MAL6 of *Saccharomyces*: A complex genetic locus containing three genes required for maltose fermentation

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**ABSTRACT** The MAL6 locus is one of five closely related unlinked loci, any one of which is sufficient for fermentation of maltose in *Saccharomyces*. Previous genetic analysis indicated that this locus is defined by two complementation groups, MALp and MALg. MALp reportedly is a regulatory gene required for inducible synthesis of the two enzymatic functions needed for fermentation: maltose permease and maltase. We have investigated the physical and genetic structure of the MAL6 locus, which has been isolated on a recombinant DNA plasmid. One subclone of the region, pDF-1, was found to encode a single transcribed region and to contain the MALp gene. A second subclone, p1, was shown to contain the MALg function but surprisingly had not one but two maltose-inducible transcripts. Subclones having only one of these transcribed regions lacked MALg activity. The three transcribed regions have been named MAL61 and MAL62, which correspond to MALg, and MAL63, which corresponds to MALp. This clustered arrangement of a regulatory gene adjacent to the sequences it controls has not previously been described in eukaryotes and is reminiscent of bacterial operons except that the messenger RNA molecules are not polycistronic.

The MAL genes from *Saccharomyces* are a repeated gene family needed for the breakdown of maltose into glucose. Strains that carry one or more MAL genes are able to grow on maltose as the sole carbon source. So far, five unlinked related genes, *MAL1*, *MAL2*, *MAL3*, *MAL4*, and *MAL6*, have been identified in different isolates of *S. cerevisiae*. However, most haploid yeast strains capable of growing on maltose contain only a single functional copy of one of these MAL genes (often referred to as the dominant copy). Most strains examined also contain one or more cryptic copies of the MAL genes, which are related to the dominant copy by nucleic acid sequence homology (2, 3). Although the cryptic copies are insufficient in themselves to permit maltose fermentation, they usually fall into one of two complementation groups capable of complementing each other to give a maltose-positive phenotype (4–7). In addition, the cryptic copies are capable of complementing mutations in the MAL6 locus (6, 8).

These genetic studies have led to the proposal that the functional (dominant) MAL gene copies are complex loci comprised of at least two cistrons, MALp and MALg, while the cryptic copies usually contain only a single functional cistron, which is either MALp or MALg. MALp is thought to encode a positive regulatory factor needed for the induction of maltose and maltase permease, the enzymes required for maltose fermentation (6, 8), while MALg has been suggested to encode the enzyme maltase (α-D-glucosidase, EC 3.2.1.20) (2, 9). Recently, it was reported that the structural gene for maltose permease maps near a functional MAL gene (refs. 10 and 11; unpublished observation).

Little is known about the physical structure of the MAL loci, and whether a functional MAL gene can be physically divided into MALp and MALg cistrons. The MAL6 locus has been isolated on a recombinant DNA plasmid (pMAL6-26) (2, 9). In this report, we describe the genetic and physical structure of this locus as well as its regulation. MAL6 DNA was found to encode a complex genetic locus that was indeed divisible into fragments containing MALp and MALg. The locations of DNA complementary to the MALp and MALg functions were determined. Surprisingly, the MALg fragment contained not one but two maltose-inducible transcripts. The arrangement of a regulatory gene adjacent to the sequences it controls has not previously been described in eukaryotes.

**MATERIALS AND METHODS**

**Strains and Growth Conditions.** Yeast strains described in Table 1 were grown on YE medium (1% [wt/vol] yeast extract/1% [wt/vol] peptone) plus various amounts of the specified carbon source. Fermentation of maltose is defined as the production of gas and acid in 1–3 days after inoculation and was determined in 5 ml of YE/2% (wt/vol) maltose medium as described (2).

*Escherichia coli* strains C600 or RR1, bearing plasmids described in the text, were grown on rich medium/ampicillin. Plasmid amplification and isolation was by standard methods (12).

**Yeast Transformation.** Transformation of yeast was done using either the glusulase spheroplasting method (13) or the lithium chloride method (14).

**R-Loop Electron Microscopy.** Quantitative electron microscopy of R-loop-containing DNA was carried out as described (15–17).

**Preparation of Poly(A)+ RNA.** Exponential phase cells were disrupted by mixing in a Vortex with glass beads in 25 mM Tris-HCl, pH 7.2/100 mM NaCl/10 mM EDTA containing heparin at 1 mg/ml and NaDodSO4 to a final concentration of 0.5%; the mixture was extracted three times with phenol and the RNA was precipitated with 2 vol of ethanol. Poly(A)+ RNA was purified by two passes through oligo(dT)-cellulose according to standard procedures (12).

