in a gene referred to as MAL64. MAL64 maps 2.3 cM to the left of MAL63. The region flanking MAL63 was cloned from the wild-type MAL6 allele of strain 332-5A and from the constitutive MAL6-C2 allele of strain 8-2B using methods described in Chapter four of this thesis. Utilizing these cloned sequences Dubin (1986) was able to localize the MAL64 gene to the region shown in Figures 1 and 2. Deletion of the MAL64 constitutive allele from strains 8-2B (carrying the MAL64-C2 allele) and R10 (carrying the MAL64-R10 allele) led to the loss of expression of the maltose fermentative enzymes (see Table 1 lines 1 and 2, 5 and 6). Dubin (1987) found that transformation of these deletion strains with episomal plasmids containing the deleted region isolated from strain 8-2B led to the recovery of constitutive expression while the same region isolated from the wild type MAL6 strain 332-5A did not. Additionally, transformants carrying the wild type MAL63 gene carried on an episomal plasmid inducibly synthesized the maltose fermentative enzymes. Because these episomal plasmids were not maintained well by the transformants the results obtained had to be evaluated

Figure 1: The wild-type MAL6 locus and plasmids containing MAL6 sequences.

A partial restriction map of the wild-type MAL6 locus is diagramed along with the location and direction of transcription of the MAL61, MAL62 and MAL63 genes. The approximate location of the MAL64 gene is shown.

Plasmids pDF-1, pMAL64 and pMAL64ΔXbaI were used in yeast transformation experiments presented in Tables 1 and 2.

(●) indicates known restriction endonuclease site polymorphisms between MAL6 and MAL6-C2 (see Figure 2).

Restriction endonuclease sites are abbreviated as follows: A, AvaI; B, BamHI; Bg, BglII; C, ClaI; H, HindIII; Hp, HpaI; M, MluI; N, NcoI; P, PstI; S, SalI; Sm, SmaI; Ss, SstI; Xb, XbaI; Xo, XhoI.

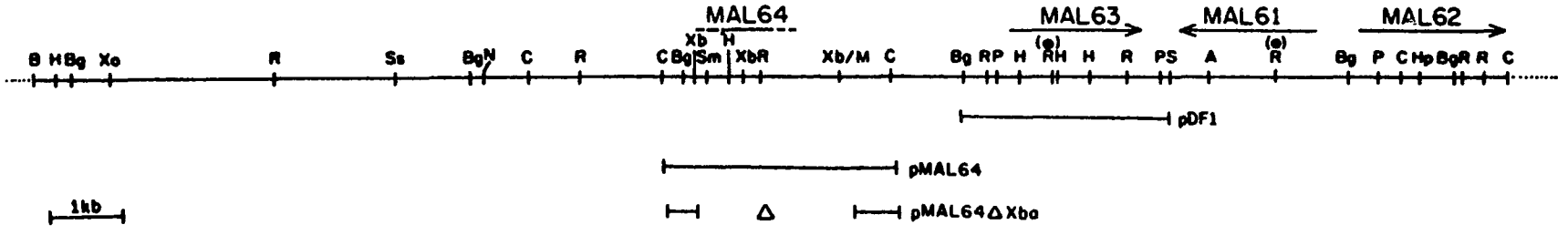

MAL64
MAL63
MAL61
MAL62
Xb
H
(φ)
(φ)
B H Bg Xo R Ss BgN C R C Bg Sm XbR Xb/M C Bg RP H RH H R PS A R Bg P C Hp BgR R C
pDF1
pMAL64
1kb
Δ
pMAL64ΔXba

Figure 2: The constitutive MAL6-C2 locus from strain 8-2B and plasmids containing MAL6-C2 sequences.

A partial restriction endonuclease map of the MAL6-C2 locus is shown along with the location and direction of transcription of MAL61, MAL62 and mal63-13 genes. The approximate location of the MAL64-C2 gene is indicated.

Plasmids pMAL64C2 and pMAL64C2 Δ XbaI were used in experiments summarized in Tables 1 and 2.

(●) indicates known restriction site polymorphisms between the MAL6 and MAL6-C2 alleles (see Figure 1).

Recognition sites for restriction endonucleases are abbreviated as in Figure 1.

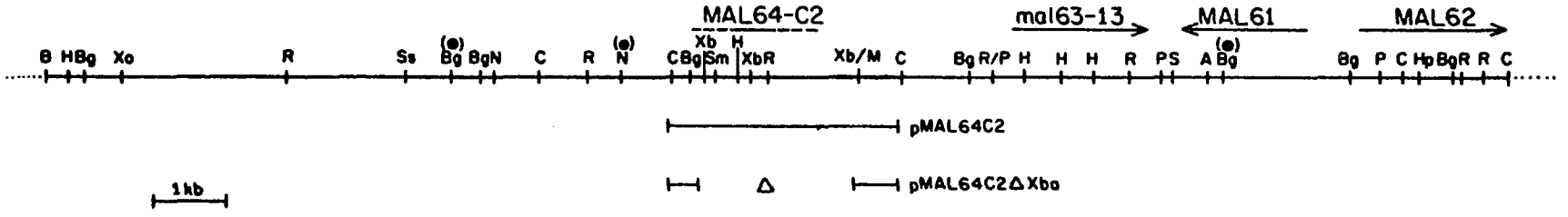

MAL64-C2
mal63-13
MAL61
MAL62
B HBg Xo R Ss (●) Bg BgN C R (●) N C Bg Xb Sm H XbR Xb/M C Bg R/P H H H R PS A (●) Bg Bg P C Hp BgR R C
pMAL64C2
pMAL64C2ΔXba
1 kb

Table 1: Alleles of the MAL64 gene and their effects on maltase and maltose permease expression.

Strain 332-5A contains the wild-type MAL64 allele and strain 8-2B carries the constitutive MAL64-C2 allele. These strains were used to clone MAL64 and MAL64-C2 by the plasmid rescue technique of Orr-Weaver et al. (1983) using plasmids pMJC6ΔC and pY6-RΔC as described in Chapter four. Maltose fermentation was determined as described previously (Appendix one). Yeast transformations were performed according to the method of Ito et al. (1983) as described in Chapter one.

[a] Measurement of the release of p-nitrophenol from p-nitrophenol-α-D-glucoside (PNPG) was as described in Appendix one.

[b] The rate of maltose transport was determined by the method of Serrano (1977).

For all assays cells were grown to mid-log phase in noninducing medium containing 2% galactose and inducing medium containing 2% maltose prior to harvesting.

