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MOLECULAR ORGANIZATION OF THE MAL6 LOCUS OF SACCHAROMYCES CARLSBERGENSIS

City University of New York PH.D. 1987

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MOLECULAR ORGANIZATION OF THE *MAL6* LOCUS OF *Saccharomyces carlsbergensis*

by

Robert Allen Dubin

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the 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.

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Abstract

Molecular Organization of the MAL6 locus of Saccharomyces carlsbergensis

by

Robert Allen Dubin

Adviser: Professor Corinne A. Michels

The MAL loci of Saccharomyces are a polymeric series of five unlinked, dominant loci, any one of which confers upon yeast the ability to ferment maltose. Maltose fermenting strains synthesize two maltose fermentative enzymes, maltase and maltose permease. In most maltose fermenting strains, synthesis of maltase and maltose permease is induced by maltose, however in certain mutants, expression of these enzymes is unregulated and constitutive.

Previous genetic evidence demonstrated that the cloned MAL6 locus was a complex locus composed of three genes: MAL61, MAL62, and MAL63. The MAL61 and MAL62 genes are responsible for the synthesis of maltose inducible transcripts while the MAL63 gene transcripts are constitutively expressed. Using in vitro mutagenesis and gene transplacement, we have selectively mutated these genes at the genomic MAL6 locus in an inducible strain and have demonstrated that all three genes are required for maltose fermentation. Further characterization of these mutants has established the functions of these three gene products. The MAL61 gene may encode the maltose permease. The MAL62 gene encodes maltase. The MAL63 gene encodes a trans-acting, positive regulatory function that is required for maltose regulated induction of maltase and maltose permease and their respective transcripts.

The mutation responsible for constitutive expression of maltase and

maltose permease in MAL6-C constitutive mutants is linked to the MAL6 locus. We have demonstrated that the positive regulatory gene MAL63 is dispensible for maltose fermentation in constitutive strains and plays no role in constitutive expression of the maltose fermentative enzymes or their respective transcripts. Instead, constitutivity is dependent upon a fourth MAL6-linked gene, MAL64. MAL64 is located 3.5 kilobase pairs to the left of MAL63 and shows at least partial homology to the MAL63 gene. The MAL64-C allele present in MAL6-linked constitutive strains encodes a trans-acting, positive regulatory function that is required for the constitutive expression of the maltose fermentative enzymes and their respective MAL61 and MAL62 transcripts. In contrast, the wild type MAL64 gene present in inducible MAL6 strains is dispensible and plays no apparent role in maltose regulated, inducible fermentation.

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I also wish to extend my gratitude to Dr. Richard Needleman for generously providing me with an uncountable number of yeast strains, for maltose permease data, discussions, and assorted plasmid constructs and other results. May your excitement for maltose fermentation never end.

I also wish to thank the many students who have passed through our lab and made this work a joyful experience for me. Most notably I should like to thank Maureen Charron for her unlimited support: for innumerable discussions, for help in unraveling the mystery of MAL64, for providing clones, and for friendship; thanks also to Yolande Sylvestre, Karen Hannenburger, Leanne Tanouye, Esther Shiffman, Joel Levine, Jeong Kim, Sheryl Haut, Qi Cheng, Marion Geras, Juan Guzman, and Sharon Calamarengo - you made all the work seem like play. I also wish to thank the faculty, staff, and graduate students in the Department of Biology at Queens College for their help and support.

To my parents, Ruth and Eugene, to my sister Leslie, to Laurie Lane and Davis Platt, and most of all, to David, for your love and support, I am forever grateful.

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INTRODUCTION

The Saccharomyces yeasts provide an excellent model organism with which to study cellular processes in a eukaryote. The budding yeast Saccharomyces cerevisiae remains the most extensively studied. Its current utility rests upon over 40 years of classical yeast genetics research which has identified hundreds of genes, many of which have been mapped (Mortimer and Shields, 1980). In conjunction with biochemical studies, the functions of many gene products have been determined or surmised. Advances in molecular biology and development of recombinant DNA techniques and transformation procedures now permit the isolation and detailed structural and functional examination of nearly any yeast gene (reviewed by Botstein and Davis, 1982). Current work focuses on gene regulation, cell cycle control (including vertebrate oncogene homologs), secretion, protein targeting, chromosome replication and segregation, recombination, and differentiation (sporulation and mating type switching). Clearly, Saccharomyces cerevisiae is a model eukaryote for examining basic biological processes at the molecular level.

The fermentation of sugars by yeasts has been extensively studied (reviewed by Barnett, 1976; 1981). Since ancient times interest in fermentation has stemmed from the practical requirements of the brewing and baking industries. Genetic studies have identified genes required for the utilization or fermentation of glucose, galactose, alpha-methyl glucopyranoside, maltose, sucrose, raffinose, trehalose, and melezitose. Some of these gene products have been identified as specific hydrolases, kinases, permeases, and regulatory molecules.

Recently, analysis of cloned genes has led to a more detailed

understanding of the regulation of gene expression. Yeast genes have contributed to these studies, including genes involved in sugar fermentation. Cis-acting regulatory sequences (promoters and regulated enhancers) have been identified upstream of the transcription initiation sites of cloned structural genes involved in sucrose and galactose fermentation (Sarokin and Carlson, 1985, 1986; Guarente et al., 1982). Genes encoding trans-acting regulatory molecules that either directly or indirectly control the expression of structural genes have also been cloned and their mechanisms of action are intensively being studied. These include the GAL4 regulatory gene, required for induction by galactose of the galactose fermentative enzymes (Laughon et al., 1984), the SNF1 gene, required for derepression of invertase (Celenza and Carlson, 1984), and the GCN4 gene, required for derepression of multiple amino acid biosynthetic structural genes (Hinnenbush, 1985).

We have chosen to study maltose fermentation because expression of the maltose fermentative enzymes is under a variety of regulatory controls. This makes maltose fermentation an important system for examining regulation at a number of different levels. Maltose fermentation in Saccharomyces requires the presence of at least one of five unlinked, dominant MAL loci: MAL1, MAL2, MAL3, MAL4, and MAL6 (Winge and Roberts, 1950; Mortimer and Hawthorne, 1969; reviewed in Barnett, 1976). The MAL1-4 loci have been mapped and are telomeric (Mortimer and Shields, 1980; Celenza and Carlson, 1985). In addition to conferring the ability to ferment maltose, the presence of a MAL locus is also correlated with the regulated synthesis of the alpha-D-glucosidase maltase (EC 3.2.1.20) and maltose permease, an active transport system required for rapid maltose uptake. Only low

basal levels of the two enzymes are detectable when maltose fermenting yeast are grown in non-inducing (and derepressing) medium, such as YEP plus galactose. However, the presence of maltose in the growth medium induces high levels of expression of both maltase and maltose permease (de Kroon and Koningsberger, 1970; Halvorson et al., 1963; Harris and Thomas, 1961; Ouwehand and van Wijk, 1972; Robertson and Halvorson, 1957; ten Berge et al., 1973a; van Wijk et al., 1969). Maltase induction is sensitive to the antibiotic Actinomycin D, suggesting induction involves de novo transcription; induced cultures are refractory to the addition of this antibiotic, further suggesting that the maltase mRNA, once synthesized, is relatively stable (van Wijk et al., 1969). Kinetic analysis of accumulation of maltase and maltose permease following maltose induction revealed coordinate increases (de Kroon and Koningsberger, 1970). These results suggested control by a common regulatory mechanism (de Kroon and Kroningsberger, 1970) and this was later demonstrated genetically (ten Berge et al., 1973a).

In addition to induction by maltose, glucose also influences the levels of maltase and maltose permease. Glucose appears to control maltase and maltose permease levels by two independent mechanisms. In fully induced cells, glucose elicits a rapid loss of maltose permease activity (Robertson and Halvorson, 1957; Gorts, 1969), even in the presence of maltose. This effect, termed glucose inactivation, appears to alter the activity of maltose permease directly, perhaps through conformational changes in the protein itself (Gorts, 1969). Glucose also inactivates fructose 1,6 diphosphatase, phosphoenolpyruvate carboxykinase, cytoplasmic malate dehydrogenase (reviewed in Switzer, 1977), and isocitrate lyase (Chapman and Bartley, 1968). In contrast,

glucose severely reduces the rate of maltase synthesis (even in the presence of maltose) although previously synthesized maltase remains stable (Gorts, 1969; van Wijk et al. 1969; Ouwehand and van Wijk, 1972). In fully induced cultures, glucose exerts its effects on maltase synthesis (at least initially) at the level of translation, since maltase levels increase following glucose removal in the presence of maltose and Actinomycin D (van Wijk et al., 1969). Additionally, basal (derepressed) levels of maltase are reduced by growth on glucose (Ouwehand and van Wijk, 1972). The effect of glucose on the rate of maltase synthesis is termed glucose or catabolite repression and affects other enzymes as well, such as secreted invertase, galactokinase, alpha-galactosidase, and numerous mitochondrial functions (see Michels et al., 1983, for references). Glucose controls the expression of secreted invertase at the level of transcription (Carlson and Botstein, 1982).

Some maltose fermenting strains constitutively synthesize high levels of maltase (and maltose permease). These strains are generally laboratory generated mutants and will be discussed below. Oftentimes, constitutive expression of maltase leads to its glucose insensitive expression (Kahn and Eaton, 1971; Zimmerman and Eaton, 1974).

Functions of a MAL locus. Identification of the function(s) of a dominant MAL locus has been the subject of numerous investigations. Mutagenic analysis of a single MAL locus as well as comparative analyses between different MAL loci have demonstrated that they play a regulatory role. Zimmerman and Eaton (1974) mutagenized an inducible MAL2 strain and isolated nonfermenting mutants that were linked to MAL2. These mal2

nonfermenters were recessive to the wild type MAL2 locus and uninducible for maltase. (Maltose permease levels were not determined.) Maltose fermenting revertants of the mal2 strains often synthesized maltase constitutively. The gene responsible for reversion/constitutivity was linked to MAL2 and dominant to both MAL2 and mal2.

Kahn et al. (1973) provided similar evidence for the existence of a regulatory function at the MAL4 locus. Following mutagenesis of a maltose fermenting MAL4-C strain (which produced high levels of maltase constitutively), maltose nonfermenters were isolated. These strains synthesized only low basal levels of maltase and the mutation responsible mapped to MAL4. Immunological reactivity, as measured by 50% neutralization of maltase activity by anti-maltase antibodies, suggested no differences between the maltases produced by the Mal^+ and mal^- strains. These results implied a quantitative, rather than qualitative change in maltase. Maltose fermenting revertants were isolated and synthesized variable, yet constitutive levels of apparently normal maltase.

Other studies have attempted to define MAL functions by comparative studies of the various MAL loci. Using synchronized yeast cultures, Tauro and Halvorson (1966) demonstrated that maltase synthesis was restricted to but a brief period of time during the cell cycle. Most interestingly, strains carrying more than one polymeric MAL locus synthesized maltase more than once during a cell cycle. For example, diploid strains carrying one MAL locus (ie.: MAL1 mal1 mal3 mal3) exhibited one burst of maltase synthesis during a cell cycle, strains with two different loci (ie.: MAL1 mal1 MAL3 mal3) exhibited two synthetic bursts, etc. Although segregational studies demonstrating

that a particular MAL locus was responsible for a particular synthetic burst were not performed, it was suggested that a MAL locus determined the timing of maltase synthesis during a cell division cycle.

Mowshowitz (1981) also demonstrated that the MAL loci regulate maltase levels. Congenic strains carrying either the MAL1, MAL2, or MAL6 locus exhibited distinctive rates of maltase induction and reached distinctive levels of maltase in fully induced cultures. Genetic analysis confirmed that both parameters co-segregated with their respective MAL locus. Since most studies have suggested that the maltase synthesized by various MAL strains are identical, these observed differences were not believed to be due to the synthesis of different maltases by different maltase structural genes (Halvorson et al., 1963; Kahn and Eaton, 1967; Needleman et al., 1978; Mowshowitz, 1981). (However, see Eaton and Zimmerman, 1976, for a slightly different view). It was therefore concluded that a MAL locus encodes a function regulating maltase levels.

The evidence presented above (recessive nonfermenters that are uninducible for maltase; dominant maltase-constitutive strains; different rates and times of maltase synthesis) demonstrate the existence of a positive regulatory function located at or linked to a dominant MAL locus. These results are consistent with two possible models: 1. a MAL locus encodes a regulatory gene whose product is required for maltase induction; or 2. a MAL locus encodes cis-acting regulatory sites controlling a closely linked maltase structural gene. Testing of the latter hypothesis was made impossible by the lack of maltase structural gene mutants.

The thorough studies of ten Berge and co-workers provide persuasive

evidence for the presence of a regulatory gene at the MAL6 locus (ten Berge et al., 1973a,b; 1974). Following mutagenesis of an inducible MAL6 strain, maltose non-fermenters were isolated (ten Berge et al., 1973a). These mutations were linked to MAL6, recessive to the wild type, and fell into a single complementation group. In the parental strain, maltose induced high levels of expression of maltase, maltose permease, and alpha-methyl glucosidase (Alpha-methyl glucosidase is induced by maltose, as well as alpha-methyl glucoside, in certain genetic backgrounds, but is not required for maltose fermentation (Ouwehand and van Wijk, 1972).) Maltose failed to induce high levels of expression of the three enzymes in the nonfermenting mal6 mutants, although low (uninduced) levels of the three enzyme activities remained detectable. Biochemical analysis (K_m, pH optimum) revealed no differences between partially purified maltases isolated from the inducible and uninducible strains.

Reversion of the nonfermenting, uninducible mal6 mutants frequently led to the isolation of strains that were temperature sensitive (ts) for growth on maltose. Some ts revertants were not complemented by mal6 strains at the restrictive temperature and it was concluded that these ts strains were revertants at mal6. In these ts revertants the three enzymes were again synthesized at high levels. Most significantly, biochemical analysis of extracts from the MAL6-ts revertants failed to detect either temperature sensitive maltase or tempertaure sensitive maltose permease, strongly suggesting that the temperature sensitive phenotype was not due to a mutation within either structural gene.

Ten Berge et al. (1973b, 1974) further reported the isolation of maltose fermenting revertants of the mal6 mutants that constitutively

expressed high levels of maltase, maltose permease, and to some extent, alpha-methyl glucosidase. Biochemical analysis of one revertant, C2, failed to reveal any differences between the inducible and constitutive maltases. Constitutivity was linked to MAL6, recessive to wild type, and designated MAL6-C. Surprisingly, the MAL6-C / mal6 heterozygotes generally exhibited "a partial or complete loss of the constitutivity and a partial or complete restoration of the induciblity" and the degree to which this occured depended upon the particular mutant combination (ten Berge et al, 1974). From these results it was proposed that one gene, MAL6 (defined by its alleles mal6 and MAL6-C), rather than two tightly linked genes, was responsible for both inducible and constitutive synthesis of the maltose fermentative enzymes (ten Berge et al., 1974).

The work of ten Berge et al. (1973a,b; 1974), particularly their demonstration of: 1. coordinate control over three enzyme activities; 2. low levels of maltose permease and apparently wild type maltase in the recessive, uninducible mal6 nonferementers; 3. temperature sensitive maltose fermenters that synthesize maltase and maltose permease with apparently wild type thermolability; and 4. constitutive strains that are recessive to the wild type, all firmly support the presence of a positive regulatory gene at the MAL6 locus. This gene product, most likely a protein, controls the level of expression of maltase and maltose permease, and thus the ability to ferment maltose.

A second MAL-linked function. Complementation analysis of a large number of maltose nonfermenting strains and naturally occuring yeast isolates demonstrated that the ability to ferment maltose depends upon

the presence of two complementary functions, MALp and MALg. (Oshima, 1967; Naumov, 1969; 1970). Strains containing either MALp or MALg functions are unable to ferment, while the MALp / MALg heterozygous diploid ferments. Linkage analysis indicated that the MALp locus and one MALg locus were tightly linked to MAL1, suggesting that a dominant MAL1 locus consisted of both MAL1p and MAL1g functions together in cis. Isolation of one other MALg gene linked to MAL2 and complemented by MAL1p suggested that all dominant MAL loci consisted of tightly linked MALp and MALg functions and at least one function was trans-acting. Maltose nonfermenting strains containing neither MALp nor MALg functions were also isolated by Naumov and these were later termed mal^0 (Needleman and Michels, 1983).

The nonfermenting mal6 mutants isolated by ten Berge et al. (1973a) were complemented by MAL1p strains but not by MAL1g or mal^0 strains (Naumov, 1976). This demonstrated the presence at MAL6 of a MAL6p function and defined MALp as encoding (at least) the positive regulatory gene function defined by the mal6 (that is to say mal6p) mutants. In addition to the dominant MAL6 locus, segregational analysis of the parental MAL6 strain used by ten Berge and co-workers revealed the presence of two cryptic MALg loci, MAL1g and MAL2g. Similar studies using one mal6 mutant demonstrated the additional presence of a MALg locus linked to MAL6 in place of the dominant MAL6 locus (Naumov, 1976). This work provided further evidence supporting the binary nature of the dominant MAL loci. Failure to obtain mutants within MAL6g could be explained by the presence of multiple MALg loci present within the MAL6 strain used by ten Berge et al. (1973a).

Naumov (1976) speculated that MALg function might encode a

structural gene(s). The obvious candidates were the maltase or maltose permease genes. Nearly 25 years ago, Halvorson and co-workers suggested that a MAL locus contained the maltase structural gene. This conclusion was based upon an apparent MAL gene dosage effect (Rudert and Halverson, 1963). As supporting evidence, Halvorson et al. (1963) cited their inability to detect even low maltase levels in nonfermenting strains lacking a MAL locus.

Both these observations have been questioned. Subsequent investigations reported low detectable levels of maltase in segregational maltose nonfermenting strains lacking a dominant MAL locus (Kahn, et al., 1973; ten Berge et al., 1973a). Also, reinvestigation of the gene dosage effect using congenic MAL2 diploids (MAL2 mal2 and MAL2 MAL2) and triploids (MAL2 mal2 mal2, MAL2 MAL2 mal2, and MAL2 MAL2 MAL2) failed to confirm a strict arithmetical increase of maltase levels with the addition of MAL loci (Mowshowitz, 1979). It was concluded instead that the non-arithmetical increase observed could result from the addition of structural or regulatory genes. Recently it was demonstrated that standard maltose fermenting laboratory strains contain one or two cryptic MALg genes in their background (Chow et al, 1983; Needleman and Michels, 1983; Michels and Needleman, 1983; 1984). Since the effect of these loci on maltase expression is difficult to predict, it is possible that conflicting results from gene dosage experiments may be due to the use of incompletely characterized strains.

The search for a maltase structural gene has been approached by attempts at isolating strains synthesizing a mutant maltase. Kahn and Eaton (1971) described a MAL4-C strain that constitutively produced a maltase with increased thermolability, specific activity, and Km as

compared to the maltase isolated from an inducible MAL4 strain. While constitutivity was linked to MAL4, it was not determined whether the altered maltase was linked as well. Eaton and Zimmerman (1976) reported heat inactivation studies indicating the presence of three species of maltase in a MAL3 strain. Temperature sensitive maltose fermenting mutants generally revealed a loss of one maltase, however the gene responsible for the ts phenotype and the missing maltase did not map to the dominant MAL locus.

Other genes, unlinked to known MAL loci, have also been implicated in maltose fermentation. Zimmerman, Kahn, and Eaton (1973) isolated 7 genes (designated DSF) that were required for maltose fermentation and unlinked to the dominant MAL4-C locus present in the parental strain. All the mutants, like the parent, continued to constitutively synthesize maltase. Four mutants exhibited reduced maltose uptake, suggesting mutations in genes involved in a multicomponent maltose permease. Kahn (1975) described three genes that were required for maltose fermentation and unlinked to the MAL3 locus present in the parental strain. One mutant synthesized reduced levels of apparently normal maltase, continued to transport maltose, and was presumed to encode some regulatory function.

Isolation of the MAL6 locus. Detailed molecular analysis of a dominant MAL locus became possible following cloning of the MAL6 locus. Plasmid YEpMAL6 (originally named pYMAL26) contains a nearly 12 kb yeast DNA insert isolated from the MAL6 MAL1g MAL3g strain CB11 and was cloned by its ability to complement a MALg strain (Federoff et al., 1982). The ability to complement a MALg strain suggested, but did not prove, that

YEpMAL6 carried MAL6p function. Further, it appeared that a maltase structural gene had been cloned since: 1. nick translated YEpMAL6 hybridized to a maltose inducible transcript; and 2. this plasmid could hybrid select RNA that directed the in vitro synthesis of a protein that was immuno-precipitable by anti-maltase antibody. It was conclusively established that the MAL6 locus had been cloned by demonstrating linkage between the YEpMAL6 sequences and the genetic MAL6 locus (Needleman and Michels, 1983). It was also observed that the other dominant MAL loci and the cryptic MALg loci hybridized to MAL6 derived probes, revealing at least partial structural homology among these loci (Chow et al., 1983; Needleman and Michels, 1983; Michels and Needleman, 1983; 1984).

Subcloning of the YEpMAL6 yeast DNA insert followed by complementation analysis using MALp, MALg, and mal^0 tester strains demonstrated that both MAL6p and MAL6g functions were located on the 12kb yeast DNA insert and the two functions were separable (Needleman et al., 1984). R-loop analysis of the sequences encoding MAL6p function (most likely the positive regulatory function) revealed a single gene that was constitutively expressed. This gene was called MAL63. Northern blot analysis of RNA isolated from a MAL6 strain detected two constitutively expressed transcripts homologous to MAL63 derived probes, 1.6 kb and 2.0 kb. The 2.0 kb transcript was further induced by maltose. R-loop analysis of the MAL6g region unexpectedly revealed the presence of two transcribed regions, the RNAs of which were induced by maltose. Subcloning and separation of the two genes abolished MAL6g activity, suggesting that MAL6g function encodes two genes. The two MAL6g genes were called MAL61 and MAL62. Northern blot analysis of MAL61 homologous

RNA detected a 2.4 kb constitutively expressed transcript and a 2.0 kb maltose inducible species. The MAL62 gene encodes a single 1.9 kb maltose inducible transcript which is detectable at only very low levels following growth in noninducing medium. The MAL61 and MAL62 genes are divergently transcribed, suggesting that a common region may regulate the expression of both genes (Needleman et al., 1984). Federoff et al. (1982) tentatively localized a maltase structural gene to the MAL61 - MAL62 region.

MAL6 encodes at least three genes, all of which are required for maltose fermentation (Needleman, et al., 1984). In order to identify the specific functions of these genes, we decided to selectively mutate each gene at the genomic MAL6 locus using the popular one-step gene disruption technique described by Rothstein (1983). With these mutant strains we hoped to determine the effects of these mutations on the synthesis of maltase, maltose permease, and the MAL61, MAL62, and MAL63 transcripts. We have demonstrated that MAL61 appears to encode the maltose permease, MAL62 encodes maltase, and MAL63 encodes a trans-acting, positive regulatory function that is required for maltose regulated induction of maltose permease and maltase and the accumulation of their respective MAL61 and MAL62 inducible transcripts. In addition, we have localized and characterized a fourth gene, linked to MAL6, that is involved in the constitutive expression of the maltose fermentative enzymes in two MAL6-C constitutive strains. This gene, called MAL64, is located nearly 3.5 kb to the left of the MAL63 gene and is partially homologous to MAL63. In constitutive strains, this gene encodes a trans-acting positive regulatory function and is required for the constitutive expression of maltase, maltose permease, and their

respective transcripts. In contrast, MAL64 is not essential for inducible maltose fermentation, as wild type strains deleted for this gene continue to induce normally.

MATERIALS AND METHODS

Strains and Growth Conditions: The yeast strains employed in this study are described in Table 1. MAL6 strains were derived from the Saccharomyces carlsbergensis strains used by ten Berge and co-workers (ten Berge, 1972; ten Berge et al., 1973a,b; Needleman and Michels, 1983). MAL6 strains, as well as MALp, MALg, and $mal1^0$ tester strains were generously provided by R. Needleman and co-workers. The spoll strains (Klapholtz and Esposito, 1982) were a gift from M. Carlson.

It has proven impossible to construct a MAL6 strain lacking all other MAL related sequences. Previous studies demonstrated that all strains thus far examined carry some MAL related information located at the MAL1 locus. The four known MAL1 alleles are the dominant MAL1 locus, the partially functional and complementary MAL1p and MAL1g loci, and the (presumably) non-functional $mal1^0$ locus (Naumov, 1976). The mal^0 strains originally described by Naumov are nonfermenters unable to complement either MALp or MALg strains. Hybridization analysis revealed that these mal^0 strains in fact contain MAL6 homologous DNA sequences and subsequent segregational analysis demonstrated these sequences to be linked to the MAL1 locus, hence the name $mal1^0$ (Chow et al., 1983; Needleman and Michels, 1983; Michels and Needleman, 1983; 1984).

Since our aim is a functional analysis of the MAL6 locus, the presence of any MAL information at the MAL1 locus could obsure the meaning of MAL6-linked mutations by providing complementary MAL functions. Presumably, ten Berge et al. (1973a) failed to isolate mutations within MAL6g due to the (cryptic) presence of two additional MALg loci in their MAL6 strain (Naumov, 1976). We decided to use a MAL6

Table 1. Yeast Strains

Strain	Genotype	Source
332-5A	MATa MAL64 MAL63 MAL62 MAL61 mal1^0 ura3-52 leu2-3,112 trp1 his	R. Needleman
612-1D	Isogenic to 332-5A except MATα	R. Needleman
2-110	Isogenic to 332-5A except mal62::LEU2	This study
2-104	Isogenic to 332-5A except mal12::LEU2	This study
2b-46	Isogenic to 332-5A except mal62::LEU2	This study
26-20	Isogenic to 332-5A except mal12::LEU2	This study
628-5B	Isogenic to 332-5A except mal62::LEU2 and mal12::LEU2	R. Needleman
aF	Isogenic to 332-5A except mal61::URA3	This study
1b-8, 1b-7	Isogenic to 332-5A except mal61::URA3	This study
332-5AΔmal1^0-7	Isogenic to 332-5A except mal1^0::LEU2	This study
RDY123-1D	Isogenic to 332-5A except mal1^0::LEU2 and mal61::URA3	This study
a9, a8	Isogenic to 332-5A except mal63::URA3	This study
612-1C	Isogenic to strain a9 except MATα	This study
9-2	MATα mal63::URA3 mal1^0 trp1	This study
332-5AΔ61/Δ62-9, 332-5AΔ61/Δ62-10	Isogenic to 332-5A except mal61/mal62::LEU2	This study
332-5AΔF-1-5, 332-5AΔF-1-15	Isogenic to 332-5A except mal64::LEU2	This study
332-5AΔF-1-29	Isogenic to 332-5A except mal64::LEU2 and mal63-13	This study
332-5AΔG-15	Isogenic to 332-5A except mal64::URA3	This study
M26	MATα MAL64 mal63-13 MAL62 MAL61 MAL1g (?) MAL3g (?) trp5	ten Berge et al. (1973a)

Strain	Genotype	Source
C2	MATα MAL64-C2 ma163-13 MAL62 MAL61 MAL1g (?) MAL3g (?) ade1 ura	ten Berge et al. (1973b)
8-2B	MATa MAL64-C2 ma163-13 MAL62 MAL61 mal1^{o} ura3-52 leu2-3,112 trp1 ade	R. Needleman
RDY101-1D	Isogenic to 8-2B except MATα	This study
8-2BΔ63-1	Isogenic to 8-2B except ma163-13::URA3	This study
8-2BΔ63-2	Isogenic to 8-2B except ma163-13::URA3	This study
RDY113-2C	Isogenic to 8-2B 63-2 except MAT	This study
8-2BΔCΔH-6	Isogenic to 8-2B except MAL6-C2::pΔCΔH	This study
8-2BΔ61/Δ62-5 8-2BΔ61/Δ62-6 8-2BΔ61/Δ62-7	Isogenic to 8-2B except ma161/ma162::LEU2	This study
RDY111-5A	Isogenic to 8-2BΔ61/Δ 62-5 except MATα	This study
8-2BΔ12-6	Isogenic to 8-2B except ma112::LEU2	This study
RDY101-1B	Isogenic to 8-2BΔ12-6 except MATα	This study
8-2BΔF-5-20 8-2BΔF-5-21	Isogenic to 8-2B except ma164-C2::LEU2	This study
8-2BΔG-10	Isogenic to 8-2B except ma164-C2::URA3	This study
8-2BΔH-1 8-2BΔH-2	Isogenic to 8-2B except ma164-C2::URA3	This study
R10	Isogenic to a9 except MAL64C-R10 (ie.: MAL64C-R10 ma163::URA3)	R. Needleman (1986)
R10u	Isogenic to R10 except 5FOA revertant (ie.: ma163::ura3)	This study
R10Δ61/Δ 62-11 R10Δ61/Δ 62-12	Isogenic to R10 except ma161/ma162::LEU2	This study
R10ΔF-9-26	Isogenic to R10u except ma164C-R10::LEU2	This study
R10ΔG-1	Isogenic to R10u except ma164C-R10::URA3	This study
100-1A	MATa MAL13 ma111::URA3 MAL12 leu2-3,112	Charron et al. (1986)
100-1C	Isogenic to RDY100-1A except MATα	Charron et al. (1986)

Strain	Genotype	Source
53-2C	MATα MAL1p met	R. Needleman
1-31	MATa MAL1p met	R. Needleman
236-2A	MATa MAL1p leu2-3,112 lys2	R. Needleman
345-4A	MATa MAL1p ura3-52 leu2-3,112 trp1 ade	R. Needleman
208-6D	MATα MAL1g lys ade	Needleman and Michels (1983)
340-1A	MATα MAL1g ura3-52 ade	R. Needleman
340-1C	MALa MAL1g ura3-52 lys2 ade	R. Needleman
340-2A	MATα MAL1g ura3-52 ade	R. Needleman
JC27	MATα MAL(?)g leu2-3,112 his	Federoff et al. (1982)
6-2A	MATα mal1^0 lys	Needleman and Michels (1983)
3-2B	MATa mal1^0 lys	Needleman and Michels (1983)
328-4A	MATα mal1^0 ura3-52 trp1 met14 ade	Needleman et al. (1984)
303-3A	MATα mal1^0 leu2-3,112 ade	R. Needleman
K382-23A	MATa spo11 ura3 can1 cyh2 ade2 his7 hom3	Klapholz and Esposito (1982)
K398-4D	MATa spo11 ura3 ade6 arg4 aro7 asp5 met14 lys2 pet17 trp1	Klapholz and Esposito (1982)
K399-7D	MATa spo11 ura3 his2 leu1 lys1 met4	Klapholz and Esposito (1982)
K396-11A	MATa spo11 ura3 ade1 his1 leu2 lys7 met3 trp5	Klapholz and Esposito (1982)

mal1^0 strain for our mutational studies since the mal1^0 locus appeared functionless, at least by the criteria available to us at the time (ie.: the inability of this locus to complement MALp or MALg). From results to be presented, it shall become clear that this assumption was incorrect.

The MAL6 mal1^0 strain 332-5A was used extensively in this study. It is inducible for maltase and maltose permease. It was derived from strain CB11, a MAL6 MAL1g MAL3g strain that had been previously characterized (Needleman and Michels, 1983). By extensive crossings to mal1^0 strains, the MALg loci were eliminated from the background. It will become clear that a more accurate genetic description of the MAL6 locus may be written as MAL64 MAL63 MAL62 MAL61; a more accurate description of the mal1^0 locus may be written as mal11 MAL12.

Strain 8-2B is one of the maltose fermenting, constitutive strains used in this study and its derivation is complex. An inducible MAL6 strain (most likely MAL6 MAL1g MAL3g) was mutagenized by ten Berge and co-workers and the nonfermenting mal6 strain, mal6-13 was isolated (mal6-13 MAL1g MAL3g) (ten Berge et al, 1973a). In addition to being a nonfermenter, strain mal6-13 failed to induce for maltase and maltose permease. The mal6-13 and mal6-10 mutations are alleles (ten Berge et al., 1973a). Chang et al. (manuscript submitted for publication) demonstrated that mal6-10 is an allele of MAL63; thus mal6-13 is also an allele of MAL63 and may be written mal63-13. Strain C2 was isolated as a maltose fermenting revertant of strain mal6-13 and strain C2 expresses maltase and maltose permease constitutively (ten Berge et al., 1973b). The mutation responsible for reversion/constitutivity is linked to MAL6 and thus strain C2 is genotypically MAL6-C2 mal63-13 MAL1g MAL3g. By

crossing strain C2 to mal1^0 strains, the MALg loci were eliminated and constitutive strain 8-2B, which is genotypically MAL6-C2 mal63-13 mal1^0, was isolated. It will become evident that the genotype of strain 8-2B is more accurately described as MAL64-C2 mal63-13 MAL62 MAL61 mal1^0.