**RNA Blot Analysis of Poly(A)+ RNA.** Poly(A)+ RNA was denatured by heating to 60°C in 6% formaldehyde/50% deionized formamide and electrophoresed on formaldehyde/1.5% (wt/vol) agarose gels as described by Ravel et al. (18). The RNA was transferred to nitrocellulose and hybridized.

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Table 1. Yeast strains

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<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
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<tbody>
<tr>
<td>W208-7C*</td>
<td>MATa MAL6 (mal&lt;sup&gt;0&lt;/sup&gt;) leu2-3,112 trp1-1</td>
<td>Ref. 3</td>
</tr>
<tr>
<td>328-4A</td>
<td>MATa mal&lt;sup&gt;0&lt;/sup&gt; ura-52 trp1-1 ade met14</td>
<td>This report</td>
</tr>
<tr>
<td>340-2B</td>
<td>MATa MALlg ura3-52 trp1-1 lys2</td>
<td>This report</td>
</tr>
<tr>
<td>2151</td>
<td>MATa MAL1G leu2-3,112</td>
<td>This report</td>
</tr>
<tr>
<td>345-4A</td>
<td>MATa MALlp leu2-3,112, trp1-, ura3-52, ade</td>
<td>This report</td>
</tr>
</tbody>
</table>

*W208-7C contains only one functional MAL gene, MAL6. However, it also contains a nonfunctional cryptic mal<sup>0</sup> locus that confers neither MALp nor MALg activity and maps at the MAL1 locus (for an explanation, see ref. 2).

ized to the 32P-labeled probes according to Thomas (19) except that the hybridized filter was washed six times for 1 hr each in 200 ml of 0.75 M NaCl/0.075 M Na citrate, pH 7.1/50% (vol/vol) formamide/0.02% Ficoll/bovine serum albumin/polyvinylpyrrolidone (PVP 40). For hybridization reactions, 1 liter of formamide was deionized by stirring with 35 g of Bio-Rad AG 501-X8 (D resin) and 10 g of acid-washed activated charcoal at 4°C for 2–4 hr. AG 501 and charcoal were then removed by repeated filtration through Whatman no. 1 filter paper. The formamide was stored frozen and protected from light.

32P-labeled DNA was obtained by nick-translation according to the procedure of Rigby et al. (20) using [α-32P]dCTP. End-labeled single strands were made according to the procedure of O'Farrell et al. (21).

RESULTS

Determining the Location of the MAL6 Locus in Plasmid YEplMAL6. Plasmid YEplMAL6 (formerly called pMAL9-26) contains the MAL6 locus of Saccharomyces carlsbergensis on an 11.4-kilobase (kb) insert (2, 9). This plasmid replicates autonomously in yeast and transforms mal<sup>0</sup> (maltose-negative) yeast into maltose fermentors. A mal<sup>0</sup> strain contains neither a functional MALp nor MALg allele (2). To determine which regions of the plasmid insert are essential for maltose fermentation, subclones were constructed that contained different parts of the 11.4-kb region inserted in either pLC544 (22) or YEp13 (23) (Fig. 1). The subclones were tested for their ability to transform a mal<sup>0</sup> strain (328-4A) to a maltose fermentor. YEplMAL6 could not be easily subdivided into a single smaller plasmid capable of transforming the mal<sup>0</sup> strain. However, two subclones, pDF-1 (a pLC544 TRP1 ars-containing plasmid) and p1 (a YEp13 LEU2 2-um-containing plasmid), which individually failed to transform the mal<sup>0</sup> strain to a maltose fermentor, did so when transformed together into the same trp1 leu2 mal<sup>0</sup> recipient strain (328-4A) (Fig. 1). Loss of either plasmid after growth on non-selective medium [YP/2% (wt/vol) dextrose], as monitored by mitotic segregation for either the TRP1 gene present on pDF-1 or for the LEU2 gene present on pL, led to loss of the ability to ferment maltose. Therefore the functional MAL6 locus is contained within the 9 kb of DNA present in the two plasmids. A map of this region is shown in Fig. 1.