Table 1

Alleles of the MAL64 gene and their effects on maltase and maltose permease expression

| Strain | MAL Related Genotype | Integrated Plasmid | Maltose Fermentation | PNPGase Activity[a] 2% Gal | PNPGase Activity[a] 2% Mal | Permease Activity[b] 2% Gal | Permease Activity[b] 2% Mal |
|---|---|---|---|---|---|---|---|
| 8-2B | mal63-13 MAL64-C2 | none | + | 265 | 498 | 3.89 | 4.46 |
| 8-2BΔF-5-21 | mal63-13 mal64::LEU2 | none | - | 3 | 0 | 0.09 | ND |
| | mal63-13 mal64::LEU2 | pMAL64 | - | 4 | 1 | | |
| | | pMAL64C2 | + | 156 | 222 | | |
| | | pMAL64C2ΔXbaI | - | 2 | 4 | | |
| | | pDF-1 | + | 4 | 201 | | |
| R10u | MAL63::ura3 MAL64-R10 | none | + | 759 | 770 | 16.16 | 6.73 |
| R10uΔF-9-26 | MAL63::ura3 MAL64::LEU2 | none | - | 4 | 0 | 0.48 | ND |
| | | pMAL64 | - | 3 | 1 | | |
| | | pMAL64C2 | + | 337 | 288 | | |
| | | pMAL64C2ΔXbaI | - | 15 | 10 | | |
| | | pDF-1 | + | 6 | 284 | | |
| 332-5A | MAL63 MAL64 | none | + | 5 | 249 | 0.23 | 9.75 |
| 332-5AΔF-1-5 | MAL63 MAL64::LEU2 | none | + | 4 | 299 | 0.28 | 9.18 |
| | | pMAL64 | + | 5 | 314 | | |
| | | pMAL64C2 | + | 212 | 292 | | |
| | | pMAL64C2ΔXbaI | + | 3 | 283 | | |

in conjunction with an analysis of plasmid retention. It was therefore decided to subclone the MAL64 and MAL64-C2 alleles into integrating plasmids and to repeat these studies using strains carrying a single integrated copy of the gene. Results of these experiments are presented in this appendix and will appear in the following article submitted for publication in Molecular and Cellular Biology: Dubin, R.J., Charron, M.J., Haut, S.R., Needleman, R.B. and Michels, C.A. Constitutive expression of the maltose fermentative enzymes in Saccharomyces carlsbergensis is dependent upon the mutational activation of MAL64, a nonessential homologue of MAL63.

Dubin (1987) also showed that deletion of the MAL64 gene had little or no effect on the inducible expression of the maltose fermentation enzymes. This result is particularly interesting especially in view of the strong sequence homology between the MAL63 and MAL64 genes (discussed in Chapter 4). The homology between MAL63 and MAL64 suggested to us that the products of these genes may also be related and could potentially interact with each other. For this reason, we undertook to determine the effect of single and multiple integrated copies of the MAL64 gene or its constitutive allele, MAL64-C2, on the expression of the maltose fermentative enzymes in various MAL6 strains.

Analysis of the expression of the maltose fermentative enzymes in MAL64 deletion / disruption strains carrying a

single integrated copy of the MAL64 or MAL64-C2 genes. Deletion/disruption F extends from the BglII site immediately flanking the 5' end of the MAL63 gene to the BglII site at the extreme left end of the map shown in Figure 1 replacing this 11.5 kb region with the LEU2 gene. A detailed description of the construction of the plasmid carrying the F deletion/disruption and the isolation of strains deleted for these sequences is presented in Dubin (1987) and Dubin et al. (submitted).

Strain 8-2B is a derivative of the constitutive revertant C2 isolated by ten Berge et al. (1973) as a fermenting revertant of the nonfermenting mutant strain mal6-13. The mutation in strain mal6-13 has been shown to lie in the MAL63 gene (Chang et al., submitted). Thus, the MAL related genotype of strain 8-2B is mal63-13 MAL64-C2. Strain R10u is a constitutive revertant of the nonfermenting MAL63 deletion/disruption strain A9. The URA3 gene used to construct the A9 deletion was mutated to ura3 using 5-fluoro-orotic acid in order to enable for the selection of $Ura^+$ transformants. The MAL-related geneotype of R10u is MAL63::ura3 MAL64-R10. Strain 332-5A is the standard MAL6 fermenting wild type strain used in the studies in our laboratory. R10 is isogenic to 332-5A except at the MAL63 and MAL64 genes.

Plasmids containing the MAL64 and MAL64-C2 genes were constructed. This was accomplished as follows. The 3.4 kb ClaI fragment from the wild type strain 332-5A and the

constitutive strain 8-2B was subcloned into YIp5 to form plasmids pMAL64 and pMAL64C2 respectively. Plasmids pMAL64 and pMAL64C2 were then individually restricted with XbaI and self ligated to form plasmids pMAL64ΔXbaI and pMAL64C2ΔXbaI respectively (Figures 1 and 2). Plasmid pDF-1 contains the 2.6 kb BglII - SalI fragment of MAL63 in the TRP1 vector pLC544 (Figure 1).

As shown in Table 1 and 2 plasmids pMAL64, pMAL64ΔXbaI, pMAL64C2, pMAL64C2ΔXbaI and pDF-1 were integrated into the yeast genome of various MAL6 strains. All YIp5-derived plasmids were targeted to the URA3 gene following linearization of the plasmid with NcoI. The pLC544-derived plasmid pDF-1 was targeted to the TRP1 gene following linearization of the plasmid with BglII. Stable transformants were then screened by the colony hybridization method of Hinnen et al. (1979) for the presence of pBR322 homologous sequences. $Ura^+$ transformants containing plasmid sequences were digested with NcoI and $Trp^+$ transformants were digested with BglII, size fractionated by agarose gel electrophoresis and subjected to Southern gel transfer analysis. Since NcoI cuts only once in the YIp5-derived subclones and BglII cuts only once in the pLC544-derived subclone, then only one pBR322-homologous fragment will be detected in strains containing a single integrated copy of the plasmid. Strains containing multiple integrated copies of the plasmid contain a second pBR322-homologous fragment that is the exact size of the entire plasmid targeted into

the yeast genome.

The results of assaying the levels of maltase and maltose permease synthesized by the single copy integrative transformants are presented in Table 1. Certain conclusions can be drawn. Firstly, the MAL64 gene is fully contained within the 3.4 kb ClaI fragment of plasmid pMAL64. Secondly, the use of integrated plasmids allows us to obtain clear cut results, but the basic conclusions are unchanged. These conclusions are: 1. mutations in the MAL64 gene in strains 8-2B and R10u are responsible for the constitutive expression of the maltose fermentative enzymes; 2. the MAL64-constitutive mutant alleles encode a trans-acting activator of the expression of the maltose fermentation structural genes; 3. in inducible strains, the deletion of the MAL64 gene has no effect on maltose fermentation or maltose regulated induction; 4. the MAL64-C2 mutant allele is dominant to the wild type MAL64 allele as well as epistatic to the wild type MAL63 allele.

Interaction between the MAL64, MAL64C2, MAL63, mal63-13 and mal63-10 gene products. Based upon the fact that MAL63 and MAL64 are highly sequence homologous yet functionally non-homologous it was of interest to see what effect (if any) an increase in the number of copies of the MAL64 or the MAL64C2 gene would have on maltose fermentation in various MAL6 strains. This would allow us to study the interaction of the MAL64 and MAL64C2 gene products as well as the

possible interaction with the MAL63 gene product and its mutant alleles. To address these questions plasmids pMAL64, pMAL64ΔXbaI, pMAL64C2 and pMAL64C2ΔXbaI (Figures 1 and 2) were transformed, in single and multiple copies, into the inducible strain 332-5A, the constitutive strain 8-2B and the non-inducible mal63-10 point mutant 348-1B using procedures described in the section above. Maltose fermentation, PNPGase, maltase, maltose permease and α-methylglucosidase activity was then assayed and reported in Table 2.