Strain R10 is another MAL6-C constitutive strain used in this study and constitutively expresses maltase and maltose permease. Strain R10 was derived as a maltose fermenting revertant of the uninducible, nonfermenting strain a9 (MAL64 mal63::URA3 MAL62 MAL61 mal1^0). Strain a9 and strain R10 are derivatives of strain 332-5A and are described fully in this text and in Dubin et al. (1986). Unlike strain 332-5A, strains a9 and R10 are Ura$^+$ by virtue of an insertion of the URA3 gene into the MAL63 gene. Strain R10 is genotypically MAL64C-R10 mal63::URA3 MAL62 MAL61 mal1^0. Strain R10u was isolated from strain R10 following selection for ura$^-$ on 5-fluoro-orotic acid containing medium as described by Boeke et al. (1984); the ura$^-$ phenotype in strain R10u was not complemented when crossed to a strain carrying ura3, thus confirming mutation within URA3. This strain spontaneously reverts to Ura$^+$ at a frequency of about 10^{-7}. Constitutive strain R10u is therefore isogenic to strain R10 except it is ura3.

Yeast strains were grown on YEP (1% [wt/vol] yeast extract-1% [wt/vol] peptone) plus various amounts of the specified carbon source at 30^{o}C. Maltose fermentation is defined by growth and the production of gas 1 to 5 days following inoculation and was determined in about 5 ml of YEP plus 2% (wt/vol) maltose medium in Durham tubes at 30^{o}C as previously described (Needleman and Michels, 1983).

Plasmids were propagated in E. coli bacterial strains C600 and RR1. Bacterial strains were grown in LB medium at 37^{o}C and antibiotics added

to the following final concentrations: ampicillin 100mcg/ml; tetracycline 25mcg/ml; chloramphenicol 50mcg/ml. Bacterial strains were also grown in the minimal medium M9CA (1x M9) (Maniatis et al., 1982) except 5g casamino acids/liter were added instead of 2g/l, one half the concentration of MgCl was substituted for $MgSO_4$, and $CaCl_2$ was omitted. When growing strain C600 in minimal medium, vitamin B_1 was added to a final concentration of 20mcg/ml.

Nucleic Acid Isolation:

Plasmid DNA: Small amounts of partially purified plasmid DNA were isolated from bacterial strains using the boiling method of Holmes and Quigley as described in Maniatis et al. (1982) except RNase was not added. Large scale isolation of plasmid DNA (following amplification in the presence of either 300mg/liter chloramphenicol or spectinomycin) was by a SDS-cleared lysate procedure and purified by centrifugation to equilibrium in a cesium chloride - ethidium bromide density gradient. Ethidium bromide was removed by two extractions with aqueous CsCl-saturated isopropanol; plasmid DNA was then precipitated by the addition of 1/10 the volume of sodium acetate (NaAc) pH 4.5 and two volumes of ethanol and redissolved in 10mM Tris HCl (pH 7.2) / 1mM EDTA (TE).

Yeast DNA: Small amounts of total genomic yeast DNA were isolated according to Winston et al. (1983) with only minor modifications. This procedure was utilized for nearly all yeast DNA preparations. Larger amounts of yeast DNA were isolated by the method of Cryer et al.(1975) and the modifications employed here have been described previously (Michels and Needleman, 1983).

Yeast RNA: Total RNA was isolated from yeast cells that had been

pregrown in YEP plus 2% (wt/vol) galactose and then inoculated into YEP plus 2% (wt/vol) of the specified carbon source, grown to mid to late log phase, and harvested. Just prior to harvesting, cycloheximide was added to a final concentration of 100mcg/ml and incubated further at 30°C for 0.25 hrs. Cells were disrupted by vortexing with glass beads in the presence of 25mM Tris HCl(pH 7.2)/ 100mM NaCl/ 10mM EDTA/ 1mg/ml heparin/ 0.5% SDS as previously described (Needleman et al., 1984). This mixture was extracted three times with phenol-chloroform-isoamyl alcohol (25:24:1) and precipitated by the addition of 1/10 volume of 1M NaCl and 2 volumes ethanol. Poly A^+ RNA was isolated by one or two chromatographic selections on oligo-dT-cellulose (Type 3 from Collaborative Research, Inc.) using a slight modification of the procedure described in Maniatis et al. (1982). The binding buffer used was 10mM Tris HCl(pH 7.5)/ 1mM EDTA/ 0.5M NaCl/ 0.25% SDS. Also, following heating to 65°C, the RNA was allowed to cool 5 minutes at room temperature and then directly loaded onto the column without the addition of 1 volume of 2x binding buffer. When two chromatographic selections were run, the precipitated poly A^+ RNA isolated from the first column passage was dissolved in 80 mcl 10mM Tris HCl(pH 7.5)/ 1mM EDTA / 0.5M LiCl, 360 mcl DMSO was added, the mixture heated to 65°C, cooled, made 0.5M with respect to NaCl, and then added to 2 ml. of 1x binding buffer prior to loading. RNA was precipitated by the addition of 2.25M NaAc (pH 4.5) to a final concentration of 0.3M and 2.2 volumes ethanol, and stored at -20°C. All aqueous solutions used in the preparation and subsequent analysis of RNA were made with diethylpyrocarbonate (DEP) treated H_2O, prepared by the addition of 2ml DEP to 20 liters distilled H_2O, followed by autoclaving for 0.3 hr. All

non-disposable glassware used in the preparation of RNA was washed in 1M NaOH, 1M HCl, tap water, distilled water, and then DEP-treated water, and autoclaved and baked overnight at 170°C.

Nucleic Acid Manipulations: Restriction endonucleases were used according to conditions suggested by the manufacturers (BRL; IBI) except the buffer used was always 1x T4 DNA Polymerase cofactors (O'Farrell et al., 1980). Yeast DNA and plasmid DNA (for integrative yeast transformations) were restricted 2-3 hours at 37°C in a total volume of 300-450 mcl, and precipitated by the addition of 1/10 volume 3M NaAc (pH4.5) and 2 volumes ethanol. The yeast DNA was redissolved in 20 - 30mcl TE and the plasmid DNA in 50 - 100mcl sterile TE. Ligations were performed at 4°C overnight, using T4 DNA ligase according to conditions suggested by the manufacturer (NEB). T4 DNA polymerase (BRL) was used to convert staggered to flush ends, and EcoRI linkers (Pharmacia) were kinased with T4 polynucleotide kinase (BRL) and ligated according to Maniatis et al. (1982).

Construction of disruption plasmids and pJunct2. Plasmid pDM2 (Fig. 1) disrupts the MAL62 gene and was constructed as follows. The 2.2 kb PstI fragment containing the MAL62 gene was subcloned from pY-6 (Needleman and Michels, 1983) into pBR322, creating plasmid p25. Plasmid pDM2 was constructed by inserting the 2.7 kb BglII fragment containing the LEU2 gene from pCV9 into the BglII site within plasmid p25 near the 3' end of the MAL62 gene.

Plasmid pDM2b (Fig. 1; Dubin et al., 1985) disrupts the MAL62 gene and was constructed by deleting the 1.4 kb BglII fragment containing the MAL62 gene from pY-6 and replacing it with the 2.7 kb BglII fragment containing the LEU2 gene from pCV9.

Plasmid pDM1 (Fig. 1) disrupts the MAL61 gene and was constructed as follows. The 3.2 kb PstI fragment containing MAL61 was subcloned from pY-6 into pBR322ΔR, creating plasmid p1C. (Plasmid pBR322ΔR was constructed by deleting the EcoRI site in plasmid pBR322 by filling in with T4 DNA polymerase.) Plasmid pDM1 was constructed by inserting a 3.0 kb EcoRI fragment from plasmid p8 (which will be described below), containing the URA3 gene (along with 375 bp of pBR322 sequences [from nucleotides 1-375] and 1.6 kb of yeast LEU2 DNA sequences) into the single EcoRI site in plasmid p1C, within the 5' region of MAL61.

Plasmid pDM1b (Fig. 1; Charron et al., 1986) disrupts the MAL61 gene and was constructed as follows. The 3.2 kb PstI fragment containing the MAL61 gene was subcloned from pY-6 into pBR322ΔRΔA, creating plasmid p(one). (Plasmid pBR322ΔRΔA is a derivative of pBR322 but is lacking its EcoRI and AvaI sites.) Plasmid p(one) was restricted with EcoRI and AvaI, which cuts only within the MAL61 gene, and sedimented through a sucrose density gradient and the 900 bp EcoRI - AvaI fragment containing a portion of MAL61 was removed; the 6.6 kb restricted plasmid was recovered, blunt ended with T4 DNA polymerase, EcoRI linkers added and religated. The plasmid formed, pB, contained a single EcoRI site and a deletion of 900 bp within the MAL61 gene. Plasmid pDM1 was constructed by inserting into the EcoRI site of pB the 3.0 kb EcoRI restriction fragment from plasmid p8 which contains the URA3 gene (and 0.375 kb of pBR322 sequences [the EcoRI - BamHI fragment containing nucleotides 1-375] and LEU2 sequences).

Plasmid p8 (not shown) was used in the construction of pDM1 and pDM1b. It contains a 3.0 kb EcoRI fragment on which is found the URA3 gene plus 375 base pairs (bp) of pBR322 sequences (from nucleotides

1-375 on the standard map of pBR322) and about 1.6 kb of yeast LEU2 DNA sequences. Plasmid p8 was constructed by subcloning the 2.7 kb BglII fragment containing the LEU2 gene from pCV9 into the BamHI site of plasmid YIp31 (Botstein et al., 1979). In this construct, the XhoI site near the yeast LEU2 gene is proximal to the BamHI site in YIp31. Thus, when p8 is restricted with EcoRI, a 3.0 kb fragment is released that appears as follows: EcoRI site - 29 bp of pBR322 from the EcoRI site to the HindIII site - 1.1 kb of the yeast URA3 gene (as a HindIII fragment) - 346 bp of pBR322 sequences from the HindIII to the BamHI site (within the tetracycline resistance gene) - 1.6 kb of the yeast LEU2 gene - EcoRI site (within the LEU2 gene).

Plasmid pD$mal1^0$ (Fig. 6) disrupts both the mal11 and MAL12 genes located at the MAL1-linked $mal1^0$ locus and was constructed as follows. The $mal1^0$ locus from strain 3-2B was cloned onto λMJCO.3 by M. Charron in this laboratory (Charron and Michels, manuscript in preparation) and generously provided for this study. The 7.0 kb HindIII fragment containing mal11 and MAL12 was subcloned into plasmid pBR325ΔPst, creating pBR325ΔPst$mal1^0$. (Plamid pBR325ΔPst was constructed by deleting the PstI site in pBR325 by filling in with T4 DNA polymerase.) Disruption plasmid pD$mal1^0$ was created by deleting three PstI fragments (a total of nearly 6.2 kb and most certainly containing the mal11 and MAL12 genes) from pBR325Δ$Pstmal1^0$ and replacing them with the 4.1 kb PstI fragment containing the LEU2 gene from pCV9.

Plasmid pDM3 (Fig. 1; Chang et al., manuscript in preparation; Dubin et al., 1986; Charron et al., 1986) disrupts the MAL63 gene and was constructed as follows. The 2.7 kb BglII - SalI fragment containing the MAL63 gene was subcloned as a 3.0 kb SalI fragment from p21-40 (see

Fig. 3; Needleman et al., 1984) into a derivative of pBR322, creating plasmid p40. Plasmid p40 contains three HindIII restriction sites, all within the MAL63 gene. Plasmid pDM3 was constructed by deleting the two HindIII fragments within MAL63 (a total of about 1.0 kb of DNA) and replacing it with the 1.1 kb HindIII fragment containing the URA3 gene from plasmid YIp31.

Plasmid pY6ΔCΔH (Fig. 1; Dubin et al., 1986) was used for targeted integration and plasmid rescue of MAL6-C2 sequences from constitutive strain 8-2B and was constructed as follows. Plasmid pY-6 (Needleman and Michels, 1983) contains the 7.3 kb HindIII fragment (containing the MAL61 and MAL62 genes) in YIp5 (Botstein et al., 1979). Plasmid pY-6 was restricted with ClaI, self-ligated, and plasmid pY6ΔC was obtained. This plasmid contains the 1.6 kb ClaI - HindIII fragment (containing sequences 3' to the MAL62 gene) in plasmid YIp5. Plasmid pY6ΔCΔH was constructed by deleting the HindIII site within pY6ΔC by restricting with HindIII, filling in with T4 DNA polymerase, and self-ligating. Plasmid pY6ΔCΔH was restricted with HpaI prior to transforming strain 8-2B in order to target this plasmid to integrate at the MAL6-C2 locus; strain 8-2BΔCΔH-6 was identified as containing the expected integration at MAL6-C2. Plasmid pRD1 (Fig. 9) was rescued from strain 8-2BΔCΔH-6 by the procedure of Orr-Weaver et al. (1983) by restricting total yeast DNA isolated from this strain with HindIII, ligating it under dilute conditions, and transforming strain RR1 to ampicillin resistance (also see below). Plasmid pRD1 contains the MAL61 and MAL62 genes isolated from constitutive strain 8-2B.

Plasmid pRD2 (not shown) contains the 7.6 kb BamHI - HindIII fragment of pRD1 (containg the MAL61 and MAL62 genes) subcloned into

pCV13. Plasmid pRD3 (Fig. 9; Dubin et al., 1986) mutates the MAL61 and MAL62 genes and was constructed by deleting the two BglII fragments within pRD1 (within the MAL61 and MAL62 genes) and replacing them with the 2.7 kb BglII fragment from pCV9 containing the LEU2 gene.

DNA sequences containing the MAL64 and MAL64-C2 genes as well as flanking regions were cloned by M. Charron in this laboratory and generously provided for this study (Charron and Michels, manuscript in preparation; M. Charron, Ph. D, Thesis, CUNY, 1987). Briefly, M. Charron constructed plasmids pMJC6ΔCla and pY6RΔC (Fig. 10) using wild type DNA isolated from strain CB11 (Federoff et a., 1982) and subcloned from YEpMAL6 or pY-6 (Needleman and Michels, 1983) into YIp5. Plasmid pMJC6ΔCla was used to isolate plasmid pBam11 (Fig. 10) from inducible strain 332-5A and also used to isolate pBamC2 (Fig. 11) from constitutive strain 8-2B by targeted integration into the appropriate strain (by cleavage with ClaI) and subsequent plasmid rescue (Orr-Weaver et al., 1983). In a similar manner, plasmid pY6RΔC was restricted with BglII and used to isolate pB3C2 (Fig. 11) from strain 8-2B.

Plasmid pBSC2 (Fig. 11) was constructed by subcloning the 14.5 kb BamHI - SalI fragment from pB3C2 (Fig. 11) into pBR325. Disruption plasmid pDMF (Fig. 11) was constructed by restricting plasmid pBSC2 with BglII, deleting the four BglII fragments (a total of nearly 11.3 kb) and replacing them with the 2.7 kb BglII fragment from pCV9 containing the LEU2 gene.

Disruption plasmid pDMG (Fig. 10) was constructed as follows. The 2.4 kb EcoRI fragment Z-2 (isolated from the wild type MAL6 DNA sequences on pBam11; see Fig. 10) was subcloned into pBR325ΔB-H, creating pZ-2 (Fig. 10). (Plasmid pBR325ΔB-H was created by deleting

the nearly 350 bp between the BamHI and HindIII sites within pBR325.) Disruption plasmid pDMG was constructed by inserting the 1.1 kb HindIII fragment containing the URA3 gene (from YIp31) into the HindIII site within pZ-2, thus mutating the MAL64 gene. Disruption plasmid pDMH (Fig. 11) was constructed by subcloning the 2.4 kb EcoRI fragment isolated from pBamC2 (and thus containing the MAL64-C2 allele) into pBR325Δ B-H, creating plasmid pZ-2C2; pDMH was constructed by inserting the URA3 gene from YIp31 into the HindIII site within pZ-2C2, thus disrupting MAL64-C2. Plasmids pBR325Δ B-H and pZ-2 were constructed by M. Charron and generously provided for this study.

Plasmid pBaml1(2M) (Fig. 10) is an episomal derivative of pBaml1 and was constructed by deleting the 1 kb BamHI - XhoI fragment from pBaml1 and replacing it with the BamHI - XhoI fragment of YEp13 which contains the yeast LEU2 gene and a portion of 2 micron circle. Plasmid pBamC2(2M) (Fig. 11) is an episomal derivative of pBamC2 (Fig. 11) and was constructed by deleting the 1 kb BamHI - XhoI fragment from pBamC2 and replacing it with the BamHI - XhoI fragment of YEp13 which contains the yeast LEU2 gene and a portion of 2 micron circle. Plasmid pBamC2Δ Xba(2M) (Fig. 11) is identical to pBamC2(2M) except it contains a deletion of two XbaI fragments (1.3 kb and 0.5 kb) within the MAL64-C2 gene. It was constructed by deleting the two XbaI fragments in pBamC2, creating pBamC2Δ Xba, and then adding the LEU2 gene and 2 micron sequences from pCV13 as in plasmid pBamC2(2M).

Plasmid pJunct2 is not a disruption plasmid; since it does not appear on any MAL6 map in this thesis and it was used to probe MAL64 disruption strains by Southern blot analysis, its construction will be described. Plasmid pBaml1 was restricted with HindIII, which cuts only

within the yeast insert, and ligated under dilute conditions, creating plasmid pBam11ΔH. The BamHI - ClaI fragment of pBam11ΔH (containing MAL64 sequences) was subcloned into pBR325ΔPst, creating plasmid pCamBam11ΔH. From pCamBam11ΔH, the 0.7 kb BamHI - EcoRI fragment (containing MAL64 sequences on the 0.4 kb HindIII - EcoRI fragment Z-1 and the 0.3 kb BamHI - HindIII fragment at the far left of the insert present in pBam11) was subcloned into pBR325, creating pJunct2.

Radiolabelling: Plasmids were labelled with ^{32}P-dCTP (Amersham) by nick-translation using DNA polymerase (IBI; Boerhinger-Mannheim) according to the procedure described by Rigby et al. (1977) or the manufacturer. Radiolabelled DNA fragments were obtained by nick-translation of restriction endonuclease digested plasmid DNA and subsequent size fractionation on a 0.8% agarose gel as described below. The appropriate DNA fragment was visualized using a hand held UV lamp, excised as a slice of agarose from the gel, and added without further purification to the hybridization mix.

DNA and RNA Analysis: Endonuclease restricted DNA was electrophoretically size fractionated on 0.8% agarose gels containing 1x Tris-acetate EDTA (TAE) buffer (0.16M Tris acetate (pH7.8)/ 0.0044M EDTA) and 1mcg/ml ethidium bromide. The running buffer was 1x TAE containing 1mcg/ml ethidium bromide. Supercoiled plasmids isolated from crude bacterial lysates (in 0.5M Tris HCl (pH6.8)/ 0.002M EDTA/ 0.4M sucrose/ 1% SDS/ 0.01% bromphenol blue) were sized using this gel system except ethidium bromide was omitted from both gel and running buffer and the gel was stained with ethidium bromide following electrophoresis. Transfer of fractionated DNA to nitrocellulose was essentially according to the method of Southern (1975). DNA blots were baked in a vacuum oven

at 80°C for 1-2 hours and hybridized to heat-denatured, ^{32}P-dCTP labeled probe in Seal-A-Meal plastic bags at 65°C for 14-24 hours in hybridization buffer (0.6M NaCl/ 0.06M NaCitrate (pH7.0)/ 0.2% Ficol/ 0.2% polyvinylpyrrolidone/ 0.2% BSA/ 0.1% SDS/ 0.1 mg/ml salmon sperm DNA). Following hybridization the probe was removed and blots were rinsed for about 1 minute at room temperature with distilled H_2O and then washed 3-4 times in a total of 1-2 liters of 0.3M NaCl/ 0.03M NaCitrate(pH 7.0)/ 0.1% SDS at 65°C for a total of 1-2 hours.

Poly A^+ RNA was denatured in 20mM $NaPO_4$(pH 7.0)/ 50% formamide/ 6% formaldehyde by heating at 65°C for 5 minutes and electrophoretically size fractionated on a 1.5% agarose gel (Seakem Agarose type ME, FMC Corp.) containing 20mM $NaPO_4$(pH 7.0)/ 6% formaldehyde. The running buffer, 20mM $NaPO_4$ (pH7.0) / 3% formaldehyde was recirculated during the run, which usually lasted 700 volt x hours (8-19 hours). RNA was transferred to nitrocellulose for at least 12 hours according to Thomas (1980) except the gel was rinsed in 3M NaCl/ 0.3M NaCitrate(pH7.0) prior to transfer and the nitrocellulose was saturated with 0.3M NaCl/ 0.03M NaCitrate(pH 7.0) prior to transfer and washed 20 minutes with 3M NaCl/ 0.3M NaCitrate (pH7.0) following transfer. Blots were baked in a vacuum oven at 80°C for 2 hours and prehybridized at 43°C overnight in 0.75M NaCl-0.075M NaCitrate(pH7.0)/ 0.02% Ficol/ 0.02% polyvinylpyrolidone/ 0.02%BSA/ 50% formamide/ 1mg/ml sheared, heat denatured, salmon sperm carrier DNA. Hybridization conditions were identical to the prehybridization conditions except for the addition of heat denatured probe and a reduction of the carrier DNA to 0.1mg/ml. Blots were washed at 43°C, 7-9 times for 8-10 hours, with a total of 100-125 ml of wash buffer which is the same as hybridization buffer except probe and

carrier DNA are omitted. Total yeast RNA was used as size markers and run on all RNA gels. This track was cut from the gel and stained with 1 mcl/ml ethidium bromide / 0.1M NH_4Ac and destained in dH_2O. The 18S and 28S rRNAs were considered to be 1.73 kb and 3.4 kb respectively. Formamide was deionized as previously described (Needleman et al., 1984).

Blots were exposed to Kodak X-AR film at -70°C with an intensifying screen for 2 hours to 21 days.

RNA integrity was confirmed by reprobing Northern blot filters with either Ty1 probe S13 (Cameron et al., 1979) or the yeast actin gene on plasmid pYactI (Ng and Abelson, 1980). No attempt was made to remove the initial probe. Early Northerns (ie.: those probed with pD-3 and pE-2) were reprobed with the Ty1 probe; later Northerns (ie.: those probed with pD-5 and pE-4) were reprobed with the yeast actin gene. Only the three Northerns presented in Fig. 9 were not reprobed.

Measurement of Maltase Activity: Maltase activity was determined as the rate of release of p-nitrophenol from p-nitrophenol-alpha-D-glucopyranoside (PNPG) according to Kahn and Eaton (1976). Cells were pregrown in 1 - 1.5ml YEP plus 2% galactose, inoculated into 50ml of fresh YEP medium containing the specified amount of carbon source, and grown 8-9 hours at 30°C, reaching mid to late log phase. In order to selectively maintain episomal plasmids and assay strains carrying such plasmids for PNPGase activity, yeast strains carrying episomal plasmids with the selectable marker gene URA3 or LEU2 (see Table 11) were grown in minimal medium (0.67% yeast nitrogen base without amino acids) plus 2% of the indicated carbon source and supplemented with tryptophan and adenine. PNPGase activity is expressed

as nmoles substrate split/min/mg protein at 30°C. All maltase levels are an average of two or more independent determinations, except for the segregants from tetrads 5, 10, and 14 listed in table 8, which were assayed only once. Protein determination was according to Lowry (1951) using BSA as standard.

Saccharomyces carlsbergensis strains are capable of synthesizing two alpha-glucosidases, maltase and alpha-methylglucosidase, both of which hydrolyse PNPG (Kahn and Eaton, 1967). Maltose induces the synthesis of maltase, and to a lesser extent, alpha-methylglucosidase (Ouwehand and van Wijk, 1972). Mostly all the PNPGase activity detected in strain 332-5A is due to maltase. As discussed in Dubin et al. (1985), "hydrolysis of alpha-methyl glucoside (in this strain) is 2.2 - 2.8% of the rate of hydrolysis of PNPG. This is consistent with the known substrate specificity of maltase and with the specificity of alpha-methyl glucosidase." Although constitutive strain 8-2B was not similarly examined, it appears, from data to be presented, that nearly all of the constitutive PNPGase activity in this strain is maltase.

Measurement of Maltose Permease Activity: The rate of maltose transport was determined by the method of Serrano (1977). Cells were grown to mid-log phase in the designated media prior to harvesting. Maltose transport activity is expressed as the number of picomoles of ^{14}C-maltose transported in 2min/mg dry weight of cells. All maltose transport determinations were performed by Dr. R. Needleman and co-workers at the Wayne State University College of Medicine, Detroit, MI.

Bacterial and Yeast Transformations: E. coli was transformed by a slight modification of the $CaCl_2$ method described by Maniatis et al. (1982).

Strains C600 and RR1 were treated with 0.5M and 0.1M $CaCl_2$, respectively. Yeast transformation was according to the method of Ito et al. (1983) using LiAc. One-step gene disruptions were according to Rothstein (1983). Integrative targeting of linearized plasmids, and integrative targeting followed by plasmid rescue were according to Orr-Weaver et al. (1983). Selectable marker stability in yeast transformants was determined following growth in non-selective (YEP plus 2% glucose) medium. Isolation of plasmid pRD1 by plasmid rescue was as follows. Yeast DNA from strain 8-2BΔCΔH-6 was restricted with HindIII, deproteinized with phenol, and fractionated on a 10 - 40% sucrose gradient (in 1M NaCl/10mM Tris HCl(pH7.4)/ 1mM EDTA). The fractions of interest were determined by Southern blot analysis, pooled, concentrated by precipitation and ligated under dilute conditions. The ligation mix was used to transform strain RR1 to ampicillin resistance.

Genetics: Standard yeast genetic analysis was according to procedures described in Mortimer and Hawthorne (1969). Isogenic strains were constructed as previously described in Dubin et al. (1985). Petite strains were isolated following growth in YEP plus 2% glucose in the presence of 10-100mcg/ml ethidium bromide and subsequent plating onto YEPDG (YEP plus 0.1% glucose and 3% glycerol) plates (Sherman et al., 1970) The chromosomal location of MAL6 was determined using the spo11 technique of Klapholtz and Esposito (1982).

RESULTS

Chapter 1: MAL62 encodes Maltase

In order to determine the role of the MAL62 gene in maltose fermentation, this gene was mutagenized by gene disruption (Rothstein, 1983) at the genomic MAL6 locus. Disruption plasmid pDM2 (for plasmid containing a Disruption of MAL62) was constructed as described (Fig. 1) and contains an insertion of the yeast selectable marker gene LEU2 into the 3' end of the cloned MAL62 gene. PstI - BamHI restricted pDM2 was used to transform the MAL6 mal1^{0} strain 332-5A to Leu^{+}. All transformants examined fermented maltose, although nearly 60% did so more slowly than the undisrupted strain (Appendix Table 3). Transformant strain 2-110 fermented maltose slowly, and this was reflected in its rate of growth in maltose containing medium. Generation times of 2.75 hr and 4.72 hr were observed for strains 332-5A and 2-110 respectively in YEP plus 2% maltose medium while almost no difference was found when the two strains were grown in YEP plus 2% glucose (1.35 hr and 1.54 hr, respectively).

Two transformants, strain 2-110 (a slow maltose fermenter) and strain 2-104 (which fermented maltose at a rate similar to the untransformed parent) were characterized by Southern blot analysis. Previous studies demonstrated that a MAL6 derived probe hybridized to a 7.3 kb MAL6-linked HindIII fragment and a 7.0 kb mal1^{0}-linked HindIII fragment in MAL6 mal1^{0} strains (Needleman and Michels, 1983) and this pattern was evident in strain 332-5A. Transformed strain 2-110 lacked the MAL6-linked HindIII fragment and instead contained a new 9.9kb fragment, indicating integration at MAL6; surprisingly, strain 2-104

Figure 1. The MAL6 locus of strain CB11 and disruption plasmids for mutating the MAL61, MAL62 and MAL63 genes.

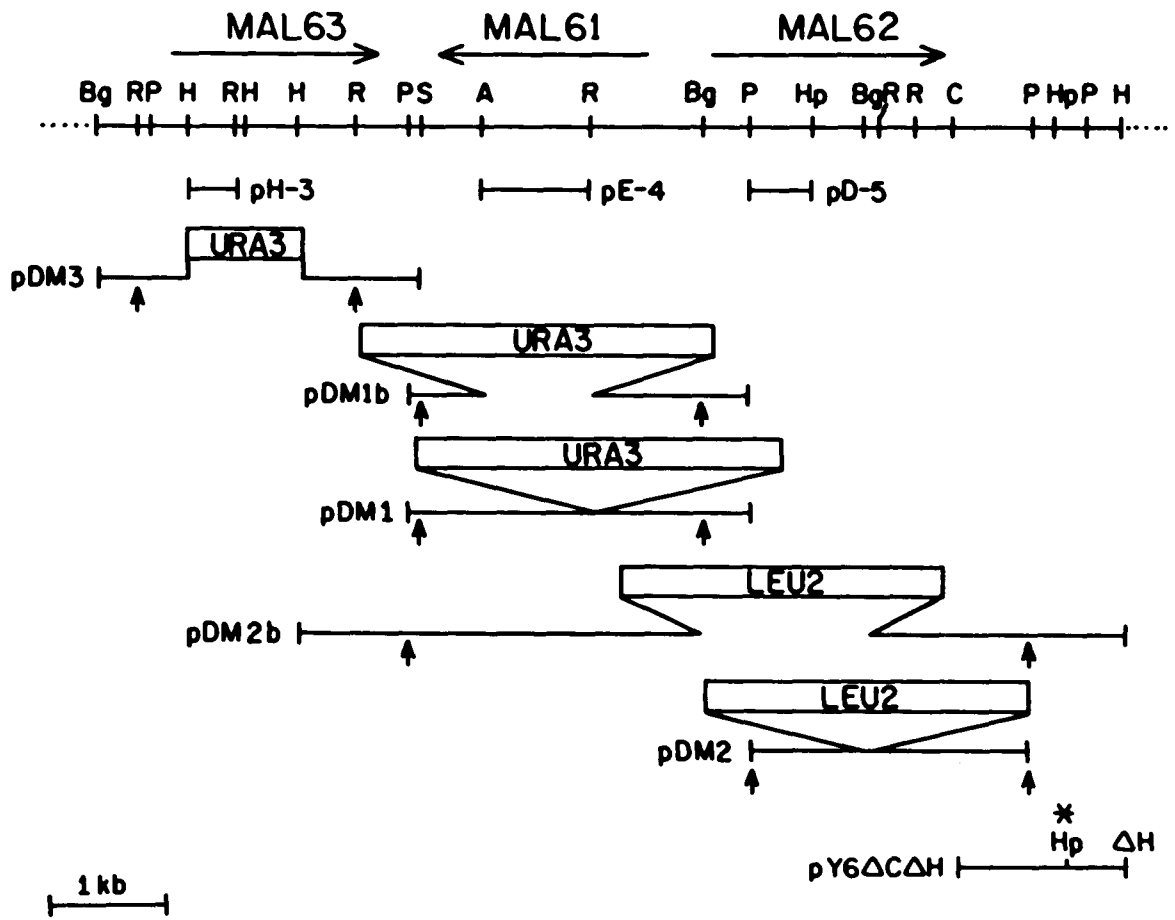
A partial restriction map of the MAL6 locus of strain CB11 (Federoff et al., 1982) is presented (———), along with the locations of three transcribed regions, MAL61, MAL62, and MAL63, and their directions of transcription (⟶ ; Needleman et al., 1984).

Plasmids pH-3, pE-4, and pD-5 contain the indicated MAL6 fragments subcloned in derivatives of plasmid pBR325. Disruption plasmids pDM2 and pDM2b mutate the MAL62 gene; disruption plasmids pDM1 and pDM1b mutate the MAL61 gene (as described in the Materials and Methods, the SalI - BglII disruption fragments from pDM1 and pDM1b that integrate at MAL61 contain not only the URA3 gene, but also 0.375 kb of pBR sequences [the EcoRI - BamHI fragement containing nucleotides 1-375] and 1.6 kb of the yeast LEU2 sequences); disruption plasmid pDM3 mutates the MAL63 gene. Plasmid pY6ΔCΔH was used to target integration to, and subsequently rescue flanking sequences from the MAL6-C2 locus of constitutive strain 8-2B. The constructions of these plasmids are detailed in Materials and Methods.