Mapping Transcribed Regions of MAL6. Electron microscopy of R-loop-containing DNA was used to map the positions of the transcribed regions and determine the relative abundance of the transcripts from the MAL6 region. Plasmids p1 and p21-40 were hybridized to poly(A)<sup>+</sup> RNA extracted from either maltose [2.0% (wt/vol)]- or glucose [2.0% (wt/vol) dextrose]-grown W208-7C cells. As a control for hybrid formation, pYact 1, a plasmid containing the yeast actin gene (ref. 24, courtesy of J. Abelson) was included in all hybridization reactions. The actin gene is essential for growth and should be expressed in all cells irrespective of carbon source (23). Indeed, R loops corresponding to the actin gene were found in hybridization experiments with all RNA samples examined.

Plasmid p1 was complementary to two maltose-inducible transcripts (transcripts 1 and 2; Fig. 2 and Table 2). Calculations based on quantitating the number of R loops (17) showed that induced cells contain about 10 copies of transcript 1 and 26 copies of transcript 2 (Table 2). Plasmid p21-40, which partially overlaps p1 (Figs. 1 and 2), also hybridizes to transcript 1 and is complementary to a third transcript

![Fig. 1. Physical structure and genetic activity of plasmid inserts from the MAL6 locus of S. carlsbergensis. The restriction map of 9.0 kb of DNA comprising the MAL6 locus and the location of the transcribed regions as obtained by electron microscopy (see Fig. 2) are shown. The restriction map outside this 9.0-kb region was not determined. Plasmid YEplMAL6 (pMAL9-26) has been described (9). The indicated regions of YEplMAL6 were subcloned into yeast–E. coli "shuttle vector" plasmids for complementation analysis. Plasmids p1 and p21-40 were constructed by insertion of the noted fragment into YEpl3. pDF-1 was obtained by insertion of the indicated fragment into pLC544 (22). pKS10 was constructed from p1 by deleting the region extending from the indicated Bgl II site in the insert to the BamHI site of the vector. pKS30 was derived from pY6 (2), which contains the HindIII yeast insert from p1 in the vector Ylp5. The construction involves rearrangement of pY6 that deleted the Sal I/EcoRI internal region of transcript 1. pKS30 retains an autonomously replicating sequence (ars) previously shown to be present in this region (2). Complementation analysis for the functions of the various plasmids was determined by transforming the MALp, MALg, and mal<sup>0</sup> strains described in the text and testing for maltose fermentation. Restriction enzymes used were A, Ava I; B, Bgl II; H2, HincII; H3, HindIII; Hp, Hpa I; K, Kpn I; Ps, Pst I; Pvu, Pvu II; R, EcoRI; S, Sal I.](image-url)
Fig. 2. R-loop electron microscopic mapping of transcripts complementary to plasmids p1 and p21-40. R-loop hybrids were formed and prepared for electron microscopy as described in the legend to Table 1. Double-stranded dX174 DNA (5385 base pairs) was included as a length standard in all samples spread for electron microscopy. Lengths are given in kb ± SD. Vector sequences are indicated by hatched lines.

(transcript 3; Fig. 2). Transcript 3 is found in equal abundance (approximately five copies per cell) in cells grown on either glucose or maltose (Table 2). Thus, under the conditions used in these experiments, the MAL6-complementing DNA appears to encode three transcripts. Transcript 1 and transcript 2 are induced by maltose while transcript 3 is constitutive.

Analysis of mRNA from Induced and Uninduced Cells. Based on the R-loop analysis, the three transcribed regions can be positioned on the restriction map of the MAL6 DNA (Fig. 3). Poly(A)⁺ RNA, prepared from strain W208-7C after growth on either maltose/3% glycerol (vol/vol) or glucose was electrophoresed on agarose, transferred to nitrocellulose, and hybridized to various probes. The probes chosen are complementary to only a single transcribed region. The probes neither overlap nor share sequence homology (unpublished observation).

The probe from region 1 hybridized to two maltose-inducible transcripts. The 2.0-kb transcript was most abundant and was found only in cells grown on maltose. The 2.4-kb transcript was found in low concentrations in glucose- and glycerol-grown cells and in higher abundance in maltose-grown cells. The probe from region 2 hybridized to a single 1.9-kb transcript that is induced to high abundance by maltose. The probe from region 3 hybridized a 2.0-kb transcript and a 1.6-kb transcript. The 1.6-kb transcript is present at similar abundance in maltose- and glucose-grown cells and at a somewhat reduced level in glycerol-grown cells. The 2.0-kb transcript is induced by maltose (Fig. 3a).