As shown in Table 2, the MAL64-C2 constitutive allele is dominant to the wild type allele and epistatic the MAL63 gene as well as two mutant alleles, mal63-13 and mal63-10. When strain 332-5A was transformed with the wild type MAL64 gene, production of the maltose fermentative enzymes is not significantly different from that found in the untransformed parental strain under both induced and uninduced conditions regardless of copy number (Table 2). In contrast, the presence of additional copies of the MAL64 gene in the constitutive strain 8-2B appeared to have a somewhat repressive effect on the level of PNPGase synthesized, particularly under non-inducing conditions (ie: transformant #6 and #24, Table 2) and the effect was stronger at higher copy number. This apparent repressive effect, noted under non-inducing conditions, is suggestive of an interaction of MAL64 and MAL64C2 gene products in a mal63-13 background. The results do not support the possibility of an interaction

Table 2: PNPGase, maltase, maltose permease and α-methylglucosidase activity of various MAL6 strains transformes with MAL64 and MAL64C2 containing plasmids.

Strains and growth conditions were as described in the text and in Table 1.

[a] Transformant # indicates the number of the individual transformant examined from the given transformation.

[b] Copy # of the integrated plasmid is indicated by s (single) or m (multiple) as determined by Southern analysis (see text for details).

[c] Maltose fermentation was determined as in Appendix one.

[d] PNPGase activity was measured as in Appendix one. Cells were grown under noninducing conditions in YP medium containing 2% galactose (GAL) and inducing conditions in YP medium containing 2% maltose (MAL).

[e] Maltase activity was measured according to the method of Lloyd and Wheland (1969). Cells were grown under inducing conditions in YP medium containing 3% glycerol 2% ethanol and 2% maltose (GE+Mal).

[f] α-Methylglucosidase activity was measured by the method of Lloyd and Wheland (1969) from cells grown under inducing conditions in YP medium plus 3% glycerol 2% ethanol 2% α-methylglucoside (GE+Mgl).

[g] Permease activity was measured as described in Table 1. Cells were grown in GE+Mal medium unless otherwise indicated.

Table 2

PNPGase, maltase, maltose permease and α-methylglucosidase activity of various MAL6 strains transformed with MAL64 and MAL64C2 containing plasmids

| Strain | MAL Related Genotype | Integrated Plasmid | Transf. #[a] | Copy #[b] | Malt. Ferm.[c] | PNPGase (Gal) | PNPGase (Mal)[d] | Maltase (GE+Mal)[e] | Permease (GE+Mal) or Mal[g] | α-MGLase (GE+Mgl)[f] |
|---|---|---|---|---|---|---|---|---|---|---|
| 332-5A | MAL63 MAL64 | none | | | + | 5 | 249 | 1246[g] | 9.75[g] | 74 |
| | | pMAL64 | 9 | m | slow | 29 | 104 | ND | ND | ND |
| | | | 11 | s | slow | 17 | 310 | 873 | 5 | 177 |
| | | | 15 | m | - | 14 | 416 | ND | ND | ND |
| | | pMAL64ΔXbaI | 2 | ND | + | 7 | 423 | 537 | 16 | 155 |
| | | pMAL64C2 | 1 | m | + | 154 | 460 | ND | ND | ND |
| | | | 4 | s | + | 94 | 371 | ND | 23 | ND |
| | | | 15 | s | + | 213 | 424 | ND | ND | ND |
| | | | 18 | m | + | 593 | 407 | 1535 | 11 | 517 |
| | | pMAL64C2ΔXbaI | 1 | s | + | 10 | 275 | 1483 | 12 | 288 |
| 8-2B | mal63-13 MAL64C2 | none | | | + | 265 | 498 | ND | 5[g] | ND |
| | | pMAL64 | 6 | m | slow | 23 | 96 | ND | 23 | ND |
| | | | 11 | s | slow | 181 | 338 | 296 | 19 | 84 |
| | | | 24 | m | slow | 75 | 325 | ND | 22 | ND |
| | | pMAL64ΔXbaI | 3 | ND | + | 261 | 445 | 513 | 14 | 131 |
| 348-1B | mal63-10 MAL64 | none | | | - | 10 | 3 | ND | ND | ND |
| | | pMAL64C2 | 3 | m | + | 192 | 285 | ND | 12 | ND |
| | | | 4 | s | + | 177 | 256 | ND | ND | ND |
| | | | 15 | s | + | 165 | 191 | 1443 | 11 | 367 |
| | | pMAL64C2ΔXbaI | 1 | ND | - | 4 | 2 | ND | ND | ND |

between the MAL63 and MAL64 proteins. When both 332-5A and 8-2B were transformed with plasmid pMAL64ΔXbaI, a $Mal^+$ phenotype was observed in 100% of the transformants scored and the expression of the fermentative enzymes appears to be unaffected supporting the proposed interaction between the products of these two MAL64 alleles. It should be noted that strains 332-5A and 8-2B transformed with the pMAL64 plasmid, regardless of copy number, were slow maltose fermenters and, in one case, appeared not to ferment at all. In all cases, the transformants grew well on maltose and were able to synthesize normal levels of the fermentative enzymes. This result suggests that an increase in the number of copies of MAL64 present in a strain slows down the process of maltose fermentation as determined by the liberation of $CO_2$ without having an obvious effect on the synthesis of maltase, permease, PNPGase and α-methylglucosidase on cells harvested in mid- to late-log phase. The rate at which these transformants liberate $CO_2$ is now being assayed directly in Dr. Richard Needleman's laboratory at Wayne State Medical College in order to confirm the results observed in Durham tubes. The meaning of this effect on fermentation is unknown.

The effect on the liberation of $CO_2$ is specific for the maltose fermentation system and is not pleiotrophic for the fermentation of other sugars. Several pMAL64 transformants were tested for the ability to ferment galactose, glucose, fructose, raffinose and sucrose. All transformants tested

maintained the phenotype of the untransformed strain (either 332-5A or 8-2B) (data not shown).

# Appendix 3

## Genetic Demonstration of the Presence of SUC1 in Strain 600-1B.

| | | | |
|---|---|---|---|
| MCY105: | 600-1B::pM1.4B#1 | x | MCY621 |
| | SUC1 SUC? | x | $suc1^0$ |
| | $Suc^+$ | | $suc^-$ |

Ratio of ($Suc^+$) : ($suc^-$) in 4 spored asci.

| 4:0 | 3:1 | 2:2 | 1:3 | 0:4 | total asci examined |
|---|---|---|---|---|---|
| 3 | 4 | 3 | 0 | 0 | 10 |

Conclusion: 600-1B contains two SUC genes.

| | | | |
|---|---|---|---|
| MCY107: | MCY105-8C | x | DBY739 |
| | SUC? | x | SUC1 |
| | $Suc^+$ | | $Suc^+$ |

Ratio of ($Suc^+$) : ($suc^-$) in 4 spored asci.

| 4:0 | 3:1 | 2:2 | 1:3 | 0:4 | total asci examined |
|---|---|---|---|---|---|
| 2 | 5 | 2 | 0 | 0 | 9 |

Conclusion: MCY105-8C carries a single SUC gene which is not SUC1.

| | | | |
|---|---|---|---|
| MCY108: | MCY105-8B | x | MCY106-3D* |
| | SUC1 (SUC?) | x | SUC1 |
| | $Suc^+$ | | $Suc^+$ |

Ratio of ($Suc^+$) : ($suc^-$) in 4 spored asci.

| 4:0 | 3:1 | 2:2 | 1:3 | 0:4 | total asci examined |
|---|---|---|---|---|---|
| 10 | 0 | 0 | 0 | 0 | 10 |

Conclusion: MCY105-8B carries at least SUC1.