(↑) indicates the restriction endonuclease sites used to restrict disruption plasmids prior to transforming.

(*) indicates the HpaI site used to restrict pY6ΔCΔH prior to transforming strain 8-2B.

Restriction endonuclease sites: AvaI, A; BglII, Bg; ClaI, C, EcoRI, R; HindIII, H; HpaI, Hp; PstI, P; SalI, S.


MAL63
MAL61
MAL62
Bg RP H RH H R PS A R Bg P Hp Bg R R C P Hp P H
pH-3
pE-4
pD-5
URA3
pDM3
URA3
pDM1b
URA3
pDM1
LEU2
pDM2b
LEU2
pDM2
*
Hp ΔH
pY6ΔCΔH
1 kb

revealed a shift in the mal1^0-linked HindIII fragment, indicating integration at mal1^0 (Appendix Table 4). Subsequent cloning and characterization of a mal1^0 locus has revealed extensive sequence homology between the MAL6 and mal1^0 loci in the region of the MAL61 and MAL62 genes (Charron and Michels, manuscript in preparation).

Poly A$^+$ RNA was isolated from the undisrupted parent strain 332-5A and transformant strain 2-110 following growth in either uninducing (YEP plus 2% galactose) or inducing (YEP plus 2% galactose/2% maltose) medium and the MAL transcripts examined by Northern blot analysis. The sizes and patterns of expression of the MAL homologous transcripts in the parental strain 332-5A appear on Fig. 2. A 1.9 kb maltose inducible MAL62 homologous RNA is expressed at low levels in the absence of maltose and is expressed at high levels when maltose is present in the growth medium. The MAL61 homologous probe pE-2 detects a constitutively expressed 2.4 kb transcript and two maltose inducible transcripts, 2.3 kb and 2.0 kb. These two inducible transcripts are undetectable in the absence of inducer. (The inducible 2.3 kb species is barely detectable when strain 332-5A is grown in YEP plus 2% maltose and probed with pE-4, a smaller MAL61 homologous probe (see Figs. 3 and 12). Since the 2.3 kb transcript is not detected in other MAL6 strains (Needleman et al, 1984; also see strain 8-2B in Fig. 8), it may be unique to the background of this strain. The origin of this 2.3 kb transcript remains unclear.) The MAL63 homologous probe pH-2 detects two constitutively expressed RNA species, 1.6 kb and 2.0 kb. The larger transcript is slightly induced by maltose. Comparison of the expression of the MAL61, MAL62, and MAL63 transcripts synthesized by strains 332-5A and 2-110 revealed no detectable differences (Fig. 2A,B,C).

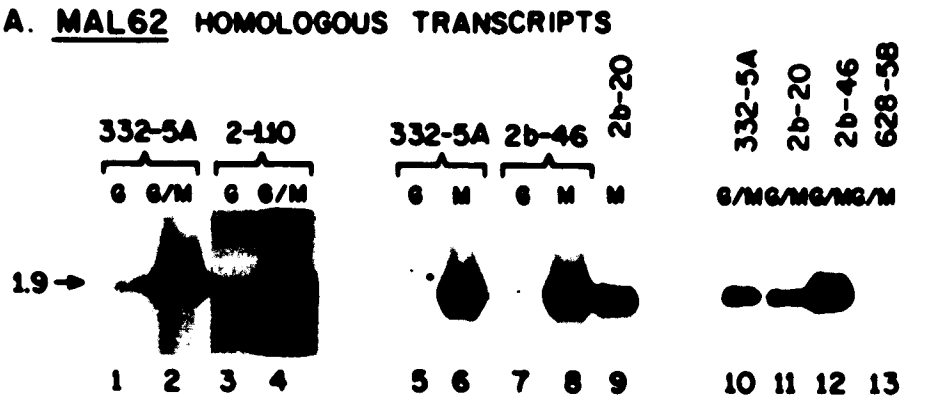
Figure 2. Disruption of the MAL62 and MAL12 genes and its effects on transcription of the MAL61, MAL62 and MAL63 genes.

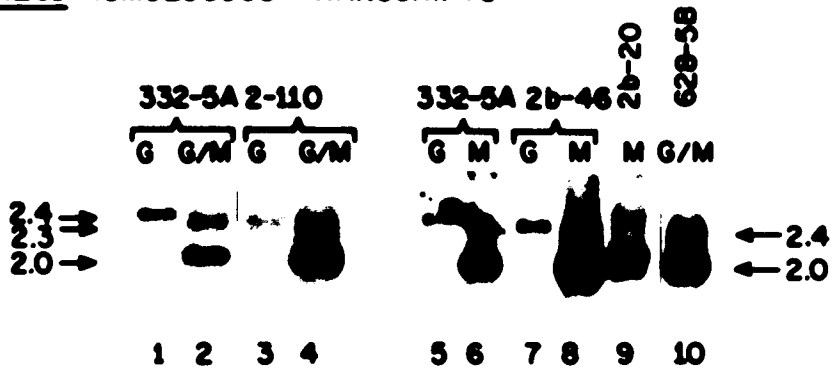
Yeast strains were grown under uninducing conditions (YEP medium plus 2% galactose [indicated by G]) or inducing conditions (YEP medium plus 2% maltose [indicated by M] or YEP medium plus 2% galactose/2% maltose [indicated by G/M]). Poly (A^+) RNA was isolated, fractionated on a formaldehyde/agarose gel, transferred to nitrocellulose, and probed with MAL61, MAL62 and MAL63 specific probes (Fig. 3). Panel A: MAL62 homologous transcripts were detected using probe pD-3 (lanes 1-4) or pD-5 (lanes 5-13). 10 micrograms (mcg) poly (A^+) RNA were loaded per track. Panel B: MAL61 homologous transcripts were detected using probe pE-2 (lanes 1-4) or pE-4 (lanes 5-10). 10 mcg poly (A^+) RNA were loaded per track. Panel C: MAL63 homologous transcripts were detected using probe pH-2. 20 mcg poly (A^+) RNA were loaded per track, except for lane 7, which contained 9 mcg.

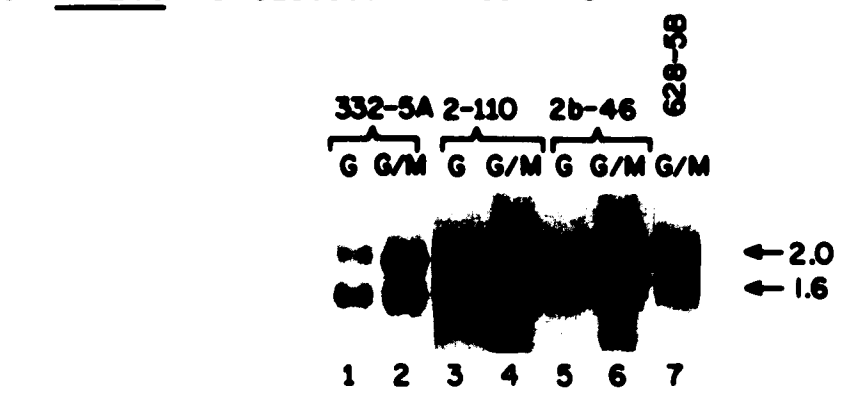
Strains:

332-5A	MAL63	MAL62	MAL61	MAL12
2-110	MAL63	mal62::LEU2	MAL61	MAL12
2b-46	MAL63	mal62::LEU2	MAL61	MAL12
2b-20	MAL63	MAL62	MAL61	mal12::LEU2
628-5B	MAL63	mal62::LEU2	MAL61	mal12::LEU2

DISRUPTION OF MAL62







The continued ability of strain 2-110 to ferment maltose and synthesize the MAL62 homologous RNA suggested the following interpretations:

1) The insertion did not disrupt the MAL62 gene. However, this conclusion is not supported by the increased generation time of strain 2-110 in maltose containing medium.

2) MAL62 function was only slightly impaired.

3) The MAL62 gene was disrupted and is not essential for maltose fermentation. However, previous complementation analysis using cloned MAL6 DNA suggested that MAL62 was a component of MAL6g function (Needleman et al., 1984).

4) The MAL62 gene was disrupted and a second functional copy of the MAL62 gene exists in the background of strain 332-5A, complementing the mutant MAL62 gene. The location of such a functional homolog would most likely be the structurally homologous mal1^{0} locus. Although no functional activity had as yet been detected at this locus, this may simply have reflected the nature of the MALp and MALg tester strains used in complementation studies.

To distinguish among these possibilities, a second disruption plasmid, pDM2b, was constructed (Fig. 1). It contains a 1.4 kb deletion within the MAL62 gene and is replaced by the yeast selectable marker gene LEU2. PstI - BamHI - HindIII restricted pDM2b was used to transform strain 332-5A to Leu^{+} and again, all transformants were maltose fermenters and nearly 60% fermented maltose somewhat more slowly than the untransformed parental strain (Appendix Table 3). Southern blot analysis of DNA isolated from transformants indicated that two slow fermenters, strains 2b-15 and 2b-46, contained deletions of MAL62 and

isolate 2b-20 (which was not a slow fermenter) contained a deletion at the homologous region of *mal1*^{0} (Appendix Table 4; also Fig. 2 in Dubin et al., 1985). Integrative disruption at *MAL6* was confirmed genetically for strain 2b-46 (Appendix Table 2, line 1) and at the *MAL1*-linked *mal1*^{0} locus for strain 2b-20 (Appendix Table 2, line 2).

Both strains 2b-46 and 2b-20 induced for maltase (Table 2). Northern blot analysis of RNA isolated from strain 2b-46 (*mal62::LEU2 mal1*^{0}) failed to reveal any changes in the transcripts of the three *MAL6* genes as compared to the undisrupted parent strain 332-5A (Fig. 2A,B,C). The continued presence of a 1.9 kb, maltose inducible, *MAL62* homologous transcript in strain 2b-46 demonstrated that this RNA is transcribed either by some gene other than *MAL62* or by some other gene and *MAL62*. The latter hypothesis was strengthened by the fact that strain 2b-20 (*MAL62 mal1*^{0}*::LEU2*) also synthesized a 1.9 kb *MAL62* homologous transcript (Fig. 2A).

If the *MAL62* gene was essential for maltose fermentation and the *mal1*^{0} locus complemented a deletion of the *MAL62* gene, then strains containing disruptions at both the *MAL62* gene and the *mal1*^{0} locus should be nonfermenters. To test this, doubly disrupted strains were genetically constructed as described in Dubin et al. (1985). Strain 612-1C was constructed by R. Needleman and co-workers and is isogenic to strain 2b-46 except at the mating type locus. Strains 612-1C (*mal62::LEU2 mal1*^{0}) and 2b-20 (*MAL62 mal1*^{0}*::LEU2*) were mated and the diploid, W628, was sporulated and dissected, and segregants tested for the ability to ferment maltose by R. Needleman and co-workers. The outcome that is expected if *mal1*^{0} complements the *MAL62* disruption appears on Table 3. Segregants from diploid W628 gave 3 parental ditype

Table 2.

Maltase Activity in a MAL6 mal1^0 strain disrupted within the MAL62 and MAL12 genes[a]

Strain[b]	MAL Genotype	Maltase Activity[c] Galactose	Maltose	Gal/Mal
332-5A	MAL6 mal1^0	5 (±1)	279 (±49)	96 (±35)
2b-46	mal62::LEU2 mal1^0	2 (2,2)	197 (190,204)	[d]N.D.
2b-20	MAL62 mal1^0::LEU2	4 (4,4)	234 (250,218)	N.D.
628-5B	mal62::LEU2 mal1^0::LEU2	1 (1,1)	12 (11,13)	3 (3,3)

(a) Cells were pregrown in YEP medium plus 2% galactose and diluted into YEP medium plus 2% galactose, or 2% maltose, or 2%galactose/2%maltose (Gal/Mal) and allowed to grow 8-9 hours (reaching mid to late log phase) prior to harvesting.

(b) Maltose fermentation was determined as described in Materials and Methods. All strains fermented maltose except 628-5B.

(c) Maltase activity was determined by measuring the rate of hydrolysis of PNPG and is expressed as nanomoles of substrate split per minute per milligram of protein at 30° C. Values are the mean and standard deviation when possible; if only two determinations were made, then the mean is given along with the two determinations.

(d) N.D., not determined.

Table 3.

Expected segregation patterns resulting from segregants of the Diploid W628 if MAL12 complements MAL62[(a)]

MAL Genotype		Maltose Fermentation	Leucine Phenotype
Parental Ditype			
mal62::LEU2	MAL12	+	+
mal63::LEU2	MAL12	+	+
MAL62	mal12::LEU2	+	+
MAL62	mal12::LEU2	+	+
Tetratype			
mal62::LEU2	MAL12	+	+
MAL62	mal12::LEU2	+	+
mal62::LEU2	mal12::LEU2	-	+
MAL62	MAL12	+	-
Nonparental Ditype			
mal62::LEU2	mal12::LEU2	-	+
mal62::LEU2	mal12::LEU2	-	+
MAL62	MAL12	+	-
MAL62	MAL12	+	-

(a) Diploid W628 was constructed by mating strains 613-1C (mal62::LEU2 MAL12) and 2b-20 (MAL62 mal12::LEU2). This table is presented in Dubin et al. (1985).

asci (PD), 4 non-parental ditypes (NPD), and 22 tetratypes (TT). Clearly, the inability of the doubly disrupted strains to ferment maltose confirms the predicted complementation. We call the mal1^0-linked MAL62 homologous gene MAL12. Subsequent work has demonstrated a functional MAL12 gene at other mal1^0 loci (results not shown; Charron and Michels, manuscript in preparation; Charron et al, 1986).

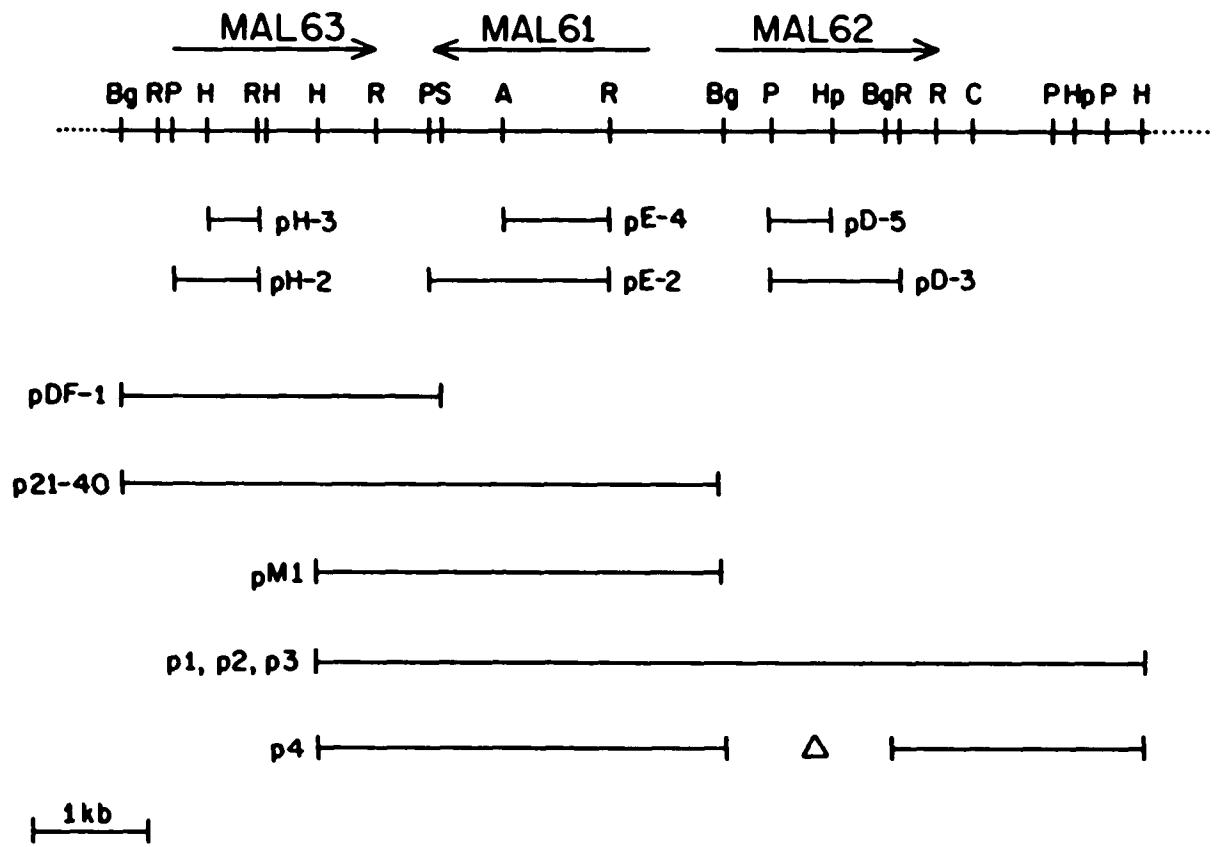
Physical confirmation of the double disruption was obtained for one mal$^-$ segregant, strain 628-5B (Appendix Table 4; also see Fig. 2 in Dubin et al., 1985). This strain is recessive to the wild type and is complemented (as a diploid) by MAL1g strains. Strain 628-5B is also complemented by the MAL62 gene; plasmid p3 (Fig. 3) was able to restore to strain 628-5B the ability to ferment maltose, while plasmid p4 (Fig. 3), which contained a deletion of the MAL62 gene, could not. Most MAL1p strains failed to complement strain 628-5B, although one MAL1p strain, 53-2C, did complement. Exceptional complementation by strain 53-2C has been observed before (Charron et al, 1986) and indicates unrecognized distinctions among MAL1p strains. Curiously, the ΔMAL62 Δ mal1^0 / mal1^0 heterozygous diploid (628-5B x 3-2B) failed to ferment maltose, despite the complementation by the mal1^0 locus observed in haploids. This particular mal1^0 strain, 3-2B, had in fact been demonstrated previously to carry a functional MAL12 gene at the mal1^0 locus (Charron et al., 1986). Although we presently do not understand this lack of complementation by a mal1^0 locus in the diploid, it is consistent with the following observation. We have noted that MAL6 mal1^0 / mal1^0 diploids either do not ferment or do so only very slowly. This is not due to diploid specific inhibition, since MAL6 mal1^0 / MAL6 mal1^0

Figure 3. 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 (⟶). Plasmids pH-3 and pH-2 contain fragments of the MAL63 gene; plasmids pE-2 and pE-4 contain fragments of the MAL61 gene; plasmids pD-3 and D-5 contain fragments of the MAL62 gene; all six fragments were subcloned into derivatives of plasmid pBR325.

Plasmid pDF1 (Needleman et al., 1984) contains the MAL63 gene subcloned into the TRP1 containing plasmid pLC544. Plasmid p21-40 (Needleman et al., 1984) contains the MAL61 and MAL63 genes subcloned into the 2 micron containing plasmid YEp13. Plasmid pM1 (Charron et al., 1986) contains the MAL61 gene subcloned into plasmid YEp13. Plasmids p1 (Needleman et al., 1984) and p2 contain the MAL61 and MAL62 genes subcloned (in both orientations) into YEp13. Plasmid p3 (Charron et al., 1986) contains the MAL61 and MAL62 genes subcloned into vector YIp5 + LEU2. (Plasmid YIp5 + LEU2 was constructed by inserting the 2.7 kb BglII fragment containing the yeast LEU2 gene from pCV9 into the BamHI site of plasmid YIp5.) Plasmid p4 (Charron et al., 1986) was constructed by deleting the 1.4 kb BglII fragment (containing the MAL62 gene) from plasmid p3. Plasmids p3 and p4 can be maintained as episomes in yeast by virtue of a functional ARS element located 3' to the MAL61 gene (Sylvestre and Michels, personnal communication).

Restriction endonuclease sites are abbreviated as in Figure 1.


MAL63
MAL61
MAL62
Bg RP H RH H R PS A R Bg P Hp BgR R C PHpP H
pH-3
pE-4
pD-5
pH-2
pE-2
pD-3
pDF-1
p21-40
pM1
p1, p2, p3
p4
Δ
1kb

diploids ferment. It is possible that the ratio of MAL6 / mal1^{0} loci may be a decisive factor but this is still unclear.

In the presence of maltose, strain 628-5B failed to synthesize maltase (Table 2). Northern blot analysis of RNA extracted from this strain following inducing growth conditions failed to detect even basal levels of the MAL62 - MAL12 homologous transcript (Fig. 2A). Clearly this RNA species is transcribed from both the MAL62 and MAL12 genes. Strain 628-5B continued to synthesize the MAL61 and MAL63 transcripts (Fig. 2B,C).

The inability of strain 628-5B to synthesize maltase suggested that the MAL62 and MAL12 genes encoded maltase. R. Needleman and co-workers have performed heat lability studies of the maltases synthesized by the MAL62 and MAL12 genes and provided evidence that the two genes encoded slightly different maltases (Dubin et al., 1985). Strain 2b-46 (ΔMAL62 MAL12) synthesized a considerably more heat labile maltase than either strains 2b-20 (MAL62 Δ MAL12) or 332-5A (MAL6 MAL12); similar results were obtained from an analysis of two tetratype tetrads derived from diploid W628 (results not shown but see Fig. 3 in Dubin et al., 1985). These results proved that the synthesis of a heat labile maltase was linked to the MAL12 gene located at the mal1^{0} locus and strongly suggested that both MAL62 and MAL12 encoded maltase structural genes.

Chapter 2: MAL61 appears to encode Maltose Permease

In order to determine the role of the MAL61 gene in maltose fermentation, this gene was disrupted from the genome of a MAL6 $mal1^0$ strain. Disruption plasmid pDM1 was constructed as described and contains an insertion of the yeast URA3 gene (along with 0.375 kb of pBR322 [nucleotides 1-375] and nearly 1.6 kb of DNA derived from the yeast LEU2 gene) into the 5' region of MAL61 (Fig. 1). SalI / BglII restricted pDM1 was used to transform strain 332-5A to Ura^+ and nearly 60% of the transformants were maltose nonfermenters (Appendix Table 3). Integrative disruption at the MAL6 locus was confirmed physically for two nonfermenters, strains aF and aG (Appendix Table 4), and this was further confirmed genetically for strain aF (Appendix Table 2, line 3). Strain aF is recessive to the wild type and is complemented (as a diploid) by MAL1g strains but not by MAL1p or $mal1^0$ strains. In addition, plasmids p21-40, p1, and pM1 (Fig. 3), which contained the MAL61 gene, restored the ability of strain aF to ferment maltose while the parental vector, YEp13 (Broach et al., 1979), did not.

Strain aF continues to induce for maltase, yet to levels somewhat lower than the undisrupted strain (Table 4). In contrast to the parent strain, which induces the synthesis of maltose permease, strain aF synthesizes intermediate levels of maltose permease constitutively, and this level does not increase in the presence of maltose (Fig. 4). The maltose nonfermenting phenotype of this strain may result from an inability to rapidly concentrate maltose. Nonetheless, sufficient maltose apparently enters the cell to effectively induce maltase.

Northern blot analysis of RNA extracted from strain aF revealed the following. In the undisrupted strain and in strain aF, the two MAL63

Table 4.

Maltase Activity in a MAL6 mal1^0 strain disrupted within the MAL61 gene and the mal1^0 locus[a]

Strain[b]	MAL Genotype	Maltase Activity[c] Galactose	Maltose	Gal/Mal
332-5A	MAL6 mal1^0	5 (±1)	279 (±49)	96 (±35)
aF	mal61::URA3 mal1^0	6 (±1)	147 (±34)	112 (118,106)
1b-8	mal61::URA3 mal1^0	7 (8,6)	11 (10,12)	8 (±1)
1b-7	mal61::URA3 mal1^0	[d]N.D.	10 (10,10)	N.D.
332-5AΔmal1^0-7	MAL61 mal1^0::LEU2	4 (4,4)	111 (143,79)	N.D.

(a) Cells were pregrown in YEP medium plus 2% galactose and diluted into YEP medium plus 2% galactose, or 2% maltose, or 2%galactose/2%maltose (Gal/Mal) and allowed to grow 8-9 hours (reaching mid to late log phase) prior to harvesting.

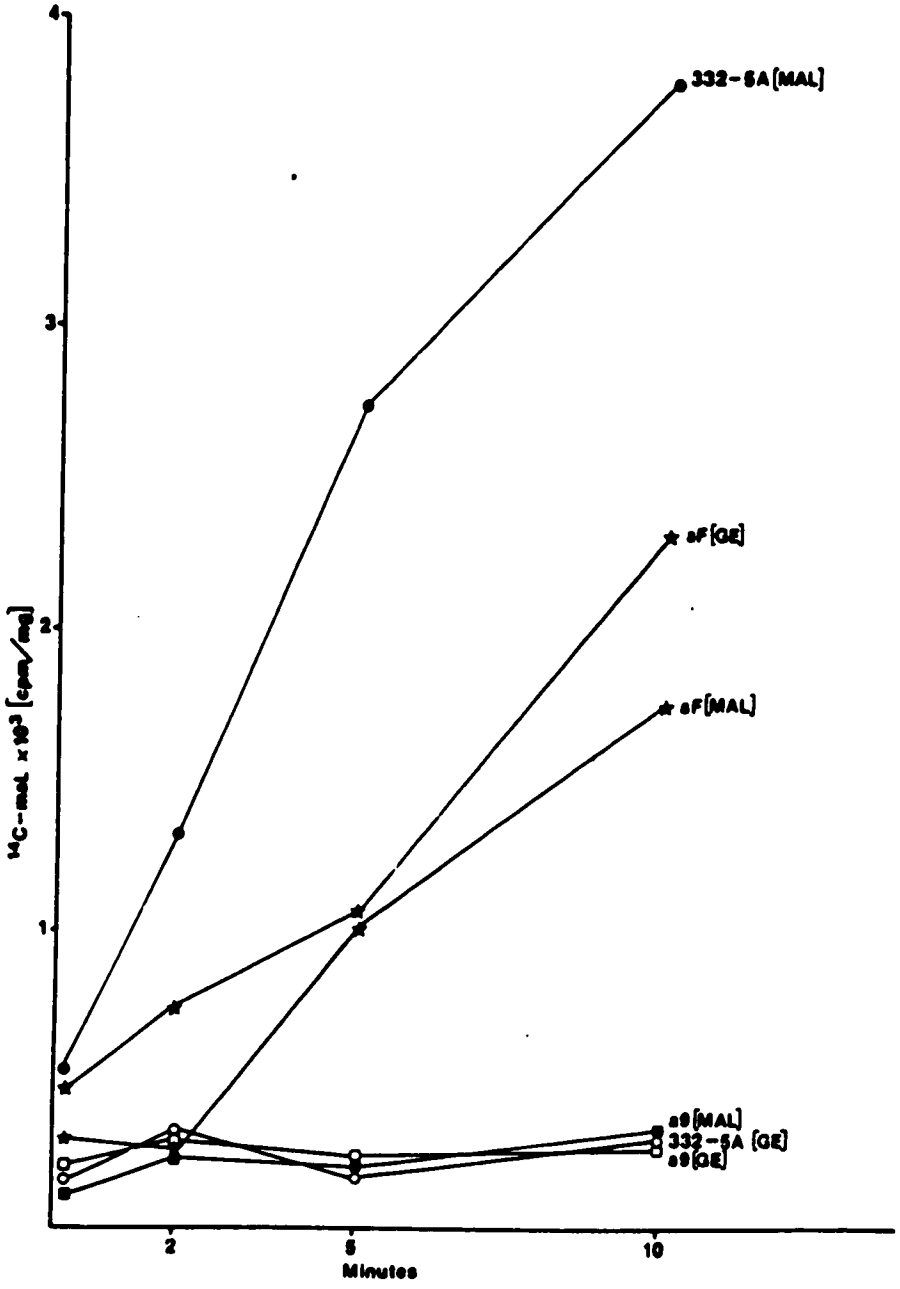
(b) Maltose fermentation was determined as described in Materials and Methods. Strains aF, 1b-7, and 1b-8 fail to ferment maltose.

(c) Maltase activity was determined by measuring the rate of hydrolysis of PNPG and is expressed as nanomoles of substrate split per minute per milligram of protein at 30°C. Values are the mean and standard deviation when possible; if only two determinations were made, then the mean is given along with the two determinations.

(d) N.D., not determined.

Figure 4. Maltose permease levels in strains 332-5A, a9, and aF.

Strains 332-5A (undisrupted MAL6 strain), a9 (mal63::URA3), and aF (which contains an insertion into the 5' region of MAL61) were grown under either uninducing (YEP plus 3% glycerol/2% ethanol [indicated by GE]) or inducing (YEP plus 2% maltose [indicated by MAL]) conditions and harvested in log phase. Maltose permease levels are expressed as picomoles of [^{14}C]maltose transported per milligram (dry weight) of cells. This figure is presented in Chang et al. (manuscript submitted for publication).


4
3
2
1
^{14}C-mol x 10^{3} [cpm/mg]
332-SA[MAL]
aF[GE]
aF[MAL]
a9[MAL]
332-SA [GE]
a9[GE]
2
5
10
Minutes

transcripts remained constitutively expressed (Fig. 5C); also, the 1.9 kb MAL62 - MAL12 transcript encoding maltase remained inducible (Fig. 5A), which is consistent with the ability of strain aF to induce maltase. The MAL61 homologous RNAs in the undisrupted parent strain 332-5A include a constitutive 2.4 kb transcript and two inducible transcripts of sizes 2.3 kb and 2.0 kb (Fig. 5B). In contrast however, strain aF synthesized two constitutively expressed MAL61 homologous transcripts, one being 2.4 kb and the other approximately 1.95 kb (Fig. 5, panel B). The level of expression of this 1.95 kb transcript did not increase in the presence of maltose and attained a level slightly less than that exhibited by the wild type, inducible 2.0 kb MAL61 RNA species. This novel 1.95 kb transcript was clearly MAL61 specific and not an RNA species homologous to the 0.375 kb of pBR322 DNA present on the integrated insert from pDM1 (and detected by pBR sequences located on probe pE-2) because 1. the novel RNA was not detected when pBR325 was used as a probe (results not shown) and 2. the novel RNA was detected when pE-4 (a plasmid which contained MAL61 sequences but lacked the 0.375 kb of pBR sequences present on the insert from pDM1) was used as probe (Fig. 5B, lanes 9 and 10). We therefore believed that the constitutive 1.95 kb MAL61 homologous transcript was an altered form of the normally inducible 2.0 kb MAL61 homologous transcript and that both its altered size and expression were directly attributable to the insertion. While we have not attempted to determine the basis for this unregulated expression of the MAL61 gene in strain aF, possible explanations are explored in the Discussion section. (I might also mention that a novel constitutively expressed, 0.6 kb transcript [not shown in Fig. 5] was also detected in strain aF, depending upon the

Figure 5. Disruption of the MAL61 gene and its effects on transcription of the MAL61, MAL62 and MAL63 genes.

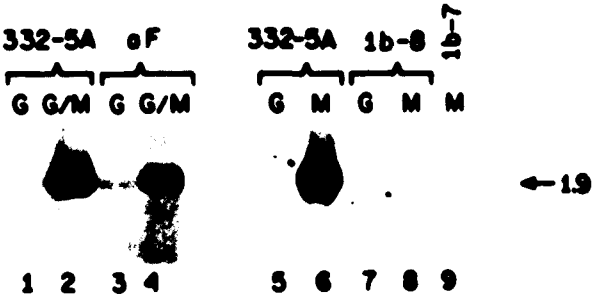
Yeast strains were grown under uninducing conditions (YEP medium plus 2% galactose [indicated by G]) or inducing conditions (YEP medium plus 2% maltose [indicated by M] or YEP medium plus 2% galactose/2% maltose [indicated by G/M]). Poly (A^+) RNA was isolated, fractionated on a formaldehyde/agarose gel, transferred to nitrocellulose, and probed with MAL61, MAL62 and MAL63 specific probes (Fig. 3). Panel A: MAL62 homologous transcripts were detected using probe pD-3 (lanes 1-4) or pD-5 (lanes 5-9). 10 mcg poly (A^+) RNA were loaded per track. Panel B: MAL61 homologous transcripts were detected using probe pE-2 (lanes 1-4) or pE-4 (lanes 5-15). Plasmids pE-2 and E-4 (Fig. 3) differ in two ways: 1. The size of the MAL61 insert is larger in pE-2, and 2. plasmid pE-2 contains the 0.375 kb EcoRI - BamHI pBR322 sequences (nucleotides 1-375) present in the pDM1 and pDM1b disruptions (and integrated at MAL61 in strains aF, 1b-7, and 1b-8) while pE-4 is deleted for these pBR sequences. 10 mcg poly (A^+) RNA were loaded per track except for lanes 12-15, which contained 25 mcg each. Panel C: MAL63 homologous transcripts were detected using probe pH-2. 20 mcg poly (A^+) RNA were loaded per track except for lane 6, which contained 10 mcg.