The direction of transcription from each region was determined by hybridizing the RNA blots to single-strand labeled probes as described in the legend to Fig. 3 and in Materials and Methods. Regions 1 and 2 were found to be divergently transcribed (Fig. 3b). This result precludes the possibility that the two maltose-inducible transcripts are derived from a single transcription unit.

Localization of the MAL6p and MAL6g Functions. Subcloned MAL6 fragments were used to determine whether the MALp and MALg complementing functions could be physically divided. Appropriate subclones were chosen that contained one or more of the transcribed regions (Fig. 1). To test for MALp function, pDF-1, which contains only transcript 3, was used to transform yeast strain 340-2B, which carries only the MAL1g allele and is malp⁻. TRP1 transformants were selected and found to ferment maltose, suggesting that the DNA containing transcript 3 contained MALp. To ensure that fermentation was the result of complementation between plasmid pDF-1 and the MAL1g allele and not with the unlinked mal⁰ or some other allele, eight MAL1g and four mal⁰ haploid segregants derived from a MAL1g (strain 2151) × mal⁰ (strain 328-4A) cross were transformed with pDF-1. Confirming our original observation, all of the MAL1g transformants and none of the mal⁰ transformants fermented maltose. Similar experiments with plasmid p1 and strains carrying the MAL1p cistron (strain 345-4A) and no MALg cistrons showed that plasmid p1 complements MAL1p and therefore is sufficient for supplying the MALg function.

Since plasmid p1 contains two transcribed regions, it is not known whether one or both transcribed regions can supply MALg functions. To determine this, a MAL1p strain (345-
Table 2. Relative abundance of transcripts complementary to MAL6

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Plasmid</th>
<th>Transcript 1</th>
<th>Transcript 2</th>
<th>Transcript 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maltose</td>
<td>p1</td>
<td>10.8 (12)</td>
<td>22.3 (26)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>p21-40</td>
<td>7.2 (8)</td>
<td>—</td>
<td>3.2 (5)</td>
</tr>
<tr>
<td>Glucose</td>
<td>p1</td>
<td>ND</td>
<td>ND</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>p21-40</td>
<td>ND</td>
<td>—</td>
<td>3.5 (5)</td>
</tr>
</tbody>
</table>

BamHI-linearized p1 or Sac I-linearized p21-40 was mixed with BamHI-linearized pYact I. The DNA mixtures were lightly crosslinked with Trioxsalen (4,5',8-trimethylpsoralen) and ultraviolet light (16) and hybridized to poly(A)⁺ RNA (18 µg/ml) isolated from cells grown on either YP medium/glucose or YP medium/maltose. Hybridizations were carried out under conditions in which all transcripts complementary to the added DNA are sequestered into R-loop structures so that the transcript level can be quantitated (17). The concentrations of DNA in the reaction mixtures were 6.0 µg/ml for p1, 6.0 µg/ml for p21-40, and 0.6 µg/ml for pYact I. The hybrids were stabilized by treatment with 1 M glyoxal (16) and prepared for electron microscopy by spreading from 50% (vol/vol) formamide according to published procedures (15). Hybrids were spread directly after treatment with glyoxal, without purification on a Sepharose 2B column. The fraction of DNA in R loops was determined by examining a minimum of 100 pYact I plasmids and 200–500 p21-40 or p1 plasmids. Plasmids were easily differentiated based on their relative size. The weight fraction (relative abundance) and copy number of each transcript was then determined as described (17) from the measured R-loop sizes and the known concentrations of added plasmid DNA and RNA. Numbers in parentheses are calculated numbers of transcripts per cell based on the assumption that the cells grown on both glucose and maltose contain the same number of poly(A)⁺ containing RNA molecules per cell (20,000) as previously reported for A364A cells grown on glucose. Measurements of actin RNA levels were included in these experiments as a control. Under both these growth conditions, 10 ± 5 copies of actin mRNA are present (data not shown). —, The transcribed region was not present within the yeast insert of the plasmid; ND, an R loop was not detectable in these experiments.

4A) was transformed with either plasmid pKS10, which contains only transcript 1 including about 800 bases upstream of the initiation site, or with plasmid pKS30, which carries only transcript 2 including about 1000 bases upstream of the initiation site. Neither pKS10 nor pKS30 complemented the MALlp-containing strain. Therefore, MAL6lp function requires either the presence of both transcripts 1 and 2 or the cis arrangement of these transcribed regions as found in plasmid p1.