* MCY106: DBY739 x MCY619

SUC1 x $suc1^0$

$Suc^+$ $suc^-$

MCY109: MCY105-8D x MCY106-3D

(SUC?) x SUC1

$Suc^+$ $Suc^+$

Ratio of ($SUC^+$) : ($SUC^-$) in 4 spored asci.

| 4:0 | 3:1 | 2:2 | 1:3 | 0:4 | total asci examined |
|---|---|---|---|---|---|
| 4 | 5 | 3 | 0 | 0 | 12 |

Conclusion: MCY105-8D carries a single SUC gene, not SUC1.

MCY110: MCY105-8A x DBY739

SUC1 (SUC?) x SUC1

$Suc^+$ $Suc^+$

Ratio of ($Suc^+$) : ($suc^-$) in 4 spored asci.

| 4:0 | 3:1 | 2:2 | 1:3 | 0:4 | total asci examined |
|---|---|---|---|---|---|
| 10 | 1 | 0 | 0 | 0 | 11 |

Conclusion: MCY105-8A carries at least SUC1. One aberrant tetrad (3:1) was isolated.

# Appendix 4

## Genetic Demonstration of the Linkage of Integrated Plasmids pM1.4B, pY6ΔCΔH, pM3.2B, pY6-RΔC and pMJC6ΔC to various MAL Loci.

1. Linkage of pM1.4B to MAL1.

| | | | |
|---|---|---|---|
| MCY105: | 600-1B::pM1.4B#1 | x | MCY621 |
| | MAL1::URA3 | x | MAL1g |
| | $Mal^+$, $Ura^+$ | | $mal^-$, $ura^-$ |

Ratio of ($Mal^+$, $Ura^+$) : ($mal^-$, $ura^-$) in 4 spored asci.

| 4:0 | 3:1 | 2:2 | 1:3 | 0:4 | total asci examined |
|---|---|---|---|---|---|
| 0 | 0 | 13 | 0 | 0 | 13 |

2. Linkage of pY6ΔCΔH to MAL1.

| | | | |
|---|---|---|---|
| MCY137: | 600-1B::pY6ΔCΔH#7 | x | 100-1B |
| | MAL1::URA3 | x | MAL12::LEU2 |
| | $Mal^+$, $Ura^+$, $leu^-$ | | $mal^-$, $ura^-$, $Leu^+$ |

Ratio of ($Mal^+$, $Ura^+$, $leu^-$) : ($mal^-$, $ura^-$, $Leu^+$) in 4 spored asci.

| 4:0 | 3:1 | 2:2 | 1:3 | 0:4 | total asci examined |
|---|---|---|---|---|---|
| 0 | 0 | 12 | 0 | 0 | 12 |

3. Linkage of pY6-RΔC to MAL4.

MCY114: MCY100-3A::pY6RΔC#2 x MCY111-2

MAL4::URA3 mal1$^0$ x MAL43::URA3 mal1$^0$

Mal$^+$, Ura$^+$ mal$^-$, Ura$^+$

Ratio of (Mal$^+$, Ura$^+$) : (mal$^-$, Ura$^+$) in 4 spored asci.

| 4:0 | 3:1 | 2:2 | 1:3 | 0:4 | total asci examined |
|---|---|---|---|---|---|
| 0 | 0 | 8 | 0 | 0 | 8 |

4. Linkage of pY6ΔCΔH to MAL4.

MCY144: MCY100-2C::pY6ΔCΔH#7 x MCY100-3A::pDM3#1

MAL4::URA3 mal1$^0$ x MAL43::URA3 mal1$^0$

Mal$^+$, Ura$^+$ mal$^-$, Ura$^+$

Ratio of (Mal$^+$, Ura$^+$) : (mal$^-$, Ura$^+$) in 4 spored asci.

| 4:0 | 3:1 | 2:2 | 1:3 | 0:4 | total asci examined |
|---|---|---|---|---|---|
| 0 | 0 | 9 | 0 | 0 | 9 |

5. Linkage to pY6-RΔC to MAL2.

MCY129: MCY101-3A::pY6-RΔC#5 x MCY120-2

MAL2::URA3 mal1$^0$ x MAL23-C::URA3 mal1$^0$

Mal$^+$, Ura$^+$ mal$^-$, Ura$^+$

Ratio of (Mal$^+$, Ura$^+$) : (mal$^-$, Ura$^+$) in 4 spored asci.

| 4:0 | 3:1 | 2:2 | 1:3 | 0:4 | total asci examined |
|---|---|---|---|---|---|
| 0 | 0 | 10 | 0 | 0 | 10 |

6. Linkage of pY6ΔCΔH#9 to *MAL2*.

MCY139: MCY101-3A::pY6ΔCΔH#9 x 347-5A

*MAL2::URA3* *mal1*$^0$ x *mal1*$^0$

Mal$^+$, Ura$^+$ mal$^-$, ura$^-$

Ratio of (Mal$^+$, Ura$^+$) : (mal$^-$, ura$^-$) in 4 spored asci.

| 4:0 | 3:1 | 2:2 | 1:3 | 0:4 | total asci examined |
|---|---|---|---|---|---|
| 0 | 0 | 7 | 0 | 0 | 7 |

MCY133: MCY101-3A::pY6ΔCΔH#9 x MCY120-2

*MAL2::URA3* *mal1*$^0$ x *MAL23-C::URA3* *mal1*$^0$

Mal$^+$, Ura$^+$ mal$^-$, Ura$^+$

Ratio of (Mal$^+$, Ura$^+$) : (mal$^-$, Ura$^+$) in 4 spored asci.

| 4:0 | 3:1 | 2:2 | 1:3 | 0:4 | total asci examined |
|---|---|---|---|---|---|
| 0 | 0 | 8 | 0 | 0 | 8 |

7. Linkage of pM3.2B to *MAL3*.

MCY143: MCY102-5A::pM3.2B#13 x 347-2A

*MAL3::URA3* *mal1*$^0$ x *mal1*$^0$

Mal$^+$, Ura$^+$ mal$^-$, ura$^-$

Ratio of (Mal$^+$, Ura$^+$) : (mal$^-$, ura$^-$) in 4 spored asci.

| 4:0 | 3:1 | 2:2 | 1:3 | 0:4 | total asci examined |
|---|---|---|---|---|---|
| 0 | 0 | 8 | 0 | 0 | 8 |

8. Linkage of pMJC6ΔC to MAL6.

MCY138: 332-5A::pMJC6ΔC#7 x RDY111-5A

MAL6::URA3 mal1$^0$ x MAL61-C2/MAL62-C2::LEU2 mal1$^0$

Mal$^+$, Ura$^+$, leu$^-$ mal$^-$, ura$^-$, Leu$^+$

Ratio of (Mal$^+$, Ura$^+$, leu$^-$) : (mal$^-$, ura$^-$, Leu$^+$) in 4 spored asci.