Strains:

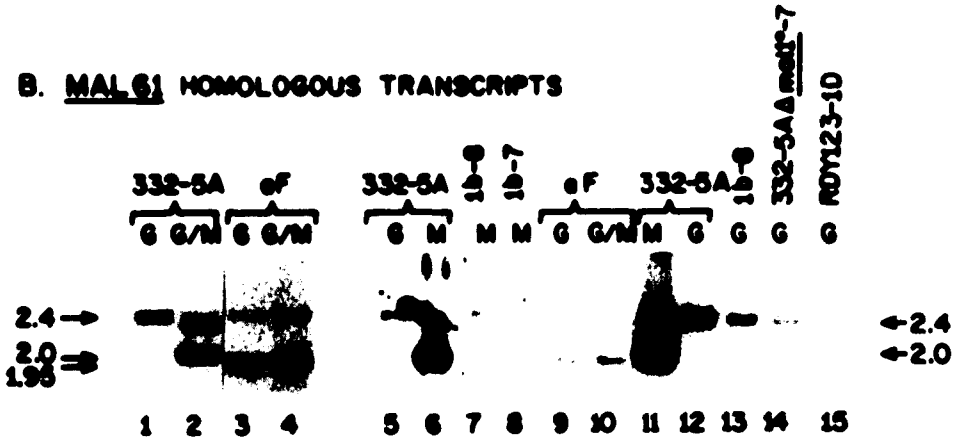
332-5A	MAL63 MAL62 MAL61 mal11 MAL12
aF	MAL63 MAL62 mal61::URA3 mal11 MAL12
1b-8 and 1b-7	MAL63 MAL62 mal61::URA3 mal11 MAL12
332-5AΔmal1^0-7	MAL63 MAL62 MAL61 mal11/mal12::LEU2
RDY123-1D	MAL63 MAL62 mal61::URA3 mal11/mal12::LEU2

DISRUPTION OF MAL61

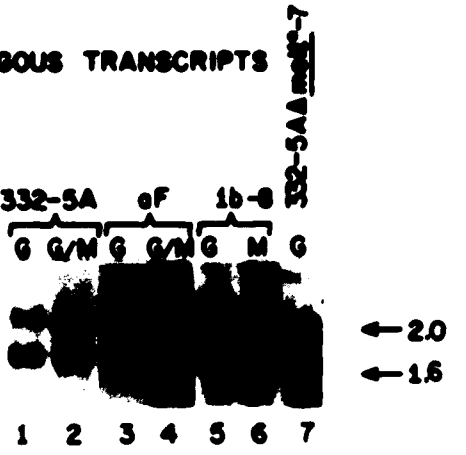
A. MAL62 HOMOLOGOUS TRANSCRIPTS



B. MAL61 HOMOLOGOUS TRANSCRIPTS



C MAL63 HOMOLOGOUS TRANSCRIPTS



probe used; this RNA species was not MAL61 specific but rather was homologous to the pBR sequences present within the disruption as this RNA was detected using pBR325 as probe but not pE-4 [results not shown]. A promoter for this 0.6 kb transcript may reside within the bacterial plasmid DNA present in this disruption and in fact this region of pBR322 has been shown to contain promoter activity in yeast (Breuning et al., 1982); it is also possible that the yeast LEU2 sequences present within the insertion could promote this pBR322 homologous transcript.)

Most conspicuous is the correlation between the novel expressions of the small MAL61 homologous RNA and maltose permease activity in strain aF. In the undisrupted strain both the 2.0 kb MAL61 homologous RNA and maltose permease are induced by maltose while in strain aF, both the novel 1.95 kb MAL61 homologous RNA and maltose permease are constitutively expressed and unresponsive to maltose. This result suggested that the MAL61 gene might encode (at least a component of) the maltose permease. Alternatively, MAL61 could encode a regulated regulator of maltose permease expression. In order to determine the effects of a deficiency of the MAL61 gene, a second disruption plasmid, pDM1b, was constructed (Fig. 1). Plasmid pDM1b contains an approximately 0.9 kb deletion within the cloned MAL61 gene which is replaced with the URA3 gene (plus 0.375 kb of pBR322 [nucleotides 1-375] and LEU2 sequences as in pDM1). SalI / BglII restricted pDM1b was used to transform strain 332-5A and greater than 90% of the Ura^+ transformants were maltose nonfermenters (Appendix Table 3). Southern analysis physically confirmed deletion of MAL61 in five nonfermenting transformants (Appendix Table 4) and integration at MAL6 was futher confirmed genetically for one isolate, strain 1b-8 (Appendix Table 2,

line 4). Strain 1b-8 is recessive to the wild type and complemented, in the diploid, by MAL1g strains but not MAL1p or mal1^0 strains. Additionally, plasmid pM1 (Fig. 3), which contains the MAL61 gene, restored the ability to ferment, while the parent vector, YEp13, did not. Plasmids p3 and p4 (Fig. 3) complemented this strain as well.

Strain 1b-8 does not induce for either maltase (Table 4) or maltose permease (the uninduced and induced levels of maltose permease were identical in strain 1b-8, being at least 15 fold lower than induced levels exhibited by the undisrupted parent strain 332-5A). A second isolate, 1b-7, similarly did not induce for maltase (Table 4). Transcript analysis of RNA isolated from strain 1b-8 revealed an inability to respond to maltose. Only basal (uninduced) levels of the MAL62 - MAL12 maltase mRNA are detectable following noninducing or inducing growth conditions (Fig. 5A); this is consistent with the inability of strain 1b-8 to synthesize high levels of maltase. As can be seen in Fig. 5B, while the 2.4 kb MAL61 homologous transcript remains constitutively expressed in strain 1b-8, the inducible 2.0 kb transcript is undetectable when grown in the presence of maltose and this is consistent with an inability to induce maltose permease. (The 2.3 kb MAL61 homologous transcript seen earlier is barely detectable in the undisrupted parental strain when probed with the MAL61 plasmid pE-4; see Fig. 5B, lanes 6 and 11). Clearly, the continued presence of the 2.4 kb MAL61 homologous species indicated that this RNA originated from some gene other than MAL61 or from some other gene and MAL61. The origin of this constitutive transcript will be discussed below. Strain 1b-8 continued to express the two MAL63 transcripts (Fig. 5C). (Like strain aF, strain 1b-8 and 1b-7 also expressed a 0.6 kb transcript that was pBR

specific.)

Results from the MAL61 deletion strain indicate that MAL61 encodes a maltose regulated, trans-acting positive function required for expression of the maltase mRNA and maltase, as well as maltose permease. The MAL61 gene could encode a transcriptional, post-transcriptional, translational, or post-translational regulator of maltose permease expression or it could encode the maltose permease. It would not be unexpected for inducible maltase expression to be dependent upon a functional maltose permease as induction is most likely an intracellular process. This hypothesis could explain the results of strain aF if it were assumed that a cis-acting alteration brought about a constitutive, intermediate level of expression of the small MAL61 homologous transcript resulting in a constitutive, intermediate level of expression of maltose permease; such a mutant would not be expected to alter the pattern of maltase expression. MAL61 most probably does not encode a positive transcriptional regulator of both maltase and maltose permease as only maltose permease is constitutively expressed in strain aF. While the MAL61 disruptions failed to conclusively demonstrate the function of the MAL61 gene, results of Cohen and co-workers (see Disucssion section; Goldenthal, et al., 1983; Cohen et al., 1984, 1985) strongly suggest that MAL61 encodes maltose permease and our results are consistent with their interpretation.

From an analysis of the MAL61 homologous transcripts in strain 1b-8, it appears that the MAL61 gene transcribes the inducible MAL61 homologous 2.0 kb mRNA which appears to encode a component of the maltose permease. However the origin and role of the constitutive 2.4 kb species remains unclear. Recent studies have revealed extensive

structural and functional homology between the MAL6 and MAL1 loci (Charron et al., 1986). Transcriptional analysis of the MAL1 locus demonstrated that the MAL1-linked homologue of MAL61, which we call MAL11, synthesized both a constitutive 2.4 kb transcript (at very low levels) and an inducible 2.0 kb transcript, and deletion disruption of MAL11 with pDM1b resulted in a loss of both RNAs (Charron et al., 1986). Like MAL1, the $mal1^0$ locus, in addition to containing sequences homologous to the MAL62 gene, also contains a region homologous to the MAL61 gene (unpublished observations; Charron and Michels, manuscript in preparation). We have also detected low levels of a 2.4 kb MAL61 homologous transcript in the $mal1^0$ strain 6-2A (results not shown). While the $mal1^0$ locus clearly does not encode a function capable of complementing a mutation within MAL61, these results suggested that the constitutive 2.4 kb RNA may be transcribed from the $mal1^0$ locus or from both the MAL61 gene and the $mal1^0$ locus.

In order to test these hypotheses, a deletion of the $mal1^0$ sequences homologous to MAL61 was required. With the recent cloning of a $mal1^0$ locus by M. Charron in this laboratory (Charron and Michels, manuscript in preparation) it became possible to construct a disruption plasmid that deleted a large portion of this locus. Disruption plasmid p$Dmal1^0$ was constructed as described (Fig. 6) and deleted nearly 6.4 kb of the $mal1^0$ locus (including the regions homologous to both MAL61 and MAL62) and replaced it with the LEU2 gene. HindIII / BamHI restricted p$Dmal1^0$ was used to transform strain 332-5A and Leu^+ transformants were obtained. All transformants fermented maltose (Appendix Table 3) and physical analysis revealed that nearly 40% (3/7) were disrupted at $mal1^0$ (Appendix Table 4). The remainder were most likely gene convertants at

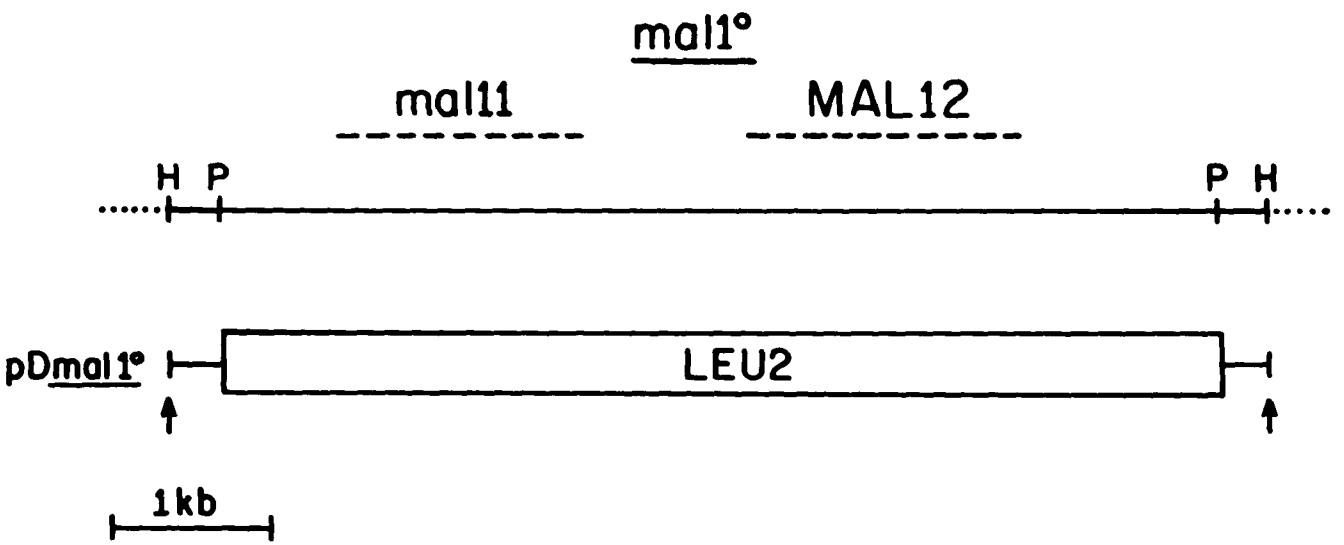
Figure 6. The MAL1-linked *mal1*^{0} locus and disruption plasmid pD*mal1*^{0}.

A partial restriction map of the MAL1-linked *mal1*^{0} locus is presented (——) along with the approximate locations of the *mal11* and MAL12 genes (as determined by homology with the MAL61 and MAL62 genes). This locus was cloned by M. Charron.

Disruption plasmid pD*mal1*^{0} is presented and its construction is described in Materials and Methods. The LEU2 gene within pD*mal1*^{0} was cloned as a 2.7 kb BglII fragment and is not drawn to scale.

(↑) indicates the restriction endonuclease sites used to restrict the disruption plasmid prior to transformation.

Restriction endonuclease sites are abbreviated as in Fig. 1.


mal1°
mal11
MAL12
H P
P H
pDmal1°
LEU2
1kb

LEU2 (see Appendix Table 4). Integration at mal1^0 was further confirmed genetically for one strain, 332-5AΔ mal1^0-7 (Appendix Table 2, line 5).

Strain 332-5AΔ mal1^0-7 (MAL61 mal1^0::LEU2) was force mated to strain 1b-8 (mal61::URA3 mal1^0) and the diploid, RDY123, was sporulated and dissected in order to obtain a Δ MAL61 Δ mal1^0 doubly disrupted strain. The Leu$^+$, Ura$^+$, mal$^-$ segregant RDY123-1D was isolated and the double disruption confirmed physically by Southern blot analysis (Appendix Table 4). In order to establish the origin of the constitutive MAL61 homologous 2.4 kb transcript, RNA was isolated from strains 332-5A (MAL6 mal1^0) , 1b-8 (Δ MAL61 mal1^0), 332-5AΔ mal1^0-7 (MAL6 Δmal1^0), and RDY123-1D (Δ MAL61 Δ mal1^0) following growth in YEP plus 2% galactose medium. Northern blot analysis of these RNAs using a MAL61 probe internal to the deleted region revealed the presence of the 2.4 kb transcript in all strains except strain RDY123-1D (Fig. 5B). This demonstrated that the 2.4 kb transcript was synthesized by both the MAL61 gene and the mal1^o locus.

Chapter 3: MAL63 Encodes a Positive Regulatory Function Required for Induction

Maltose permease and maltase and their respective MAL61 and MAL62 mRNAs are induced by maltose. Ten Berge et al. (1973) isolated maltose nonfermenting mutants from a MAL6 strain that were uninducible for both maltase and maltose permease but synthesized basal (uninduced) levels of both proteins. These mutations were recessive to the wild type and linked to MAL6, suggesting that MAL6 encoded a positive regulatory function required for induction by maltose. Naumov (1976) demonstrated that these mal6 nonfermeters were complemented by the MAL1p locus, concluding that MAL1p and MAL6p (the latter defined by the mal6 mutants) represented a polymeric series of positive regulatory genes. Needleman et al. (1984) later showed that the cloned MAL63 gene, like the MAL1p locus, could complement a MAL1g strain, strongly suggesting allelism between the mal6 gene identified by ten Berge et al. (1973a) and MAL63.

In order to directly demonstrate the role of this gene, MAL63 was disrupted from the genome of a MAL6 $mal1^0$ strain. Disruption plasmid pDM3 was constructed as described (Fig. 1) and contains a deletion of nearly 1 kb within MAL63 which is replaced by the URA3 gene. EcoRI / SalI restricted pDM3 was used to transform strain 332-5A to Ura^+ and 90% of the transformants were maltose nonfermenters (Appendix Table 3). Integration at MAL6 was confirmed physically for six nonfermenting transformants (Appendix Table 4) and further confirmed genetically for one isolate, strain a9 (Appendix Table 2, line 1). Physical confirmation of the MAL63 disruption in strain a9 appears on Fig. 13 (tracks 1 and 2).

Strain a9 is recessive to the undisrupted parent strain and

complemented by MAL1p strains but not by MAL1g or mal1^0 strains. Other transformants tested followed a similar complementation pattern. Additionally, the episomal plasmid p21-40, (which contains the MAL63 and MAL61 genes), restored the ability of strain a9 to ferment maltose, while plamids pM1 (which contains MAL61), p2 (which contains MAL61 and MAL62), and YEp13 (vector alone) did not (Fig. 3). Integration of pDF1, which contains the MAL63 gene (Fig. 3), was directed to the TRP1 locus (by restriction with BglII prior to transformation) and also restored the ability of strain a9 to ferment, while similar integration of the parental vector, pLC544 (Clarke and Carbon, 1980), did not.

Strain a9 is uninducible for maltase (Table 5) and maltose permease (Fig. 4). Another isolate, a8, was also uninducible for maltase (Table 5). As seen in Table 5, transformation of strain a9 with pDF1 (containing MAL63) restored not only the ability to ferment maltose but also the ability to induce for maltase (maltose permease activity was not measured). Northern blot analysis of RNA isolated from strain a9 reveals an inability to induce the maltose inducible transcripts (Fig. 7). MAL62 homologous probes detect only basal (uninduced) levels of the 1.9 kb MAL62 - MAL12 transcript under noninducing or inducing conditions (Fig. 7A). Clearly, the mal1^0- linked MAL12 gene is also under the control of the MAL63 gene product. MAL61 homologous probes detect only the 2.4 kb constitutive transcript, while neither the 2.3 kb nor 2.0 kb inducible transcripts are detected (Fig. 7B). These results correlate with an inability to induce the two maltose fermentative enzymes. The MAL63 homologous probe pH-2 detects a very low constitutive level of the 2.0 kb mRNA and, in place of the 1.6 kb transcript, a constitutive diffuse band centered around 1.4 kb is visible (Fig. 7C). The

Table 5.

Maltase Activity in a *MAL6* *mal1*^{0} strain disrupted within the *MAL63* gene[a]

Strain[b]	MAL Genotype	Maltase Activity[c] Galactose	Maltose	Gal/Mal
332-5A	*MAL6* *mal1*^{0}	5 (±1)	279 (±49)	96 (±35)
a9	*mal63::URA3* *mal1*^{0}	5 (4,6)	8 (10,6)	5 (5,5)
a8	*mal63::URA3* *mal1*^{0}	[d]N.D.	7 (6,8)	6 (5,7)
a9::pLC544-4	*mal63::URA3* *mal1*^{0} *trp1::pLC544(TRP1)*	7 (7,7)	9 (8,10)	N.D.
a9::pDF1-3	*mal63::URA3* *mal1*^{0} *trp1::pDF1(TRP1 MAL63)*	6 (6,6)	165 (161,169)	N.D.
a9::pDF1-4		7 (8,6)	206 (±151)	N.D.
a9::pDF1-6		4 (4,4)	124 (101,147)	N.D.

(a) Cells were pregrown in YEP medium plus 2% galactose and diluted into YEP medium plus 2% galactose, or 2% maltose, or 2%galactose/2%maltose (Gal/Mal) and allowed to grow 8-9 hours (reaching mid to late log phase) prior to harvesting.

(b) Maltose fermentation was determined as described in Materials and Methods. Strains a8, a9, and a9::pLC544 fail to ferment maltose.

(c) Maltase activity was determined by measuring the rate of hydrolysis of PNPG and is expressed as nanomoles of substrate split per minute per milligram of protein at 30° C. Values are the mean and standard deviation when possible; if only two determinations were made, then the mean is given along with the two determinations.

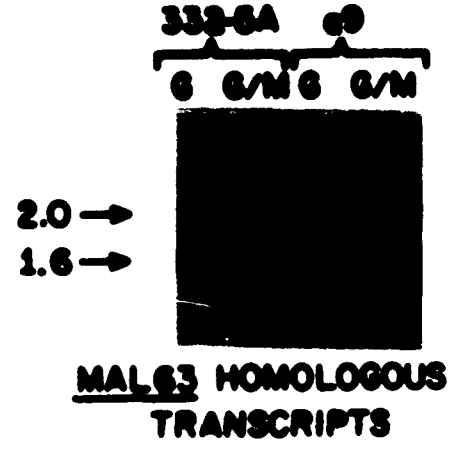
(d) N.D., not determined.

Figure 7. Disruption of the MAL63 gene in an inducible MAL6 strain and its effects on transcription of the MAL61, MAL62, and MAL63 genes.

Yeast strains were grown under uninducing conditions (YEP medium plus 2% galactose [indicated by G]) or inducing conditions (YEP medium plus 2% galactose/2% maltose [indicated by G/M]). Poly (A^+) RNA was isolated, fractionated on a formaldehyde/agarose gel, transferred to nitrocellulose, and probed with MAL specific probes. Strain 332-5A is the inducible, undisrupted parental MAL63 MAL62 MAL61 MAL12 strain and strain a9 is a derivative of strain 332-5A and contains a deletion of the MAL63 gene.

MAL62 homologous transcripts were detected using probe pD-3 (Fig. 3); 10 mcg poly (A^+) RNA were loaded per track. MAL61 homologous transcripts were detected using probe pE-2 (Fig. 3); 10 mcg poly (A^+) RNA were loaded per track. MAL63 homologous transcripts were detected using probe pH-2 (Fig. 3); 20 mcg poly (A^+) RNA were loaded per track.

DISRUPTION OF MAL63



transcripts centered around 1.4 kb may represent transcription initiation at the MAL63 promoter site and termination at various points within the insertion. These results demonstrate that the MAL63 gene encodes a trans-acting, positive regulatory function that is required for the maltose induced expression of maltase, maltose permease, and their respective transcripts.

This data also suggests that the 2.0 kb MAL63 homologous transcript may originate from sequences adjacent to the MAL63 gene (and detected by the MAL63 probe pH-2) or may originate from elsewhere in the genome. Clearly, its reduced expression in strain a9 suggests control by the MAL63 gene product. MAL63 sequences are not unique to the MAL63 gene. Southern blot analysis of genomic DNA isolated from strain 332-5A and probed with the MAL63 containing plasmid pH-2 or pH-3 (Fig. 3) reveal one strong signal (which is MAL63) and two weaker signals (Fig. 13, lane 2). Analysis of the cloned $mal1^0$ locus revealed no homology to MAL63 sequences (Charron and Michels, manuscript in preparation). The 2.4 kb fragment with homology to MAL63 (Fig. 13) has recently been found to be linked to MAL6 and located about 3.5 kb to the left of MAL63 and will be discussed below. This MAL63 homologous region encodes a gene required for constitutive expression of the maltose fermentative enzymes in MAL6-linked constitutive strains.

Strain a9 was used to determine the chromosomal linkage group to which MAL6 belonged. Using the procedure described by Klapholtz and Esposito (1982) and their spoll tester strains, it was demonstrated that the MAL6 locus maps to chromosome VIII (see Appendix Table 1). This confirmed the mapping results of H. Stark, who demonstrated linkage of MAL6 to chromosome VIII using a chromosome loss technique (Ph.D Thesis, Columbia University, 1981).

Chapter 4: Identification of a New MAL6-linked Regulatory Function

Constitutive Expression of the Maltose Fermentative Enzymes Does Not Require MAL63. Maltose fermenting strains that constitutively express high levels of maltase and maltose permease have been isolated and the mutation responsible for constitutivity is linked to a dominant MAL locus. Constitutive mutations have been isolated at all five dominant MAL loci. As described, these strains were generally isolated as maltose fermenting revertants of maltose nonfermenting (uninducible) mutants (Zimmerman and Eaton, 1974; ten Berge et al., 1973b,1974) although constitutive strains have also been isolated directly from inducible strains (Needleman and Eaton, 1974; perhaps Kahn and Eaton, 1971).

Reversion of the mal6 nonfermenting mutants (ten Berge et al., 1973a) led to the isolation of maltose fermenting strains that constitutively expressed both maltase and maltose permease (ten Berge et al, 1973b;1974). Constitutivity was linked to MAL6 and recessive to the inducible MAL6 locus.

Strain 8-2B is a MAL6-C mal1^0 derivative of one of these constitutive strains, strain C2, and carries the MAL6-C2 allele (ten Berge et al., 1973b). Its construction is described in the Materials and Methods and it is genotypically MAL6-C2 mal6-13 mal1^0; mal6-13 refers to the original mal6 mutation conferring an uninducible phenotype and this has been demonstrated by Chang et al. (manuscript submitted for publication) to be mutant within MAL63 and is thus mal63-13. MAL6-C2 refers to the second mutation restoring the ability to ferment and conferring constitutive expression of the maltose fermentative enzymes. Strain 8-2B expresses maltase and maltose

permease constitutively (Table 6). To determine whether this phenotype reflects unregulated transcription or accumulation of the MAL61 (most likely maltose permease) and MAL62 (maltase) transcripts, RNA was isolated from strain 8-2B and examined by Northern blot analysis. This study revealed that the 2.0 kb MAL61 and 1.9 kb MAL62 homologous mRNAs were expressed at high levels under noninducing and inducing growth conditions (Fig. 8). This is in contrast to the inducible nature of these transcripts in wild type strains (Fig. 7). The two MAL63 transcripts as well as the 2.4 kb MAL61 homologous transcript continued to be expressed constitutively in strain 8-2B (Fig. 8), as in the inducible wild type strain.

MAL6-C / mal6 heterozygous diploids exhibited various degrees of suppression of constitutivity and restoration of inducibility, depending upon the allele combination used (ten Berge et al, 1973b,1974). These results suggested to ten Berge and co-workers that the observed variability was due to interallelic complementation between interacting mal6 and MAL6-C gene products (ten Berge et al., 1974). Chang et al. (manuscript submitted for publication) have demonstrated that the mal6 nonfermenting mutants of the type isolated by ten Berge et al (1973a) were mutant within the MAL63 gene. MAL63 encodes a positive trans-acting regulatory function required for the induction of maltase, maltose permease, and their respective transcripts (ten Berge et al., 1973a; Chang et al., manuscript submitted for publication; data presented above). Since deletion of MAL63 results in an uninducible phenotype (see strain a9), the MAL63 gene product is not a pure repressor. If MAL6-C were an allele of the MAL63 gene it would likely encode a positive activator that was no longer dependent upon maltose.

To test this directly, the MAL63 gene was deleted from constitutive strain 8-2B.

EcoRI restricted pDM3 (Fig. 1) was used to transform strain 8-2B and Ura^+ transformants isolated. All transformants continued to ferment maltose (Appendix Table 3). Disruption of MAL63 was confirmed physically for five isolates and a sixth isolate underwent gene conversion at URA3 (Appendix Table 5). Disruption was further confirmed genetically for two of these isolates, 8-2BΔ63-1 and 8-2BΔ63-2 (Appendix Table 2, lines 6 and 7). Deletion of MAL63 in strain 8-2B does not alter the constitutive expression of maltase or maltose permease (Table 6). Northern blot analysis of RNA isolated from strains 8-2BΔ63-1 (Fig. 8) and 8-2BΔ63-2 (results not shown) revealed constitutive expression of the MAL61 and MAL62 homologous transcripts. This is consistent with constitutive expression of maltase and maltose permease. The MAL63 homologous probe pH-2 continued to detect the 2.0kb transcript, but not the 1.6 kb transcript (Fig. 8). In place of the 1.6 kb RNA is a faint diffuse band at about 1.5 kb. and a band at about 1.4 kb. The presence of the 2.0 kb species suggests, but does not prove, that the 2.0 kb MAL63 homologous RNA species is derived from a gene outside of MAL63. In contrast, deletion of MAL63 in the inducible strain 332-5A resulted in maltose nonfermenters that were uninducible for the normally maltose inducible transcripts and enzymes (see strain a9 above). Together, these results demonstrated that MAL63 is not required for constitutivity in strain 8-2B and that the MAL6-C2 mutation lies outside of the MAL63 gene.

If MAL63 is not required for MAL6-linked constitutivity, then it was believed possible to revert the uninducible ΔMAL63 strain a9 to a

Table 6.

Maltase and maltose permease levels in constitutive strains 8-2B and 8-2B Δ63[a]

Strain[b]	Maltase Activity[c]		Maltose Transport[d]	
	Uninduced	Induced	Uninduced	Induced
8-2B	265 (±130)	498 (±44)	365	381
8-2B Δ63-1	218 (212,224)	N.D.	N.D.[e]	N.D.
8-2B Δ63-2	170 (178,163)	509 (432,587)	268	432
332-5A	5 (±1)	279 (±49)	10	460
a9	5 (4,6)	8 (8,10)	(f)	(f)

(a) For maltase assays, cells were pregrown in YEP medium plus 2% galactose and diluted into YEP medium plus 2% galactose (uninduced) or 2% maltose (induced) and allowed to grow 8-9 hours (reaching mid to late log phase) prior to harvesting. For maltose transport assays, cells were grown about 14 hours to mid-log phase in either YEP medium plus 2% galactose (uninduced) or 2% maltose (induced).

(b) Of the five strains presented, all but strain a9 ferment maltose.

(c) Maltase activity was determined by measuring the rate of hydrolysis of PNPG and is expressed as nanomoles of substrate split per minute per milligram of protein at 30° C. Values are the mean and standard deviation when possible; if only two determinations were made, then the mean is given along with the two determinations.

(d) Maltose transport is reported as picomoles of [^{14}C]maltose transported per 2 min per milligram (dry weight) of cells.

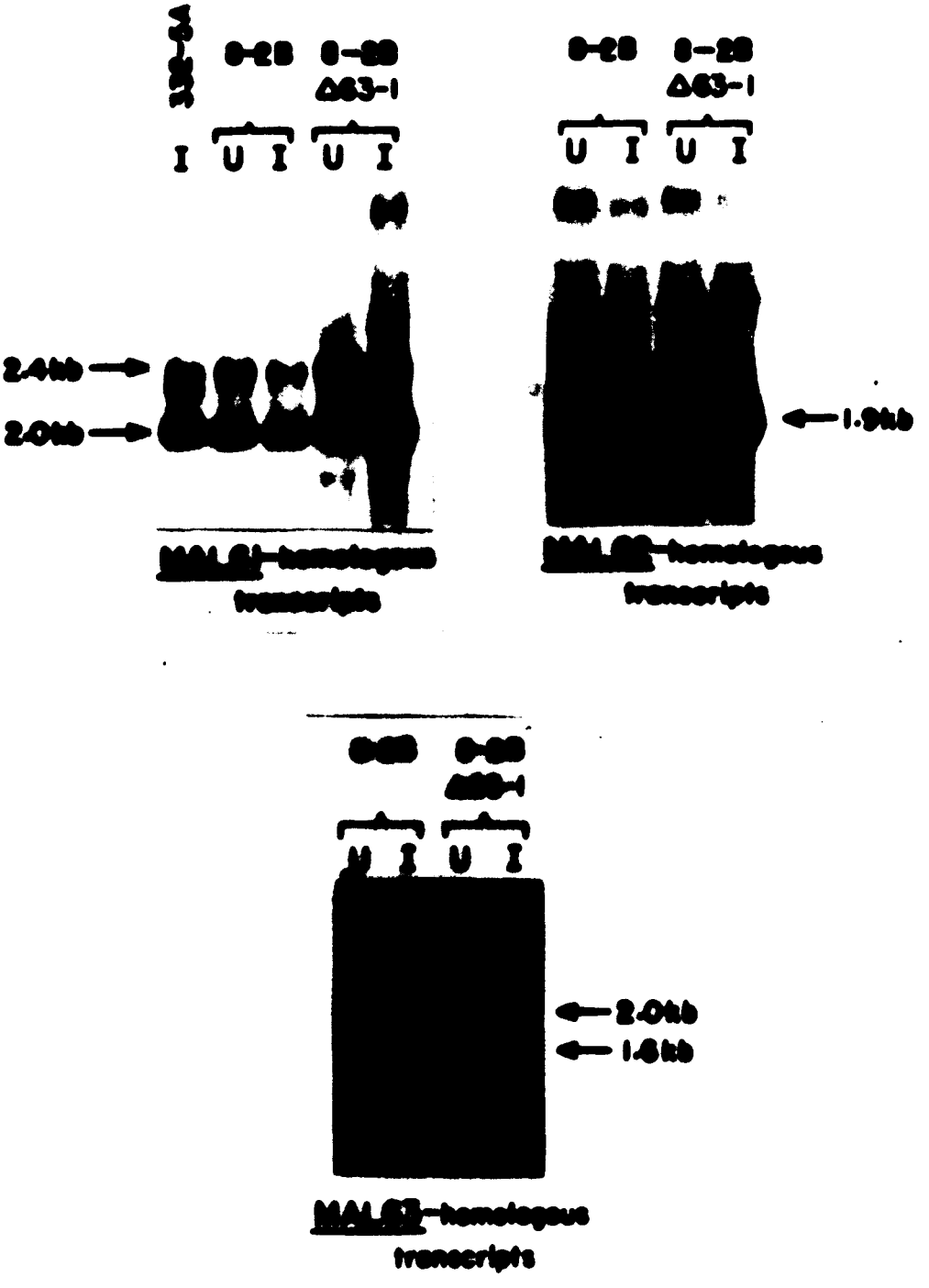
(e) N.D., not determined.