DISCUSSION

We have investigated the organization of the MAL6 locus from S. carlsbergensis. All genes necessary for maltose fermentation were contained on 9.0 kb of DNA (Fig. 1). Three transcribed regions were identified on this DNA. Plasmid subclones lacking any of these regions were unable to ferment maltose. We now name these genes MAL61, MAL62, and MAL63, corresponding to transcribed regions 1, 2, and 3, respectively (Fig. 3).

The results reported here indicate that the MAL63 gene is MAL6p, the putative regulatory gene. MAL6p appears to be more complex than anticipated from previous genetic analysis, which defined it as a single complementation group (4–6). Surprisingly, the MAL6p DNA contained two transcribed regions (MAL61 and MAL62) both of which appear essential for function. Previous studies show that the structural gene for maltase is encoded within the MAL6 region of MAL6. Only one of the transcribed regions of MAL6p should correspond to the structural gene for maltase, which is known to be a single polypeptide (26). We have recently obtained evidence that MAL62 codes for maltase (unpublished results). However, the function of MAL61 remains to be defined. One study of MAL1 suggests that a maltose permease gene resides at this locus (10, 11). All of the MAL loci appear to be related, suggesting that a permease gene would also reside at MAL6. Recent evidence indicates that MAL61 indeed encodes this permease gene. Mutations that cause low basal levels of maltose transport are complemented by plasmids.
containing DNA from only the MAL61 region. In addition, mal0 strains transformed with this same DNA show a greatly increased ability to transport maltose but no increase in maltase activity (unpublished work).

RNA blots detected two different size transcripts complementary to the MAL61 probe. Both are maltose inducible but appear to be differentially regulated. The smaller (2.0-kb) species is highly inducible and not detectable in glucose- or glycerol-grown cells whereas the larger species, while inducible, is present under all growth conditions. If both the 2.4- and 2.0-kb transcripts are the product of the MAL61 gene, it would imply either differential transcript initiation, termination, or processing under the varied growth conditions. The size obtained for this transcribed region by electron microscopy is consistent with either or both transcripts forming R-loops. However, R-loop electron microscopy did not detect any MAL61 transcript from glucose grown cells. We believe this failure is most likely a result of the low abundance of the 2.4-kb transcript in glucose-grown cells. Nevertheless, we cannot exclude the possibility that the 2.4-kb transcript is undetectable using R loops because it is the product of another related, yet nonidentical genomic copy (for example, the mal0 locus present in strain W208-7C). Forming R loops may require a high degree of sequence homology. Minor sequence variation might prevent R-loop formation yet still allow for hybridization on nitrocellulose filters.

Likewise, two size classes of transcript were seen from MAL63. Again, both transcripts were differentially regulated. The smaller (1.6-kb) species was constitutively synthesized and the larger (2.0-kb) species was induced by maltose. Unlike MAL61, MAL63 transcripts were found in equal abundance in glucose- and maltose-grown cells by R loop electron microscopy. For the MAL63 transcripts we are unable to conclude whether or not both the 1.6-kb and 2.0-kb transcripts are products of just the MAL63 gene. As with the MAL61 gene, additional nonfunctional genomic sequences homologous to the MAL63 gene are also found in strain W208-7C and one of these transcripts could be the product of this DNA. This would also explain the apparent discrepancy in the quantitation between R-loop electron microscopy and the RNA blot analysis, where it appears there could be twice as much total MAL63 complementary transcript in the induced cells.

Given the extensive sequence and functional homology between MAL6 and the other MAL loci (2, 3), we suggest that the MAL genes mapping to other loci resemble those at MAL6. We therefore propose a model for the structure of the MAL family of functional loci. Each functional MAL locus is a cluster of three genes: a regulatory gene linked to the two divergently transcribed structural genes under its control. The structural genes probably code for maltase and maltose permease. This arrangement is reminiscent of bacterial sugar catabolism operons such as ara, lac, and gal, which also contain structural genes adjacent to regulatory ones (27). The MAL6 locus is the first example we know in Saccharomyces of such an arrangement. The divergent transcription of the coordinately regulated MAL61 and MAL62 genes is not unique to this locus. Similar arrangements of other coordinately expressed divergently transcribed regions have been reported in yeast (28, 29). This type of arrangement might represent a common feature relating to the mechanism of achieving coordinate expression.

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