| 4:0 | 3:1 | 2:2 | 1:3 | 0:4 | total asci examined |
|---|---|---|---|---|---|
| 0 | 0 | 10 | 0 | 0 | 10 |

9. Linkage of pY6ΔCΔH to MAL6.

MCY136: 332-5A::pY6ΔCΔH#7 x RDY111-5A

MAL6::URA3 mal1$^0$ x MAL61-C2/MAL62-C2::LEU2 mal1$^0$

Mal$^+$, Ura$^+$, leu$^-$ mal$^-$, ura$^-$, Leu$^+$

Ratio of (Mal$^+$, Ura$^+$, leu$^-$) : (mal$^-$, ura$^-$, Leu$^+$) in 4 spored asci.

| 4:0 | 3:1 | 2:2 | 1:3 | 0:3 | total asci examined |
|---|---|---|---|---|---|
| 0 | 0 | 9 | 0 | 0 | 9 |

10. Linkage of pMJC6ΔC to MAL6-C2.

MCY118: 8-2B::pMJC6ΔC#6 x RDY111-5A

MAL6-C2::URA3 mal1$^0$ x MAL61-C2/MAL62-C2::LEU2 mal1$^0$

Mal$^+$, Ura$^+$, leu$^-$ mal$^-$, ura$^-$, Leu$^+$

Ratio of ($Mal^+$, $Ura^+$, $leu^-$) : ($mal^-$, $ura^-$, $Leu^+$) in 4 spored asci.

| 4:0 | 3:1 | 2:2 | 1:3 | 0:4 | total asci examined |
|---|---|---|---|---|---|
| 0 | 0 | 12 | 0 | 0 | 12 |

11. Linkage of pY6-RΔC to MAL6-C2.

MCY116: 8-2B::pY6-RΔC#47 x RDY111-5A

MAL6-C2::URA3 $mal1^0$ x MAL61-C2/MAL62-C2::LEU2 $mal1^0$

$Mal^+$, $Ura^+$, $leu^-$ $mal^-$, $ura^-$, $Leu^+$

Ratio of ($Mal^+$, $Ura^+$, $leu^-$) : ($mal^-$, $ura^-$, $Leu^+$) in 4 spored asci.

| 4:0 | 3:1 | 2:2 | 1:3 | 0:4 | total asci examined |
|---|---|---|---|---|---|
| 0 | 0 | 11 | 0 | 0 | 11 |

12. Linkage of pM1.4B to $mal1^0$.

MCY126: 328-4A::pM1.4B#1 x 203-7D

$mal1^0$::URA3 x MAL1

$mal^-$, $Ura^+$ $Mal^+$, $ura^-$

Ratio of ($mal^-$, $Ura^+$) : ($Mal^+$, $ura^-$) in 4 spored asci.

| 4:0 | 3:1 | 2:2 | 1:3 | 0:4 | total asci examined |
|---|---|---|---|---|---|
| 0 | 0 | 7 | 0 | 0 | 7 |

13. Linkage of pY6ΔCΔH to mal1$^0$.

MCY127: 328-4A::pY6ΔCΔH#8 x 203-7D

mal1$^0$::URA3 x MAL1

mal$^-$, Ura$^+$ Mal$^+$, ura$^-$

Ratio of (mal$^-$, Ura$^+$) : (Mal$^+$, ura$^-$) in 4 spored asci.

| 4:0 | 3:1 | 2:2 | 1:3 | 0:4 | total asci examined |
|---|---|---|---|---|---|
| 0 | 0 | 15 | 0 | 0 | 15 |

14. Linkage of pM1.4B to MAL1p.

MCY112: 345-4A::pM1.4B#1 x 203-7D

MAL1p::URA3 x MAL1

mal$^-$, Ura$^+$ Mal$^+$, ura$^-$

Ratio of (mal$^-$, Ura$^+$) : (Mal$^+$, ura$^-$) in 4 spored asci.

| 4:0 | 3:1 | 2:2 | 1:3 | 0:4 | total asci examined |
|---|---|---|---|---|---|
| 0 | 0 | 15 | 0 | 0 | 15 |

15. Linkage of pY6ΔCΔH to MAL1g.

340-2B::pY6ΔCΔH #4 x 203-7D

MAL1g::URA3 x MAL1

mal$^-$, Ura$^+$ Mal$^+$, ura$^-$

Ratio of (mal$^-$, Ura$^+$) : (Mal$^+$, ura$^-$) in 4 spored asci.

| 4:0 | 3:1 | 2:2 | 1:3 | 0:4 | total asci examined |
|---|---|---|---|---|---|
| 0 | 0 | 8 | 0 | 0 | 8 |

# Appendix 5

## Genetic Demonstration of Disruption Sites

1. Disruption of MAL43-C in strain MCY111-2.

MCY113: MCY111-2 x MCY100-3A

MAL43-C::URA3 mall$^0$ x MAL4 mall$^0$

mal$^-$, Ura$^+$ Mal$^+$, ura$^-$

Ratio of (mal$^-$, Ura$^+$) : (Mal$^+$, ura$^-$) in 4 spored asci.

| 4:0 | 3:1 | 2:2 | 1:3 | 0:4 | total asci examined |
|---|---|---|---|---|---|
| 0 | 0 | 18 | 0 | 0 | 18 |

2. Disruption of MAL23 in strain MCY101-3A.

MCY141: MCY101-3A::pDM3#1 x MCY120-2

MAL23::URA3 mall$^0$ x MAL2-C mall$^0$

mal$^-$, Ura$^+$ Mal$^+$, ura$^-$

Ratio of (mal$^-$, Ura$^+$) : (Mal$^+$, ura$^-$) in 4 spored asci.

| 4:0 | 3:1 | 2:2 | 1:3 | 0:4 | total asci examined |
|---|---|---|---|---|---|
| 0 | 0 | 6 | 0 | 0 | 6 |

3. Disruption of MAL23-C in strain MCY120-1.

MCY140: MCY120-1::pDM3#1 x MCY101-3A::pY6-RΔC#5

MAL23-C::URA3 mall$^0$ x MAL2::URA3 mall$^0$

mal$^-$, Ura$^+$ Mal$^+$, Ura$^+$

Ratio of ($mal^-$, $Ura^+$) : ($Mal^+$, $Ura^+$) in 4 spored asci.

| 4:0 | 3:1 | 2:2 | 1:3 | 0:4 | total asci examined |
|---|---|---|---|---|---|
| 0 | 0 | 7 | 0 | 0 | 7 |

4. Disruption of MAL41/MAL43 in strain MCY133-1C.

MCY146: MCY133-1C::pRD#8 x MCY111-2

MAL43-C MAL41/MAL42::LEU2 $mal1^0$

$mal^-$, $Leu^+$, $ura^-$

x

$mal1^0$ MAL43::URA3 MAL41 MAL42

$mal^-$, $leu^-$, $Ura^+$

Ratio of ($mal^-$, $Leu^+$, $ura^-$) : ($mal^-$, $leu^-$, $Ura^+$) in 4 spored asci.

| 4:0 | 3:1 | 2:2 | 1:3 | 0:4 | total asci examined |
|---|---|---|---|---|---|
| 0 | 0 | 6 | 0 | 0 | 6 |

# Appendix 6

## Genetic Demonstration of the Random Segregation of the Maltase Gene in Strain 53-2C

MCY148: 53-2C x 100-1B

MAL13 mal11 mal12 x MAL13 MAL11 MAL12::LEU2

$mal^-$ $mal^-$

Ratio of ($Mal^+$) : ($mal^-$) in 4 spored asci.

| 4:0 | 3:1 | 2:2 | 1:3 | 0:4 | total asci examined |
|---|---|---|---|---|---|
| 0 | 0 | 3 | 2 | 3 | 8 |

Ratio of ($Mal^+$) : ($mal^-$) in random spores.

| 41 : 49 | total spores examined |
|---|---|
| | 100 |

## Bibliography

Abraham, J., K.A. Nasmyth, J.N. Strathern, A.J.S. Klar and J.B Hicks. 1984. Regulation of mating-type information in yeast. Negative control requiring sequences both 5'and 3' to the regulated region. J. Mol. Biol. 176: 307-331.