(f) The rate of maltose transport by strain a9 is presented in Figure 4. Strain a9 is a derivative of 332-5A and does not induce due to a deletion of the MAL63 gene.

Figure 8. Analysis of the MAL61, MAL62, and MAL63 transcripts in constitutive strains 8-2B and 8-2BΔ63-1.

Yeast strains were grown under uninducing conditions (YEP medium plus 2% galactose [indicated by U]) or inducing conditions (YEP medium 2% galactose/2% maltose [indicated by I]). Poly (A^+) RNA was isolated from constitutive strains 8-2B and 8-2BΔ63-1, fractionated on a formaldehyde/agarose gel, transferred to nitrocellulose, and probed with MAL61, MAL62, and MAL63 specific probes (Fig. 3). Strain 8-2B contains the undisrupted MAL6-C2 locus and is thus genotypically MAL6-C2 mal63-13; strain 8-2BΔ63-1 is a derivative of 8-2B, contains a deletion of the mal63-13 gene, and is thus genotypically MAL6-C2 mal63-13::URA3.

MAL62 homologous transcripts were detected using probe pD-3; 10 mcg poly (A^+) RNA were loaded per track. MAL61 homologous transcripts were detected using probe pE-2; 10 mcg poly (A^+) RNA were loaded per track. MAL63 homologous transcripts were detected using probe pH-2; 20 mcg poly (A^+) RNA were loaded per track.


U I U I
2.4kb
2.0kb
1.9kb
transcripts
U I U I
2.0kb
1.6kb
transcripts

maltose fermenting constitutive strain. As described in Dubin et al. (1986), R. Needleman and co-workers were able to select for Mal^+ revertants of strain a9, and 16/16 fermenting revertants tested expressed maltase constitutively. One strain, R10, was further characterized by them. R10 was further shown to express maltose permease constitutively and genetic analysis demonstrated that constitutivity was linked to MAL6. Constitutivity in R10 is partially dominant to the wild type MAL6 (inducible) and Δ MAL63 (uninducible) loci when assayed for maltase and fully dominant when assayed for maltose permease (see Table 5 in Dubin et al., 1986).

Southern blot analysis of strain R10 confirmed the continued presence of the original mal63::URA3 gene disruption from strain a9 (Appendix Table 6). Northern blot analysis of RNA isolated from this strain revealed constitutive expression of the MAL61 and MAL62 transcripts (Fig. 12A). The MAL63 homologous probe pH-2 (Fig. 3) detected four transcripts, 2.0kb, 1.5 kb, 1.4 kb, and 0.65 kb. (Fig. 12B); a smaller probe, pH-3 (Figs. 1 and 3), which contains a DNA fragment deleted in the MAL63::URA3 disruption, detects only the 2.0 kb transcript and a trace of the 1.4 kb. transcript (results not shown). Clearly, these 2.0 kb and 1.4 kb transcripts must originate from a gene outside of MAL63 and will be discussed in greater detail below.

Constitutivity is Trans-Acting and Not Within the MAL61 or MAL62 Genes. MAL6-linked constitutivity could be due to an alteration in a (putative) common, upstream activating sequence located within the MAL61-MAL62 intergenic region. Since the two structural genes are divergently transcribed and both transcripts initiate within a 0.8 kb region, it is

possible that a single cis-acting mutation could activate both genes. However, such a mutation would not be expected to result in a recessive constitutive allele like MAL6-C. Alternatively, constitutivity may reside within either structural gene, or within a new, as yet uncharacterized regulatory gene or site.

To begin to localize the MAL6-C2 mutation, we isolated the region containing the MAL61 and MAL62 genes from constitutive strain 8-2B. Southern analysis of DNA isolated from strain 8-2B (using the maltase gene as probe) revealed two MAL homologous HindIII fragments, 7.3 kb and 7.0 kb. Since strains 8-2B and the original constitutive isolate, strain C2, had only the 7.3 kb HindIII band in common (results not shown) and all mal1^0 strains examined to date contained a MAL1-linked 7.0 kb HindIII band (Needleman and Michels, 1983; Michels and Needleman, 1983, 1984; unpublished results), we assumed, and later genetically demonstrated, that the 7.3 kb HindIII fragment was linked to MAL6 in this constitutive strain (see strain 8-2BΔCΔH-6 below). Since the wild type MAL6 locus is also linked to a 7.3 kb HindIII fragment (Needleman and Michels, 1983), we further assumed that the MAL6 locus cloned by Federoff et al (1982) and the MAL6-C2 locus were basically homologous. We made use of this homology and cloned the MAL61-MAL62 region from constitutive strain 8-2B by targeted integration to the MAL6-C2 locus followed by plasmid rescue as described by Orr-Weaver et al. (1983).

The MAL61-MAL62 region from strain 8-2B was recovered as follows. Plasmid pY6ΔCΔH was constructed as described (Dubin et al., 1986; see Fig. 1) and contains a 1.6 kb ClaI - HindIII fragment derived from the wild type MAL6 locus (located 3' to MAL62) subcloned into YIp5 (Botstein

et al., 1979). Integration of pY6ΔCΔH was directed to MAL6 by restriction with HpaI prior to transforming strain 8-2B. Ura$^+$ transformants were examined (Appendix Table 3) and integration at MAL6 was confirmed for one isolate, 8-2BΔCΔH-6, by both physical (Appendix Table 5) and genetic analyses (Appendix Table 2, line 9). This strain is a maltose fermenter and continues to synthesize maltase constitutively (Table 7). Total genomic DNA isolated from strain 8-2BΔCΔH-6 was digested with HindIII, ligated under dilute conditions, and used to transform bacterial strain RR1 to ampicillin resistance. In this way, the MAL61-MAL62 genes flanking the plasmid insert in strain 8-2BΔCΔH-6 were recovered on plasmid pRD1 (Fig. 9).

Restriction mapping of pRD1 revealed only two minor differences between the constitutive and wild type sequences. The EcoRI site near the 5' end of the MAL61 gene was missing and a new BglII site within MAL61 was acquired in pRD1 (Fig. 9). From the data presented below, it appears that these site differences are not functionally significant and represent polymorphisms between the two MAL6 loci. The MAL61 and MAL62 genes on pRD1 were localized by hybridization to cloned MAL6 probes (data not shown).

MAL6-linked constitutivity in strain 8-2B does not require the MAL63 gene function (see 8-2BΔ63-1 above). Therefore, if the MAL6-C2 constitutive gene or site had been cloned on the yeast DNA insert of plasmid pRD1, it might be expected to complement a nonfermenting mal63 strain. Plasmid pRD2 was constructed by subcloning the 7.6 kb HindIII - BamHI insert of pRD1 (containing the MAL61 and MAL62 genes isolated from the MAL6-C2 locus) into YEp13 in order to construct a more stable episomal yeast plasmid. Plasmids pRD2 and p2 (Fig. 3; plasmid p2

Figure. 9. The MAL6 locus of constitutive strain 8-2B and restriction maps of plasmids pRD1 and pRD3.

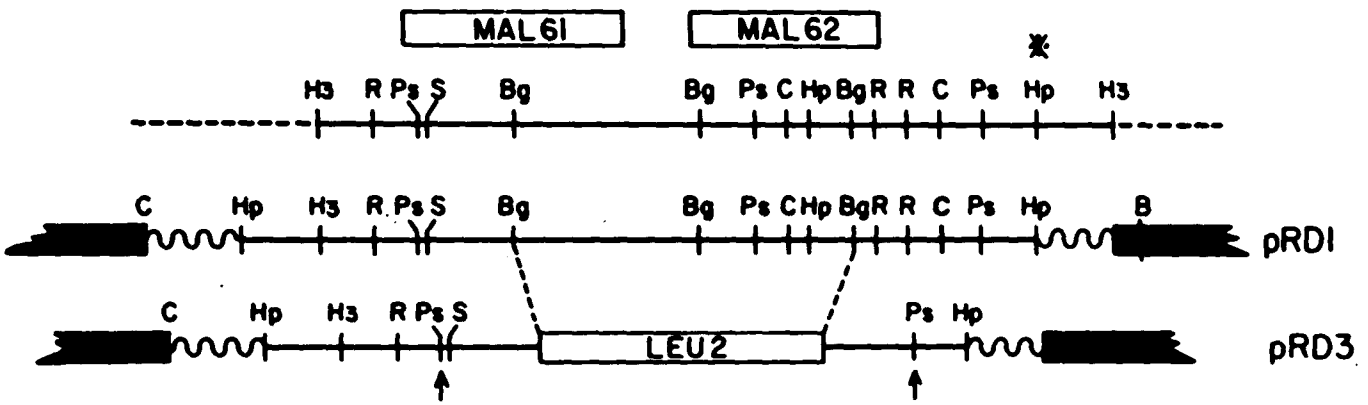
A partial restriction map of the MAL6-C2 locus of strain 8-2B is presented (——). Regions of homology to the MAL61 and MAL62 genes are indicated and were determined by Southern blot analysis using wild type MAL6 specific probes. Plasmid pRD1 contains the MAL6-C2 locus of strain 8-2B and was recovered following targeted integration at the MAL6-C2 locus (using homology to the wild type MAL6 sequences present on plasmid pY6ΔCΔH [Fig. 1]) and subsequent plasmid rescue as described in the Materials and Methods. Disruption plasmid pRD3, a derivative of plasmid pRD1, was constructed as described in the Materials and Methods and contains a deletion within the MAL61 and MAL62 genes. Its restriction map is also presented.

(✕) indicates the HpaI restriction site used to restrict plasmid pY6ΔCΔH prior to transforming strain 8-2B, thus directing integration to the MAL6-C2 locus.

(∿∿) indicates the wild type sequences derived from pY6ΔCΔH and present on plasmids pRD1 and pRD3.

(↑) indicates the restriction endonuclease sites used to restrict plasmid pRD3 prior to transformation.

Restriction endonuclease sites are abbreviated as in Fig. 1, except HindIII is represented by H3.


MAL61
MAL62
H3 R Ps S Bg
Bg Ps C Hp Bg R R C Ps Hp H3
C Hp H3 R Ps S Bg
Bg Ps C Hp Bg R R C Ps Hp B
pRD1
C Hp H3 R Ps S
LEU2
Ps Hp
pRD3

contains the wild type MAL61 and MAL62 genes in YEp13) were separately transformed into the ΔMAL63 strain a9, as well as the MALp strain 236-2A. Plasmids pRD2 and p2 were unable to complement strain a9 but both complemented the MAL1p strain and restored the ability to ferment. While complementation of the MAL1p strain indicated that both plasmids contained functional MAL6g activity, the inability of pRD2 to complement strain a9 suggested that the constitutive mutation MAL6-C2 had probably not been cloned.

In order to eliminate possible cloning artifacts or other unforeseen technical problems (ie.: cis requirements or high copy plasmids) we returned to studying constitutivity at the genomic MAL6-C2 locus. Constitutive strain 8-2B contains in its background the MAL1-linked $mal1^0$ locus and thus a second functional maltase structural gene (MAL12). In the wild type MAL6 $mal1^0$ strain 332-5A, we demonstrated that an unlinked MAL12 gene may be induced by maltose (data presented above; Dubin et al., 1985). Since strain 8-2B also contains the MAL62 and MAL12 genes, we attempted to determine whether one or both maltase genes were constitutively expressed. If MAL12 was constitutively expressed then constitutivity would be a trans-acting function. As described below, the experimental design used to determine whether constitutivity was trans-acting also allowed us to examine whether the MAL61 or MAL62 genes were required for constitutivity.

To determine whether the MAL12 gene was constitutively expressed in strain 8-2B, the MAL62 gene was deleted at the genomic MAL6-C2 locus. This was accomplished using disruption plasmid pRD3 (Fig. 9). Plasmid pRD3 was constructed by deleting about 3.1 kb from pRD1 and replacing it with the LEU2 gene. Both the MAL61 and MAL62 genes should be disrupted

in this construct. PstI restricted pRD3 was used to transform strain 8-2B to Leu^+ and 25% were maltose nonfermenters (Appendix Table 3). Five mal^- isolates were examined physically and shown to carry the predicted MAL6 disruption (Appendix Table 5). (Five Mal^+ transformants were not disrupted at MAL6 and were assumed to have undergone gene conversion at LEU2). Disruption at MAL6 was further confirmed genetically for one mal^- transformant, 8-2BΔ61/Δ62-5 (Appendix Table 2, lines 8 and 9). While the uninduced maltase level found in strain 8-2BΔ61/Δ62-5 was reduced compared to that of the undisrupted parent strain, a significant amount of constitutively expressed maltase remained (Table 7), suggesting that the MAL12 gene was constitutively expressed. Two other transformants exhibited a similar phenotype (Table 7). If this interpretation was correct, then deletion of the MAL12 gene in strain 8-2BΔ61/Δ62-5 should significantly reduce the level of uninduced maltase even further.

As a first step in constructing the doubly disrupted strain, the MAL12 locus was deleted from strain 8-2B. PstI - HindIII - BamHI restricted pDM2b (Fig. 1) was used to transform strain 8-2B to Leu^+ and the maltose fermenting strain 8-2BΔ12-6 was isolated (Appendix Table 3). Disruption of the MAL12 locus in this strain was confirmed by physical analysis (Appendix Table 5). Strain RDY101-1B was constructed and is isogenic to 8-2BΔ12-6 except at the mating type locus. The mal12::LEU2 disruption was confirmed genetically in strain RDY101-1B (Appendix Table 2, line 10). Strain RDY101-1B is a maltose fermenter and continues to synthesize high levels of maltase constitutively (Table 7).

The two single disruption strains, 8-2BΔ61/Δ62-5 and RDY101-1B,

Table 7.

Uninduced Maltase Activity in constitutive strain 8-2B containing deletions within the MAL61/MAL62 and MAL12 genes[a]

Strain[b]	MAL Genotype	Uninduced[c] Maltase Activity
8-2B	MAL6-C2 mal1^0	265 (±130)
8-2BΔCΔH-6	MAL6-C2::pY6ΔCΔH mal1^0	298 (331,266)
8-2BΔ61/Δ62-5	MAL6-C2 mal61/mal62::LEU2 mal1^0	77 (±36)
8-2BΔ61/Δ62-6	MAL6-C2 mal61/mal62::LEU2 mal1^0	57 (±15)
8-2BΔ61/Δ62-7	MAL6-C2 mal61/mal62::LEU2 mal1^0	72 (71,73)
RDY101-1B[d]	MAL6-C2 mal1^0::LEU2	192 (192,192)

(a) Cells were pregrown in YEP medium plus 2% galactose and diluted into YEP medium plus 2% galactose and allowed to grow 8-9 hours (reaching mid to late log phase) prior to harvesting.

(b) Maltose fermentation was determined as described in Materials and Methods. Strains 8-2BΔ61/Δ62-5, -6, and -7 fail to ferment maltose.

(c) Maltase activity was determined by measuring the rate of hydrolysis of PNPG and is expressed as nanomoles of substrate split per minute per milligram of protein at 30° C. Values are the mean and standard deviation when possible; if only two determinations were made, then the mean is given along with the two determinations.

(d) Strain RDY101-1B is isogenic to strain 8-2BΔ12-6 except at the mating type locus.

were crossed and the resulting diploid, RDY103, was sporulated and dissected. By monitoring the leucine and maltose phenotypes of the segregants it is (often) possible to determine whether the undisrupted or disrupted MAL6 and MAL12 genes are present. Tetrad 1 (Table 8) is a non-parental ditype ascus and therefore both Leu^+ mal^- spores, 1A and 1C, should contain the ΔMAL61/ΔMAL62 and ΔMAL12 disruptions (provided no gene conversion occured). Constitutive maltase levels in these spore colonies were nearly zero (Table 8). These findings indicated that the MAL12 gene is constitutively expressed in strain 8-2B and is responsible for the constitutively expressed maltase in 8-2BΔ61/Δ62-5. The results from three other tetratype asci were consistent with these findings. Clearly, constitutivity at MAL6-C2 is trans-acting. In addition, constitutivity at MAL6-C2 does not require the MAL61 or MAL62 genes.

Constitutivity in strain R10 is also a trans-acting function and does not require the MAL61 or MAL62 genes. The MAL61 and MAL62 genes were deleted from strain R10 using PstI - BamHI restricted pRD3 (Fig. 9) as above. Leu^+ transformants were isolated (Appendix Table 3) and Southern analysis confirmed disruption at MAL6 in three mal^- isolates (Appendix Table 6). One transformant, R10Δ61/Δ62-12, was further characterized genetically and the disruption at MAL6 was confirmed (Appendix Table 2, line 11). When grown under noninducing conditions, this strain constitutively synthesizes nearly 320 units of maltase (Table 9), presumably from the MAL12 locus. A second transformant also continued to synthesize maltase constitutively (Table 9). Most likely, these strains are unable to ferment maltose due to an absence of the presumed maltose permease (MAL61) gene. To be certain of this however,

Table 8.

Uninduced maltase activity in Segregants from diploid RDY103[a]

Tetrad Segregant	Leucine Phenotype	Maltose Fermentation[b]	Uninduced Maltase Activity[c]
1A	+	-	4 (2,7)
B	-	+	222 (132,312)
C	+	-	3 (2,4)
D	-	+	228 (281,175)
5A	-	+	159
B	+	+	103
C	+	-	2
D	+	-	149
8A	-	+	226 (266,186)
B	+	-	4 (3,6)
C	+	-	93 (82,104)
D	+	+	217 (219,215)
10A	+	-	4
B	-	+	311
C	+	-	70
D	+	+	199
14A	+	-	87
B	+	+	200
C	+	+	226
D	+	-	91

(a) Diploid RDY103 was constructed by mating strains 8-2BΔ61/Δ 62-5 (MAL6-C2 mal61/mal62::LEU2 MAL12) and RDY101-1B (MAL6-C2 mal12::LEU2). Strain RDY101-1B is isogenic to strain 8-2BΔ12-6 except at the mating type locus. The resulting diploid was sporulated and dissected, and segregants analyzed. Cells were pregrown in YEP medium plus 2% galactose, diluted into YEP medium plus 2% galactose, and allowed to grow 8-9 hours (reaching mid to late log phase) prior to harvesting.

(b and c) Maltose fermentation and maltase activity were determined as described in Table 7 and Materials and Methods. Maltase activities were assayed twice for tetrads 1 and 8 and the mean (along with the two determinations in parentheses) is given.

Table 9.

Uninduced maltase activity in constitutive strain R10 and inducible strain 332-5A containing a deletion of the MAL61/MAL62 genes[a]

Strain	MAL Genotype	Maltose Ferm.[b]	Uninduced Maltase Activity[c]
R10	MAL6C-R10 mal63::URA3 mal1^0	+	1151 (1287,1016)
R10Δ61/Δ62-11	MAL6C-R10 mal63::URA3 mal61/62::LEU2 mal1^0	-	341 (±279)
R10Δ61/Δ62-12	MAL6C-R10 mal63::URA3 mal61/62:::LEU2 mal1^0	-	320 (±177)
332-5A	MAL6 mal1^0	+	5 (±1)
332-5AΔ61/Δ62-9	mal61/mal62::LEU2 mal1^0	-	2 (2,2)
332-5AΔ61/Δ62-10	mal61/mal62::LEU2 mal1^0	-	2 (2,2)

(a) Cells were pregrown in YEP medium plus 2% galactose, diluted into YEP medium plus 2% galactose, and allowed to grow 8-9 hours (reaching mid to late log phase) prior to harvesting.

(b) Maltose fermentation was determined as described in Materials and Methods.

(c) Maltase activity was determined by measuring the rate of hydrolysis of PNPG and is expressed as nanomoles of substrate split per minute per milligram of protein at 30°C. Values are the mean and standard deviation when possible; if only two determinations were made, then the mean is given along with the two determinations.

maltose permease levels must be determined in these strains; this would confirm that constitutive expression of maltase does not require a functional maltose permease.

These results contrast to disruption of the MAL61 and MAL62 genes in an inducible MAL6 $mal1^0$ strain. PstI - BamHI restricted pRD3 (Fig. 9) was used to transform inducible strain 332-5A to Leu^+ and maltose nonfermenters isolated (Appendix Table 3). Disruption at MAL6 was confirmed physically for two mal^- isolates (Appendix Table 4) and for one of these, strain 332-5A Δ61/Δ 62-9, the disruption was also confirmed genetically (Appendix Table 2, line 6). Disruption of these two genes in the inducible strain does not lead to constitutive synthesis of maltase. Rather, uninduced maltase levels for this strain was 1.7 units (Table 9) and induced levels (in YEP plus 2% galactose/ 2% maltose) was only 2.7 units. Another transformant, 332-5A Δ61/Δ62-10, also did not constitutively express maltase.

Together, these results demonstrate that the two MAL6-linked mutations, MAL6-C2 and MAL6C-R10, control the expression of an unlinked maltase structural gene and are thus trans-acting functions. In addition, MAL6-linked constitutivity does not reside within the MAL61, MAL62, or MAL63 genes, or the MAL61-MAL62 intergenic region. This strongly supports the existence of a second trans-acting gene whose product controls the expression of maltase and maltose permease, at least in constitutive strains. We call this second MAL6-linked regulatory gene MAL64.

Since the constitutive mutations in strains 8-2B and R10 are linked to MAL6, epistatic to mal63, and trans-acting, it is likely that they both lie within the same gene. Due to the partial dominance of strain R10 (Dubin et al., 1986) it is not possible to confirm allelism by the

standard complementation test; however, results to be presented below strongly suggest that both MAL6-C2 and MAL6C-R10 are alleles of MAL64.

Chapter 5: The MAL64 Gene

Localization and Characterization of MAL64. The evidence presented above strongly suggests the presence of a new MAL6-linked gene, MAL64, that is involved in constitutive expression of the maltose fermentative enzymes. Its identification has thus far been based upon negative evidence (ie.: it is not encoded by the three previously identified MAL6 genes). To fully characterize this new gene, it must be mapped, cloned, and disrupted.

Initial mapping studies performed by ten Berge et al. (1973b) failed to detect recombination between MAL6-C2 and mal6-13 (that is to say MAL64-C2 and mal63-13) in twenty tetrads. However, in a similar analysis, R. Needleman and co-workers demonstrated recombination between MAL64-R10 and the mal63::URA3 disruption in strain R10. Examination of 142 complete tetrads gave a map distance of 2.3 centimorgans (cM). Additionally, a three point test cross performed by them further demonstrated that MAL64 was located to the left of MAL63 (Dubin et al., 1986).

In Saccharomyces, 2.3 cM corresponds roughly to 6 kb of DNA (Strathern et al., 1979). The original MAL6 clone YEpMAL6 (Federoff et al., 1982) extended only about 3.5 kb to the left of MAL63. Further characterization of MAL64 at the molecular level therefore required additional cloning of flanking MAL6 sequences. Nearly 12 kb of DNA to the left of MAL63 was cloned from the MAL6 $mal1^0$ strain 332-5A and the MAL64-C2 mal63-13 $mal1^0$ strain 8-2B by M. Charron in this laboratory. Cloning was accomplished by targeted integration at the MAL6 and MAL6-C2 loci (using small fragments of wild type MAL6 DNA indicated on Fig. 10) followed by plasmid rescue as described by Orr-Weaver et al. (1983). A

more complete description of these plasmids and their cloning will appear in Dubin, Charron, Perkins, Needleman, and Michels (manuscript in preparation), Charron and Michels (manuscript in preparation), and M. Charron (Ph.D. Thesis, CUNY, 1987).

Three plasmids were isolated by M. Charron in this manner and a partial restriction map, generated by M. Charron and myself, appears on Figs. 10 and 11. Plasmids pBam11 and pBamC2 (Figs. 10 and 11) contain the identical 12 kb of MAL6 DNA except the former was isolated from the wild type MAL6 strain 332-5A while the latter was isolated from the MAL6-C2 locus of constitutive strain 8-2B. Plasmid pB3C2 (Fig. 11) and a subclone of this, plasmid pBSC2, contain even larger fragments isolated from constitutive strain 8-2B. Restriction endonuclease mapping of these plasmids revealed only a few restriction site polymorphisms between the MAL6 and MAL6-C2 loci. It is perhaps significant that the EcoRI site internal to the wild type MAL63 gene has been lost from the mal63 gene (mal63-13) isolated from strain 8-2B. This confirms results from genomic Southern blot analysis of DNA isolated from strain 8-2B (MAL64-C2 mal6-13 $mal1^0$) (compare lanes 5 and 6 in Fig. 13) and strain M26 (MAL64 mal6-13 MAL1g(?) MAL3g(?)) (results not shown) and suggests, but does not prove, that loss of this EcoRI site defines the original mal6-13 mutation.

In order to establish the location and function of MAL64, a total of eight gene disruption plasmids were constructed and used to transform wild type and constitutive MAL6 strains. The results of three disruption experiments will be presented below.

Plasmid pDMF (Fig. 11) was constructed using DNA cloned from the MAL6-C2 locus and contains an 11.5 kb deletion of the region to the left

Figure 10. The wild type MAL6 locus, including the MAL64 gene, and disruption plasmid pDMG.

A partial restriction map of the wild type MAL6 locus is presented (——), along with the locations and directions of transcription of the MAL61, MAL62, and MAL63 genes (⟶) and approximate location of the MAL64 gene (··——··). Plasmids pZ-1, pZ-2, and pC-1 contain sequences derived from the wild type MAL64 gene in pBR325; pH-2, pH-3, pE-4, and pD-5 contain wild type MAL6 DNA sequences and were described in Fig. 3.

Plasmids pMJC6ΔCla and pY6RΔC contain fragments derived from the wild type MAL6 locus and were used by M. Charron to clone plasmid pBam11 (from the MAL6 strain 332-5A) and plasmids pBamC2 and pB3C2 (from the MAL6-C2 strain 8-2B; see Fig. 12) by targeted integration and subsequent plasmid rescue (Charron and Michels, manuscript in preparation).

Plasmid pBam11(2M) is an episomal derivative of pBam11; disruption plasmid pDMG, a derivative of pZ-2, contains an insertion of the URA3 gene into the MAL64 gene. Plasmid pDF1 was described in Fig. 3.

(●) indicates known restriction site polymorphisms between the MAL6 and MAL6-C2 loci (compare to Fig. 12)

(O) indicates the ClaI site used to restrict pMJC6ΔCla prior to transforming strains 332-5A and 8-2B.

(✳) indicates the BglII site used to restrict plasmid Y6RΔC prior to transforming strain 8-2B.

(↑) indicates the restriction endonuclease sites used to restrict disruption plasmid pDMG prior to transformation.

Restriction endonuclease sites are abbreviated as in Fig. 1, with the following additions: BamHI, B; MluI, M; NcoI, N; SmaI, Sm; SstI, Ss; XbaI, Xb; XhoI, Xo.

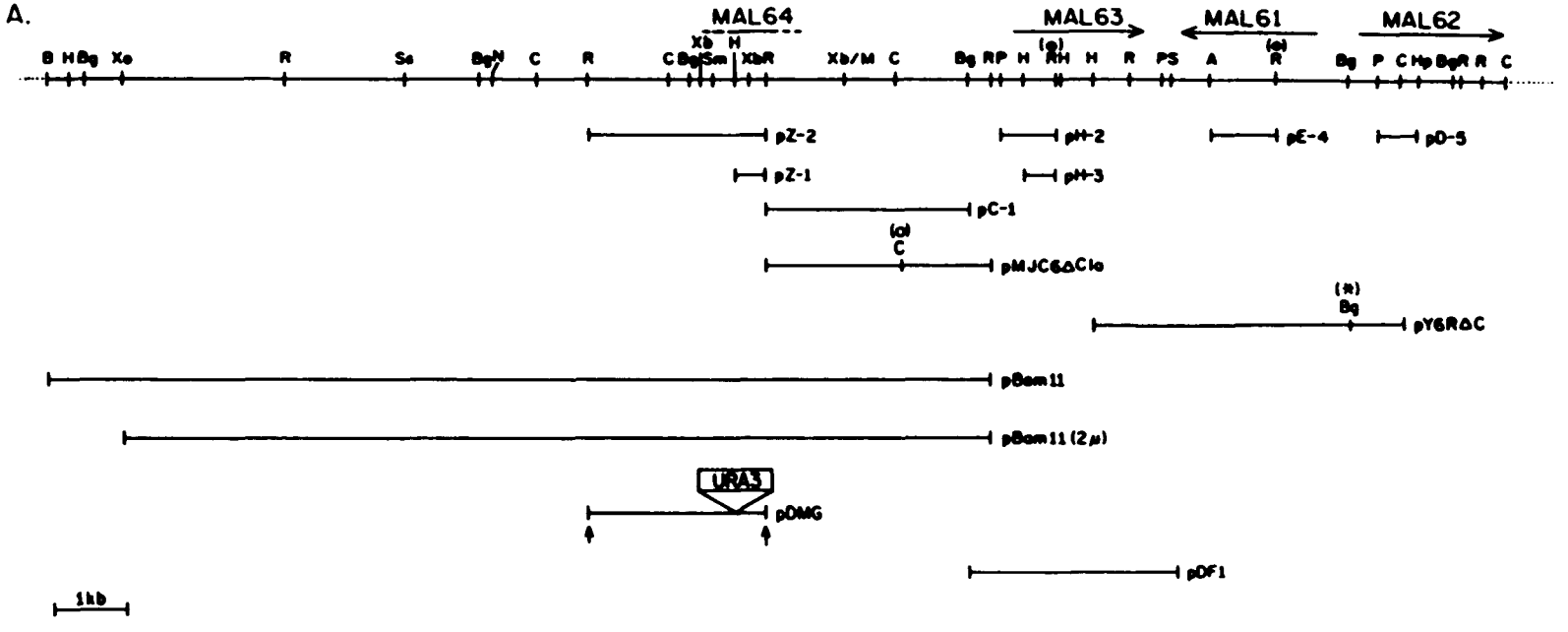

A.
MAL64
MAL63
MAL61
MAL62
B H Bg Xo R Se BgN C R C Bg Sm Xb H XbR Xb/M C Bg RP H (g) RH H R PS A (a) R Bg P C Hp BgR R C
pZ-2
pH-2
pE-4
pD-5
pZ-1
pH-3
pC-1
(a) C
pMJC6ΔC1o
(R) Bg
pY6RΔC
pBam11
pBam11(2μ)
URA3
pDMG
pDF1
1kb

Figure 11. The constitutive MAL6-C2 locus isolated from strain 8-2B, and disruption plasmids pDMF and pDMH.

A partial restriction map of the MAL6-C2 locus is presented (——), along with the locations and directions of transcription (⟶) of the MAL61, MAL62, mal63-13 genes and approximate location of the MAL64-C2 gene (··——··).

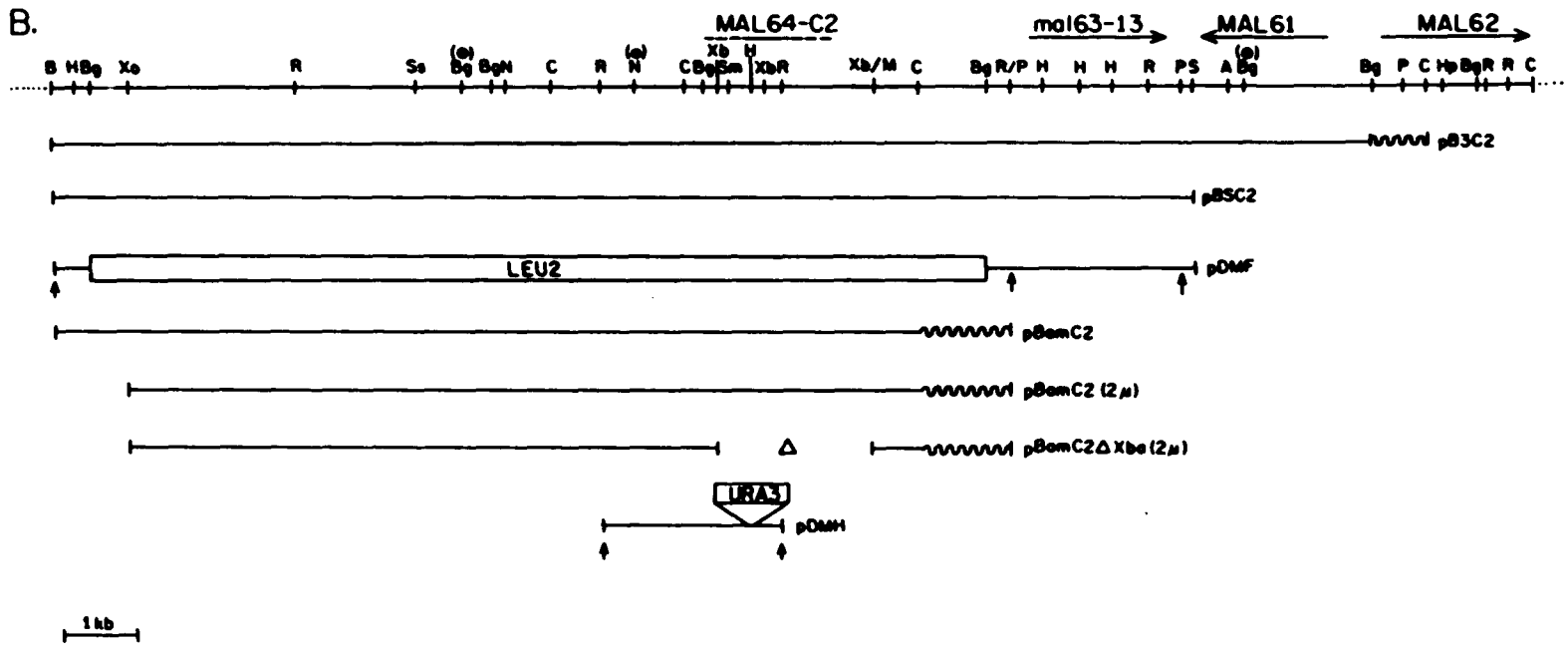
Plasmids pB3C2 and pBamC2 were cloned from constitutive strain 8-2B by M. Charron as described in Fig. 11 and Materials and Methods. Plasmid pBSC2 contains a 14.5 kb BamHI - SalI fragment subcloned into pBR325 from plasmid pB3C2 . Disruption plasmid pDMF was derived from plasmid pBSC2 and contains a deletion of the MAL64-C2 gene. The LEU2 gene present on pDMF is contained on a 2.7 kb BglII fragment and is not drawn to scale. Plasmids pBamC2(2M) and pBamC2ΔXba(2M) are episomal derivatives of pBamC2. Disruption plasmid pDMH was constructed using the 2.4 kb EcoRI fragment derived from the cloned MAL6-C2 locus and contains an insertion of the URA3 gene into the MAL64-C2 gene.

(●) indicates known restriction site polymorphisms between the MAL6 and MAL6-C2 loci (compare to Fig. 11)

(∿∿) indicates the wild type sequences derived from pMJC6ΔCla and pY6RΔC (Fig. 11) and present on pBamC2 and pB3C2, respectively.

(↑) indicates the restriction endonuclease sites used to restrict disruption plasmids pDMF and pDMH prior to transformation.

Restriction endonuclease sites are abbreviated as in Fig. 1, with the following additions: BamHI, B; MluI, M; NcoI, N; SmaI, Sm; SstI, Ss; XbaI, Xb; XhoI, Xo.


B.
MAL64-C2
mal63-13
MAL61
MAL62
B HBg Xo
R
Ss
(e) Bg BgN
C
R
(e) N
C Bg Xb Sm H XbR
Xb/M C
Bg R/P H H H R PS A (e) Bg
Bg P C Hp Bg R R C
pB3C2
pBSC2
LEU2
pDMF
pBamC2
pBamC2 (2μ)
pBamC2ΔXba (2μ)
URA3
pDMH
1 kb

of the mutant MAL63 gene found in this strain, replacing it with the LEU2 gene. We specifically chose a plasmid carrying DNA isolated from the constitutive locus in order to avoid the possibility of gene conversion of MAL64-C2 to MAL64 which could have occurred had wild type MAL6 derived sequences been used. PstI - BamHI restricted pDMF was used to transform strain 8-2B to Leu^+ and nearly 50% of the transformants were maltose nonfermenters (Appendix Table 3). The predicted disruption was confirmed physically for two mal^- isolates, strains 8-2BΔF-5-20 and 8-2BΔF-5-21 (Appendix Table 5), and this was further confirmed genetically in the latter strain (Appendix Table 2, line 12). One maltose fermenting transformant contained an intact MAL6 locus when analyzed physically and no doubt underwent gene conversion at LEU2. Unlike the parental strain 8-2B, strains 8-2BΔF-5-21 and 8-2BΔF-5-20 no longer constitutively synthesized maltase (Table 10); further, strain 8-2BΔF-5-21 no longer constitutively synthesized maltose permease (maltose transport levels in this strain were 12 fold lower than the parental strain 8-2B following growth in YEP plus 2% galactose medium). Northern blot analysis of RNA extracted from strain 8-2BΔF-5-21 revealed that the 2.0 kb MAL61 homologous (presumed maltose permease) mRNA and the 1.9 kb MAL62 homologous (maltase) mRNA were no longer constitutively expressed, as they had been in strain 8-2B (Fig. 12A). The 2.4 kb MAL61 homologous transcript continued to be detected, but at reduced levels. As expected, strain 8-2BΔF-5-21 did not respond to maltose regulated induction since growth in the presence of maltose failed to stimulate maltase synthesis (Table 10).

While these results demonstrated that the region deleted in plasmid pDMF contains a gene(s) required for the constitutive expression of the

Table 10 Legend

(a) Cells were pregrown in YEP medium plus 2% galactose, diluted into YEP medium plus 2% galactose or 2% maltose and allowed to grow 8-9 hours (reaching mid to late log phase) prior to harvesting.

(b) Maltose fermentation was determined as described in Materials and Methods. Only strains 8-2B and 8-2BΔF-5-21::pDF1 fermented maltose.

(c) Maltase activity was determined by measuring the rate of hydrolysis of PNPG and is expressed as nanomoles of substrate split per minute per milligram of protein at 30° C. Values are the mean and standard deviation when possible; if only two determinations were made, then the mean is given along with the two determinations. When grown in YEP plus 2% galactose/2% maltose, strain 8-2B, strain 8-2BΔF-5-21, and strain 8-2BΔH-1 had maltase values of 181 (157, 206), 4 (5, 3) and 5 (5,5), respectively.

(d) N.D., not determined.

Table 10.

Disruption of the MAL64-C2 gene in constitutive strain 8-2B and its effects on maltase activity[a]

Strain[b]	MAL Genotype	Maltase Activity[c] Galactose	Maltose
8-2B	MAL64-C2 ma163-13 mal1^0	265 (±130)	498 (±44)
8-2BΔF-5-20	ma164-C2::LEU2 ma163-13 mal1^0	3 (3,3)	N.D.[d]
8-2BΔF-5-21	ma164-C2::LEU2 ma163-13 mal1^0	3 (3,3)	8 (7,9)
8-2BΔF-5-21 ::pLC544 -1-1	ma164-C2::LEU2 ma163-13 mal1^0 trp1::pLC544(TRP1)	1 (2,0)	6 (6,5)
8-2BΔF-5-21 ::pDF1 -2-2	ma164-C2::LEU2 ma163-13 mal1^0 trp1::pDF1(TRP1 MAL63)	5 (5,5)	201 (209,194)
8-2BΔG-10	ma164-C2::URA3 ma163-13 mal1^0	4 (4,4)	N.D.[d]
8-2BΔH-1	ma164-C2::URA3 ma163-13 mal1^0	5 (6,4)	10 (9,11)
8-2BΔH-2	ma164-C2::URA3 ma163-13 mal1^0	4 (4,4)	N.D.

Figure 12. Disruption of the MAL64 gene in inducible and constitutive MAL6 strains and its effects on transcription of the MAL61, MAL62, and MAL63 genes.

Yeast strains were grown under uninducing conditions (YEP medium plus 2% galactose [indicated by G]) or inducing conditions (YEP medium plus 2% maltose [indicated by M]). Poly (A^+) RNA was isolated, fractionated on a formaldehyde/agarose gel, transferred to nitrocellulose, and probed with MAL61, MAL62, and MAL63 specific probes (Fig. 11). Panel A: The MAL62 specific probe pD-5 was used to detect maltase specific transcripts and the MAL61 specific probe pE-4 was used to detect maltose permease specific transcripts; two different, yet identical Northern blots were used; 10 micrograms (mcg) poly (A^+) RNA were loaded per track. Panel B: The MAL63 specific probe pH-2 was used to detect MAL63 homologous transcripts; 20 mcg poly (A^+) RNA were loaded per track.

Strains:

332-5A	MAL64 MAL63
a9	MAL64 mal63::URA3
332-5AΔF-1-5	mal64::LEU2 MAL63
332-5AΔG-15	mal64::URA3 MAL63
R10	MAL64C-R10 mal63::URA3
R10u	MAL64C-R10 mal63::ura3
R10uΔF-9-26	mal64C-R10::LEU2 mal63::ura3
8-2B	MAL64-C2 mal63-13
8-2BΔ63-1	MAL64-C2 mal63-13::URA3
8-2BΔF-5-21	mal64-C2::LEU2 mal63-13
8-2BΔG-10	mal64-C2::URA3 mal63-13

DISRUPTION OF MAL64

maltose fermentative enzymes, it did not prove that the disrupted gene was MAL64-C2; the disrupted region could possibly encode an unrecognized gene that is epistatic to MAL64-C2 and required for expression of the maltose fermentative enzymes. To demonstrate that MAL64-C2 had indeed been disrupted, strain 8-2B Δ F-5-21 was transformed with either pBam11(2M), which restores nearly all of the wild type sequences that had been deleted, or pBamC2(2M), which restores those sequences isolated from the MAL6-C2 locus (Figs. 10 and 11). Plasmids pBam11(2M) and pBamC2(2M) are episomal derivatives of plamids pBam11 and pBamC2, respectively, and were constructed by the addition of the LEU2 gene and portions of 2 micron circle isolated from YEp13. Plasmid pBamC2(2M) restored to strain 8-2BΔF-5-21 the ability to both ferment maltose and constitutively synthesize maltase while pBam11(2M) could do neither (Table 11). This proves that the MAL64-C2 gene lies within the 11 kb XhoI - EcoRI fragment and is trans-acting. To localize the complementing function, plasmid pBamC2Δ Xba(2M) was constructed and is identical to p64C2(2M), except for a 1.7 kb deletion within the MAL6-C2 insert (Fig. 11). Strain 8-2BΔF-5-21 was transformed with plasmid pBamC2 Δ Xba(2M) and it failed to complement (Table 11), strongly suggesting, but not proving, that MAL64-C2 is located approximately 2 - 4 kb to the left of MAL63.

In order to further demonstrate that the MAL64-C2 gene was located within this region and to further localize it, disruption plasmid pDMG was constructed (Fig. 10). This plasmid contains an insertion of the URA3 gene into the HindIII site of the wild type derived 2.4 kb EcoRI fragment located about 3.5 kb to the left of MAL63. This 2.4 kb EcoRI fragment has been named fragment Z-2 (Fig. 10). EcoRI restricted pDMG

Table 11 Legend.

(a) In order to select for the maintenance of the episomal plasmids, cells were grown in minimal medium containing tryptophan and adenine plus either 2% galactose or 2% galactose/2% maltose (Gal/Mal). Cells were harvested at mid to late log phase. The 8-2BΔF-5-21 transformants were grown for nearly 46 hours prior to harvesting; the 8-2BΔH-1 transformants were grown for only 30 hours due to the fact that they received a greater concentration of cells in the initial inoculum. This difference could partially explain the reduced maltase values observed in the 8-2BΔF-5-21[pBamC2(2M)] transformants as compared to the 8-2BΔH-1[pBamC2(2M)] transformants.

(b) and (c) Maltose fermentation and maltase activity were determined as described in Table 2. Only strain 8-2BΔF-5-21[BamC2(2M)] and strain 8-2BΔH-1[BamC2(2M)] ferment maltose. For the maltase assays, the mean is given along with the two determinations in parentheses ( ).

(d) The percentage values presented directly beneath the maltase levels in the [ ] brackets represent the percent of cells containing the episomal plasmid at the time of harvesting. This was determined as the fraction of cells that were Ura^+ (for the 8-2BΔF-5-21 transformants) or Leu^+ (for the 8-2BΔH-1 transformants).

(e) N.D., not determined.

Table 11.

Complementation of strains 8-2BΔF-5-21 and 8-2BΔH-1 by the MAL64-C2 gene[a]

Strain[b]	MAL Genotype	Maltase Activity Galactose	Gal/Mal
8-2BΔF-5-21 [pBaml1(2M)] -3-3	mal64-C2::LEU2 mal63-13 mal1^0 plus [MAL64]	1 (1,1) (d) [60%]	1 (1,1) [66%]
8-2BΔF-5-21 [pBamC2(2M)] -4-1	mal64-C2::LEU2 mal63-13 mal1^0 plus [MAL64-C2]	67 (65,69) [32%]	59 (61,56) [20%]
8-2BΔF-5-21 [pBamC2ΔXba (2M)] -X-4	mal64-C2::LEU2 mal63-13 mal1^0 plus [MAL64-C2ΔXba]	1 (1,1) [55%]	1 (1,1) [46%]
8-2BΔH-1 [pBaml1(2M)] -1	mal64-C2::URA3 mal63-13 mal1^0 plus [MAL64]	1 (1,1) [56%]	1 (1,1) [66%]
8-2BΔH-1 [pBamC2(2M)] -22	mal64-C2::URA3 mal63-13 mal1^0 plus [MAL64-C2]	211 (236, 187) [50%]	161 (186, 136) [40%]
8-2BΔH-1 [pBamC2ΔXba (2M)]	mal64-C2::URA3 mal63-13 mal1^0 plus [MAL64-C2ΔXba]	(e) N.D.	N.D.

was used to transform strain 8-2B to Ura$^+$ and greater than 95% of the transformants were maltose nonfermenters (Appendix Table 3). Disruption at MAL6 was confirmed physically for four nonfermenting isolates (Appendix Table 5) and further confirmed genetically for one, strain 8-2BΔG-10 (Appendix Table 2, line 13). Strain 8-2BΔG-10 no longer constitutively synthesized maltase (Table 10) or the 2.0 kb MAL61 homologous (presumed maltose permease) mRNA and the 1.9 kb MAL62 homologous (maltase) mRNA (Fig. 12A).

Since plasmid pDMG was constructed using the wild type derived fragment Z-2, it remained uncertain whether the URA3 insert disrupted MAL64-C2 or whether gene conversion to MAL64 occurred, or both. To determine whether insertional disruption at the HindIII site within fragment Z-2 was sufficient to mutate MAL64-C2, plasmid pDMH was constructed. Plasmid pDMH (Fig. 11) is identical to plasmid pDMG, except the 2.4 kg EcoRI fragment used in its construction was derived from the cloned MAL6-C2 constitutive locus. EcoRI restricted pDMH was used to transform strain 8-2B to Ura$^+$ and greater that 95% of the transformants were maltose nonfermenters (Appendix Table 3). Disruption at MAL6 was confirmed physically for seven mal$^-$ isolates (Appendix Table 5), and further confirmed genetically for one strain, 8-2BΔH-1 (Appendix Table 2, line 14). Strain 8-2BΔH-1 and another isolate, 8-2BΔH-2 no longer constitutively synthesized maltase (Table 10); additionally, strain 8-2BΔH-1 did not induce in the presence of maltose (Table 10). Most significantly, transformation of strain 8-2BΔH-1 with plasmid pBamC2(2M) restored the ability to ferment maltose and synthesize maltase constitutively, while plasmid pBam11(2M) did neither (Table 11). Plasmid pBamC2ΔXba(2M) also failed to restore the ability

of strain 8-2BΔH-1 to ferment maltose. This clearly proves that the HindIII site within fragment Z-2 lies within the MAL64-C2 gene.

The same region within constitutive strain R10 also encodes a positive activating function required for constitutive expression of the maltose fermentative enzymes. Strain R10u is a 5 fluoro-orotic acid derived ura3 revertant of strain R10 (see Materials and Methods), and like strain R10, it too constitutively synthesized maltase (Table 12) and the MAL62 (maltase) and MAL61 (presumably maltose permease) transcripts (Fig. 12) and still contained the MAL63 deletion (results not shown). (Maltose permease activity is constitutively expressed in strain R10 [Dubin et al., 1986] but was not determined for strain R10u.) PstI - BamHI digested pDMF was used to transform strain R10u to Leu^+ and 37% of the transformants were maltose nonfermenters (Appendix Table 3). Physical analysis of two nonfermenters confirmed disruption at MAL6 as well as the continued absence of MAL63 (Appendix Table 6); disruption at MAL6 was further confirmed genetically for one isolate, transformant R10uΔF-9-26 (Appendix Table 2, line 15). One Mal^+ isolate was also analyzed physically and contained an intact MAL6 locus having no doubt undergone gene conversion at LEU2. Strain R10uΔF-9-26 is unable to constitutively synthesize maltase or induce for maltase (Table 12) and fails to constitutively express the MAL62 (maltase) and MAL61 (presumably maltose permease) mRNAs (Fig. 12A). This strain is not complemented by the wild type plasmid pBam11(2M) but is complemented, somewhat, by pBamC2(2M). These results suggest that MAL64C-R10 has been deleted in strain R10uΔF-9-26. To determine whether MAL64C-R10 is located within fragment Z-2, EcoRI restricted pDMG was used to transform strain R10u to Ura^+ and nearly 90% of the transformants were

Table 12 Legend

(a) Cells were pregrown in YEP medium plus 2% galactose, diluted into YEP medium plus 2% galactose (Gal) or 2% maltose (Mal) and allowed to grow 8-9 hours (reaching mid to late log phase) prior to harvesting.

(b) Maltose fermentation was determined as described in Materials and Methods. Only strains R10, R10u, and R10u Δ F-9-26::pDF1 ferment maltose.

(c) Maltase activity was determined by measuring the rate of hydrolysis of PNPG and is expressed as nanomoles of substrate split per minute per milligram of protein at 30°C. For the maltase assays, the mean is given along with the two determinations in parentheses.

(d) N.D., not determined.

Table 12.

Disruption of the MAL64C-R10 gene in constitutive strain R10 and its effects on maltase levels[a]

Strain[b]	MAL Genotype	Maltase Activity[c] Gal	Mal
R10	MAL64C-R10 mal63::URA3 mal1^0	1151 (1287, 1016)	[d]N.D.
R10u	MAL64C-R10 mal63::ura3 mal1^0	759 (812, 707)	770 (787, 754)
R10uΔF-9-26	mal64C-R10::LEU2 mal63-13::ura3 mal1^0	4 (4,4)	3 (4,2)
R10uΔF-9-26 ::pLC544	mal64C-R10::LEU2 mal63-13::ura3 mal1^0 trp1::pLC544(TRP1)	N.D.	N.D.
R10uΔF-9-26 ::pDF1 -12	mal64C-R10::LEU2 mal63-13::ura3 mal1^0 trp1::pDF1(TRP1 MAL63)	5 (5,5)	284 (324, 244)
R10uΔG-1	mal64C-R10::URA3 mal63-13::ura3 mal1^0	5 (5,5)	N.D.

nonfermenters (Appendix Table 3). Physical analysis of five mal$^-$ isolates confirmed the expected MAL6 disruption (Appendix Table 6). One nonfermenting isolate, R10u ΔG-1, no longer constitutively synthesized maltase (Table 12). Since plasmid pDMG was constructed using wild type MAL6 derived sequences, it is uncertain whether MAL64C-R10 was disrupted and/or gene converted by this transformation. The definitive experiment awaits the cloning of fragment Z-2 from strain R10. It is clear though that MAL64C-R10 is located within fragment Z-2, making it highly likely that MAL64-C2 and MAL6C-R10 are alleles.

From these results it may be concluded that the MAL64-C gene encodes a positive, trans-acting product that is required for constitutive expression of the maltose fermentative enzymes and their transcripts in constitutive strains. However the wild type MAL64 gene does not play a similar positive regulatory role in inducible maltose fermentation. To demonstrate the role played by the wild type MAL64 gene, it was deleted from the inducible MAL6 mal1^0 strain 332-5A. PstI - BamHI restricted pDMF was used to transform strain 332-5A to Leu$^+$ and all but one out of 36 transformants tested continued to ferment maltose (Appendix Table 3). The predicted disruption was confirmed physically for 5 maltose fermenting isolates as well as the lone nonfermenting isolate (Appendix Table 4). Disruption at MAL6 was further confirmed genetically for one Mal$^+$ transformant, isolate 332-5AΔF-1-5 (Appendix Table 2, line 16). Like the undisrupted parent, strain 332-5AΔF-1-5 continued to induce for maltase (Table 13) as well as the MAL62 (maltase) and MAL61 (presumably maltose permease) mRNAs (Fig. 12). Maltose permease activity was not determined. Another Mal$^+$ transformant, 332-5AΔF-1-15 also continued to induce for maltase (Table

Table 13.

Disruption of the MAL64 gene in the inducible strain 332-5A and its effects on maltase activity[a]

Strain[b]	MAL Genotype	[c]Maltase Activity Galactose	Maltose
332-5A	MAL64 MAL63 mal1^0	5 (±1)	279 (±49)
332-5AΔF-1-5	mal64::LEU2 MAL63 mal1^0	4 (4,4)	299 (269,330)
332-5AΔF-1-15	mal64::LEU2 MAL63 mal1^0	6 (7,5)	224 (228,221)
332-5AΔF-1-29	mal64::LEU2 mal63-13 mal1^0	4 (4,4)	7 (6,7)
332-5AΔG-15	mal64::URA3 MAL63 mal1^0	4 (5,3)	215 (182,248)

(a) Cells were pregrown in YEP medium plus 2% galactose, diluted into YEP medium plus 2% galactose or 2% maltose (Mal) and allowed to grow 8-9 hours (reaching mid to late log phase) prior to harvesting.

(b) Maltose fermentation was determined as described in Materials and Methods. Only strain 332-5AΔF-1-29 failed to ferment maltose.

(c) Maltase activity was determined by measuring the rate of hydrolysis of PNPG and is expressed as nanomoles of substrate split per minute per milligram of protein at 30° C. Values are the mean and standard deviation when possible; if only two determinations were made, then the mean is given along with the two determinations.

13).

The single mal$^-$ transformant, strain 332-5AΔF-1-29, did not behave like the other Leu$^+$ transformants. It neither fermented maltose nor induced for maltase (Table 13). This phenotype suggested that the MAL61 or MAL63 genes may also have been disrupted in this strain. PstI - BamHI restricted pDMF releases a 2.2 kb PstI fragment that presumably encodes the MAL63 allele mal63-13, as well as the 3.6 kb BamHI - PstI disruption fragment containing the LEU2 gene and the MAL64 disruption (Fig. 11). It was possible that the 2.2 kb PstI fragment could occasionally co-transform along with the 3.6 kb disruption fragment, or more likely, a partially restricted 5.8 kb BamHI - PstI fragment could integrate at MAL6. If either of these two events occurred, then MAL63 should be converted to mal63-13 and the resulting strain should be uninducible. This difficulty can be controlled for as follows. Due to a fortuitous EcoRI site polymorphism between MAL63 and mal63-13 (compare Figs. 10 and 11), it is possible to physically distinguish the two alleles. DNA isolated from the 5 maltose fermenting transformants (including strains 332-5AΔF-1-5 and 332-5AΔF-1-15), as well as the mal$^-$ transformant, 332-5AΔF-1-29, were restricted with EcoRI and physically analyzed using a MAL63 derived probe. All 5 fermenters previously analyzed contained the wild type MAL63 gene, as determined by the presence of an EcoRI site within MAL63; the single nonfermenting strain lacked this EcoRI site and appeared to carry the mal63-13 allele of MAL63 (Appendix Table 4). This strongly suggested that the mal63-13 mutation lies 3' to the PstI site within MAL63. Like the other mal6 mutants isolated by ten Berge et al. (1973a), (including the mal63-13 strain M26), strain 332-5AΔF-1-29 was complemented by MAL1p strains.

Plasmid pDMG (Fig. 10) was also used to disrupt the wild type MAL64 gene in strain 332-5A by insertional mutagenesis. EcoRI restricted pDMG was used to transform strain 332-5A to Ura^+ and 100% of the transformants were maltose fermenters (Appendix Table 3). Disruption at MAL6 was confirmed physically for 5 isolates (Appendix Table 4) and further confirmed genetically for strain 332-5AΔG-15 (Appendix Table 2, line 17). Strain 332-5AΔG-15 continued to induce for maltase (Table 13).

The results of these two disruptions in the inducible MAL6 $mal1^0$ strain 332-5A clearly demonstrate that the MAL64 gene does not play an essential role in maltose fermetation in inducible MAL6 strains; nor is MAL64 an essential cellular function under these growth conditions. In fact, only the MAL61, MAL62, and MAL63 genes are required for maltose regulated fermentation and induction of the maltose fermentative enzymes. This conclusion is further supported by the following observations. Strains 8-2BΔF-5-21, 8-2BΔH-1, and R10uΔF-9-26 no longer ferment maltose and fail to constitutively or inducibly synthesize maltase (Tables 10 and 12); these three strains carry a mutant MAL63 gene (mal63-13 in the 8-2B derivatives and mal63::ura3 in the R10u derivative) and a disrupted MAL64-C gene. Heterozygous diploids created by crossing the above strains to the MAL1p strains 53-2C or 1-31 are able to ferment maltose. Additionally, a functional MAL63 gene also restored the ability to ferment. This was demonstrated by the following experiments. Strains 8-2BΔF-5-21 and R10uΔF-9-26 were transformed with either plasmid pDF1, which contains the MAL63 gene (Fig. 3), or with the parental vector pLC544, which lacks MAL6 sequences. Plasmids were targeted to integrate at the TRP1 locus by

restricting with BglII prior to transformation. In both cases plasmid pDF1 restored the ability to ferment maltose and maltase synthesis was now induced by maltose (Tables 10 and 12); plasmid pLC544 failed to restore the ability to ferment maltose. This was also supported by the genetic analysis performed on strain 8-2BΔG-10::pDF1-1 (Appendix Table 2, line 13). Clearly, the MAL64 gene is not required for maltose regulated induction in the presence of the MAL63 gene. While MAL64-C is a positive activator of the MAL61 and MAL62 genes, the wild type MAL64 gene appears to function only following mutation, playing no apparent role in inducible maltose fermentation.

Homology between MAL63 and MAL64. EcoRI restricted DNA isolated from the MAL6 mal1^0 strain 332-5A and the MAL6-C2 mal1^0 strain 8-2B and probed with MAL63 derived sequences revealed three fragments with homology to probe pH-3. In strain 332-5A, the strongest signal with homology to this probe is a 0.9 kb fragment corresponding to MAL63 and in strain 8-2B the strongest signal is a 1.9 kb frangment corresponding to mal63-13 (Fig. 13, lanes 5 and 6). Two signals of slightly weaker intensity are due to cross hybridization to a 2.4 kb EcoRI fragment and a 5.6 kb EcoRI fragment. It had been determined previously that the 5.6 kb fragment was unlinked to MAL6 (unpublished observations). Unexpectedly, physical analysis of DNA isolated from various MAL64 disruption strains probed with the MAL63 derived probe pH-3 revealed changes in the 2.4 kb MAL63 homologous EcoRI fragment. Insertional disruption of MAL64 or MAL64-C caused this 2.4 kb MAL63 homologous EcoRI fragment to increase in size and deletion of MAL64 or MAL64-C produced a loss of this signal (Fig. 13, lanes 3,4,7, and 8). These results

Figure 13. Structural homology between the MAL63 and MAL64 genes.

DNA was isolated, restricted with EcoRI, fractionated on an agarose gel, transferred to nitrocellulose, and probed with the MAL63 specific probe pH-3 (Figs. 1, 3, 11).

Strain a9 is a derivative of strain 332-5A and contains a deletion of the MAL63 gene; strain 332-5A contains the undisrupted, inducible MAL6 locus; strain 332-5AΔF-1-5 is a derivative of 332-5A and contains a deletion of the MAL64 gene; strain 332-5AΔG-15 is a deriative of 332-5A and contains an insertion of the URA3 gene into the MAL64 gene; strain 8-2B contains the undisrupted constitutive MAL6-C2 locus; strain 8-2BΔG-8 and 8-2BΔG-10 are derivatives of strain 8-2B and contain an insertion of the URA3 gene into the MAL64-C2 gene.

The 0.9 kb MAL63 homologous fragment corresponds to the MAL63 gene in strain 332-5A; the 1.9 kb fragment corresponds to the mal63-13 allele of the MAL63 gene in strain 8-2B; the 2.4 kb fragment corresponds to the MAL64 and MAL64-C2 genes; the 3.5 kb fragment corresponds to the MAL64 and MAL64-C2 genes disrupted by insertion of the URA3 gene; the location of the 5.6 kb fragment is unknown, although it is unlinked to MAL6.

STRUCTURAL HOMOLOGY BETWEEN MAL63 AND MAL64

PROBE: MAL63 SPECIFIC SEQUENCES

suggested that the 2.4 kb EcoRI fragment containing sequences partially structurally homologous to MAL63 was identical to the 2.4 kb EcoRI fragment containing MAL64 (fragment Z-2). From both my own work and that of M. Charron (personal communication) it was directly demonstrated that the MAL63 specific 0.4 kb HindIII - EcoRI fragment on plasmid pH-3 hybridized to the cloned fragment Z-2 and this homology was restricted to the 0.4 kb HindIII - EcoRI fragment (designated Z-1; Fig. 10) within fragment Z (results not shown). While we have not yet determined whether the structural homology continues further to the left, sequences adjacent and to the right of Z-1 and H-3 also share some degree of sequence conservation, as determined by Southern blot hybridization (L. Tanouye and C. Michels, personal communication). As was suggested by the insertional disruptions of MAL64-C2 above and as will be demonstrated below by analysis of the MAL64 transcripts, at least a portion of the MAL64 gene is encoded by the sequences within fragment Z-1. It must be concluded that the MAL63 and MAL64 genes are at least partially structurally homologous.

The MAL64 mRNAs. In order to characterize the MAL64 transcript(s), plasmid pZ-2 (Fig. 10), which contains the 2.4 kb EcoRI fragment Z-2 (and the MAL64 gene), was used to probe Northern blots containing RNAs isolated from various strains (Fig. 14A). When probed with pZ-2, the uninduced wild type MAL6 $mal1^0$ strain 332-5A expresses low levels of two transcripts, 2.3 kb and 2.0 kb. Maltose induced RNA isolated from this strain no longer expresses the 2.3 kb species but instead high levels of the 2.0 kb species and low levels of four other transcripts, 3.0 kb, 1.4 kb, 1.1 kb, and 0.65 kb are detected (Fig. 14A). In contrast, strains

R10 and R10u express high constitutive levels of the 3.0 kb, 2.0 kb, 1.4 kb, 1.1 kb, and 0.65 kb RNAs (Fig. 14A). Despite the fact that plasmid pZ-2 contains sequences homologous to MAL63 (as discussed above), this probe fails to detect the two MAL63 homologous transcripts under the hybridization conditions used. This was demonstrated by examining transcripts synthesized by strain 332-5AΔF-1-5 (which contains a deletion of MAL64). While strain 332-5AΔF-1-5 continues to express the 2.0 kb and 1.6 kb MAL63 homologous transcripts when probed with plasmid pH-2 (Fig. 12B), no transcripts are detected in this strain when pZ-2 is used as probe (Fig. 14A).