Ashley, T. 1979. Specific end-to-end attachment of chromosomes in Ornithogalum virens. J. Cell Sci. 38:357-367.

Barnett, J.A. 1976. The utilization of sugars by yeasts. Adv. Carbohydr. Chem. Biochem. 32:125-234.

Barnett, J.A. 1981. The utilization of disaccharides and some other sugars by yeasts. Adv. Carbohydr. Chem. Biochem. 39:347-404.

Benton, W.D. and R.W. Davis. 1977. Screening λgt recombinant clones by hybridization to single plaques in situ. Science 196:180-182.

Blackburn, E.H. and J.W. Szostak. 1984. The molecular structure of centromeres and telomeres. Ann. Rev. Biochem. 53:163-194.

Blattner, F.R., B.G. Williams, A.E. Blechl, K. Denniston-Thompson, H.E. Faber, L. Furlong, D.J. Grunwald, D.O. Kiefer, D.D. Moore, J.M. Schumm, E.L. Sheldon and O. Smithies. 1977. Charon phages: Safer derivatives of bacteriophage lambda for DNA cloning. Science 196:161-169.

Boeke, J.D., D.J. Garfinkel, C.A. Styles and G.R. Fink. 1985. Ty elements transpose through an RNA intermediate. Cell 40:491-500.

Boeke, J.D., F. LaCroute and G.R. Fink. 1984. A positive selection for mutants lacking orotidine-5'-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. Mol. Gen. Genet. 197:345-346.

Buckle, V., C. Mondello, S. Darling, I.W. Craig and P.N. Goodfellow. 1985. Homologous expressed genes in the human sex chromosome pairing region. Nature 317:739-741.

Button, L.L. and C.R. Astell. 1986. The Saccharomyces cerevisiae chromosome III left telomere has a type X, but not a type Y', ARS region. Mol. Cell. Biol. 6:1352-1356.

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.

Carlson, M. and D. Botstein. 1983. Organization of the SUC gene family in Saccharomyces. Mol. Cell. Biol. 3:351-359.

Carlson, M., J.L. Celenza and F.J. Eng. 1985. Evolution of the dispersed SUC gene family of Saccharomyces by rearrangements of chromosome telomeres. Mol. Cell. Biol. 5:2894-2902.

Carson, M.J. and L. Hartwell. 1985. CDC17: An essential gene that prevents telomere elongation in yeast. Cell 42:249-257.

Celenza, J.L. and M. Carlson. 1985. Rearrangement of the genetic map of Chromosome VII of Saccharomyces cerevisiae. Genetics 109:661-664.

Chan, C.S.M. and B.-K. Tye. 1983. Organization of DNA sequences and replication origins at yeast telomeres. Cell 33: 563-573.

Chan, C.S.M. and B.-K. Tye. 1983. A family of Saccharomyces cerevisiae repetitive autonomously replicating sequences that have very similar genomic environments. J. Mol. Biol. 168:505-523.

Chang, Y.S., R.A. Dubin, C.A. Michels and R.B. Needleman. 1986. MAL63 encodes a positive regulator of maltose fermentation in Saccharomyces carlsbergensis. (Manuscript submitted for publication).

Charron, M.J. 1983. Molecular cloning and characterization of a MAL3g locus present in Saccharomyces cerevisiae. M.A. Thesis, Queens College.

Charron, M.J., R.A. Dubin and C.A. Michels. 1986. Structural and functional analysis of the MAL1 locus of Saccharomyces cerevisiae. Mol. Cell. Biol. 6:3891-3899.

Charron, M.J. and C.A. Michels. 1987. The constitutive, glucose repression-insensitive mutation of the yeast MAL4 locus is an alteration of the MAL43 gene. Genetics 116:23-31.

Chow L. and T. Broker. 1981. Mapping RNA:DNA heteroduplexes by electron microscopy. In: Electron Microscopy in Biology, vol. 1. (ed. J. D. Griffith) p. 140. John Wiley and Sons, Inc., New York.

Chow, T., M.J. Goldenthal, J.D. Cohen, M. Hegde and J. Marmur. 1983. Identification and physical characterization of yeast maltase structural genes. Mol. Gen. Genet. 191:366-371.

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

Clarke, L. and J. Carbon. 1980. Isolation of the centromere-linked CDC10 gene by complimentation in yeast. Proc. Natl. Acad. Sci. USA 77:2173-2177.

Cohen, J., M.J. Goldenthal, B. Buchferer, and J. Marmur. 1984. Mutational analysis of the MAL1 locus of Saccharomyces: Identification and functional characterization of three genes. Mol. Gen. Genet. 196:208-216.

Cohen, J., M.J. Goldenthal, T. Chow, B. Buchferer, and J. Marmur. 1985. Organization of the MAL loci of Saccharomyces: Physical identification and functional characterization of three genes at the MAL6 locus. Mol. Gen. Genet. 200:1-8.

Cooke, H.J., W.R.A. Brown and G.A. Rappold. 1985. Hypervariable telomeric sequences from the human sex chromosomes are pseudoautosomal. Nature 317:687-692.

Craig, I.W. and E. Tolley. 1986. Steroid sulphatase and the conservation of mammalian X charomosomes. Trends in Gen.:210-204.

Dawes, I.W. and I.D. Hardie. 1974. Selective killing of vegetative cells in sporulated yeast cultures by exposure to diethyl ether. Mol. Gen. Genet. 131: 281-289.

de Kroon, R.A. and V.V. Kroningsberger. 1970. An inducible transport system for alpha-glucosides in protoplasts of Saccharomyces carlsbergensis. Biochim. Biophys. Acta 204:590-609.

DeLange, T., A.Y.C. Liu, L.H.T. van der Ploeg, P. Borst, M.C. Tromp, and J.H. van Boom. 1983. Tandem repetitiion of the 5' mini-exon of variant surface glycoprotein genes: A multiple promoter for VSG gene transcription? Cell 34:891-900.

Denis, C.L. and C. Gallo. 1986. Constitutive RNA synthesis for the yeast activator ADR1 and identification of the ADR1-5$^{C}$ mutation: Implications in post-translational control of ADR1. Mol. Cell. Biol. 6:4026-4030.

Douglas, H.C. and D.C. Hawthorne. 1966. Regulation of genes controlling synthesis of the galactose fermentative enzymes in yeast. Genetics 54:911-916.