An insertion of the URA3 gene into the HindIII site within fragment Z-2 is sufficient to disrupt MAL64-C2 (see strain 8-2BΔH-1 above). Insertional disruption at this HindIII site would be expected to result in a loss of or alteration in the MAL64 mRNA(s). To determine which transcript(s) belong to MAL64, RNA synthesized by strain 332-5AΔG-15 (which contains an insertion of the URA3 gene into the HindIII site within fragment Z-2) was examined by Northern blot analysis. RNA was isolated from strain 332-5AΔG-15 following inducing growth conditions and probed with plasmid pZ-2. As in the undisrupted parental strain, the 3.0 kb, 1.1 kb, and 0.65 kb transcripts continued to be expressed, yet unlike the parental strain, the 2.0 kb and the 1.4 kb transcripts were undetectable and were replaced by a novel 1.65 kb species and a very faint, diffuse band centered around 0.4 kb (Fig. 14A). While the origin of the novel 1.65 kb RNA species is unclear, it is possible that the diffuse 0.4 kb band represents various points of termination within the insertion. Absence of the 2.0 kb and 1.4 kb transcripts strongly suggest that one or both species represent the MAL64 mRNA.

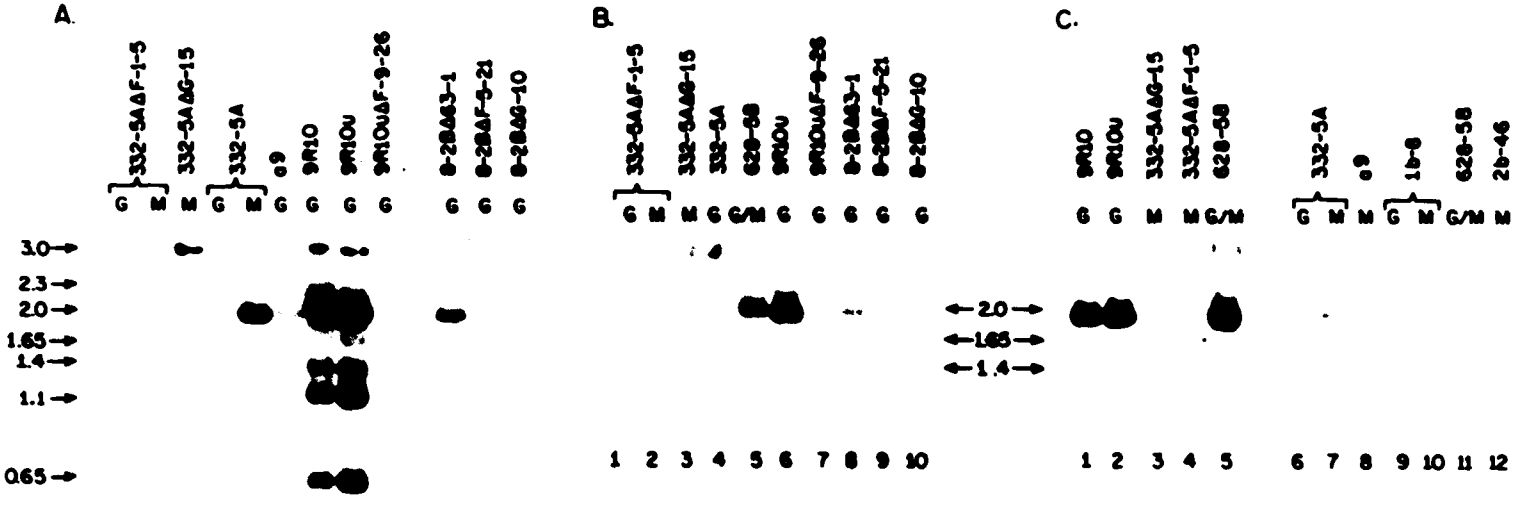
Figure 14. The MAL64 transcripts.

Growth conditions and RNA analysis are as described in Figs. 2 and 11, except MAL64 specific probes (Fig. 11) were used. Panel A: Probe pZ-2 was used to detect MAL64 homologous transcripts; 20-25 mcg poly (A^+) RNA loaded per track. Panel B: Probe pZ-1 was used to detect MAL64 homologous transcripts; 15 mcg poly (A^+) RNA loaded per track except for lanes 9-10, which contained 10 mcg each. Panel C: Probe pC-1 was used to detect MAL64 homologous transcripts; 25 mcg poly (A^+) RNA loaded per track in lanes 1-3, 15 mcg in lanes 4-5, and 10 mcg in lanes 6-12.

Strains:

332-5A	MAL64 MAL63 MAL62 MAL61 MAL12
a9	MAL64 mal63::URA3 MAL62 MAL61 MAL12
332-5AΔF-1-5	mal64::LEU2 MAL63 MAL62 MAL61 MAL12
332-5AΔG-15	mal64::URA3 MAL63 MAL62 MAL61 MAL12
1b-8	MAL64 MAL63 MAL62 mal61::URA3 MAL12
2b-46	MAL64 MAL63 mal62::LEU2 MAL61 MAL12
628-5B	MAL64 MAL63 mal62::LEU2 MAL61 mal12::LEU2
R10	MAL64C-R10 mal63::URA3 MAL62 MAL61 MAL12
R10u	MAL64C-R10 mal63::ura3 MAL62 MAL61 MAL12
R10uΔF-9-26	mal64C-R10::LEU2 mal63::ura3 MAL62 MAL61 MAL12
8-2B	MAL64-C2 mal63-13 MAL62 MAL61 MAL12
8-2BΔ63-1	MAL64-C2 mal63-13::URA3 MAL62 MAL61 MAL12
8-2BΔF-5-21	mal64-C2::LEU2 mal63-13 MAL62 MAL61 MAL12
8-2BΔG-10	mal64-C2::URA3 mal63-13 MAL62 MAL61 MAL12

(Strains 332-5AΔF-1-5, R10ΔF-9-26, and 8-2BΔF-5-21 contain deletions of MAL64, MAL64C-R10 and MAL64-C2, respectively; strains 332-5AΔG-15 and 8-2BΔG-10 contain insertions in MAL64 and MAL64-C2, respectively.)


MAL64 TRANSCRIPTS
A.
B.
C.
332-5AAF-1-5
332-5AAG-15
332-5A
o.9
9R10
9R10U
9R10UAF-9-26
8-28A13-1
8-28AF-5-21
8-28AG-10
G
M
3.0
2.3
2.0
1.65
1.4
1.1
0.65
1 2 3 4 5 6 7 8 9 10
1 2 3 4 5 6 7 8 9 10 11 12

Fewer transcripts are detected when the probe is reduced in size. When plasmid pZ-1 (Fig. 10; containing the 0.4 kb HindIII - EcoRI fragment subcloned from fragment Z-2) is used to probe Northern blots, only the 2.0 kb and 1.4 kb transcripts are detected in constitutive strains R10u and 8-2BΔ63-1. When pZ-1 is used to probe RNA isolated from strains 332-5AΔG-15 and 8-2BΔG-10 (both of which contain the URA3 gene inserted into MAL64 and MAL64-C2, respectively), only the novel 1.65 kb species is detected (Fig. 14B). This probe fails to detect any transcripts from strains 332-5AΔF-1-5, 8-2BΔF-5-21, and R10uΔF-9-26 (which contain deletions of MAL64, MAL64-C2 and MAL64C-R10, respectively; Fig. 14B). These results demonstrate that the 0.4 kb HindIII - EcoRI fragment Z-1 (that is structurally homologous to MAL63) is internal to the MAL64 gene.

Plasmid pC-1 contains the 2.7 kb EcoRI - BglII fragment adjacent and to the right of fragment Z-2 (Fig. 10). When pC-1 was used to probe Northern blots of RNA isolated from the inducible MAL6 strain 332-5A, a 2.0 kb transcript and a faint 1.4 kb trainscript were detected in maltose grown cells while only a trace level of the 2.0 kb species was observed in galactose grown cells (Fig. 14C). Both transcripts were detected with probe pC-1 in constitutive strains R10 and R10u and were constitutively expressed at high levels. These results suggested that the MAL64 gene extended into the next EcoRI fragment to the right of fragment Z-2. Analysis of the transcripts synthesized by disruption strains 332-5AΔF-1-5 (deletion of MAL64) and 332-5AΔG-15 (insertion into MAL64) and probed with pC-1 were consistent with this conclusion (Fig. 14C). Preliminary results suggest that induction of the MAL64 transcripts, in wild type MAL6 strains, requires a functional MAL63 gene

and MAL61 gene (for inducer transport). Northern blot analysis of RNA isolated from disruption strains a9 (Δ MAL63) and 1b-8 (Δ MAL61) following inducing growth conditions demonstrated an inability to induce for the MAL64 RNAs when probed with pC-1 (Fig. 14C). In contrast, pC-1 and pZ-1 detect the MAL64 transcripts in the maltase-less strain 628-5B (Δ MAL62 Δ MAL12) when grown in maltose containing medium (Fig. 14B and C).

We have demonstrated that the MAL63 and MAL64 genes are partially homologous (H-3 and Z-1) yet MAL64 sequences (Z-2 and Z-1) fail to detect the 2.0 kb and 1.6 kb MAL63 homologous RNAs (strain 332-5AΔF-1-5 in Figs. 14A and B). In contrast, the MAL63 derived probe pH-3 detects a 2.0 kb transcript (and perhaps a 1.4 kb transcript) in strains R10 and R10u, despite the fact that the MAL63 sequences on pH-3 are deleted in these strains (results not shown). This probe could in fact be cross hybridizing to the 2.0 kb MAL64C-R10 transcript, and this would be consistent with the fact that this 2.0 kb RNA species is not detected in strain 9R10uΔF-9-26 with probe pH-3 (results not shown). We believe that MAL63 probes detect this (presumed) 2.0 kb MAL64 transcript but MAL64 probes do not detect the MAL63 transcripts simply because of the high level of expression of this MAL64 transcript in strains R10 and R10u and the normally low level of expression of the MAL63 transcripts. These results suggested that the 2.0 kb MAL63 transcript might be transcribed soley from the MAL64 gene, however, this is not the case. As seen in strain 332-5AΔF-1-5 (which contains a deletion of MAL64), both the 2.0 kb and 1.6 kb MAL63 homologous transcripts continue to be detected when probed with pH-2 (Fig. 12B). Thus, the MAL63 homologous 2.0 kb transcript detected by probe pH-2 may be a mixture of MAL63 and

MAL64 encoded transcripts of singular size; the MAL64 gene may be responsible for the maltose inducible component of this heterogeneous transcript (see Fig. 2C). We still remain uncertain as to whether any component of the 2.0 kb MAL63 homologous transcript originates from the MAL63 gene. Given that the MAL1-linked MAL13 gene (which is structurally and functionally homologous to MAL63) encodes only a 1.6 kb mRNA (Charron et al., 1986), it is possible that the MAL63 gene also encodes only a 1.6 kb transcript; the 2.0 kb MAL63 homologous RNA species may originate from MAL64 and some third structurally homologous locus, and simply be controlled by MAL63 (Fig. 7C) or it may be derived from MAL63 itself. Clearly, this point remains unresolved.

DISCUSSION

Maltose fermentation in Saccharomyces requires the presence of at least one dominant MAL locus which confers the ability to synthesize high levels of maltase and maltose permease in the presence of the inducer maltose (reviewed in Barnett, 1976; 1981). Previous physical and genetic analysis demonstrated that the cloned MAL6 locus encoded three genes, all of which were required for maltose fermentation (Needleman et al., 1984). The MAL61 and MAL62 genes encode maltose inducible mRNAs; the MAL63 gene encodes two constitutively expressed mRNAs and results of previous studies suggested that it encoded a positive regulatory function required for the induction of maltase and maltose permease (ten Berge et al., 1973; Needleman et al., 1984). In order to determine the functions of these three MAL6 genes, we have selectively mutagenized these genes at the genomic MAL6 locus using the gene disruption technique described by Rothstein (1983). These studies established the following: MAL61 appears to encode maltose permease; MAL62 encodes maltase; MAL63 encodes a trans-acting, positive regulatory function required for the maltose dependent induction of the maltase and maltose permease transcripts and proteins. In addition, we identified and characterized a fourth MAL6-linked gene, MAL64, which is responsible for constitutive expression of the maltose fermentative enzymes in MAL6-linked constitutive strains but appears to play no role in maltose regulated induction.

Deletion of the MAL62 gene in the MAL6 $mal1^0$ strain 332-5A failed to affect maltose fermentation due to complementation by the structurally and functionally homologous MAL12 gene located at the MAL1-linked $mal1^0$

locus. These results, and subsequent analyses (unpublished observations; Charron and Michels, manuscript in preparation; Charron et al., 1986) demonstrated that the mal1^0 locus was not functionally inert, as previous genetic studies suggested. The MAL12 gene, like the MAL62 gene, also encoded a 1.9 kb MAL62 homologous transcript whose synthesis was induced by maltose. Deletion of all MAL62 homologous sequences in the MAL6 mal1^0 strain (that is to say both the MAL62 and MAL12 genes) resulted in a maltose nonfermenter that did not synthesize maltase or any MAL62 homologous transcripts when grown in the presence of maltose. These results suggested that the MAL62 and MAL12 genes encode the maltase structural gene and this was borne out through subsequent biochemical analysis of these disruption strains. R. Needleman and co-workers performed heat inactivation studies on the maltase synthesized by the MAL62 gene (using extracts from MAL62 ΔMAL12 strains) and the maltase synthesized by the MAL12 gene (using extracts from the ΔMAL62 MAL12 strains) and were able to prove that the MAL12 gene was responsible for the synthesis of a more thermolabile species of maltase than the MAL62 derived maltase. This difference may be explained by assuming that the MAL62 and MAL12 genes, while nearly identical, have diverged slightly following gene duplication.

Other studies have indicated that the MAL62 gene encodes maltase. Standard genetic analysis of the MAL1 locus led to the isolation of a MAL1-linked mutation responsible for temperature sensitive maltose fermentation and biochemical analysis revealed the lesion to be due to the synthesis of a more thermolabile maltase (Cohen et al., 1984). It was subsequently demonstrated that the cloned MAL62 gene complemented this mutation and restored the ability to synthesize wild type maltase

(Cohen et al., 1985). Charron et al. (1986) have also isolated a MAL1-linked maltose non-fermenting mutation by disrupting the MAL1-linked homologue of the MAL62 gene. This mutant failed to synthesize maltase, was complemented by the MAL62 gene, and continued to induce for the MAL61 homologous (presumed maltose permease) mRNA. Most convincingly, the sequence of the MAL62 gene has recently been determined and the predicted protein sequence corresponds with the previously published percent amino acid composition of maltase (Hong and Marmur, 1986).

Evidence suggesting that the MAL61 gene encodes a component of the maltose permease comes from strains carrying deletion and insertion mutations in this gene. Deletion of the MAL61 gene resulted not only in an inability to induce the 2.0 kb MAL61 homologous transcript but also an inability to induce for maltose permease and the maltase transcript and protein. Chang et al. (manuscript submitted for publication) also found that strains containing chemically induced mutations within the MAL61 gene failed to induce for both maltose permease and maltase. Further, deletion of the MAL61 homologue located at the dominant MAL1 locus (what we call the MAL11 gene) also resulted in a nonfermenter that was complemented by the MAL61 gene and failed to induce for the maltase transcript or protein. Clearly, MAL61 formally encodes a maltose inducible positive regulator that is required for induction of maltose permease, maltase mRNA, and maltase. This is consistent with the MAL61 gene encoding a transcriptional or post-transcritpional function controlling accumulation of the maltase mRNA and perhaps the maltose permease transcript, or encoding a regulated positive regulator of the maltose permease, or else directly encoding a component of maltose

permease.

Evidence exists supporting a model of MAL61 encoding a component of the maltose permease or a regulator of maltose permease. Insertion into the 5' region of the MAL61 gene produced a non-fermenting strain, aF, that continued to induce for maltase and the maltase mRNA yet synthesized intermediate levels of both maltose permease and a novel 1.95 kb MAL61 homologous transcript in the absence and presence of maltose. In contrast, the undisrupted parent strain failed to express the 2.0 kb MAL61 homologous transcript and synthesized only low basal levels of maltose permease in derepressing growth conditions and both were highly induced by maltose. We believe that in strain aF, the MAL61 gene has been constitutively activated by a cis-acting mutation leading to constitutive activation of maltose permease expression. To be certain that the constitutve maltose permease activity is due to enzyme mediated transport and not simply an increased permeability to maltose, this constitutive maltose permease must be studied further. The mechanism by which this insertion into the 5' region of the MAL61 gene results in constitutive and maltose unresponsive expression of the normally inducible MAL61 transcript remains unclear. This insertion may have placed the MAL61 gene under the control of a new promoter located within the insert. Alternatively, the insert may have separated the MAL61 promoter from a cis-acting silencer element that is maltose regulated; silencers have been described previously at the HMR locus in yeast (Brand et al., 1985). Identification of the transcription initiation sites of the small MAL61 homologous transcripts in strains 332-5A and aF could help clarify this point. If indeed maltose permease is constitutively expressed in strain aF, then these results are

consistent with MAL61 directly encoding maltose permease or a regulatory function controlling maltose permease activity (at some level of transcriptional, translational, or post-translational control). Deletion of MAL61 would then be expected to result in an inability to express maltase if it were assumed that only intracellular maltose effectively acts as inducer. Pleiotropic effects have been reported for other transport mutations in Saccharomyces and E. coli. Mutations within the PHO84 (PHOT) gene of yeast, which is required for inorganic phosphate uptake, leads to non-repressible expression of the normally phosphate repressible acid phosphatase (Ueda and Oshima, 1975; Ueda et al., 1975). Similarly, while ornithine carbamoyltransferase is normally inactivated when grown in the presence of arginine, its activity remains high in mutant yeast strains carrying the defective arginine permease gene can1 (Grenson et al., 1966). Urea fails to induce the synthesis of allophanate hydrolase in yeast lacking the DUR3 gene product urea permease (Sumrada et al., 1976). Finally, galactose permease mutants of E. coli fail to induce for beta-galactosidase unless the external concentration of inducer is considerably increased, presumably allowing for inducer entry by diffusion (Cohen and Monod, 1957).

Supporting evidence that MAL61 encodes maltose permease or a maltose permease regulator comes from the work of Cohen et al. (1985) and Chang et al. (manuscript submitted for publication). Both groups report an increase in maltose permease activity, but not maltase activity, in strains containing the MAL61 gene on multicopy plasmids. However, Cohen and co-workers provide convincing evidence that MAL61 encodes the maltose permease. Standard mutagenic analysis of a MAL1 strain led to the isolation of a maltose transport mutant that

synthesized a biochemically altered maltose permease; this strain failed to ferment maltose and the mutation was linked to MAL1 (Goldenthal et al., 1983). Other alleles of this gene (MALT) failed to induce maltase and the maltase mRNA and were shown to be complemented by the cloned MAL61 gene (Cohen et al., 1984; 1985). Assuming that maltose might enter the malT mutant more effectively in a more highly concentrated maltose containing growth medium, these workers observed a slight induction (3.3 fold) of maltase in their maltose permease mutants following growth in 10% maltose containing medium compared to the low level expressed in 2% maltose medium. We too grew our Δ MAL61 strain 1b-8 in 10% maltose containing medium and saw a 2.2 fold increase over 2% maltose grown 1b-8 cells (results not shown). The MAL61 disruption mutants presented here support the results of Cohen et al. (1984, 1985). Sequencing of the MAL61 gene and its structural and functional MAL1-linked homologue is currently in progress in this laboratory and will reveal whether these genes exhibit characteristics common to other integral membrane and transport proteins.

MAL61 homologous probes detect a 2.4 kb constitutively expressed transcript as well as the functional 2.0 kb maltose permease transcript and both RNAs are transcribed from the same strand (Needleman et al., 1984). We demonstrated that the 2.4 kb RNA species is synthesized by both the MAL61 gene and sequences located at the $mal1^0$ locus that are homologous to the MAL61 gene. The MAL11 gene is located at the dominant MAL1 locus and is structurally and functionally homologous to the MAL61 gene (Charron et al., 1986). The MAL11 gene is required for maltose fermentation and also synthesizes two transcripts: a constitutively expressed 2.4 kb species that is detected at very low levels and a

maltose inducible 2.0 kb species. We do not yet know know whether the 2.4 and 2.0 kb MAL61 and MAL11 transcripts utilize different promoters or different termination sites or whether they have a precursor and product relationship (ie.: the smaller transcript being expressed following a maltose regulated splicing event). Two differentially regulated promoters control the expression of the SUC2 gene in yeast, the expression from one promoter being unregulated while expression from the other is catabolite repressed (Sarokin and Carlson, 1984). Synthesis of a 2.4 kb MAL61 homologous transcript by the $mal1^0$ locus was not entirely unexpected, considering the sequence homology to MAL61 and the adjacent functional maltase gene, however its function is not immediately apparent. Clearly this RNA species is unable to complement the MAL61 gene function. It is possible that at one time the $mal1^0$ locus may have encoded a functional maltose permease gene and its current lack of function may represent an inability to express the 2.0 kb transcript in response to maltose. This could be tested by attempting to revert the ΔMAL61 $mal1^0$ mutant.

The MAL63 gene encodes a trans-acting, positive regulatory function that is required for maltose fermentation and maltose induced accumulation of maltose permease and maltase and their respective mRNAs. Deletion of this gene results in a nonfermenter that fails to induce for maltose permease, maltase, and their respective transcripts. This confirms and extends previous genetic analysis of the MAL6 locus in which ten Berge et al. (1973a) demonstrated the presence of a positive regulatory gene. These results also complement those of Chang et al. (manuscript submitted for publication). Using standard genetic analysis they isolated MAL6-linked nonfermenting mutants that were uninducible

for the maltose fermentative enzymes, recessive to wild type, and complemented by MAL1p strains and the cloned MAL63 gene. They also demonstrated that a derivative of one of the original mal6 mutants isolated by ten Berge et al. (1973a), mal6-10, was complemented by the cloned MAL63 gene, confirming that the previously identified MAL6-linked positive regulatory gene was MAL63. Cohen et al. (1984) isolated MAL1-linked mutations in a gene (MALR) that was required for maltose fermentation and induction of maltase and its mRNA, and maltose permease; the malR mutants were complemented by the cloned MAL63 gene, which restored the ability to ferment and synthesize the two structural proteins. Charron et al. (1986) have also demonstrated the existence of a MAL1-linked gene that is structurally and functionally homologous to MAL63; deletion of this gene, MAL13, at the genomic MAL1 locus resulted in a nonfermeter that failed to induce for the maltase transcript and protein and the maltose permease mRNA and both the cloned MAL63 and MAL13 genes complemented this mutant.

We are confident, as were ten Berge and co-workers, that the MAL63 gene does not simply encode a second structural component of the maltose permease. This is clear from the fact that the uninducible mal6 (mal63) nonfermenters, including a strain containing a deletion of the MAL63 gene, typically revert to fermenters that constitutively express maltase, maltose permease, and their respective transcripts (ten Berge et al., 1973b; Dubin et al., 1986).

The mechanism by which the MAL63 gene product induces the accumulation of the MAL61 (maltose permease) and MAL62 (maltase) mRNAs, as well as the role played by the inducer maltose, remains to be determined. It is possible that the MAL63 gene product acts by directly

stimulating the rate of transcription from the MAL61 and MAL62 genes. In this regard, Federoff et al. (1983) demonstrated that maltose induced accumulation of the maltase mRNA is due (at least in part) to an increase in transcription rate. Alternatively, MAL63 could function at a post-transcriptional level such as modifying transcript stability and regulating splicing. The MAL63 gene product need not reside in the nucleus and may influence transcript accumulation indirectly. The SNF1 gene product, which is required for derepression of SUC2 (Celenza and Carlson, 1984), is a protein kinase located throughout the cell (Celenza and Carlson, 1986). In addition, our results do not exclude an additional role of the MAL63 gene product in translation stimulation.

Preliminary sequence analysis of the MAL63 gene reveals a short sequence that is conserved in four other known positive regulatory proteins (Kim and Michels, personal communication). These include the Saccharomyces genes ADR1, GAL4, and PPR1, as well as the Xenopus transcription factor TFIIIA (Hartshorne et al., 1986). TFIIIA is required for accurate in vitro transcription of the ribosomal 5S genes (Engelke et al., 1980) and binds to an internal promoter region of these genes (Sakonju et al., 1981). The GAL4 gene product has also been demonstrated to bind to DNA at galactose regulated promoters (Giniger et al., 1985). This suggests that the MAL63 gene product plays a direct role in regulating transcription of the maltose inducible transcripts. Isolation and characterization of the MAL63 gene product is currently underway in the laboratory of R. Needleman (personal communication) and should further clarify its role in maltose fermentation.

Clearly, MAL61, MAL62 and MAL63 represent three independent genes as deletions within each gene in a MAL6 $mal1^0$ strain produced mutants

with a characteristic phenotype and distinctive complementation patterns with the MAL1p, MAL1g and mal1^0 tester strains. While deletion disruption mutants were recessive to the wild type MAL6 mal1^0 strain, they failed to complement each other when maltose fermentation was assayed in Durham tubes (results not shown). Chang et al. (manuscript submitted for publication) isolated mal63 and mal61 mutants in another MAL6 mal1^0 strain and observed a lack of complementation when assayed in Durham tubes but found variable degrees of complementation when assayed on maltose containing plates. This contrasts with genetic analyses of the MAL1 locus. The MAL1 locus encodes three genes that are structurally and functionally homologous to MAL61, MAL62, and MAL63. Point and deletion mutations within the three MAL1-linked genes complemented each other fully (Cohen et al., 1984; Charron et al., 1986). It is unclear whether this difference represents valid distinctions between the MAL1 and MAL6 loci or whether it simply reflects differences in the backgrounds of the strains used. Preliminary evidence suggests that no intrinsic difference exists between the two dominant MAL loci. The diploid created by mating a MAL6 mal1^0 nonfermenting strain containing a deletion of the MAL63 gene (ΔMAL63 MAL62 MAL61 MAL12) with a MAL1 nonfermenting strain containing a deletion of the MAL61 homologous, MAL1-linked gene MAL11 (MAL13 ΔMAL11 MAL12) failed to ferment maltose; while the diploid did not ferment, the ΔMAL63 MAL62 MAL61 MAL13 Δ MAL11 MAL12 haploid segregants did ferment (results not shown). We further favor the idea that background differences may be responsible for the lack of complementation between the MAL6 deletion mutants as we have observed that while MAL6 mal1^0 x MAL6 mal1^0 diploids ferment, MAL6 mal1^0 x mal1^0

diploids either do not ferment or do so only very slowly (unpublished results; R. Needleman, personal communication). We are unable to explain these results although it is possible that the ratio of MAL6 : mal1^{0} loci may be involved.

An exciting aspect of this work has been the identification (Dubin et al., 1986) and characterization of a fourth MAL6-linked gene, MAL64. The MAL64 gene is located nearly 3.5 kb to the left of MAL63 and is at least partially homologous to the MAL63 gene. The MAL64-C allele of this gene is responsible for constitutive expression of maltase, maltose permease, and their repective transcripts in the two MAL6-linked constitutive strains examined. Deletion of the MAL64-C gene in these constitutive strains resulted in nonfermenters that no longer constitutively expressed the maltose fermentative enzymes or their transcripts. Since these constitutive strains were derived from mal63 mutants, the ΔMAL64-C strains failed to respond to maltose. These and other results clearly demonstrate that MAL64-C encodes a trans-acting, positive regulatory function responsible for the constitutive expression of the MAL61 (maltose permease) and MAL62 (maltase) genes in MAL6-linked constitutive strains. In contrast to the MAL64-C gene, the wild type MAL64 allele appears to play no essential role in maltose fermentation or maltose regulated induction in inducible MAL6 strains. Deletion of MAL64 in a wild type inducible MAL6 strain elicits no phenotypic effect and the strain continues to ferment and induce normally. This demonstrates that only the MAL61, MAL62, and MAL63 genes are necessary for maltose regulated induction and fermentation. This is consistent with analyses of the other MAL loci, in which it has been demonstrated that the four other dominant MAL loci also contain three

genes that are structurally and functionally equivalent to the MAL61, MAL62 and MAL63 genes yet in contrast, by hybridization analysis all loci do not appear to contain a MAL64 homologous gene (M. Charron and C. Michels, manuscript in preparation; Charron et al, 1986). This underscores a possible unique role for the MAL64 gene.

Ten Berge et al. (1974) proposed that the MAL6-linked gene responsible for constitutivity was an allele of the positive regulatory gene required for maltose regulated induction, (ten Berg et al., 1973a), namely MAL63. This was concluded from their observation that uninducible x constitutive (mal63 / MAL6-C) heterozygous diploids were generally inducible for maltase and they proposed that interallelic complementation restored inducibility by the production of an oligomeric protein composed of the two gene products. We have clearly demonstrated that the MAL63 gene plays no role in MAL6-linked constitutivity in the two constitutive strains examined (Dubin et al, 1986), one of which was a derivative of constitutive strain C2 (ten Berge et al, 1973b); rather, constitutivity requires the MAL64-C gene. Restoration of inducibility in the uninducible x constitutive (mal63 MAL64 / mal63 MAL64-C) heterozygous diploid strains described by ten Berge et al. (1973b, 1974) cannot yet be unambiguously explained since interactions in these diploids between the MAL64 and MAL64-C gene products or interactions among the mal63, MAL64, and MAL64-C gene products could be responsible for this effect. It was reported that the Δ MAL63 MAL64 / Δ MAL63 MAL64C-R10 diploid is partially constitutive and partially inducible for maltase (see Table 5 in Dubin et al., 1986), suggesting that the MAL64 and MAL64-C gene products interact. With the recent cloning of the MAL64, MAL64-C2, and mal63-13 genes, and the construction of a

MAL64-C2 Δmal63-13 strain, such interactions can be tested directly.

The MAL63 and MAL64 genes are at least partially homologous and may have originated by a gene duplication event. As the wild type MAL64 gene plays no essential role in maltose regulated induction, the evidence is consistent with MAL64 having accumulated one or more mutations, rendering it functionless at least as far as maltose fermentation is concerned. While this does not exclude the possibility that the wild type MAL64 gene plays some unknown, non-essential function in yeast that is only distantly related or completely unrelated to maltose fermentation, MAL64 may instead currently serve as genetic reserve information, becoming active only following mutation and appearance of the MAL64-C allele. Similar cryptic genes have been described in bacterial systems (Hall et al., 1983). It remains to be determined whether activation of MAL64 to MAL64-C is due to a mutation within the coding sequence or within a transcriptional or translational regulatory sequence controlling MAL64 expression. Sequencing the MAL64 gene and its alleles will clarify this point and reveal the extent of homology between it and the MAL63 gene. Given the structural homology between the MAL63 and MAL64-C genes, it is likely that both products regulate the levels of the maltase and maltose permease mRNAs by a similar mechanism. MAL-linked constitutive mutations, including MAL6-C, also confer the ability to accumulate trehalose in glucose grown cells (Panek et al., 1979; Oliveira et al., 1981). It will be interesting to determine whether the MAL6-C gene product mediates its effect on trehalose accumulation in a manner similar to its control of the maltose fermentative enzymes.

The MAL64 gene appears to encode an inducible 2.0 kb and a 1.4 kb

transcript. When plasmid pZ-2 was used as a probe, it detected four other transcripts as well. While preliminary evidence is consistent with the 3.0, 2.3, 1.1, and 0.65 kb RNA species being transcribed from this region, one or more may actually originate from a structurally homologous region of the genome. Although we have no proof at this time, we favor the latter hypothesis as it is strikingly atypical for so small a sequence of yeast DNA to encode so many transcripts. The 3.0, 2.0, 1.4, 1.1, and 0.65 kb transcripts are induced by maltose in inducible MAL6 strains and constitutively expressed in constitutive MAL6 strains. Preliminary evidence indicated that induction of the two MAL64 transcripts depends upon the MAL63 gene. We are in the process of determining whether this is true for the other three RNA species. It is difficult to explain the existence of five maltose inducible transcripts that are dispensible for inducible maltose fermentation.

In addition to MAL6-C, constitutive alleles have been isolated at the other four dominant MAL loci (references cited in Results section). In order to investigate the genetic basis for constitutivity at another MAL locus, M. Charron deleted the MAL43 gene (the MAL63 homologous gene located at the MAL4 locus) in a derivative of the MAL4-C constitutive strain isolated by Kahn and Eaton (1971). In contrast to the results presented here for MAL6-C constitutive strains, deletion of MAL43 resulted in an *nonfermenter* that neither constitutively nor inducibly synthesized maltase. Further work clearly demonstrated that constitutivity was trans-acting and was encoded within the MAL43 gene (Charron and Michels, manuscript submitted for publication). Preliminary results suggest this is also the case in one MAL2-C constitutive strain (Charron and Michels, personal communication).

Given these results, it is possible that some MAL6-C constitutive mutations could reside within the MAL63 gene and this possibility is actively being explored by R. Needleman and co-workers. Together, these results demonstrate that constitutive expression of the maltose fermentative enzymes can occur by two mechanisms: mutational alteration within the MAL-linked, MAL63 homologue (for example at MAL4, the MAL43 gene, normally the maltose dependent positive regulatory gene), or by mutational alteration within the MAL-linked MAL64 homologue (like MAL64, which is partially homologous to MAL63 and normally plays no essential role in maltose fermentation). This does not imply that both mechanisms for constitutivity are always available to the cell, since not all dominant MAL loci contain a MAL-linked MAL64 homologue (M. Charron and C. Michels, manuscript in preparation). The MAL1 locus has been cloned and does not appear to contain a MAL64-homologous gene (Charron et al., 1986; M. Charron and C. Michels, personal communication; unpublished observations), suggesting that MAL1-linked constitutive strains may be mutant in the MAL1-linked MAL63 homologue, namely the MAL13 gene. This is consistent with the observation that an uninducible mal1 strain containing a deletion of the MAL13 gene fails to revert to a maltose fermenter (unpublished observation).

Extensive genetic analyses have indicated that maltose regulated induction of the maltose fermentative enzymes depends upon a single MAL-linked, positive regulatory gene (ie.: MAL63 at the MAL6 locus) (Zimmerman and Eaton, 1972; ten Berge et al., 1973a; Cohen et al., 1984; Chang et al., manuscript submitted for publication). While we hoped that MAL64 might define a second regulatory component involved in maltose regulated induction, we have gone on to demonstrate that this

gene is only involved in constitutive expression of the maltose fermentative enzymes. Since mutant isolation depends upon the selection scheme and the absence of repeated genes, it is always possible that other regulatory genes involved in inducible maltose fermentation may exist, however as yet no others have been detected in this system. The requirement for a single positive regulatory gene in the MAL system contrasts with the more complex hierarchy of regulatory controls exhibited by other eukaryotic systems. While induction of the galactose fermentative enzymes, repression of the repressible acid phosphatase, and derepression of amino acid biosynthetic enzymes in S. cerevisiae all appear to be directly controlled by positive activating regulatory gene products (GAL4, PHO2 and PHO4, GCN4, respectively), these positive activators are themselves controlled by one or more other regulatory gene products: the GAL80 gene product regulates the activity of the GAL4 gene product (reviewed in Oshima, 1982); PHO4 is controlled by PHO80, PHO81, and PHO85 (reviewed by Oshima, 1982); translation of the GCN4 mRNA is directly controlled by GCD1, which is itself controlled by GCN1, GCN2, and GCN3 (Hinnenbush, 1985). While inducible maltose fermentation appears to require a single positive regulatory gene function, a more complex control is involved in constitutive maltose fermentation. In addition to the MAL6-linked MAL64-C gene, constitutive expression of the maltose fermentative enzymes also depends upon an unlinked gene, MALx (ten Berge et al., 1974). While MALx mal63-13 MAL64-C2 strains constitutively synthesize express maltase and maltose permease, the malx mal63-13 MAL6-4C2 strains are nonfermenters and do not constitutively express these two maltose fermentative enzymes. One could imagine the MALx gene product being necessary for MAL64-C

transcription (which can now be tested) or influencing the MAL64-C2 gene product by acting as an internal inducer. Despite this apparent interaction in constitutive strains, neither MALx (ten Berge et al., 1974) nor MAL64 play a role in inducible maltose fermentation.

In contrast to the MAL loci, the eukaryotic regulatory genes mentioned above that have been mapped (GAL4, GAL80, PHO80, PHO81, PHO85) are unlinked to the structural genes they control. The only other eukaryotic system known to contain linked regulatory and structural genes is the qa locus in Neurospora crassa. Induction in Neurospora crassa of the quinic acid catabolic enzymes by quinic acid requires the product of a positive regulatory gene, qa-1F, which is controlled by a putative repressor, qal-S (Patel and Giles, 1985). The implication here is that linkage between structural and regulatory genes may be selectively maintained. Since the MAL loci appear to move about the genome (as the dispersed family of loci suggests) it would be advantageous to move as a unit, tending to ensure that no essential functions become separated and later segregate. While the mechanism of dispersal is unknown, a size limitation may exist, thus forcing the MAL loci to be compact and therefore simply regulated.

Studies on the MAL6 locus (Federoff et al., 1982; Needleman et al, 1984; Dubin et al., 1985; Dubin et al., 1986; Chang et al., manuscript submitted for publication; data presented above), as well as analyses of the MAL1 locus (Cohen et al., 1984, 1985; Charron et al., 1986) and the MAL2-4 loci (Charron and Michels, manuscript in preparation; Chow and Marmur, personal communication) demonstrate that a MAL locus encodes three structurally and functionally related genes required for inducible maltose fermentation: MAL61, which appears to encode maltose permease,

MAL62, which encodes maltase, and MAL63, which encodes a trans-acting, positive regulatory function required for maltose regulated induction of the maltase and maltose permease mRNAs and proteins. A fourth gene, MAL64, present at least at the MAL6 locus, plays no role in inducible maltose fermentation, but following mutation acts as a trans-acting, positive regulatory function responsible for consititutive expression of the genes encoding the two maltose fermentative enzymes. MAL64 is structurally homologous to the maltose dependent, positive regulatory gene MAL63. Now that the MAL6 locus has been characterized and its component genes identified and their functions basically understood, fine structure analysis of these genes has begun. Analysis of the MAL63, mal63, MAL64, and MAL64-C genes and gene products may clarify the mechanism(s) by which these proteins act, define the regions that are essential for their proper functioning, and perhaps clarify the role played by the inducer maltose. Identification of the cis-acting sequences controlling expression of the maltase and maltose permease genes may lead to the characterization of a maltose regulated enhancer (UAS_{Mal}) as well as sequences involved in catabolite repression. Analysis of the MAL61 gene could reveal the role played by its 2.4 kb constitutively expressed transcript and establish its relationship (if any) to the inducible 2.0 kb (presumed) maltose permease transcript. Studies on the maltose permease gene could provide a clearer understanding of active maltose transport, glucose inactivation, and protein localization. Clearly much work lies ahead.

Appendix Table 1 Legend.

(a) The mal63::URA3 strain a9 was crossed to strain 328-4A, the resulting diploid sporulated, and strain 9-2 (MATα mal63::URA3 mal1^0 trp1) was isolated from random spores (strain 9-2, like strain a9, was complemented by MAL1p strains). Strain 9-2 was crossed to strain K382-23A, the diploid sporulated, and strains 7 (MATα mal63::URA3 cyh2 spo11 trp1) and 58 (MATα mal63::URA3 cyh2 spo11 his7) were isolated as random spores. Strain 7 was crossed to the spo11 strains K399-7D and K396-11A and strain 58 was crossed to strains K396-11A and K398-4D. Strains 7 and 58 were thus mal6::URA3 ARG4 and strain K398-4D was ura3 arg4. The lack of ARG4 ura3 segregants and arg4 URA3 segregants indicates linkage of MAL6 to chromosome VIII.

(b) All segregants were cyh2, ADE6, LEU1, and TRP5 (except for the two ade6 spores indicated) due to selection for the Chromosome VII marker cyh2 which confers cycloheximide resistance.

(c) Linkage groups XIV and XVII have been shown to actually be the same chromosomes (Klapholtz and Esposito, 1982).

(d) The aro phenotype was determined by the ability or inability to grow on minimal medium plates containing all required amino acids except phenylalanine.

Appendix Table 1.

Chromosomal Assignment of MAL6 [a]

Chromosome	Marker	URA3 [a]	ura3	Strain [a]
I	ADE1:ade1	22:9	7:9	58 x K396-11A
II	HIS7:his7	45:20	19:8	58 x K398-4D
	LYS2:lys2	39:25	12:14	58 x K398-4D
III	LEU2:leu2	24:8	12:4	58 x K396-11A
IV	TRP1:trp1	39:27	14:12	58 x K398-4D
V	HIS1:his1	13:11	12:12	7 x K396-11A
VI	HIS2:his2	20:12	8:7	7 x K399-7D
(b) VII	CYH2:cyh2	0:211	0:101	all four
	ADE6:ade6	64:1	28:1	58 x K398-4D
	LEU1:leu1	32:0	15:0	7 x K399-7D
	TRP5:trp5	32:0	16:0	58 x K396-11A
VIII	ARG4:arg4	123:0	0:44	58 x K398-4D
IX	LYS1:lys1	22:8	9:3	7 x K399-7D
X	MET3:met3	22:9	8:8	58 x K396-11A
XI	MET14:met4	35:29	18:8	58 x K398-4D
XII	ASP4:asp4	87:11	29:7	58 x K398-4D
XIII	LYS7:lys7	27:5	13:3	58 x K396-11A
(c) XIV	PET8:pet8	N.D.	N.D.	N.D.
XV	PET17:pet17	39:26	11:16	58 x K398-4D
(d) XVI	ARO7:aro7	86:45	21:23	58 x K398-4D
XVII	MET4:met4	17:14	8:7	7 x K399-7D

Appendix Table 2.

Genetic Demonstration of Disruption Sites

1. Disruption of *MAL62* in strain 2b-46 and *MAL63* in strain a9.

RDY126:	a9	x	2b-46
	mal63::URA3 MAL62 mal1^0	x	*MAL63 mal62::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	9	1	0	10

(N.B.: all mal$^-$ segregants complemented by *MAL1p* strains)

2. Disruption of *mal1*^{0} in strain 2b-20.

RDY110:	2b-20	x	RDY100-1C
	MAL6 mal1^0*::LEU2*	x	*mal11::URA3*
	Mal$^+$, Leu$^+$, ura$^-$		mal$^-$, leu$^-$, Ura$^+$

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

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

3. Disruption of *MAL61* in strain aF.

RDY122:	aF	x	RDY111-5A
	MAL64 mal61::URA3 MAL62	x	*MAL64-C2 mal61/mal62::LEU2*
	mal$^-$, Ura$^+$, leu$^-$		mal$^-$, ura$^-$, Leu$^+$

Ratio of (Ura$^+$, leu$^-$: ura$^-$, Leu$^+$) 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 MAL61 in strain 1b-8.

RDY107: 1b-8 x RDY101-1D

mal61::URA3 x MAL6-C2

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	12	0	0	13

A single ascus contained the following: mal^-, Ura^+ : mal^-, Ura^+ : Mal^+, ura^- : mal^-, ura^- .This could be due to a conversion of MAL64-C2 to MAL64; in support of this interpretation, the mal^-, ura^- spore was complemented by MAL1p.

RDY123: 1b-8 x 332-5AΔmal1^0-7

mal61::URA3 mal1^0 x MAL61 mal1^0::LEU2

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	1	7	0	0	8

5. Disruption of mal1^0 in strain 332-5AΔmal1^0-7.

RDY131: 332-5AΔmal1^0-7 x RDY100-1C

MAL6 mal1^0::LEU2 x mal11::URA3

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

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

4:0	3:1	2:2	1:3	0:4	total asci examined
0	0	16	0	0	17

One of the 17 asci was a tetratype (Ura^+, leu^- : Ura^+, Leu^+ : ura^-, leu^- : ura^-, Leu^+), indciating a single recombination event.

6. Disruption of mal63-13 in strain 8-2BΔ63-1 and MAL61/MAL62 in strain 332-5AΔ61/Δ62-9.

RDY117: 8-2BΔ63-1 x 332-5AΔ61/Δ62-9

MAL64-C2 mal63-13::URA3 MAL61 MAL62 x MAL64 MAL63 mal61/mal62::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	10	0	0	12

Two of the 12 asci contained the following: Mal^+, Ura^+, leu^- : mal^-, Ura^+, leu^- : mal^-, ura^-, Leu^+ : mal^-, ura^-, Leu^+). This can be explained by assuming a conversion of MAL64-C2 to MAL64.

7. Disruption of mal63-13 in strain 8-2BΔ63-2.

RDY134: 8-2BΔ63-2 x 612-1D

MAL64-C2 mal63-13::URA3 x MAL64 MAL63

Mal^+, Ura^+ Mal^+, ura^-

Ratio of (Ura^+ : ura^-) in four spored asci

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

RDY105: 8-2BΔ63-2 x a9

MAL64-C2 ma163-13::URA3 x MAL64 ma163::URA3

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

Ratio of (Mal^+, Ura^+ : mal^-, Ura^+) in four spored asci

4:0	3:1	2:2	1:3	0:4	total asci examined
0	0	13	1	0	14

The 1:3 ascus could be due to conversion of MAL64-C2 to MAL64.

8. Disruption of MAL61/MAL62 in strain 8-2BΔ61/Δ62-5.

RDY111: 8-2BΔ61/Δ62-5 x RDY101-1D

ma161/ma162::LEU2 x MAL61 MAL62

mal^-, Leu^+ Mal^+, leu^-

Ratio of (mal^-, Leu^+ : Mal^+, leu^-) in four spored asci

4:0	3:1	2:2	1:3	0:4	total asci examined
0	1	5	0	0	6

9. Disruption of MAL61/MAL62 in strain 8-2BΔ61/Δ62-5 (isogenic to RDY111-5A) and integration of pY6ΔCΔH adjacent to MAL6-C2.

RDY115: RDY111-5A x 8-2BΔCΔH-6

ma161/ma162::LEU2 x MAL61 MAL62 ::pY6ΔCΔH

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

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

4:0	3:1	2:2	1:3	0:4	total asci examined
0	1	8	2	0	11

10. Disruption of *mal1*^{0} in strain RDY101-1B (which is isogenic to strain 8-2BΔ12-6).

RDY114: RDY101-1B x RDY100-1A

MAL6-C2 mal1^{0}*::LEU2* x *mal11::URA3*

Mal^{+}, Leu^{+}, ura^{-} mal^{-}, leu^{-}, Ura^{+}

Ratio of (Leu^{+}, ura^{-} : leu^{-}, Ura^{+}) in four spored asci

4:0	3:1	2:2	1:3	0:4	total asci examined
0	0	11	0	0	12

One of the asci was a tetratype (Leu^{+}, ura^{-} : Leu^{+}, Ura^{+} : leu^{-}, ura^{-} : leu^{-}, Ura^{+}), indicating a single recombination event.

11. Disruption of *MAL61/MAL62* in strain R10Δ61/Δ62-12.

RDY121: R10Δ61/Δ62-12 x RDY113-2C

MAL64C-R10 mal63::URA3 mal61/mal62::LEU2 x *MAL64-C2 mal63-13::URA3 MAL61 MAL62*

mal^{-}, Ura^{+}, Leu^{+} Mal^{+}, ura^{-}, leu^{-}

Ratio of (mal^{-}, Ura^{+}, Leu^{+} : Mal^{+}, ura^{-}, leu^{-}) in four spored asci

4:0	3:1	2:2	1:3	0:4	total asci examined
0	0	5	0	0	5

RDY118: R10Δ61/Δ62-12 x 2b-20

mal63::URA3 mal61/mal62::LEU2 mal1^{0} x *MAL6 mal1*^{0}*::LEU2*

mal^{-}, Ura^{+}, Leu^{+} Mal^{+}, ura^{-}, Leu^{+}

Ratio of (mal^{-}, Ura^{+} : Mal^{+}, ura^{-}) in four spored asci

4:0	3:1	2:2	1:3	0:4	total asci examined
0	0	14	0	0	14

12. Disruption of MAL64-C2 in strain 8-2BΔF-5-21. (Strain 612-1C is isogenic to strain a9).

RDY127: 8-2BΔF-5-21 x 612-1C

mal64-C2::LEU2 ma163-13 x MAL64 ma163::URA3

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

Ratio of (Leu^+, ura^- : leu^-, Ura^+) in four spored asci

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

13. Disruption of MAL64-C2 in strain 8-2BΔG-10.

RDY130: 8-2BΔG-10::DF1-1 x 332-5AΔ61/Δ62-9

mal64-C2::URA3 ma163-13 MAL61 MAL62 trp1::TRP1/MAL63 x

MAL64 MAL63 mal61/mal62::LEU2 trp1

Mal^+, Ura^+, leu^-, Trp^+ mal^-, ura^-, Leu^+, trp^-

Ratio of (Ura^+, leu^- : ura^-, Leu^+) in four spored asci

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

The trp phenotype was unlinked to both Ura^+ and Leu^+ (and thus MAL6). All the (ura^-, Leu^+) spores were mal^-, regardless of the trp phenotype. One-half of the (Ura^+, leu^-) spores were Trp^+ and Mal^+, and the other half were trp^- and mal^-. This further demonstrates that mal64-C2 ma163-13 is complemented by an unlinked MAL63 gene.

14. Disruption of MAL64-C2 in strain 8-2BΔH-1.

RDY129: 8-2BΔH-1 x 332-5AΔ61/Δ62-9

mal64-C2::URA3 ma163-13 MAL61 MAL62 x MAL64 MAL63 mal61/mal62::LEU2

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

Ratio of (Ura^+, leu^- : ura^-, Leu^+) in four spored asci

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

15. Disruption of MAL64C-R10 in strain R10uΔF-9-26. (Strain RDY113-2C is isogenic to strain 8-2BΔ63-2).

RDY128: R10uΔF-9-26 x RDY113-2C

mal64C-R10::LEU2 ma163::ura3 x MAL64-C2 ma163-13::URA3

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

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

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

16. Disruption of MAL64 in strain 332-5AΔF-1-5. (Strain RDY113-2C is isogenic to strain 8-2BΔ63-2).

RDY132: 332-5AΔF-1-5 x RDY113-2C

mal64:LEU2 MAL63 x MAL64-C2 ma163-13::URA3

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

Ratio of (Leu^+, ura^- : leu^-, Ura^+) in four spored asci

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

17. Disruption of MAL64 in strain 332-5AΔG-15.

RDY133: 332-5AΔG-15 x 8-2BΔF-5-21

ma164::URA3 MAL63 x ma164::LEU2 ma163-13

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

Ratio of (Ura^+, leu^- : ura^-, Leu^+) in four spored asci

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

Appendix Table 3 Legend

(a) Between 5 - 30 micrograms restricted DNA were used per transformation.

(b) 40% of the transformants fermented maltose as rapidly as the undisrupted parent, 60% fermented more slowly.

(c) 44% of the transformants fermented maltose as rapidly as the undisrupted parent, 56% fermented more slowly.

(d) This transformant was not stable for the URA3 marker nor disrupted at MAL63.

(e) This transformant was disrupted at MAL64 but also gene converted from MAL63 to mal63-13 (see text).

(f) This maltose non-fermenting class was unexpected. One mal^- transformant was analyzed physically and shown to be altered at MAL6, but was not pursued further.

Appendix Table 3.

Phenotypic Effect of Transformation with Disruption Plasmids on Maltose Fermentation

Strain	Disruption Plasmid[a]	Total Transformants	Tested for Maltose Ferm.	Mal^+	mal^-
332-5A	pDM2	174	10	10[b]	0
	pDM2b	660	62	62[c]	0
	pDM1	7	7	3	4
	pDM1b	13	13	1	12
	pD*mal1*^{0}	140	20	20	0
	pDM3	9	9	1[d]	8
	pRD3	130	35	20	15
	pDMF	101	36	35	1[e]
	pDMG	344	30	30	0
8-2B	pDM3	8	8	8	0
	pY6ΔCΔH	22	22	22	0
	pRD3	51	44	33	11
	pDM2b	16	16	7	9[f]
	pDMF	77	30	16	14
	pDMG	432	35	3	32
	pDMH	148	30	1	29
R10	pRD3	800	35	21	14
R10u	pDMF	65	32	20	12
	pDMG	729	35	4	31

Appendix Table 4.

Physical Confirmation of Gene Disruptions in Strain 332-5A

Strain	Disruption Plasmid	Restriction Enzyme	Probe	Observations
332-5A	-	HindIII	pD-1	A MAL6-linked 7.3 kb fragment, a 7.0 kb mal^0-linked fragment and three smaller faint fragments of unknown linkage.
332-5A	-	HindIII	pD-5	A MAL6-linked 7.3 kb fragment and a 7.0 kb $mal1^0$-linked fragment.
332-5A	-	HindIII	pE-4	A MAL6-linked 7.3 kb fragment, a 7.0 kb $mal1^0$-linked fragment, and an approximately 2.7 kb fragment of unknown linkage.
332-5A	-	EcoRI	pH-2 or pH-3	A 0.9 kb fragment linked to MAL63, a 2.4 kb fragment linked to MAL64, and a 5.6 kb fragment unlinked to MAL6.
332-5A	-	EcoRI/BamHI	[a] pJunct2	A 2.4 kb fragment linked to MAL64, a 3.0 kb fragment linked to MAL6 (to the left of MAL64), a faint 0.9 kb fragment linked to MAL63 and a faint 5.3 kb fragment of unknown linkage.
332-5A	-	EcoRI	pJunct2	A 2.4 kb fragment linked to MAL64, a 9.0 kb fragment presumably linked to the left of MAL64, a faint 0.9 kb fragment linked to MAL63, and a faint 6.0 kb fragment of unknown linkage.
332-5A	pDM2	HindIII	pD-1	Loss of the 7.3 kb fragment and appearance of a 9.9 kb fragment in strain 110. Loss of the 7.0 kb fragment and appearance of a 9.6 kb fragment in strain 104.

Strain	Disruption Plasmid	Restriction Enzyme	Probe	Observations
332-5A	pDM2b	HindIII	pD-1	Loss of the 7.3 kb fragment and appearance of a 8.5 kb fragment in strains 2b-15 and 2b-46. Loss of the 7.0 kb fragment and appearance of a 8.2 kb fragment (Fig. 2 in Dubin et al., 1985).
332-5A	pDM2b	HindIII	pD-5	Loss of 7.3 kb fragment in 2b-46 and loss of 7.0 kb fragment in 2b-20.
628-5B	Double Disruption	HindIII	pD-1	Loss of 7.3 kb and 7.0 kb fragments and appearance of 8.5 kb and 8.2 kb fragments.
628-5B	Double Disruption	HindIII	pD-5	loss of 7.3 and 7.0 kb fragments.
332-5A	pDM1	HindIII	pD-1 or pD-5	Loss of the 7.3 kb MAL6-linked fragment and appearance of a new fragment about 6.8 kb in strains aG and aF. One maltose fermenting transformant contained a new fragment of 6.8 kb and two other maltose fermenting transformants, while missing the 7.0 kb fragment, contained a new fragment of 6.8 kb.
332-5A	pDM1b	HindIII	pE-4	loss of the 7.3 kb fragment in five mal^- transformants, including 1b-7 and 1b-8. One maltose fermenting transformant was probed with pE-3 (which is very similar to pE-2 but lacking the pBR sequences present on the insert) and lacked the 7.3 kb fragment but contained a new 7.5 kb fragment.
332-5A	pDmal^0	HindIII	pE-4	Three isolates lost the 7.0 kb fragment, (including isolate number 7), and four isolates were identical to the untransformed parent, appearing to have undergone gene conversion at LEU2.

Strain	Disruption Plasmid	Restriction Enzyme	Probe	Observations
RDY123-1D	Double Disruption	HindIII	pE-4	loss of 7.3 and 7.0 kb fragments.
332-5A	pDM3	EcoRI	pH-2	loss of 0.9 kb fragment and appearance of a 2.0 kb fragment in six nonfermenting transformants, including strains a8 and a9. The one maltose fermenting transformant, a5, contained an intact MAL63 fragment and a novel band.
332-5A	pDM3	EcoRI	pH-3	loss of the 0.9 kb MAL63 fragment (checked in strain a9; see Fig. 13).
332-5A	pRD3	HindIII	pD-5	7.3 kb fragment lost in two nonfermenting transformants. One maltose fermenting transformant lost the 7.3 kb fragment but contained a novel larger fragment, suggesting plasmid integration at MAL6 but not deletion disruption.
332-5A	pDMF	EcoRI/BamHI	pJunct2	Loss of the 2.4 and 3.0 kb fragments and appearance of a 1.7 kb fragment in five maltose fermenting transformants (including 1-5 and 1-15); this was expected. The sole nonfermenter, 1-29, was also missing the 2.4 and 3.0 kb fragments and contained a new 1.7 kb fragment but was also missing the faint 0.9 kb MAL63 fragment and contained a new faint 1.9 kb fragment. Other maltose fermenting transformants were either undisrupted or were disrupted at MAL64 and also contained extra fragments so they were discarded.

Strain	Disruption Plasmid	Restriction Enzyme	Probe	Observations
332-5A	pDMF	EcoRI	pH-3	The five Mal$^+$ transformants containing the predicted MAL64 deletion all contained the 0.9 kb MAL63 fragment and lacked the 2.4 kb MAL64 fragment; the maltose nonfermenting strain 1-29 not only lacked the 2.4 kb fragment but was missing the 0.9 kb fragment and instead contained a 1.9 kb MAL63 homologous fragment, thus appearing to contain the mal63-13 allele.
332-5A	pDMG	EcoRI	pJunct2	Loss of the 2.4 kb fragment and appearance of a 3.5 kb fragment.
332-5A	pDMG	EcoRI	pH-3	Loss of the 2.4 kb fragment and appearance of a 3.5 kb fragment; the 0.9 kb MAL63-linked fragment was still present.

The following plasmid does not appear on any MAL6 maps in this thesis: (a) Plasmid pJunct2 contains the 0.4 kb HindIII - EcoRI fragment found within pZ-1 and the 0.3 kb BamHI - HindIII fragment present at the far left end of the yeast insert in plasmid pBam11 (see Fig. 10). These two fragments were subcloned together into pRB325.

Appendix Table 5.

Physical Confirmation of Gene Disruptions in Strain 8-2B

Strain	Disruption Plasmid	Restriction Enzyme	Probe	Observations
8-2B	-	HindIII	pD-1	A MAL6-linked 7.3 kb fragment, a 7.0 kb $mal1^0$-linked fragment and two smaller faint fragments of unknown linkage.
8-2B	-	HindIII	pD-5	A MAL6-linked 7.3 kb fragment and a 7.0 kb $mal1^0$-linked fragment.
8-2B	-	EcoRI	(a) pH or pH-2 or pH-3	A 1.9 kb fragment linked to mal63-13, a 2.4 kb fragment linked to MAL64, and a 5.6 kb fragment unlinked to MAL6.
8-2B	-	EcoRI/BamHI	(b) pJunct2	A 2.4 kb fragment linked to MAL64, a 3.0 kb fragment linked to MAL6 (to the left of MAL64), a faint 1.9 kb fragment linked to mal63-13, and a faint 5.3 kb fragment of unknown linkage.
8-2B	-	EcoRI	pJunct2	A 2.4 kb fragment linked to MAL64, a 9.0 kb fragment presumably linked to the left of MAL64, a faint 1.9 kb fragment linked to mal63-13, and a faint 6.0 kb fragment of unknown linkage.
8-2B	pDM3	EcoRI	pH	The 1.9 kb mal63-13 fragment was lost and a 2.0 kb fragment appeared in five of the maltose fermenting transformants. One maltose fermenting transformant was like the untransformed parent and had undergone a conversion to URA3 (confirmed with YIp31 as probe).
8-2B	pDM3	EcoRI	pH-2	Loss of 1.9 kb fragment and appearance of 2.0 kb fragment; checked in isolates 1 and 2.

Strain	Disruption Plasmid	Restriction Enzyme	Probe	Observations
8-2B	pDM3	EcoRI	pH-3	Loss of the 1.9 kb fragment; checked in isolates 1 and 2.
8-2B	pY6ΔCΔH	HindIII	pD-1	One transformant, number 6, contained the expected upshift of the MAL6-linked fragment (the loss of the 7.3 kb fragment and gain of a 14.3 kb fragment). The other five transformants examined contained extra fragments and were discarded.
8-2B	pDM2b	HindIII	pD-1	Two Mal^+ isolates (including isolate 12) contained the expected loss of the 7.0 kb fragment and the appearance of a new 8.2 kb fragment; one fermenting isolate was like the parent strains and possibly was a gene convertant. One nonfermenting transformant appeared to lack the 7.3 kb fragment and gained an 8.5 kb fragment; while the nonfermenting phenotype was unexpected it was not pursued.
8-2B	pRD3	HindIII	pD-5	Loss of the 7.3 kb fragment in five nonfermenting isolates. Five fermenting transformants were identical to the parent strain and were probably gene convertants at LEU2.
8-2B	pDMF	EcoRI/BamHI	pJunct2	Two mal^- isolates (5-20 and 5-21) lost the 3.0 and 2.4 kb fragments and gained a novel 1.7 kb fragment as expected for this disruption; one fermenting transformant was not disrupted.
8-2B	pDMF	EcoRI	pH-3	Transformants 5-20 and 5-21 lacked the 2.4 kb fragment and still retained the 1.9 kb fragment.

Strain	Disruption Plasmid	Restriction Enzyme	Probe	Observations
8-2B	pDMG	EcoRI	pJunct2	All four nonfermenting isolates examined lost the 2.4 kb fragment and gained a 3.5 kb fragment.
8-2B	pDMG	EcoRI	pH-3	The 2.4 kb fragment was lost and a 3.5 kb fragment was present; the 1.9 kb fragment was retained.
8-2B	pDMH	EcoRI	pJunct2	All seven maltose nonfermenting transformants examined lost the 2.4 kb fragment and a 3.5 kb fragment appeared.
8-2B	pDMH	EcoRI	pH-3	The mal63-13 sized fragment (1.9 kb) was still present when checked; the 2.4 kb fragment was lost and a 3.5 kb fragment appeared.

The following plasmids do not appear on any MAL6 maps in this thesis:

(a) Plasmid pH contains the 0.9 kb EcoRI fragment containing the 5' region of MAL63 subcloned into pBR325.

(b) Plasmid pJunct2 contains the 0.4 kb HindIII - EcoRI fragment found within pZ-1 and the 0.3 kb BamHI - HindIII fragment present at the far left end of the yeast insert in plasmid pBam11 (see Fig. 10). These two fragments were subcloned together into pRB325.

Appendix Table 6.

Physical Confirmation of Gene Disruptions in Strains R10 and R10u

Strain	Disruption Plasmid	Restriction Enzyme	Probe	Observations
R10	-	HindIII	pD-5	A MAL6-linked 7.3 kb fragment and a 7.0 kb $mal1^0$-linked fragment.
R10	pRD3	HindIII	pD-5	Loss of the 7.3 kb fragment in three maltose nonfermenting transformants. No maltose fermenting transformants were analyzed physically.
R10u	-	EcoRI	pH-3	A 2.4 kb MAL64-linked fragment and a 5.6 kb fragment of unknown linkage. (The 0.9 kb MAL63-linked fragment is missing since this strain was derived from strain a9.)
R10u	-	EcoRI/BamHI [(a)]	pJunct2	A 2.4 kb fragment linked to MAL64, a 3.0 kb fragment linked to MAL6 (to the left of MAL64), and a faint 5.3 kb fragment of unknown linkage.
R10u	-	EcoRI	pJunct2	A 2.4 kb fragment linked to MAL64, a 9.0 kb fragment presumably linked to the left of MAL64, and a faint 6.0 kb fragment of unknown linkage.
R10u	pDMF	EcoRI/BamHI	pJunct2	Three maltose nonfermenting isolates (including 9-26) lost the 3.0 and 2.4 kb fragments and contain a new 1.7 kb fragment as expected for this disruption. One maltose fermenting transformant was unchanged compared to the undisrupted parent strain.
R10u	pDMF	EcoRI	pH-3	The 2.4 kb fragment is lost (as is the 0.9 kb fragment).

Strain	Disruption Plasmid	Restriction Enzyme	Probe	Observations
R10u	pDMG	EcoRI	pJunct2	Five maltose nonfermenting transformants examined lost the 2.4 kb fragment and gained a 3.5 kb fragment, as expected.
R10u	pDMG	EcoRI	pH-3	The 2.4 kb fragment is lost and a 3.5 kb fragment appears; (the 0.9 kb fragment is still missing).

The following plamsid does not appear on any MAL6 maps in this thesis:

(a) Plasmid pJunct2 contains the 0.4 kb HindIII - EcoRI fragment found within pZ-1 and the 0.3 kb BamHI - HindIII fragment present at the far left end of the yeast insert in plasmid pBam11 (See Fig. 10). These two fragments were subcloned together into pRB325.

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