Dubin, R.A. 1987. Molecular organization of the MAL6 locus of Saccharomyces carlsbergensis. Ph. D. Thesis. City University of New York.

Dubin, R.A., M.J. Charron, S.R. Haut, R.B. Needleman and C.A. Michels. Constitutive expression of the maltose fermentative enzymes in Saccharomyces carlsbergensis is dependent upon the mutational activation of MAL64, a nonessential homologue of MAL63. (Manuscript submitted for publication).

Dubin, R.A., R.B. Needleman, D. Gossett and C.A. Michels. 1985. Identification of the structural gene encoding maltase within the MAL6 locus of Saccharomyces carlsbergensis. J. Bacteriol. 164:605-610.

Dubin, R.A., E.L. Perkins, R.B. Needleman and C.A. Michels. 1986. Identification of a second trans-acting gene controlling maltose fermentation in Saccharomyces carlsbergensis. Mol. Cell. Biol. 6:2757-2765.

Duesberg, P.H. 1983. Retroviral transforming genes in normal cells? Nature 304:219-226.

Englund, P.T., S.L. Hajduk and J.C. Marini. 1982. The molecular biology of Trypanosomse. Ann. Rev. Biochem. 51: 695-726.

Enquist, L. and N. Sternberg. 1979. In Vitro packaging on λDam vectors and their use in cloning DNA fragments. Meth. Enzymol. 68:281-298.

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

Federoff, H.J., J.D. Cohen, T.R. Eccleshall, R.B. Needleman, B.A. Buchferer, J. Giacalone and J. Marmur. 1982. Isolation of a maltase structural gene from Saccharomyces carlsbergensis. J. Bacteriol. 149:1064-1070.

Frohlich, K.-U., K. -D. Entian and D. Mecke. 1984. Cloning and restriction analysis of the hexokinase PII gene of the yeast Saccharomyces cerevisiae. Mol. Gen. Genet. 194:144-148.

Garfinkel, D.J., J.D. Boeke and G.R. Fink. 1985. Ty element transposition: Reverse transcriptase and virus-like particles. Cell 42: 507-517.

Goldenthal, M.J., J.D. Cohen and J. Marmur. 1983. Isolation and characterization of a maltose transport mutant in the yeast Saccharomyces cerevisiae. Curr. Genet. 7:195-199.

Gorts, C.P.M. 1969. Effect of glucose on the activity and the kinetics of the maltose-uptake system and of alpha-glucosidase in Saccharomyces cerevisiae. Biochim. Biophys. Acta 184:299-305.

Green, M.M. 1980. Transposable elements in Drosophila and other Diptera. Ann. Rev. Genet. 14:109-120.

Grunstein, M. and D.G. Hogness. 1975. Colony hybridization: A method for the isolation of cloned DNA's that contain a specific gene. Proc. Natl. Acad. Sci. 72:3961-3965.

Haber, J.E. and P.C. Thorburn. 1984. Healing of broken linear dicentric chromosomes in yeast. Genetics 106:207-226.

Hall, B.G., S. Yokoyama and D.H. Calhoun. 1983. Role of cryptic genes in microbial evolution. Mol. Biol. and Evol. 1:109-124.

Halvorson, H. and L. Ellias. 1958. The purification and properties of an alpha-glucosidase of Saccharomyces italicus Y1225. Biochim. Biophys. Acta 30:28-40.

Hicks, J., J.N. Strathern and A.J.S. Klar. 1979. Transposable mating type genes in Saccharomyces cerevisiae. Nature 282:478-483.

Hinnen, A., J.B. Hicks and G.R. Fink. 1978. Transformation in yeast. Proc. Natl. Acad. Sci. 75:1929-1933.

Horowitz, H., P. Thorburn and J.E. Haber. 1984. Rearrangements of highly polymorphic regions near telomeres of Saccharomyces cerevisiae. Mol. Cell. Biol. 4:2509-2517.

Ito, H., Y. Fukuda, K. Murata and K. Kimura. 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. 153:163-168.

Ivy, J.M., J.B. Hicks and A.J.S. Klar. 1985. Map positions of yeast genes SIR1, SIR3 and SIR4. Genetics 111:735-744.

Ivy, J.M., A.J.S. Klar and J.B. Hicks. 1986. Cloning and characterization of four SIR genes of Saccharomyces cerevisiae. Mol. Cell. Biol. 6:688-702.

Jones, K.W. and L. Singh. 1982. Conserved sex-associated repeated DNA sequences in vertebrates. In: Genome Evolution. (eds. G.A. Dover and R.B. Flavell) p. 135. Academic Press, New York.

Kahn, N.A. and N.R. Eaton. 1967. Purification and characterization of maltase and alpha-methyl glucosidase from yeast. Biochim. Biophys. Acta. 146:173-180.

Kahn, N.A. and N.R. Eaton. 1971. Genetic control of maltase formation in yeast. I. Strains producing high and low basal levels of enzyme. Mol. Gen. Genet. 112:317-322.

Klar, A.J.S., J.N. Strathern, J.R. Broach and J.B. Hicks. 1981. Regulation of transcription in expressed and unexpressed mating type cassettes of yeast. Nature 289: 239-244.

Kleckner, N. 1981. Transposable genetic elements. Ann. Rev. Genet. 15:341-404.

Kostricken, R., J.N. Strathern, A.J.S. Klar, J.B. Hicks and F. Hefferon. 1983. A site-specific endonuclease essential for mating-type switching in Saccharomyces cerevisiae. Cell 35:167-174.

Li, W.-H. 1984. Retention of cryptic genes in microbial populations. Mol. Biol. Evol. 1:213-219.

Lloyd,J.B. and W.J. Wheland. 1969. An improved method for enzymic determination of glucose in the presence of maltose. Anal. Biochem. 30:467-470.

Lustig, A.J. and T.D. Petes. 1986. Identification of yeast mutants with altered telomere structure. Proc. Natl. Acad. Sci. USA 83:1398-1402.

Mainx, F. 1964. The genetics of Megaselia scalaris Loew (Phoridae): A new type of sex determination. Amer. Nat. 98:415-430.

Maniatis, T., E.F. Fritch and J. Sambrook. 1982. Molecular Cloning: A laboratory handbook. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

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.

Matsumoto, K., Y. Adachi, A. Toh-E and Y. Oshima. 1980. Function of positive regulatory gene gal4 in the synthesis of galactose pathway enzymes in Saccharomyces cerevisiae: Evidence that the GAL81 region codes for part of the gal4 protein. J. Bacteriol. 141:508-527.

Michels, C.A. and R.B. Needleman. 1983. A genetic and physical analysis of the MAL1 and MAL3 standard strains of Saccharomyces cerevisiae. Mol. Gen. Genet. 191:225-230.

Michels, C.A. and R.B. Needleman. 1984. The dispersed, repeated family of MAL loci in Saccharomyces spp. J. Bacteriol. 157:949-952.

Mortimer, R.K. and D.C. Hawthorne. 1966. Genetic mapping in Saccharomyces. Genetics 53:165-173.

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

Nasmyth, K.A. 1983. Molecular analysis of a cell lineage. Nature 209:67-676.

Nasmyth, K.A. and K. Tatchell. 1980. The structure of the transposable yeast mating type loci. Cell 19:753-764.

Naumov, G.I. 1971. Comparative genetics in yeast. V. Complementation in the MAL1 locus in Saccharomyces which do not utilize maltose. Genetika 7:141-148.

Naumov, G.I. 1972. Comparative genetics of yeast. VII. Identification of mutations which block the utilization of maltose in natural Saccharomyces mutants. Vestn. Mosk. Gos. Univ. Biol., Pochvovodenie 3:34-38.

Naumov, G.I. 1976. Comparative genetics of yeast. XVI. Genes for maltose fermentation in Saccharomyces carlsbergensis N.C.Y.C. 74. Genetika 12:87-100.

Needleman, R.B. and N.R. Eaton. 1974. Selection of yeast mutants constitutive for maltase synthesis. Mol. Gen. Genet. 133:135-140.

Needleman, R.B. and C. Michels. 1983. Repeated family of genes controlling maltose fermentation in Saccharomyces carlsbergensis. Mol. Cell. Biol. 3:796-802.

Needleman, R.B., D.B. Kaback, R.A. Dubin, E.L. Perkins, N.G. Rosenberg, K.A. Sutherland, D.B. Forrest and C.A. Michels. 1984. MAL6 of Saccharomyces: A complex locus containing three genes required for maltose fermentation. Proc. Natl. Acad. Sci. USA 81:2811-2815.

Ng, R. and J. Abelson. 1977. Isolation and sewuence of the gene for actin in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA 77:3912-3916.

Orr-Weaver, T.L., J.W. Szostak and R.J. Rothstein. 1983. Genetic applications of yeast transformation with linear and gapped plasmids. Methods Enzymol. 101:228-245.

Oshima, Y. 1967. The inter-cistronic complementation of the polymeric genes for maltose fermentation in Saccharomyces. J. Fermen. Technol. (Japan) 45:550-565.

Ouwehand, J. and R. van Wijk. 1972. Regulation of maltase and alpha-methylglucosidase synthesis in genetically defined strains of Saccharomyces carlsbergensis. Mol. Gen. Genet. 117:30-38.

Pretorius, I.S., T. Chow, D. Modena and J. Marmur. 1986. Molecular cloning and characterization of the STA2 glucoamylase gene of S. cerevisiae. Mol. Gen. Genet. 203:29-35.

Rigby, P.W.J., M. Dieckmann, C. Rhodes and P. Berg. 1977. Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. J. Mol. Biol. 113:237-251.

Rodicio, R. and F.K. Zimmerman. 1985. Cloning of maltase regulatory genes in Saccharomyces cerevisiae. II. Isolation of the MAL4 regulatory gene. Curr. Genet. 547-551.

Roeder, G.S. 1983. Unequal crossing-over between yeast transposable elements. Mol. Gen. Genet. 190:117-121.

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

Serrano, R. 1977. Energy requirements for maltose transport in yeast. Eur. J. Biochem. 80:97-102.

Simmler, M., F. Rouyer, G. Vergnaud, M. Nystrom-Lahti, K.Y. Ngo, A. de la Chapelle and J. Weissenbauch. 1985. Pseudoautosomal DNA sequences in the pairing region of the human sex chromosomes. Nature 317: 692-697.

Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503-517.

Strathern, J.N., A.J.S. Klar, J.B. Hicks, J.A. Abraham, J.M. Ivy, K.A. Nasmyth and C. McGill. 1982. Homothallic switching of yeast mating type cassettes is initiated by a double-stranded cut in the MAL locus. Cell 31:183-192.

Strathern, J.N., E. Spatola, C. McGill and J.B. Hicks. 1980. Structure and organization of transposable mating type cassettes in Saccharomyces yeasts. Proc. Natl. Acad. Sci. USA 77:2839-2843.

Szostak, J.W. and E.H. Blackburn. 1982. Cloning yeast telomeres on linear plasmid vectors. Cell 29:245-255.

ten Berge, A.M.A. 1972. Genes for the fermentation of maltose and alpha-methylglucoside in Saccharomyces carlsbergensis. Mol. Gen. Genet. 115:80-88.

ten Berge, A.M.A., G. Zoutewelle and R.B. Needleman. 1974. Regulation of maltose fermentation in Saccharomyces carlsbergensis. III. Constitutive mutations at the MAL6-locus and suppressors changing a constitutive phenotype into a maltose negative phenotype. Mol. Gen. Genet. 131:113-121.

ten Berge, G. Zoutewelle and K.W. van de Poll. 1973. Regulation of maltose fermentation in Saccharomyces carlsbergensis. I. The function of the gene MAL6, as recognized by mal6-mutants. Mol. Gen. Genet. 123:233-246.

ten Berge, A.M.A., G. Zoutewelle, K.W. van de Poll and H.P.J. Bloemers. 1973. Regulation of maltose fermentation in Saccharomyces carlsbergensis. II. Properties of a constitutive MAL6-mutant. Mol. Gen. Genet. 125:139-146.

van der Ploeg, L.H.T., A.Y.C. Liu and P. Borst. 1984. Structure of the growing telomeres of Trypanosomes. Cell 36:459-468.

van Wijk, R., J. Ouwehand, T. van den Bos, and V.V. Koningsberger. 1969. Induction and catabolite repression of alpha-glucosidase synthesis in protoplasts of Saccharomyces carlsbergensis. Biochim. Biophys. Acta 186:178-191.

Wagenaar, E.B. 1969. End-to-end chromosome attachments in mitotic interphase and their possible significance to meiotic chromosome pairing. Chromosoma 26:410-426.

Walmsley, R.W., C.S. Chan, B. -K. Tye and T.D. Petes. 1984. Unusual DNA sequences associated with the ends of yeast chromosomes. Nature 310:157-160.

Walmsley, R.W. and T.D. Petes. 1985. Genetic control of chromosome lenght in yeast. Proc. Natl. Acad. Sci. 82:506-510.

Walmsley, R.W., J.W. Szostak and T.D. Petes. 1983. Is there left-handed DNA at the ends of yeast chromosomes? Nature 302:84-86.

Winge, O. and C. Roberts. 1950. The polymeric genes for maltose fermentation in yeasts, and their mutability. C. R. Trav. Lab. Carlsberg 25:35-83.

Yamashita, I., T. Maemura, T. Hatano and S. Fukui. 1985. Polymorphic extracellular glucoamylase genes and their evolutionary origin in the yeast Saccharomyces diastaticus. J. Bacteriol. 161:574-582.

Zakian, V.A., H.M. Blanton and L. Wetzel. 1986. Distribution of telomere associated sequences in yeast. In: Extrachromosomal Elements in Lower Eukaryotes. (eds. R. Wickner, A. Hinnenbusch, L. Mets, A. Lambowitz, I.C. Gunsalus and A. Hollaender) Plenum Press, New York.

Zimmerman, F.K. and N.R. Eaton. 1974. Genetics of induction and catabolite repression of maltase synthesis in Saccharomyces cerevisiae. Mol. Gen. Genet. 134:261-272.

Zimmerman, F.K., N.A. Kahn, and N.R. Eaton. 1973. Identification of new genes involved in disaccharide fermentation in yeast. Mol. Gen. Genet. 123:29-41.